A STUDY OF THE AGGREGATING CATIONIC ANTIBACTERIAL PROTEINS AND PEPTIDES IN BOVINE SEMINAL PLASMA

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This thesis is dedicated to my loving parents,
Abdul Hamid Azhar and Ruqqaiyya Hamid,
for their continuing encouragement, understanding and love.

ABSTRACT

Several non-specific defence mechanisms are known to be involved in the protection of the mammalian host against invading microorganisms. Among them are well researched proteins like lysozyme and lactoferrin, systems like the myeloperoxidase- and lactoperoxidase-mediated system, and two complement systems. Also, a number of mammalian cationic proteins and peptides have been found to have antibacterial properties.

The antibacterial activity in bovine seminal plasma has been reported to be due to proteins which appeared to be present in aggregated forms (Eschenbruch, 1980; Shannon *et al.*, 1987). This investigation was designed to account for the aggregation and disaggregation of the antibacterial activity in bovine seminal plasma.

The overall study demonstrated that most or all of the high molecular weight antibacterial proteins of bovine seminal plasma are aggregates. Three major antibacterial aggregates with molecular weights of around 500 kDa, 250 kDa and 20 kDa were identified and isolated using gel filtration chromatography at neutral pH.

Acidic gel filtration chromatography, cation exchange chromatography using citrate buffer and anion exchange chromatography at pH 12.0 and pH 13.0 were found to be successful techniques for disaggregation of the antibacterial aggregates. FPLC of diluted samples using acetonitrile and trifluoroacetic acid was found to give complete disaggregation.

The aggregates were found to be made up of two peptides, a basic antibacterial peptide with a molecular weight of about 1.2 kDa and an inactive acidic peptide with a molecular weight of about 1 kDa. However, the molecular weights estimated of the components and the aggregates could be larger than estimated in this study because of possible retardation on gel filtration chromatography.

Both the peptides were shown to be necessary for the formation of the antibacterial aggregate. These peptides were found to form a stable antibacterial aggregate of about 20 kDa mainly through

hydrophobic interactions. The aggregation beyond the 20 kDa aggregate, resulting in the formation of the 500 kDa and 250 kDa aggregates, was found to occur through ionic interactions. Furthermore, lysozyme was found to occur both in a free form and bound with the antibacterial aggregates.

The possible significance of the aggregation on the antibacterial activity is discussed.

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CHAPTER ONE

INTRODUCTION

For several decades researchers have frequently demonstrated the existence of naturally occurring non-specific antimicrobial substances in mammalian tissues and secretions. There is a great deal of confusion regarding the identities of these substances and their role in supplementing the immune response in natural resistance to invading microorganisms. A great deal of this confusion is due to the lack of characterisation of these various antimicrobial agents as they are very difficult to obtain in a homogeneous state. The non-specific natural defence mechanisms involved in the protection of the host against invading microorganisms have been reviewed by Eschenbruch (1980) and Shackell (1980).

One of these mechanisms is the phagocytic defence system. After ingestion of a microorganism a number of antibacterial factors within the phagocytic vacuole can lead to the killing and lysis of the parasite. Among these factors are low pH, lactic acid, hydrogen peroxide, the myeloperoxidase-mediated system, cationic proteins, lysozyme and lactoferrin (Hirsch, 1960a, 1972). Some of these substances are also present in body fluids such as saliva, tears, milk, blood and seminal plasma. Blood also has the complement system as an additional antibacterial constituent.

Another defence mechanism is due to polyamines, spermine and spermidine. Studies by Razin and Rozansky (1957) indicated that the antimicrobial action present in semen could be attributed to the presence of spermine and spermidine. However, the antibacterial activity present in prostate fluid and amniotic fluid was only partially due to these polyamines (Fair and Wehner 1971; Miller *et al.*, 1976), and Shackell (1984) demonstrated that the antibacterial activity of bovine seminal plasma was not due to any polyamine, spermine or spermidine. Instead, the antibacterial activity was found to be protein in nature. Detailed studies of the antimicrobial action of spermine and spermidine were carried out by Razin and Rozansky (1959). In general, spermine was found to be the most effective in inhibiting bacterial growth, spermidine being somewhat less active. Exponentially

growing cultures of *Escherichia coli* were studied after exposure to a lethal concentration of spermine (Mills and Dublin, 1966). The first changes noted were a slowing of protein synthesis, stimulation of RNA synthesis and increased potassium fluxes. This was followed by a decline in viable numbers. Polyamines have been also shown to inactivate certain viruses (Bachrach, 1973).

Razin and Rozansky (1959) also found that nucleic acids, lecithin, basic organic compounds and inorganic salts antagonise the antibacterial action of spermine. The antibacterial action of various drugs is inhibited by polyamines which suggests a common binding site. Such effects have been reported for streptomycin (Bragg and Polgase, 1963) and a number of other drugs. In cases of pneumonia, tuberculosis, and carcinoma, the diseased tissue consistently contained approximately twice as much spermine as a normal portion of the same organ. This pointed to the possible biological significance of these compounds (Tabor and Tabor, 1964).

Polyamines can also affect the cell surface of bacteria. This can happen either by direct attack on the membrane permeability properties or by causing cell clumping by neutralising the polyanionic nature of the cell walls (Silver *et al.*, 1970). It appears that the bactericidal effect occurs only in a nutritionally adequate medium. Glucose and a temperature of 37°C are required to inhibit growth (Razin and Rozansky, 1959). These results are consistent with the finding that energy is required for the binding of polyamines to microbial cells (Tabor and Tabor, 1966).

Several proteins have been isolated which specifically bind spermine. Mezzetti *et al.* (1982) have found that human peripheral lymphocytes contain a cytosol factor able to bind spermine non-covalently and with a high affinity. This factor is heat sensitive and appears to be a protein which is an intracellular, specific binder for spermine. Roch *et al.* (1979) also identified the existence of a polyamine conjugate of high molecular weight in which spermine is bound to immunoglobulin. The chemical structure of the spermine-immunoglobulin complex is not known.

Many of the non-specific substances have been found to be cationic peptides and proteins. One of the best characterised cationic proteins is lysozyme. It is found in tears, nasal mucus, saliva, blood serum and plasma, in many other tissues and secretions of human and other animal origin, in microorganisms and plants.

Chicken egg-white lysozyme has an isoelectric point of 10.5 - 11.0 and a molecular weight of 14.7 kDa (Salton, 1957). It is very stable under acidic conditions and withstands heating at 100°C at pH 4.5 for two minutes without losing activity. It is, however, labile at alkaline pH. Human lysozyme with three cystine residues per molecule is a little more heat-sensitive, and in goose lysozyme, which only contains two cystine residues, the heat stability is almost lost (Jollès, 1969).

Lysozyme exhibits both enzymic and non-enzymic action. As an enzyme, lysozyme specifically attacks the β 1-4 glycosidic linkage that joins N-acetyl muramic acid and N-acetyl glucosamine in the murein backbone of peptidoglycan (Strominger and Ghuysen, 1967). Therefore, to be active, lysozyme must reach the critical glycosidic bonds and cleave a sufficient number to result in fracture of the murein backbone of the peptidolglycan.

The non-enzymic action of lysozyme involves flocculation of bacterial suspensions owing to the basic properties of the protein. Other basic substances such as protamine, histones and basic polypeptides do this also. Negatively charged groups on the bacterial surface are likely to attract the basic compounds thus causing neutralisation of the charges and agglutination of the cells (Salton, 1957).

The role of lysozyme in resistance to bacterial infections has never been clearly established (Skarnes and Watson, 1957). While most bacteria of clinical significance are resistant to lysozyme itself, the major function of this enzyme may be to act in concert with other factors (Root and Cohen, 1981). Lysozyme has been shown, for example, to act cooperatively in killing organisms with the serum antibody-complement system (Muschel and Jackson, 1963), with a combination of hydrogen peroxide and ascorbate (Miller, 1968), and with both high and low pH (Saint-Blancard *et al.*, 1970). Elsbach (1980) suggested that

the most important role of lysozyme in neutrophil function may be to digest phagocytosed bacteria once they are killed rather than to act as a primary bactericidal agent. However, Spitznagel (1979 and 1980) supports the view that lysozyme plays an important role in killing as well as degradation of susceptible bacteria.

As well as lysozyme, another mammalian antibacterial system consisting of enzymes is the peroxidase system. The peroxidase of the granules of the neutrophilic polymorphonuclear leukocytes, or PMNs, is myeloperoxidase, or MPO, which, together with hydrogen peroxide and a halide as an oxidizable cofactor forms a potent microbicidal system. It is effective against bacteria (Klebanoff, 1968; McRipley and Sbarra, 1967), fungi (Lehrer, 1969), viruses (Belding *et al.*, 1970) and mycoplasma (Jacobs *et al.*, 1972).

Myeloperoxidase was first characterised by Agner (1941), who isolated it from pus and called it, due to its green colour, verdoperoxidase. It is present in the PMNs of a wide variety of species (Jain, 1967) and in high concentrations, estimated to be up to 5 % of the dry weight of human leukocytes (Schulz and Kaminker, 1962). MPO is a basic protein with an isoelectric point of about 10 and a molecular weight of 150 kDa. It is a haemprotein, containing two haem prosthetic groups per molecule, and the iron content of the molecule is 0.075 % (Ehrenberg and Agner, 1958; Schulz and Kaminker, 1962).

There are two potential sources of hydrogen peroxide for MPO - the leukocytic metabolism and the microbial metabolism. It is well established that phagocytosis by PMNs is associated with a striking burst of oxidative metabolism (Karnovsky, 1962). An increase in oxygen consumption is detected within seconds of contact between cells and the particle to be ingested. The consumed oxygen is converted, in part at least, to hydrogen peroxide. Microorganisms can also serve as the source of hydrogen peroxide. Lactic acid bacteria, to which the Pneumococci, Streptococci and Lactobacilli belong, are a typical example. They are in general devoid of haem and thus do not contain the cytochrome system. Terminal oxidations are catalyzed by flavoproteins which reduce oxygen to hydrogen peroxide. Usually they also lack catalase. Thus, hydrogen peroxide accumulates which

contributes alone (Klebanoff, 1975) or together with MPO to the microbicidal activity of the leukocytes.

In the MPO-mediated system iodide, bromide or chloride may act as the oxidizable cofactor (Klebanoff and Smith, 1969). Chloride is present in the leukocytes at a concentration which is over 100 times that required for activity in the isolated MPO system. Iodide is considerably more efficient than chloride, but its concentration in the leukocytes is rather low. Thus, it can be assumed that the concentration of cofactors is high enough and should not be a limiting factor in the intact leukocyte.

The mechanism of action of the MPO-mediated system is not completely known. Hydrogen peroxide reacts with the iron of the haem prosthetic group of MPO to form a complex with strong oxidative capacity. The halide is oxidized and might thus form a strong antimicrobial agent. The nature of this agent is likely to vary with the halide employed (Klebanoff and Smith, 1969).

When iodide is the cofactor, iodination of the bacterial proteins may occur as well as oxidation of essential sulphydryl groups or tryptophan residues in bacterial enzymes (Klebanoff, 1967). Lipid peroxidation (Welton and Aust, 1972) and cleavage of tryptophanyl peptide bonds (Alexander, 1974) during the peroxidase-catalyzed iodination reaction have also been described. The mechanism is similar when bromide serves as a cofactor, resulting in bromination and oxidation of essential compounds.

The MPO-hydrogen peroxide-chloride system, which generates highly reactive products of chlorine oxidation, may act in several different ways. Oxidation of sulphyryl groups may occur as with iodide and bromide. Also, the active chlorine component can oxidize existing iodide to iodine, which reacts as mentioned above. Furthermore, the chlorine derivatives can react with nitrogenous compounds to form chloramines, which contribute to the death of the organism in a number of ways. They are directly toxic in forming organic chloramine derivatives, or their toxicity can be due to decomposition products which are believed to cause deamination and decarboxylation of amino acids and formation of corresponding aldehydes (Zgliczynski *et al.*, 1971). The killing of *Escherichia coli* by isolated human

myeloperoxidase plus hydrogen peroxide plus chloride ions has been shown to proceed via an increased permeability of the bacterial cell wall (Sips and Hamers, 1981).

The bactericidal action of the MPO system can also be due to highly reactive intermediates of oxygen reduction. There are several possibilities which were summarized by Rosen and Klebanoff (1979) for PMN leukocytes and by Johnston (1978) for macrophages. The one-electron reduction product of oxygen is the superoxide anion, which reacts in the following way : it can dismutate with a second radical to form hydrogen peroxide, a reaction which is catalyzed by superoxide dismutase. However, when hydrogen peroxide is further reduced, a highly reactive and toxic hydroxyl radical is produced. This reaction is called the Haber-Weiss reaction (Haber and Weiss, 1934). Another potentially harmful oxygen metabolite is singlet oxygen. Singlet oxygen is molecular oxygen in an excited state, where an electron has been shifted to an orbital of higher energy with inverted spin. It could be produced by the MPO/hydrogen peroxide/Cl^- system with HOCl as an intermediate (Harrison and Schulz, 1976). This reaction is favoured by the low pH which exists in the phagocytosing cell. However, Thomas (1979) demonstrated that the bactericidal activity is due to HOCl rather than to the very short-lived singlet oxygen.

Another peroxidase enzyme with antibacterial activity is lactoperoxidase, which has been found to be present in milk, saliva, and tears. Lactoperoxidase, which can be isolated from raw milk by ion exchange chromatography, is a haem protein which is unique in that it has the highest protein to haem ratio of any of the peroxidases yet isolated, with a single haem per molecule. The iron content is 0.073%. The enzyme is heat-stable and has a molecular weight of 78 kDa. It is a glycoprotein with 28 moles of carbohydrate per mole of enzyme (Rombauts *et al.*, 1967).

The concentration of lactoperoxidase in tears, milk, and saliva is very different and also varies widely among different species (Gothefors and Marklund, 1975). For example, in humans the peroxidase activity is much higher in saliva than in milk, whereas in cows the quantities are reversed.

Although it was believed for a long time that only Gram-positive bacteria are inhibited, Bjorck *et al.* (1975) have shown that the lactoperoxidase system is also active against Gram-negative bacteria such as *Pseudomonas* and *Escherichia coli*.

Studies have shown that the inhibitory system requires thiocyanate or another oxidizable factor (Reiter *et al.*, 1963). Increasing the concentration of thiocyanate to a constant 0.25 mmol/l and adding an equimolar amount of hydrogen peroxide to milk have been shown to cause a substantial reduction of the bacterial flora (Björck, 1978).

Hydrogen peroxide is not known to occur naturally in bacteria-free milk or saliva. It can, however, be provided by catalase-negative bacteria. Also, the autooxidation of ascorbic acid and the oxidation of glucose by glucose oxidase leads to hydrogen peroxide production and may thus contribute to the antibacterial effect.

Although it had long been known that the antibacterial compound of the lactoperoxidase system is an oxidation product of thiocyanate, its identification caused considerable difficulties. The final products of the lactoperoxidase-catalysed oxidation of thiocyanate by hydrogen peroxide are sulphate, carbon dioxide and ammonia, none of which are inhibitory for bacteria. The antibacterial compound thus had to be an intermediate of the thiocyanate oxidation. Oram and Reiter (1966a) compared the antibacterial activity of the lactoperoxidase-mediated system with that of sulphur dicyanide and found similarities in the effect of both on catabolic enzymes of Streptococci. They found that hexokinase was completely inhibited and glucose-6-phosphate dehydrogenase was partially inhibited; phosphohexokinase was little affected in both cases. Hogg and Jago (1970) established from their spectrophotometric and polarographic studies that the inhibitor was either cyanosulphurous acid or cyanosulphuric acid. Hoogendoorn *et al.*, (1977) succeeded in identifying the oxidation product of the lactoperoxidase system as being the hypothiocyanite ion, $OSCN^-$. This was also confirmed by Tenovuo *et al.* (1985). They showed that the growth of *Bacillus cereus* is inhibited by the lactoperoxidase as well myeloperoxidase system and that the growth inhibition was directly proportional to the amount of $OSCN^-$. The $OSCN^-$, which is the principle

oxidation product of lactoperoxidase (and myeloperoxidase)-SCN^--H_2O_2 system at neutral pH, was also found to be a normal component of human saliva.

Many factors, namely, pH, amount of of H_2O_2, cell sulfhydryl content, and stored-carbohydrate content have been found to determine the antibacterial efficiency of lactoperoxidase (Thomas *et al.*, 1983). Furthermore, IgA has also been shown to enhance the antibacterial efficiency of the lactoperoxidase system (Tenovuo *et al.*, 1982).

Gothefors and Marklund (1975) have shown that the system could provide a protection against infection in newborn babies, because the concentration of lactoperoxidase and thiocyanate, although being low in breast milk, are high in the infant's saliva, and the enzyme is not inactivated by the gastric juice. The antibacterial lactoperoxidase system has been reviewed by Reiter and Harnulv (1984).

Another high molecular weight antibacterial protein of mammalian origin is lactoferrin. It was discovered when Sorenson and Sorenson (1939) found a red protein in bovine milk. Later, Wolin and Kosikowski (1959) described an inhibitor for *Bacillus subtilis* in raw milk, which subsequently was shown to withstand the ultra-high temperatures used for sterilizing milk (momentary heating at 135°C) (Franklin *et al.*, 1958). Extending these studies, Cheeseman and Jayne-Williams (1964) showed that the antibacterial activity was not abolished by heating at 90°C for one hour but was destroyed by digestion with trypsin and prevented by addition of Fe^{2+} or Mg^{2+}.

Lactoferrin is an iron-binding glycoprotein, its carbohydrate content being about 7 %. It can also be isolated as the iron-free apolactoferrin (Gordon *et al.*, 1962). The molecular weight was calculated to be 77 kDa (Klebanoff, 1975). The basic character of the protein is evidenced by an excess of 27 cationic residues over anionic residues. Lactoferrin binds two iron ions per protein molecule as shown by metal-ion titration studies by Aisen and Leibmann (1972).

The antibacterial properties of lactoferrin have been established independently by Masson *et al.* (1966) and Oram and Reiter (1966b). The bacteriostatic effect appears to be due to the complexing of

metabolically required iron since the inhibition was prevented by addition of iron to the growth medium. As the concentration of free iron in external secretions is low and the stability constant of the protein-iron complex exceeds 10^{30} (Weinberg, 1975), microorganisms are likely to compete unsuccessfully for the available iron.

The antibacterial activity is enhanced by certain divalent cations such as Mn^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} as demonstrated by Oram and Reiter (1968). These authors concluded that the bacteriostatic activity, although primarily due to the depletion of iron in the medium below the requirement of bacterial growth, nevertheless could be augmented by the associated alteration of the cation balance (e.g. Fe^{2+} to Co^{2+} ratio) in the medium and thus potentiate the cytotoxic effects of Co^{2+} and Zn^{2+} by the sequestering of Fe^{2+}.

In vitro experiments indicate that lactoferrin is bacteriostatic for a wide variety of microorganisms, including *Staphylococcus aureus*, *Staphylococcus albus* and *Pseudomonas aeruginosa* (Masson *et al.*, 1966), *Bacillus stearothermophilus* and *Bacillus subtilis* (Oram and Reiter, 1968) and *Escherichia coli* (Bullen *et al.*, 1972). Arnold *et al.* (1977), in studies with *Streptococcus mutans* and *Vibrio cholera*, found that apolactoferrin in concentrations below that necessary for total inhibition reduced markedly the number of viable colonies. They concluded that lactoferrin has a bactericidal rather than bacteriostatic effect on some microorganisms which is contingent upon its metal-chelating properties. However, Arnold *et al.* (1981) suggested that human apolactoferrin is capable of exerting a bactericidal effect on *Streptococcus mutans* by a process which is independent of simple iron deprivation.

It is known that the growth of some fungi, protozoa and Gram-positive and Gram-negative bacteria in body fluids, cells and tissue is stimulated by excess iron, as summarized by Weinberg (1978). Furthermore, the resistance to microbial invasion was reported to become seriously impaired in humans when stressed with excess iron (Barry and Reeve, 1977; Becroft *et al.*, 1977), or in diseases which result in hyperferraemia, e.g. destruction of liver cells in viral

hepatitis, or haemolysis in malaria or leukaemia (Weinberg, 1975). This suggests that iron-binding proteins are of some importance in the host's defence against infections.

In vivo experiments show that, in patients with bacterial meningitis or pneumonia, the lactoferrin concentration in the plasma increased about four-fold (Hansen *et al.*, 1976) probably due to the release by degranulation of neutrophils rather than to *de novo* synthesis (Leffell and Spitznagel, 1975). Increase in the rate of lactoferrin production in a tissue exposed to microbial invasion was shown by Harmon *et al.* (1976): 90 hours after the onset of experimental or natural mastitis of the bovine mammary gland, the concentration of secreted lactoferrin was increased 30-fold. Taking into account the high number of neutrophils that accumulated in the infected area, it was calculated that only 10 % of the extra lactoferrin could have been derived from these cells. The remainder was considered to be synthesized by the tissue in response to the infection. It may therefore be concluded that lactoferrin contributes to the antimicrobial activity of secretions and cells alone or in combination with lactoperoxidase and lysozyme, owing to the similar distribution of these proteins in the host.

Besides lactoferrin, several other antibacterial cationic proteins and peptides have been isolated from mammalian tissues and secretions. One group of these proteins is histones. In recent years, much work has been carried out on the separation and characterisation of histones. Various fractions have been obtained, and they are designated as follows: H1 is a histone with a very high proportion of lysine (lysine/arginine ratio: 22.0), H2a and H2b are lysine-rich histones, whereas H3 and H4 are arginine-rich histones (Elgin and Weintraub, 1975).

The antimicrobial character of histones was observed originally by Vaughan *et al.* (1893) and Kossel (1896). Their findings were later confirmed by Miller *et al.* (1942), Negroni and Fischer (1944), Weissman and Graf (1947) and Hirsch (1958). These authors also described the bactericidal action on certain microorganisms of protamines, a group of cationic polypeptides associated with the nucleic acids of sperm.

Hirsch (1958) studied the antibacterial action of histones in some detail. Using *E. coli* K_{12} as a test organism, he found histone "fraction B", which contained about equal amounts of lysine and arginine, to be bactericidal at concentrations as low as 0.35 μg/ml, whereas the lysine-rich "fraction A" was inactive at concentrations up to 25 μg/ml. Fraction B was also active against some strains of *Salmonella*, *Shigella*, *Pseudomonas*, *Klebsiella* and *Micrococcus*.

The antibacterial activity of the histone fraction was heat-stable under acidic conditions, unstable in neutral phosphate buffer and sensitive to the proteolytic action of pepsin. It was little affected by pH values between 5 and 7 but was reduced by salt concentrations higher than 0.2 mol/l. Inhibition of the bactericidal action by protamine and spermine was considered to be due to the higher basicity of these substances, which would compete successfully for acid receptor sites on the bacteria and protect them from the lethal action of histones. (Protamine itself was not found active under the conditions of the experiment).

Ashmarin *et al.* (1970) found that the lysine-rich fractions, too, of histones were antibacterial against certain microorganisms. They were able to select cultures of one species which differed in susceptibility to the action of histone fractions: *Pseudomonas fluorescens VKMV50* was most sensitive to the lysine-rich histone fraction, whereas *Ps. fluorescens MGU* was sensitive to the action of the arginine-rich histone fractions.

It is not known whether histones have any antibacterial effect *in vivo*. Hirsch (1960) believes that the inhibition of action by salt concentrations only slightly higher than physiological levels and by acidic polymers render the role of histones as antibacterial agents in mammalian tissue unlikely.

Protamines contain high amounts of arginine. Unlike histones, they are resistant to pepsin digestion, but are inactivated by trypsin (Kossel, 1928) and inhibited by acidic polymers (Weissman and Graf, 1947). They are more active against Gram-positive bacteria, with an optimum pH of 8.1 for the activity (Miller *et al.*, 1942).

Another cationic antibacterial compound of protein nature is beta-lysin. It was Pettersson (1924) who termed a serum bactericidal agent effective against Gram-positive bacteria as beta-lysin. Donaldson and Marcus (1958) demonstrated that blood coagulation mechanisms exert a mediating effect on the serum bactericidal activity, and Hirsch (1960b) established that beta-lysin originates from blood platelets. Blood coagulation was shown to be essential for the liberation of beta-lysin from the platelets. Tew *et al.* (1974) demonstrated that thrombin is involved in the release of beta-lysin. Inflammatory exudates are also rich in free beta-lysin, indicating its involvement in infections (Jenson *et al.*, 1967).

Beta-lysin is a cationic protein with a molecular weight of 6 kDa (Johnson and Donaldson, 1968). It is heat-stable, remaining active after incubation at 97°C for 30 minutes. According to Johnson and Donaldson, there are other substances like beta-lysin in serum. One of them, which they called staphylocidal beta-lysin, is very similar to the original beta-lysin: it is heat-stable, of similar molecular weight and can be isolated using almost the same techniques, yet it differs in several other aspects and is not released from platelets.

Further investigation (Carroll and Martinez, 1979) revealed that purified beta-lysin isolated from normal rabbit serum by the classical procedure is a heterogeneous mixture of compounds. It was also demonstrated that the kinetics of action, cellular source and relative concentrations of serum bactericides (beta-lysins) vary between animal species.

The cell membrane is considered to be the primary site of actions of beta-lysin as it combines with purified cell membranes (Gooch and Donaldson, 1974), and also prevents the development of the space between the wall and the plasma membrane which normally occurs in cells suspended in hypertonic sucrose solutions (Matherson and Donaldson, 1968). Beta-lysin was also found to rupture protoplasts (Matherson and Donaldson, 1970).

Another antibacterial defence mechanism present in serum include antibacterial proteins of blood cells. Rabbit granulocytes have been shown to contain microbicidal lysosomal cationic proteins (Zeya and Spitznagel, 1968, 1969, and 1971).

Patterson-Delafield *et al.* (1980) reported the isolation of two highly cationic microbicidal proteins from rabbit alveolar macrophages. They were shown to be distinct from histones and not to arise from granulocyte contamination. Both microbicidal cationic proteins 1 and 2 (MCP-1 and MCP-2) were highly active against diverse Gram-positive and Gram-negative organisms under conditions of near neutral pH and relatively low ionic strength (Lehrer *et al.*, 1983). Molecular characterisation of the peptides revealed them to be small, rich in arginine and cystine, and devoid of carbohydrate or reduced sulphydryl groups (Patterson-Delafiled *et al.*, 1981). Each peptide is composed of 33 amino acid residues. MCP-1 differs from MCP-2 only by containing arginine instead of leucine at the 13th position from the amino terminus (Selsted *et al.*, 1983). Of the various bacterial species studied, only *Bacillus bronchiseptica* appeared to be resistant to MCP-1 and MCP-2. As bronchopulmonary colonisation of rabbits by B. bronchiseptica is extremely common, they suggested that the resistance of this microorganism to MCP-1 and MCP-2 may be an important factor allowing its persistence in the rabbit respiratory tract.

Other studies have shown that *Streptococcus aureus* is not killed by alveolar macrophages unless the bacterial cells are first exposed to alveolar lining material (LaForce *et al.*, 1973). They demonstrated that it is the surfactant-containing fraction of alveolar lining material that enhances intracellular killing of *S. aureus*. Coonrod and Yoneda (1983) also demonstrated that the alveolar lining material from rats caused rapid killing and lysis of Pneumococci. Killing by the surfactant fraction was associated with increased bacterial cell membrane permeability. It was suggested that the surfactant fraction may be a lysophospholipid, palmitoyl lysophosphatidylcholine. It has many similar properties such as antibacterial activity against several Gram-positive bacteria and alters cell membrane permeability. The antibacterial functions of alveolar macrophages has been reviewed by Hocking and Golde (1979).

Many antibacterial cationic proteins have been isolated from polymorphonuclear leukocyte granules. They have dissimilar antibacterial spectra and vary from one species of animal to another, differing in molecular weight, amino acid composition, and enzymic

properties. The human cationic proteins have been reported to range in molecular weight from 10 kDa to 25 kDa (Odeberg and Olsson, 1975).

Purification and characterisation of the bactericidal proteins had been described and compared with a cationic protein isolated under the same conditions from granules of human polymorphonuclar leukocytes (Weiss *et al.*, 1978; Elsbach *et al.*, 1979). These proteins have been named "bactericidal/permeability-increasing proteins" (BPI). The molecular weight of human BPI is 59 kDa and of rabbit BPI is 50 kDa. BPI is larger than any previously described antibacterial protein isolated from neutrophil granules. Both proteins are strongly basic (isoelectric points greater than 9.6) and relatively heat-resistant. The amino acid compositions of the two proteins are very similar.

The antibacterial actions of BPI are highly specific for certain Gram-negative bacterial species. This specificity seems to be closely related to the bacterial envelope structure that determines whether or not, and with what degree of affinity, BPI binds to bacterial surface structures (Elsbach and Weiss, 1981, 1983). Binding is an absolute requirement for the actions of BPI (Weiss *et al.*, 1980), but is not sufficient to generate the antibacterial effects of BPI (Weiss *et al.*, 1983). Apparently, saturation binding is required to trigger "postbinding" steps that are actually responsible for the membrane-active and bactericidal effects of BPI. The hydrophobic interactions with the outer membrane irreversibly trigger the bactericidal actions of BPI. The BPI can be removed from the bacterial surface by 80 mmol/l $MgCl_2$ or by trypsin. This results in repair of the envelope alteration, but viability is irreversibly lost even after only 15 seconds exposure to BPI (Elsbach *et al.*, 1983). This suggested that BPI is the principle bactericidal agent in the intact neutrophil against *Escherichia coli* and *Salmonella typhimurium*.

A new protein with bactericidal activity was discovered from leukemic PMNs. The extract, fractionated on a column of Sephadex G-100, gave a highly active fraction in a region of low protein concentration (Modrzakowski *et al.*, 1979). Further studies of granule extracts from normal human polymorphonuclear leukocytes revealed differences in the chromatographic fractions from Sephadex G-

100 (Modrzakowski *et al.*, 1981). The physiochemical properties of the granule extracts have not yet been described.

The bactericidal capacity of human neutrophil PMNs is shown to depend on oxidative as well as non-oxidative mechanisms. Under certain conditions, non-oxidative (i.e. oxygen-independent) systems may form the principal antimicrobial system available to PMN. For example, human PMNs can mediate killing under strictly anaerobic conditions (Mandell, 1974; Okamura and Spitznagel, 1982).

Two antibacterial proteins with molecular weights of 37 kDa and 57 kDa have been isolated from human PMNs (Shafer *et al.*, 1984; Casey *et al.*, 1985a, 1985b). Shafer *et al.* (1984) also demonstrated that the bacterial cell wall could bind and block the antibacterial activity of the proteins from human PMNs. Furthermore, Shafer *et al.* (1986a) showed that mutation(s) resulting in different lipopolysaccharide subunits resulted in resistant *Neisseria gonorrhoea* strains resistant to the antibacterial proteins of PMNs.

Casey *et al.* (1986) demonstrated that the majority of ingested *N. gonorrhoea* were killed by the antibacterial proteins of human PMNs. Later, Shafer *et al.* (1986b) established that the antibacterial activity of the 37 kDa protein from human PMNs is increased at the acidic pH (pH 5.5 - 6.0) of maturing phagolysosomes.

Shafer *et al.* (1986b) also demonstrated non-enzymic antibacterial activity of an isozyme of cathepsin G. Spitznagel and Shafer (1985) reviewed the cationic proteins that participate in non-oxidative killing of Gram-negative bacteria by human PMNs and suggested that the ability of the cationic proteins to kill susceptible bacteria requires that they bind to the microbial cell surface. Shafer and Morse (1987) further demonstrated that the outer membrane proteins are involved in the binding of antimicrobial protein and that the variation in the level of resistance reflects the degree to which these outer-membrane proteins are exposed.

Hovde and Gray (1986b) isolated and characterised a 55 kDa bactericidal protein from the cytoplasmic granules of normal human PMNs.

Three cationic proteins from rabbit PMNs with molecular weight from 4 kDa to 8 kDa have also been reported (Zeya and Spitznagel, 1968). Extracts of cationic proteins from rabbit PMNs that kill *E. coli* by increasing the permeability of the microbial envelope have been reported by another group of research workers (Berkerdite *et al.*, 1974; Weiss *et al.*, 1975, 1976). Six antibacterial peptides, each with a molecular weight of about 4 kDa have also been isolated from rabbit peritonial granulocytes (Selsted *et al.*, 1984).

Hodinka and Modrzakowski (1983) separated extracts of rat PMNs granules by column chromatography on Sephadex G-100 into three major peaks. The peak of the lowest molecular weight (Peak C) contained lysozyme and two components more cationic than lysozyme on cationic electrophoresis. The Peak C fraction was heat-stable, maintaining bactericidal activity after boiling for 30 minutes. This is consistent with the findings of Walton (1978) and Odeberg and Olsson (1975) who reported the existence of heat-stable cationic proteins from rabbit and human polymorphonuclear leukocytes. Treatment with trypsin substantially abolished the antibacterial activity, suggesting the component(s) are of a protein nature.

The importance of cationic proteins may vary between animal species but nonetheless they have an important function. The weight of evidence suggests that cationic proteins of different kinds play a role in the antibacterial activities of polymorphonuclear leukocytes (Spitznagel, 1980).

A number of antibacterial cationic proteins have also been isolated from bovine teat canal keratin by acid extraction of the keratin (Hibbitt and Cole, 1968; Hibbitt *et al.*, 1969; Hibbitt, 1970). After further purification, electrophoresis resolved the isolated proteins into six principal bands at pH 3.0. The cationic proteins inhibited the growth of mastitis-causing strains of *staphylococci* and *streptococci*, causing marked changes in the cell wall and plasma membrane. Similar changes were also seen in *staphylococci* recovered

from the teat canal of a healthy cow, and in the presence of calf thymus histones (Macmillan and Hibbitt, 1969).

Cationic proteins with antimicrobial activity have been isolated from the cells in bulk milk samples (Hibbitt *et al.*, 1971). Polyacrylamide gel electrophoresis studies showed at least nine components with isoelectric points between 7 and 9 and one component with an isoelectric point above 9. The antimicrobial activity was not destroyed after heating at temperatures up to 70°C for 30 minutes, whereas at higher temperatures the activity diminished and was almost completely lost at 100°C. The cationic proteins isolated from milk cells had a greater antimicrobial activity than similarly charged proteins isolated from teat canal keratin.

An antimicrobial extract has also been obtained from normal bovine whey. This heat-stable, dialysable fraction was capable of killing several species of Mycoplasmas (Brownlie *et al.*, 1974; Howard *et al.*, 1975). It was distinguished from other bovine antibacterial agents such as lactenin (Wilson and Rosenblum, 1952), the cationic protein fraction from bovine teat canal keratin (Hibbitt *et al.*, 1969), the basic fraction present in cervical mucus (Brownlie and Hibbitt, 1972), and lactoferrin on the basis of dialysability and heat stability. However, the lack of further characterisation makes comparison with other antimicrobial substances difficult.

Bloom and coworkers obtained anthracidal peptides from the thymus, thyroid, pancreas, spleen and caecum of different animal species (Bloom *et al.*, 1947, 1953; Bloom and Blake, 1948; Bloom and Prigmore, 1952). Verdos *et al.* (1966) isolated a bacterial inhibitor from human throat washings and found it to be of low molecular weight (5 kDa or less), relatively heat-stable and with cationic properties. Ellison *et al.* (1985) also isolated an antibacterial peptide from human lung lavage fluid. Antibacterial polypeptides present as complexes have also been isolated (Timoney and Trachman, 1985).

Shackell (1980) developed a method of extraction on a larger scale for isolation of the antibacterial factor from bovine rumen. The

molecular weight of the cationic antibacterial peptide estimated on thin layer gel filtration chromatography was found to be from 2 kDa to 3.2 kDa depending on the eluent used. The antibacterial peptide was found to be heat-stable (1 hour at 100°C), sensitive to trypsin and chymotrypsin and inactive in the presence of 0.7 mol/l NaCl. Sahl (1985) has reviewed the bactericidal cationic peptides involved in host defence mechanism.

A variety of biological functions has been ascribed to the action of cationic proteins and peptides. They cause mast cell degranulation and release of histamine as reported by Seegers and Janoff (1966), and increase vascular permeability directly, and indirectly as a result of histamine release (Ranadive and Cochrane, 1966). They have also been shown to possess anticoagulation activity (Saba *et al.*, 1967) and pyrogenicity (Herion *et al.*, 1966). They can induce inflammatory changes (Janoff and Zweifach, 1964; Golub and Spitznagel, 1965) and neutralise some inhibitors of the complement system (Baker *et al.*, 1976). Pruzanski and Saito (1978) reported the enhancement of phagocytosis by several cationic proteins.

The bactericidal action of cationic proteins and peptides has been attributed to the following: combination of the proteins with acidic components on the cell surface and the cytoplasmic membrane (Burger and Stahlmann, 1952; Spitznagel, 1961; MacMillan and Hibbitt, 1973; Gooch and Donaldson, 1974; Elsbach and Weiss, 1981, 1983); combination with anionic sites, such as phosphate groups of nucleic acids, inside the cell (Sugimura and Ono, 1956); detergent action (Few and Schulman, 1953; Spitznagel, 1961); interference with cations (Newton, 1954); inhibition of respiration (Gladstone *et al.*, 1974; Walton and Gladstone, 1976; Walton, 1978); inhibition of energy-dependent membrane transport (Odeberg and Olsson, 1976; Olsson *et al.*, 1977); inhibition of r-RNA transcription and of the enzyme RNA polymerase (Reddy and Bhargava, 1979; Scheit *et al.*, 1979, 1985, 1986; Scheit and Zimmer, 1984; Scheit and Bhargava, 1985; incorporation of the protein into lipid bilayer membranes (Galla and Warncke, 1985)

There are various antibacterial substances in seminal plasma which have been identified in other mammalian tissues and secretions. As well as the amines spermine and spermidine there are the proteins,

lysozyme, complement, seminalplasmin, and the prostate antibacterial fraction. The presence of lysozyme in bovine seminal plasma has been demonstrated (Eschenbruch, 1980). However, the presence of lysozyme in the secretions of the human male reproductive tract has not been convincingly demonstrated (Shivaji, 1984).

In experiments with human semen it was reported that the antibacterial activity present in the system was due to spermine and spermidine (Rozansky *et al.*, 1949; Gurevitch *et al.*, 1951; Razin and Rozansky, 1957). The antimicrobial role of spermine *in vivo* was viewed by Hirsch (1960a) as speculative since spermine in tissues may be firmly bound or its action on microorganisms may be influenced by the presence or absence of other material in the body. Tabor *et al.* (1961) also showed that an alkaline medium is needed for optimum activity. Fair and Wehner (1971) studied the effects of spermine against a variety of microorganisms in an effort to determine the role of spermine in human male. They demonstrated that spermine would have little, if any, effect against the majority of organisms normally responsible for urinary tract infections, at the normal acidic pH of prostatic secretions. Rusk *et al.* (1973) did not attempt to characterise the antibacterial factor of human semen. However, they suggested that it is of protein nature, as, after electrophoresis, semen samples without activity were lacking one protein band when compared with the active samples. The antibacterial spectrum of human prostatic fluid has also been established (Gupta *et al.*, 1967).

A fraction possessing antibacterial activity against Gram-negative and Gram-positive bacteria was isolated from canine prostatic fluid by Stamey *et al.* (1968). On gel filtration of the prostatic fluid on Sephadex G-10, the proteins of high molecular weight were eluted in a single peak that had no antibacterial activity. Another peak of proteins, named the prostatic antibacterial fraction (PAF), was eluted after the large peak. PAF had no lysozymal activity and was unrelated to spermine. Fair *et al.* (1973, 1976) further studied the antibacterial factor of human and canine prostatic fluid. They determined its molecular weight to be 1.5 kDa or below, and also showed it to be a potent bactericidal cationic substance which is heat-stable and water-soluble. They also found that 90 % of the organisms

responsible for urinary tract infection were sensitive to this antibacterial agent. In further work, the prostatic antibacterial factor (PAF) responsible for the activity of normal prostatic fluid was identified as free zinc (Fair *et al.*, 1976; Fair and Parrish, 1981). They suggested that the bactericidal activity of the prostatic secretions is related to the amount of zinc present in the fluid and may play a role in the natural resistance of the male urinary tract to infections.

An antibacterial agent isolated from bovine seminal plasma was seminalplasmin (Reddy and Bhargava, 1979). It was heat-stable, had an isoelectric point of 9.8 and a molecular weight of between 8 kDa and 19.8 kDa depending on the method used. Seminalplasmin was found to have potent antibacterial activity against a variety of Gram-positive and Gram-negative bacteria. They also showed that the microbicidal action of seminalplasmin was due to the inhibition of RNA synthesis. Protein synthesis in *E. coli* started to decline linearly 10 minutes after incubation with seminalplasmin. They showed that r-RNA and t-RNA were inhibited 85 % and 10 % respectively.

Scheit *et al.* (1979) extended the investigations and found that seminalplasmin was also a potent inhibitor of RNA polymerase of *E. coli*. Later, a highly purified seminalplasmin with a molecular weight of 6.3 kDa - 6.5 kDa was isolated and its amino acid sequence determined (Theil and Scheit, 1983a, 1983b). Reddy *et al.* (1983) also showed seminalplasmin to be a potent inhibitor of several reverse transcriptases, purified or in viral lysates. Further investigations on seminalplasmin showed that it inhibits gene expression in *E. coli*, and *in vitro* RNA synthesis in *Candida albicans* and *Saccharomyces cerevisiae* (Scheit and Zimmer, 1984; Scheit and Bhargava, 1985; Scheit *et al.*, 1985). Seminalplasmin was also shown to be incorporated into bacterial lipid bilayer membranes (Galla and Warncke, 1985). Very recently, Scheit *et al.* (1986) have shown that seminalplasmin inhibits both RNA synthesis as well as growth in a variety of bacterial species.

Rao and Bhargava (1985) isolated a weakly acidic protein, antiseminalplasmin with a minimum molecular weight of about 39 kDa, which inhibited the antibacterial activity of seminalplasmin.

Furthermore, Scheit (1986), when investigating the basic proteins from bull seminal vesicle secretion, identified an antimicrobial protein with a molecular weight (about 6 kDa) and amino acid composition similar to seminalplasmin.

Shannon *et al.* (1974, 1975) have also studied the antibacterial activity of bovine seminal plasma. The activity was heat-stable and dialysable at pH 3.0 but not at pH 7.0. Consequently, the material was described as being heterogeneous, existing in various forms ranging from a monomer to polymers of large molecular weight. Shannon *et al.* (1975) suggested that the association of the peptide with larger proteins may be advantageous, and would perhaps be less susceptible to inhibition by anionic substances. Preliminary characterisation by Schollum *et al.* (1977) of the antibacterial factor in bovine seminal plasma confirmed the heat stability, and showed it to be destroyed by some proteolytic enzymes, thus indicating protein nature of the antibacterial factor.

Shannon *et al.* (1975) also found antibacterial activity in cell-free extracts of pancreas, spleen, liver and lungs by dialysis at pH 3.0. Bactericidal activity was also obtained from bovine kidney, saliva, intestinal mucosa, leukocytes and teat canal epithelium dialysed at pH 3.0. All diffusates were found to have a major component with the same electrophoretic mobility as the peptide from seminal plasma. The substance was shown by gel filtration chromatography at pH 1.7 or pH 12.0 to have a molecular weight of 3.5 kDa.

Eschenbruch (1980) also studied antibacterial extracts from a number of bovine tissues and secretions. One of these secretions, bovine seminal plasma, was found to contain an antibacterial peptide with a molecular weight of about 2.2 kDa. She also found different high molecular weight antibacterial proteins, which were thought to be the aggregates of the antibacterial peptide. Shannon *et al.* (1987) continued the investigations on the antibacterial proteins of bovine seminal plasma. They too suggested that small antibacterial component(s) appeared to be present in aggregated forms in bovine seminal plasma.

The investigation described in this thesis was undertaken to examine more closely the antibacterial proteins found by Eschenbruch (1980) and Shannon *et al.* (1987), and find if they were the antibacterial aggregates and if so then whether all the aggregates were made up of the same small component(s). Other main aims of this investigation were to identify and isolate the antibacterial aggregates in bovine seminal plasma, to develop a reliable and reproducible method for the complete disaggregation of the aggregates and to isolate and characterise the component(s) of the antibacterial aggregates using that method. Other aims of this investigation were to determine the roles of different component(s) in the formation of different high molecular weight antibacterial aggregates if more than one component were present and to find out what types of interactions were involved in the assembly of the antibacterial aggregates.

CHAPTER TWO

MATERIALS AND METHODS

Bovine Seminal Plasma

Bovine seminal plasma was kindly supplied by the NZ Dairy Board Artificial Breeding Centre, Newstead. Freshly collected samples from a number of vasectomized bulls were collected by means of an artificial vagina, pooled and stored frozen at -20°C.

Buffers

Tris, phosphate and citrate buffers were prepared by the methods given by Plummer (1971). Specific buffer concentrations and pH values are described in the individual experiments in the following chapter.

Cationic Electrophoresis

The method for cationic polyacrylamide electrophoresis was that of Reisfeld *et al* (1962). Some modifications are described below.

Gel slabs were used instead of tubes. Gels were prepared using the apparatus supplied with the LKB 2001 vertical electrophoresis unit. Two chromic acid-cleaned glass plates (16 X 18 cm) were separated by two 1.5 mm thick spacers, and clamped together. This was then clamped in the gel casting stand which sealed the bottom edge. After polymerisation the glass plates were removed from the casting stand and clamped in the LKB vertical electrophoresis unit.

Two polyacrylamide gel zones were used : Zone 1, a 15 % acrylamide small-pore gel in which electrophoretic separation is accomplished, Zone 2, a 3.3 % acrylamide large-pore gel in which electrophoretic concentration takes place.

The unpolymerised gel mixture, Zone 1, was pipetted into the template to the required level and overlaid with distilled water. After Zone 1 had polymerised, the remaining surface liquid was washed

off with a small quantity of unpolymerised Zone 2 gel and the mould filled with this mixture. After polymerisation, the unpolymerised surface of the Zone 2 gel was washed with tray buffer.

Stock solutions were stored in brown glass bottles at 4°C for several weeks. With most of the commercially available acrylamide it was found impossible to prepare a solution of 60 % (Solution A of Reisfeld *et al*, 1962), therefore stock Solution A (60 % acrylamide) was replaced with 30 % acrylamide. Thus, in mixtures twice the volume specified by Reisfeld *et al* (1962) for Solution A was required. The extra volume was added in place of water in the given volumes.

Samples were mixed with a drop of glycerol and applied to wells on the top of the large pore gel (generated by insertion of a Gradipore plastic applicator strip). The samples were applied to wells formed by insertion of a comb during the polymerisation of the large pore gel. Methyl green was used as a tracking dye.

Electrophoresis was carried out in a LKB 2001 vertical electrophoresis unit at a constant current of 40 mA for approximately 2 hours.

Electrophoresis was also carried out at pH 6.8 (Thomas and Hodes, 1981) using a method similar to the one described above.

Cellulose acetate electrophoresis

The samples were applied by an 8 sample applicator (Cat.No.4084) supplied by Helena Laboratories and cellulose acetate electrophoresis was carried out in a locally manufactured apparatus for 10-20 minutes at 200 volts using pre-coated cellulose acetate plates with a size of 60 x 76 mm (Titan 111, Cat.No.3023) supplied by Helena Laboratories. The samples were stained for 20 minutes with 0.05% Ponceau S. in 10% aqueous solution of salphosalicylic acid and destaining was done by repeated washings with 5% acetic acid.

Dialysis

Dialysis was carried out at 4°C using Spectra/Por 3 dialysis tubing with a molecular weight cut-off of 3.5 KDa supplied by Spectrum Medical Industries.

FPLC

Fast protein liquid chromatography (FPLC) was performed on a 10 x 300 mm analytical column of Superose 12 (Pharmacia) at a constant flow rate of 0.7 ml/minute at room temperature.

Freeze-drying

Samples were freeze-dried under vacuum using Virtis apparatus (the Virtis Company, Inc.).

Gel Filtration Chromatography

Gel filtration chromatography was carried out using "Sephadex" (Pharmacia) cross-linked dextrans of various pore sizes at 4°C. Columns of Sephadex were prepared and used according to the instructions given in the booklet "Sephadex Gel Filtration in Theory and Practice", published by Pharmacia. Pharmacia K 26 Chromatographic tubes with an internal diameter of 2.6 cm and lengths varying from 20 to 50 cm were used. Void volumes (Vo) and bed volumes (Vt) are marked in figures showing the elution profiles. The void volume was determined using a marker of high molecular weight such as blue dextran. Columns were eluted by upward flow using a peristaltic pump, the flow rates being 80 ml/h for Sephadex G-10, Sephadex G-15, Sephadex G-25SF and the Sephadex G-25SF-G-10 mixture column (two gels mixed in equal proportions), 60 ml/h for Sephadex G-50SF, 40 ml/h for Sephadex G-100SF and 20 ml/h for Sephadex G-200F. The composition of elution buffers and eluents is described with the individual experiments in the following chapter.

The absorbance of the eluent was usually monitored at a wavelength of 220 nm using a Cecil 272 Spectrophotometer (with a 1 mm flow-cell) and recorded on an Omniscribe chart recorder. Fractions were collected with an automatic fraction collector (LKB Bromma 2111 Multirac Fraction

Collector). The fractions were marked on the chart by means of an event marker connected to the fraction collector.

Sometimes concentrated samples with high antibacterial activity were loaded on to the columns so that the activity could easily be picked up on subsequent chromatography. In these cases, to keep the peaks within the scale, fractions were monitored at different wavelengths, away from 220 nm, depending on the concentration of the samples.

Gel Stains

Initially, two different staining methods were used in cationic electrophoresis to stain the protein samples of bovine seminal plasma.

(1) Staining using Coomassie Brilliant Blue R

The following solution was used :

Coomassie Brilliant Blue R 1.37 g Methanol 250 ml Glacial Acetic Acid 50 ml Distilled Water 250 ml.

Gels were stained for approximately 2 hours followed by destaining in 35 % ethanol until a clear background was attained.

(2) Staining using Amido Black 10B

The following solution was used:

Amido Black 10B 1 g Glacial Acetic Acid 70 ml Distilled Water 930 ml.

The staining solution was heated to near boiling immediately before use (Ritchie *et al.*, 1966). After staining for approximately 5 hours, gels were destained in a 7 % acetic acid solution.

The Amido Black method was found to be a better method for the staining of the proteins of seminal plasma, therefore the Amido Black staining method was subsequently used for the staining.

HPLC

High-pressure liquid chromatography (HPLC) was carried out with an analytical Brownlee RP C-18 reversed phase column (particle size, 5 μm spherical silica based) at room temperature. The column dimension was 4.6 mm ID X 22 mm. Columns were eluted by a high pressure pump (Waters M-45), and a Waters Associates Model 660 Solvent Programmer was used to establish solvent gradients using gradient curve 6 or 7. The gradient curves are described in the individual experiments. The absorbance of the eluent was continuously monitored at a wavelength of 220 nm using Millipore Waters Lamda-Max LC Spectrophotometer Model 481 and recorded on a Sekonic Chart recorder SS.250F. Fractions were collected with an automatic fraction collector (FRAC-100 Pharmacia Fine Chemicals).

The following solvent system was employed in the HPLC : Solvent A, 0.1 % trifluoroacetic acid; Solvent B, 80 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid. (Flow rate 1 ml/min)

Ion Exchange Chromatography

Ion exchange chromatography was performed at 4°C using Pharmacia chromatographic tubes with an inner diameter of 2.6 cm and lengths varying from 20 cm to 40 cm. The ion exchange gels was packed into the columns giving approximate bed volumes of 60-80 ml. For cation exchange chromatography, SP-Sephadex C-50, with Sulphopropyl functional groups was used. For anion exchange chromatography, QAE-Sephadex A-25 containing diethyl-(2-hydroxypropyl)aminoethyl groups, was used. Buffers, eluents and conditions used for eluting the columns are described with the experiments in the following chapter. The eluted fractions were monitored at a wavelength away from 220 nm in order to keep the peaks within the scale.

Rotary Evaporation

Samples were evaporated by rotary evaporation under vacuum using a Buchi Rotovapor, at a temperature of 35° to 40° C.

The Antibacterial Assays

Routine testing was performed against *Escherichia coli* K_{12} and in some cases against *Staphylococcus aureus*. The bacteria were grown for 18 hours in 10 ml of Nutrient Broth (Oxoid No. 2). Out of that 100 µl was then added to 100 ml of sterile nutrient broth containing 1 % agarose (Sigma Chemical Company) at a temperature of about 45°C. Plates were poured immediately in a Laminar Flow Cabinet. The plates were stored for up to 3 weeks at 4°C. Wells, 8 mm in diameter, were punched in the plates. The wells were numbered and filled with the appropriate test samples. The bacterial plates were then incubated at 37°C for about 18 hours. The extent of the zone of bacterial growth inhibition of neutralised fractions was measured in millimeters with a ruler. Data was expressed as the distance between the edge of the well and the edge of the clearing. Antibacterial assays were done in triplicate and the means were calculated.

When testing samples for lysozyme activity, 1 % agarose plates containing 0.02 % dried *Micrococcus lysodeikticus* cells (Sigma Chemical Company) were used as described by Gosnell *et al.* (1975). The plates were incubated at 37°C for 18 hours after which time a bacterial growth had covered the plates. The plates were stored at 4°C for no longer than 2 weeks. Lytic activity was tested by the well diffusion technique as described above. After incubation for 12 - 18 hours, the size of the clear zones, formed by the lytic action of lysozyme, gave an indication of the concentration of lysozyme in the sample.

CHAPTER THREE

EXPERIMENTAL

Introduction

Shannon *et al.* (1987) demonstrated that low molecular weight antibacterial compound(s) of bovine seminal plasma, which passed through 10 kDa cut-off dialysing membrane at pH 7.0 citrate and at pH 3.0, later re-aggregated to the molecular weight of 30 kDa or over at neutral pH. This antibacterial activity was reported to be due to a basic peptide of about 3.5 kDa (Shannon *et al.*, 1974, 1975). However, another antibacterial agent, also basic in nature, isolated from bovine seminal plasma is seminalplasmin and was reported to have a molecular weight between 8 kDa - 19.8 kDa depending on the method used (Reddy and Bhargava, 1979).

Later, Theil and Scheit (1983) isolated a highly purified seminalplasmin and its molecular weight was estimated to be 6.3 kDa by ultracentrifugation and 6.5 kDa from amino acid analysis. As 10 kDa cut-off dialysing membrane would have allowed the diffusion of both seminalplasmin as well as the antibacterial peptide, therefore to find out whether the antibacterial peptide is responsible for the aggregation, it was decided to do some acid dialysis of seminal plasma in 3.5 kDa cut-off dialysing membrane.

It was thought since seminalplasmin is too big to pass through the 3.5 kDa cut-off dialysing membrane and only the antibacterial peptide can pass across the 3.5 kDa cut-off dialysing membrane, therefore information could be obtained which of these is responsible for aggregation. Also, as Shannon *et al.* (1987), when studying re-aggregation, even though most of the acid diffusate had re-aggregated up to void volume on Sephadex G-50SF, did not run the sample beyond Sephadex G-50SF gel. Thus, it was not known to what extent re-aggregation occurred

Therefore, when studying re-aggregation, it was decided to run acid diffusates onto successively more porous Sephadex gels at pH 7.0, until re-aggregates eluted within the separating range of a gel. It

was hoped that this would give an idea about the molecular weights of different aggregates, and later would help to identify different antibacterial aggregates of high molecular weight when whole seminal plasma was applied onto an appropriate Sephadex gel.

Acid Dialysis and Re-aggregation of Acid Diffusates

Although the pH of bovine seminal plasma is known to be 7.4, it was found to be about 6.5 for stored seminal plasma, probably owing to the high amount of lactic acid secreted from sperm. The pH of about 200 ml bovine seminal plasma was adjusted to 7.0 with NaOH, and in order to get rid of small molecules which have nothing to do with antibacterial activity, was dialysed in 3.5 kDa cut-off dialysing membrane at 4°C against equal amount of distilled water. The diffusate was collected after 2 days and then the dialysis was stopped. Dialysis against distilled water was done for a short duration to avoid any excessive loss of salts which could have resulted in the denaturation and/or precipitation of the proteins.

The pH of the seminal plasma was then adjusted to 3.0 with HCl, and was subsequently dialysed in 3.5 kDa cut-off dialysing membrane at 4°C against equal amount of HCl-water, pH 3.0. The pH of 3.0 was chosen for acid dialysis because maximum activity is shown to pass through the dialysing membrane at pH 3.0 (Shannon *et al.*, 1987). Again, the diffusates were collected after every 2 days, stored at -20°C, and then the diffusates changed for a fresh solution. The pH of all the diffusates was adjusted to 7.0 with NaOH, and each was concentrated down to about 5 ml by rotary evaporation and tested for antibacterial activity against *Escherichia coli*. As can be seen in Table 3.1, the activity diffused slowly across the membrane at pH 3.0 and did not diffuse at pH 7.0.

Table 3.1: Diffusion of antibacterial activity of seminal plasma on dialysis

Diffusate No.	pH of Dialysis	Dialysed Against	Antibacterial Activity
1	7.0	distilled water	0 mm
2	3.0	HCl-water, pH 3.0	3.5 mm
3	3.0	HCl-water, pH 3.0	3.0 mm
4	3.0	HCl-water, pH 3.0	3.0 mm
5	3.0	HCl-water, pH 3.0	2.5 mm
6	3.0	HCl-water, pH 3.0	2.5 mm
7	3.0	HCl-water, pH 3.0	3.0 mm
8	3.0	HCl-water, pH 3.0	2.5 mm
9	3.0	HCl-water, pH 3.0	2.0 mm
10	3.0	HCl-water, pH 3.0	2.5 mm
11	3.0	HCl-water, pH 3.0	2.0 mm
12	3.0	HCl-water, pH 3.0	2.5 mm
13	3.0	HCl-water, pH 3.0	1.5 mm
14	3.0	HCl-water, pH 3.0	1.0 mm
15	3.0	HCl-water, pH 3.0	1.0 mm
16	3.0	HCl-water, pH 3.0	0 mm

The acid diffusates are reported to re-aggregate at pH 7.0 (Shannon *et al.*, 1987). Therefore, to investigate the sizes of different antibacterial aggregates it was decided to run the acid diffusates on different Sephadex gels at pH 7.0. In order to study re-aggregation, gel filtration was done at pH 7.0 because it is a near physiological pH of bovine seminal plasma and the antibacterial activity was tested at pH 7.0. Thus, to find whether the pH 7.0 diffusate has component(s) required for aggregation, a concentrated sample of 5 ml of the pH 7.0 diffusate was applied onto Sephadex G-50SF equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. All the samples eluted on the bed volume indicating it had a molecular weight of 1.5 kDa or lower. Therefore, aggregating component(s) did not seem to dialyse at neutral pH across the 3.5 kDa cut-off dialysing membrane.

Next, the acid diffusates were investigated. When pH of first change of the acid diffusate was adjusted to 7.0 with NaOH, precipitate formed at about pH 4.5. The precipitate was removed by centrifugation at 4,000 g (r av.10 cm) for 10 minutes and a part of the precipitate was re-dissolved in 0.1 mol/l Tris-HCl buffer, pH 7.0. The pH of the supernatant was readjusted to 7.0 with NaOH. Both the supernatant and the precipitate were tested for antibacterial activity (against *E. coli*) and were found active. A 5 ml sample of the supernatant of the first acid diffusate was incubated at 37°C for 2 hours, and then chromatographed on a column of Sephadex G-50SF, equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. The run was repeated twice, identical results were obtained and a typical result is shown in Fig. 3.1.

As can be seen in Fig. 3.1, there was a major active void volume peak indicating it had a molecular weight of 30 kDa or over. When a part of the precipitate of the acid diffusate formed at about pH 4.5 was dissolved in 5 ml of 0.1 mol/l Tris-HCl buffer, pH 7.0, held at 4°C for 2 h and then chromatographed on Sephadex G-50SF at pH 7.0, an identical elution and activity profile to that shown in Fig. 3.1 was obtained, indicating the similar composition of the supernatant and the precipitate. Therefore, further work was done on the supernatant. The column of Sephadex G-50SF was calibrated by running standards of Carbonic anhydrase (molecular weight 29 kDa), Cytochrome C (molecular weight 12.9 kDa), Trypsin Inhibitor (molecular weight 6.5 kDa) and Insulin B chain (molecular weight 3.495 kDa). The estimated molecular weights given to three active peaks were 30 kDa or over (void volume peak), 12 ($\pm$ 1.2) kDa (middle peak) and 1.5 kDa or lower (bed volume peak).

It was thought that long exposure of 3.5 kDa cut-off dialysing membrane to low temperature and/or acidic condition could have altered the properties of the membrane and the activity on the void volume shown in Fig. 3.1 could be because of large molecules which passed through the membrane rather than the aggregates formed from small molecules. An experiment was undertaken to investigate this possibility. A 1 mg standard of egg-white lysozyme (molecular weight 14 kDa) was dissolved in 50 ml HCl-water, pH 3.0, put in 3.5 kDa cut-off dialysing membrane and dialysed for 4 days at 4°C against equal

amount of HCl-water, pH 3.0. After that time the diffusate was concentrated down to 5 ml by rotary evaporation and chromatographed on Sephadex G-50SF column equilibrated with HCl-water, pH 3.0. No lysozyme peak was detected. By running a control of lysozyme standard on the same column it was calculated that even if 1/500 of the standard in the dailysis membrane had passed into the diffusate it would have been easily picked up on the monitoring conditions used. Therefore, it was concluded that the active void volume peak shown in Fig. 3.1 was actually an aggregate rather than a large molecule passed into the diffusate owing to any physical alteration to the membrane.

It was also feared that storing the diffusates at -20°C could have caused aggregation of the sample during freezing. Therefore, to check this possibility a second batch of diffusate was loaded onto Sephadex G-50SF at pH 7.0 as soon as it was collected (without storing it in freezer) and an identical elution and activity pattern to that shown in Fig. 3.1 was obtained. Therefore, it was concluded that the activity on void volume peak shown in Fig. 3.1 was due to a spontaneous aggregation rather than any physical aggregation due to storage in the freezer.

Most of the sample was eluted on the void volume, meaning it had a molecular weight of 30 kDa or over. It was considered that the active void volume of Fig. 3.1 could be a small molecular weight insoluble active molecule(s) eluting on void volume as a colloidal suspension rather than a high molecular weight aggregate(s).

To investigate this, a part of the acid diffusate was centrifuged at 6,000 g (r av.10 cm) for 20 minutes and only very clear material from the top of centrifuge tube was applied onto a Sephadex G-50SF column at pH 7.0, and an identical elution and activity pattern to that shown in Fig. 3.1 was obtained. Therefore, the void volume peak shown in Fig. 3.1 was concluded to be a high molecular weight material rather than a small molecular weight insoluble material. The activity had earlier passed across the 3.5 kDa cut-off dialysing membrane but was now present as a void volume peak on Sephadex G-50SF (molecular weight 30 kDa or over), thus indicating aggregation of the activity at neutral pH.

Several acid diffusates were separately run on Sephadex G-50SF at pH 7.0, and the later diffusates gave a similar elution and activity pattern to that shown in Fig. 3.1. Therefore, the same antibacterial compound(s) seemed to dialyse in all the diffusates.

The activity had re-aggregated up to the void volume on Sephadex G-50SF, meaning it had a molecular weight of 30 kDa or over. Therefore, to have a better idea about the degree of aggregation, the void volume peak (fractions 14 - 20) of Fig. 3.1 was concentrated down to 5 ml by rotary evaporation and chromatographed through a column of Sephadex G-100SF equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. The results are shown in Fig. 3.2.

Most of the re-aggregated material eluted on the void volume on Sephadex G-100SF, indicating a molecular weight of 100 kDa or over (Fig. 3.2), therefore in order to find out the actual molecular weight of the aggregate(s), the void volume peak (fraction 14 - 20) shown in Fig. 3.2 was concentrated down to 5 ml by rotary evaporation, and was chromatographed on a column of Sephadex G-200F at pH 7.0. Sephadex G-200F gel was preferred over Sephadex G-200SF because Sephadex G-200F, with a fractionation range of 5 kDa - 600 kDa, has a wider fractionation range than Sephadex G-200SF (fractionation range 5 kDa - 250 kDa), also it could be run at a faster flow rate (maximum flow rate of Sephadex G-200F is 1.0 ml/min. compared with that of 0.25 ml/min. for Sephadex G-200SF). As can be seen in Fig. 3.3 the sample was eluted within the separating range of Sephadex G-200F.

The column of Sephadex G-200F was calibrated by running Urease tetramer (molecular weight 480 kDa), Urease dimer (molecular weight 240 kDa), Alcohol dehydrogenase (molecular weight 150 kDa), Bovine serum albumin (molecular weight 66 kDa) and Carbonic anhydrase (molecular weight 29 kDa) as standards. The molecular weights estimated from the calibration curve for the peaks were 500 kDa for the peak eluted in fractions 16 - 22 (Fig. 3.3), 250 kDa for the peak eluted in fractions 23 - 28 (Fig. 3.3), and 20 kDa for the peak eluted in fraction 29 - 41 (Fig. 3.3).

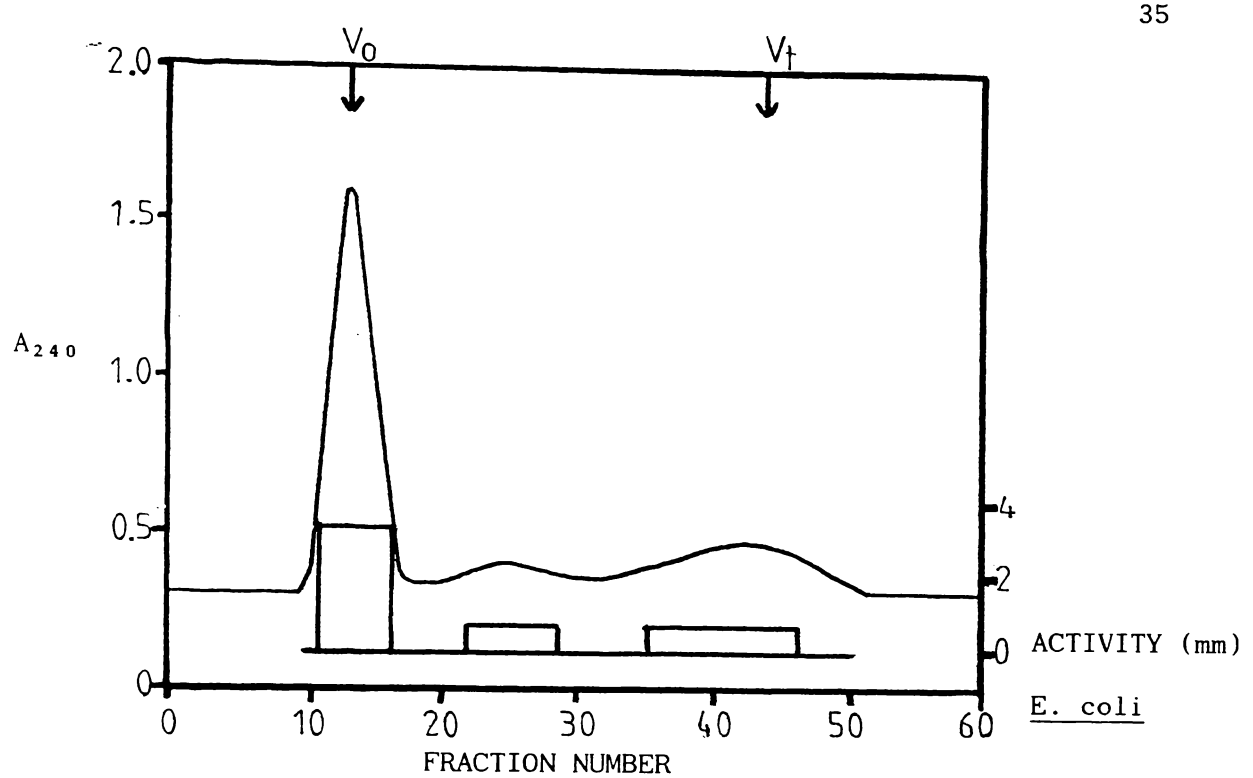


Fig. 3.1: Gel Filtration Chromatography of First Acid Diffusate
Gel: Sephadex G-50SF
Buffer: 0.1 mol/l Tris-HCl, pH 7.0
Sample: Concentrate of first acid diffusate (5 ml)
Fraction Volume: 4 ml

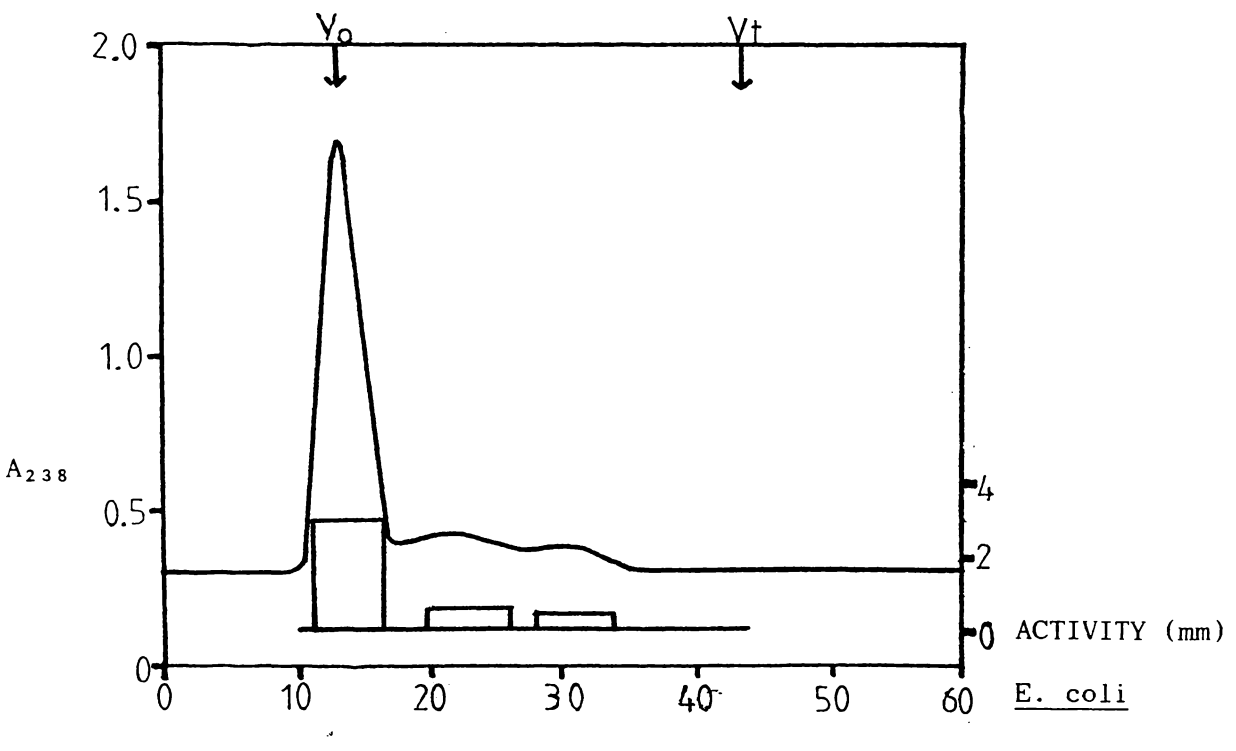


Fig. 3.2: Gel Filtration Chromatography of Fractions 14-20 from Fig. 3.1
Gel: Sephadex G-100SF
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Concentrate of fractions 14-20 from Fig. 3.1 (5 ml)
Fraction Volume: 4 ml

Disaggregation of the Acid Diffusates

Antibacterial activity had dialysed at pH 3.0 across the 3.5 kDa cut-off dialysing membrane, thus indicating disaggregation at acidic pH. Therefore, in order to study the components, gel filtration at acidic pH was tried as a disaggregation technique. The pH of a concentrated sample of acid diffusate was adjusted to 3.0 with HCl, the sample was held at 4°C for 2 h and then chromatographed on Sephadex G-50SF eluted with HCl-water, pH 3.0. This was repeated twice. A typical elution profile is shown in Fig. 3.4.

As can be seen in Fig. 3.4, the activity was spread over the wide range of molecular weight, ranging from the void volume peak (molecular weight of 30 kDa or over) to the bed volume peak (molecular weight of 1.5 kDa or lower). Complete disaggregation of the acid diffusate was not achieved by gel filtration at pH 3.0, and it was thought that some strong acidic group(s) may be involved in the assembly of aggregates through ionic interactions which may not be suppressed at pH 3.0.

Therefore, to check this possibility, the pH of some acid diffusate of seminal plasma was adjusted to 1.7 with HCl, the sample was held at 4°C for 2 h and then chromatographed on to Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. Almost all the activity eluted on the void volume, meaning it had a molecular weight of 5 kDa or over. The activity had earlier passed through 3.5 kDa cut-off dialysing membrane, therefore the component(s) responsible for the formation of aggregates should be of size of less than 3.5 kDa. Thus, acidic gel filtration, even at pH 1.7, could not cause complete disaggregation of the aggregates for study of the component(s).

Conclusions

Antibacterial activity did not diffuse across 3.5 kDa cut-off dialysing membrane at pH 7.0 nor did the pH 7.0 diffusate form aggregates, but at pH 3.0 activity slowly passed through the membrane. The disaggregated material that diffused out formed antibacterial peaks of up to 500 kDa at pH 7.0, thus indicating that the antibacterial component(s) of less than 3.5 kDa molecular weight(as the activity had earlier dialysed across 3.5 kDa cut-off dialysing membrane) can form antibacterial aggregates of up to 500 kDa molecular weight.

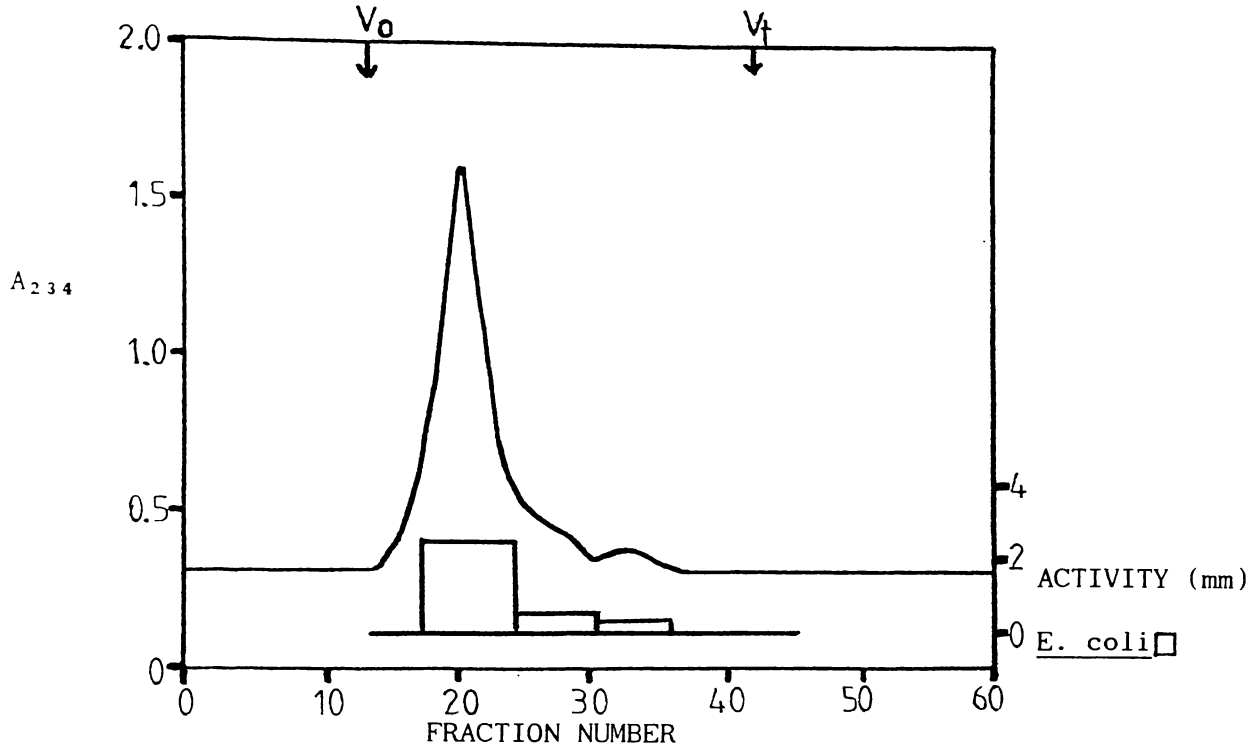


Fig. 3.3: Gel Filtration Chromatography of Fractions 14-20 from Fig. 3.2
Gel: Sephadex G-100SF
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Concentrate of fractions 14-20 from Fig.3.2 (5 ml)
Fraction Volume: 4 ml

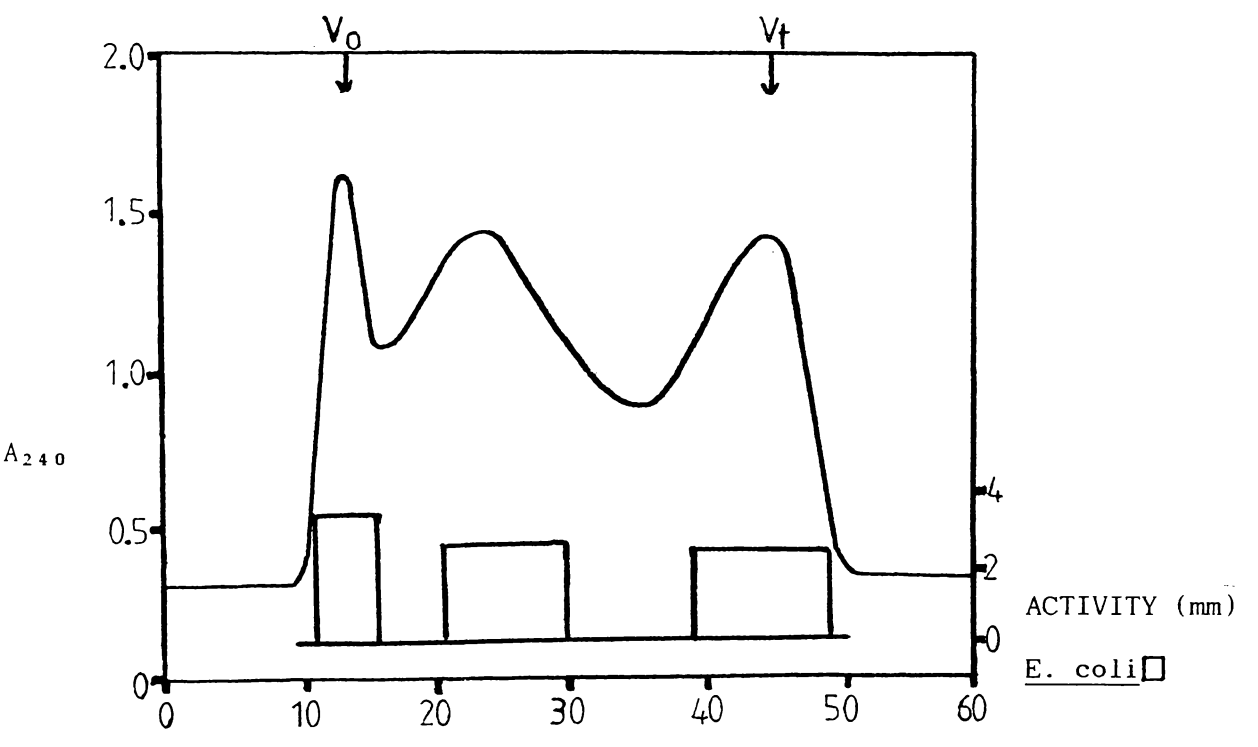


Fig. 3.4: Gel Filtration Chromatography of Acid Diffusate
Gel: Sephadex G-50SF
Eluent: HCl-water, pH 3.0
Sample: Concentrate of acid diffusate (5 ml)
Fraction Volume: 4 ml

Subsequently gel filtration chromatography of the acid diffusate at acidic pH did not cause complete disaggregation as only a small amount of activity was present in the low molecular weight region, and high activity was still present in the peak with the size of 30 kDa or over. The re-aggregated aggregate could not be disaggregated by gel filtration chromatography at acidic pH, therefore, in order to study the component(s) of the aggregates, other treatments had to be used to dissociate the aggregates.

Identification of Different High Molecular Weight Antibacterial Aggregates in Bovine Seminal Plasma

It was considered possible that the large aggregates (up to 500 kDa) that formed from the acid diffusate may have been an artifact. Therefore, in order to see whether the large antibacterial aggregates also exist in the physiological conditions of bovine seminal plasma, it was decided to chromatograph seminal plasma on Sephadex G-200F at pH 7.0. It was also considered that work with whole seminal plasma rather than the diffusates would reveal any antibacterial component(s) that were larger than 3.5 kDa and thus would not have been present in the diffusates.

Accordingly, a 5 ml sample of centrifuged untreated seminal plasma was chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. The results are shown in Fig. 3.5. Four antibacterial peaks with estimated molecular weights of 500 ($\pm$ 50) kDa (fractions 16 - 22), 250 ($\pm$ 25) kDa (fractions 23 - 28) and 20 ($\pm$ 2) kDa (fractions 29 - 41) were obtained. However, the molecular weight of the last peak (fractions 42 - 53) could not be estimated because it was eluted just after the bed volume. In order to see the reproducibility and to prepare a concentrated sample with enough activity to be picked up in subsequent chromatography the experiment was repeated six times. As can be seen in Fig. 3.6, the 250 kDa peak (fractions 23 - 28, Fig. 3.6) was sometimes more pronounced than as shown in Fig. 3.5.

The chromatography was also repeated at physiological pH of the bovine seminal plasma, pH 7.4, and the elution and activity pattern obtained was identical to that shown in Fig. 3.5.

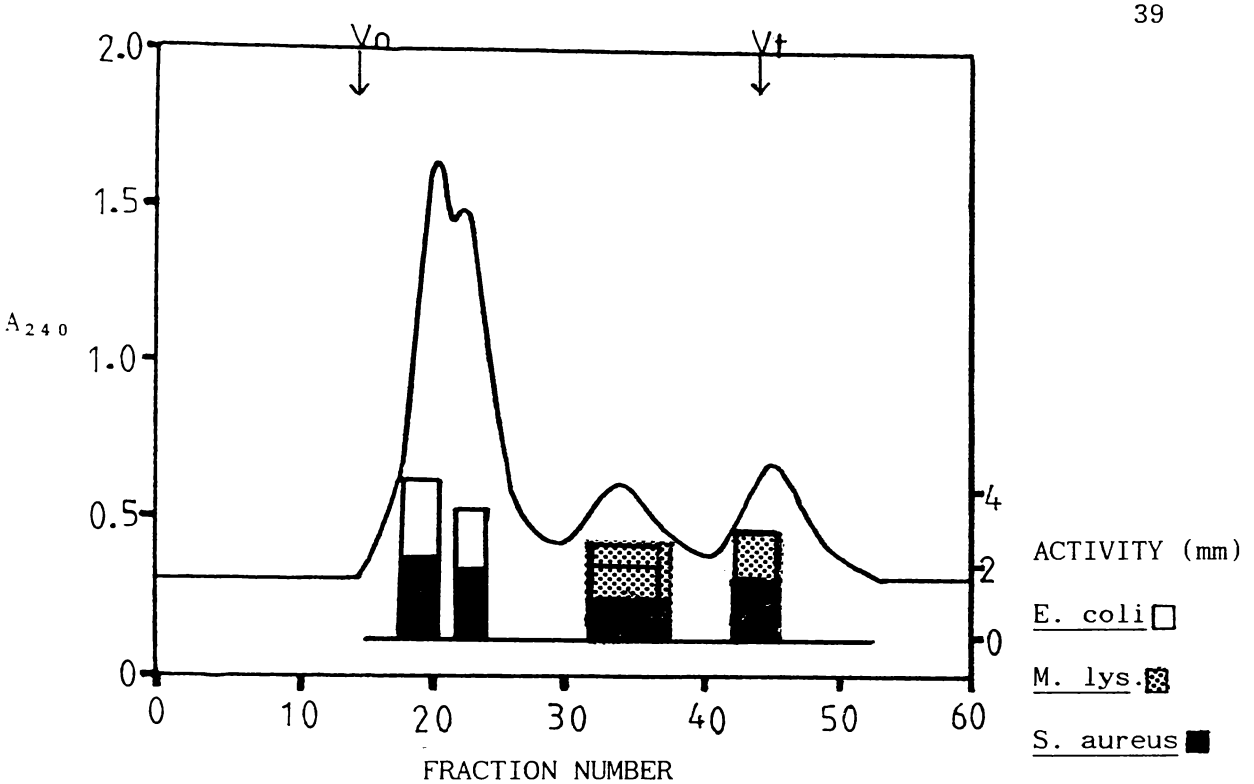


Fig. 3.5: Gel Filtration Chromatography of Bovine Seminal Plasma
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Bovine seminal plasma (5 ml)
Fraction Volume: 4 ml

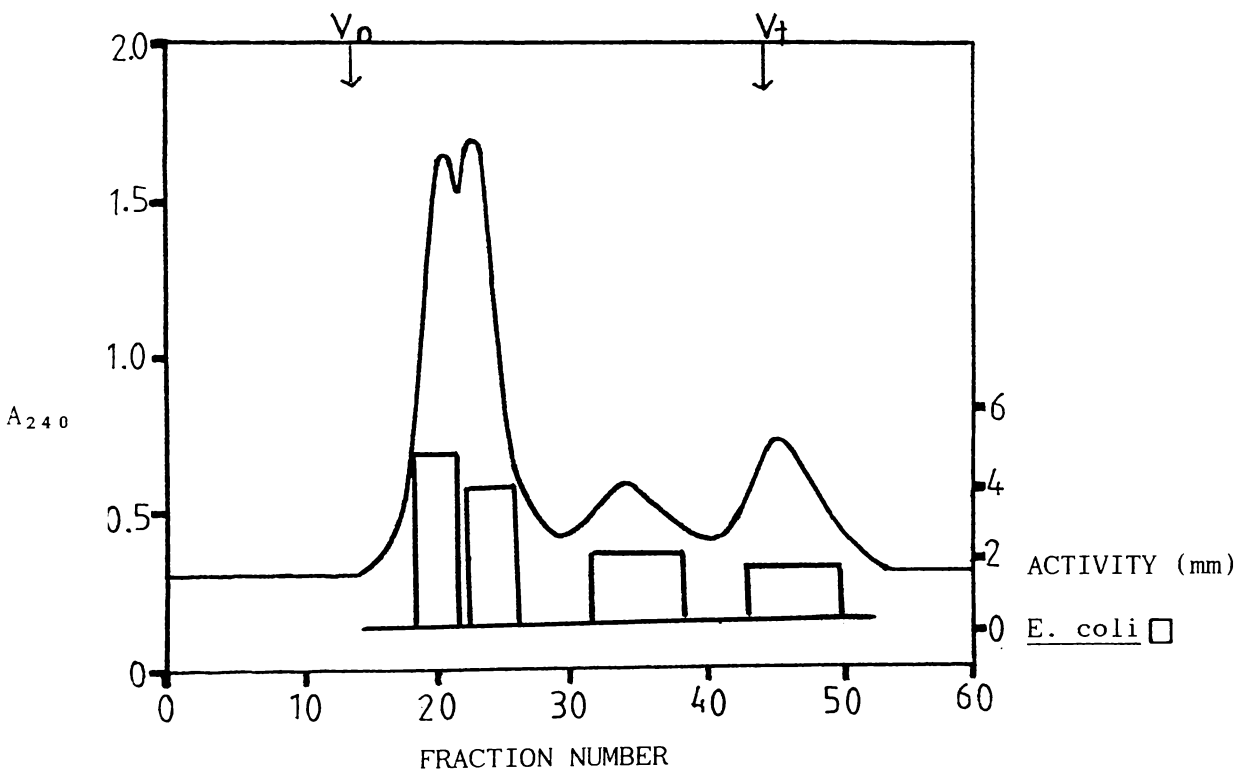


Fig. 3.6: Gel filtration Chromatography of Bovine Seminal Plasma
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Bovine seminal plasma (5 ml)
Fraction Volume: 4 ml

The antibacterial activity of all peaks of Fig. 3.5 was tested against *E. coli*, *S. aureus* and *M. lysodeikticus* and the results are shown in Fig. 3.5. When lysozyme standard was tested against these three organisms, it was found that lysozyme is highly active against *M. lysodeikticus*, weakly active against *E. coli* and inactive against *S. aureus*. As can be seen in Fig. 3.5 there was no lysozyme activity in the 500 kDa and 250 kDa peaks of Fig. 3.5. Thus, the antibacterial activity of these peaks appeared to be due to antibacterial compound(s) other than lysozyme.

In order to compare the high molecular weight peaks from acid dialysis with untreated seminal plasma, a sample of acid diffusate was neutralised and chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0 (Fig. 3.7). The three antibacterial peaks eluting in fractions 16 - 22, 23 - 28 and 29 - 41 as shown in Fig. 3.7 had the identical molecular weights to the first three antibacterial peaks of Fig. 3.5. Also, the relative sizes of these peaks were quite similar. It was, therefore, anticipated that the first three antibacterial peaks in untreated seminal plasma as shown in Fig. 3.5, were probably aggregates of the same small component(s). The broadness of the different antibacterial peaks shown in Figs. 3.5 - 3.7 could be because of continuous aggregation/disaggregation during chromatography.

It was thought that the high molecular weight antibacterial peaks of Fig. 3.5 could have been the low molecular weight antibacterial molecules eluting in high molecular weight region because of interactions between $Tris^+$ and the low molecular weight molecules. Thus, to check this possibility, pH of a 5 ml sample of seminal plasma was adjusted to 7.0 and the sample was then chromatographed on a column of Sephadex G-200F equilibrated with 5 mmol/l Tris-HCl buffer, pH 7.0 (very low concentration of $Tris^+$). An identical elution and activity profile to that shown in Fig. 3.5 was obtained, indicating that the high molecular weight antibacterial peaks of Fig. 3.5 were probably aggregates of low molecular weight component(s) rather than a result of interaction between $Tris^+$ and low molecular weight antibacterial compounds.

In order to purify the 500 kDa peak shown in Fig. 3.5, it was reduced to 5 ml by rotary evaporation and re-chromatographed on Sephadex G-200F at pH 7.0. As can be seen in Fig. 3.8, re-chromatography of the 500 kDa peak at pH 7.0 resulted in the formation of antibacterial peaks with elution profile and relative sizes similar to those of the peaks shown in Fig. 3.5. The finding of peaks other than the 500 kDa peak on re-chromatography could not have been because of poor resolution of the peaks shown in Fig. 3.5. The overlapping would not have resulted in the elution of different peaks with similar relative size, and also the 20 kDa peak was too well separated from the 500 kDa peak in Fig. 3.5 to be a contaminant.

Similarly, The 250 kDa peak as shown in Fig. 3.5 was also re-chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. The experiment was repeated twice and a typical result is shown in Fig. 3.9. Again, an elution and activity profile similar to that shown in Fig. 3.5 was obtained. This could not be because of poor resolution of neighboring peaks, as overlapping of the 500 kDa peak with the 250 kDa peak of Fig. 3.5 could not have caused formation of a larger 500 kDa peak than that of the 250 kDa peak when the 250 kDa peak was re-chromatographed.

Conclusion

Acid diffusate formed three antibacterial peaks of high molecular weight on Sephadex G-200F at pH 7.0 (Fig. 3.7). Untreated seminal plasma chromatographed through the same column at pH 7.0 also gave three antibacterial peaks at the identical position to those of Fig. 3.7 (Fig. 3.5). Also, re-chromatography of the 500 kDa and the 250 kDa peaks of Fig. 3.5 formed other peaks as well (Figs. 3.8 and 3.9), which did not appear to be because of overlapping. Thus, it was concluded that the 500 kDa, 250 kDa and 20 kDa antibacterial peaks as shown in Fig. 3.5 were probably the aggregates of the same component(s) of low molecular weight. Therefore, in order to study the component(s) of these aggregates attempts were made to disaggregate these peaks.

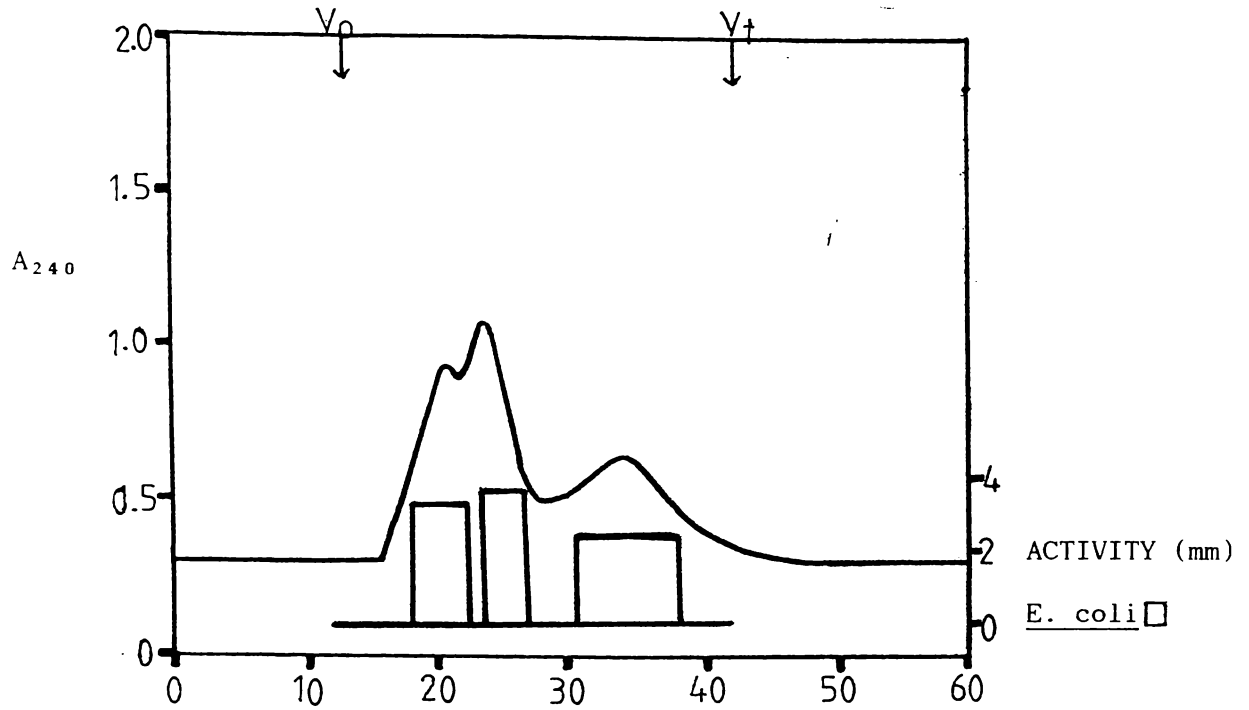


Fig.3.7: Gel Filtration Chromatography of Acid Diffusate
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Concentrate of acid diffusate (5 ml)
Fraction Volume: 4 ml

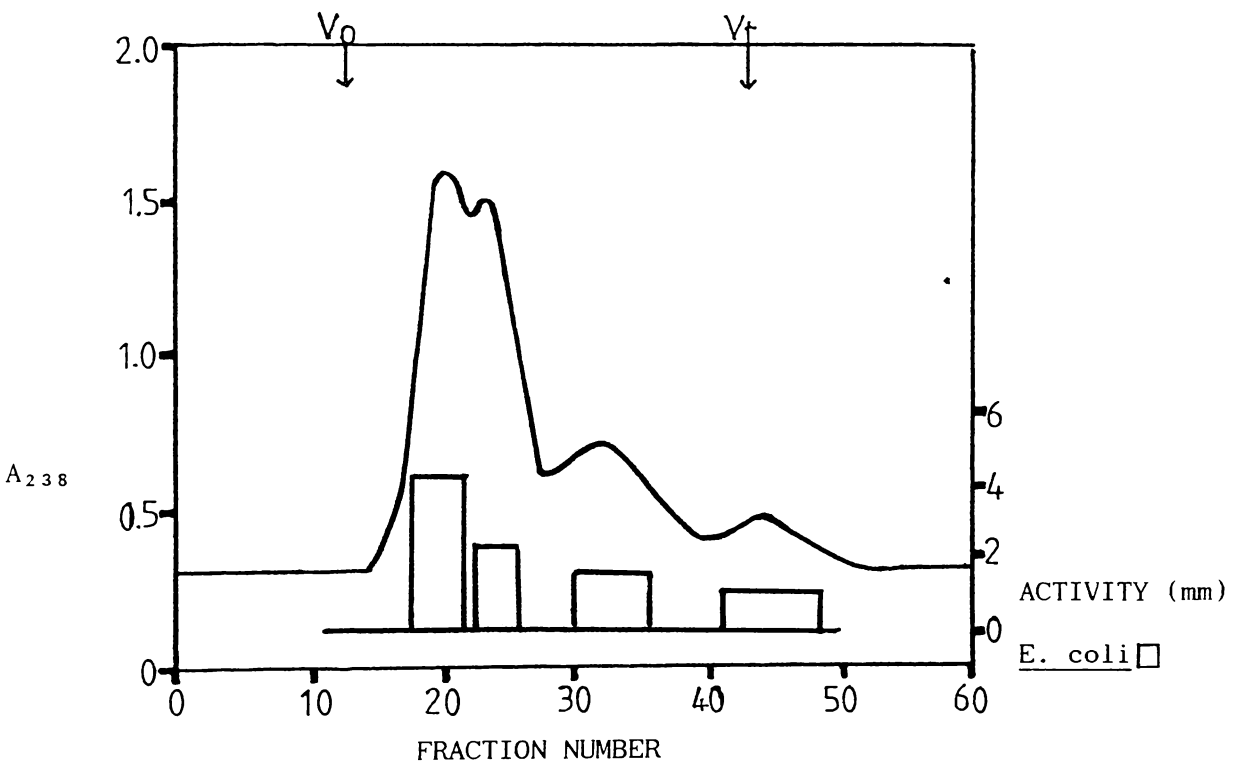


Fig. 3.8: Gel Filtration Chromatography of Fractions 16-22 from Fig. 3.5
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 16-22 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

Disaggregation of the 500 kDa Aggregate

Introduction

As described in the preceding sections, the 500 kDa aggregate as shown in Fig. 3.5 appeared to be an aggregate made up of small component(s), thus, in order to confirm that the peak was an aggregate, and to study the component(s) of the aggregate, attempts were made to disaggregate the 500 kDa aggregate of Fig. 3.5.

Disaggregation of the 500 kDa Aggregate Using Acidic Gel Filtration Chromatography

Eschenbruch (1980) found that although high pH seems to cause more disaggregation than low pH, unfortunately activity was unstable at high pH. Also, as found earlier, acid dialysis had apparently caused at least partial disaggregation. Therefore, gel filtration chromatography at acidic pH was initially used as a disaggregating technique.

As it was thought that ionic interactions might be involved in the aggregation, gel filtration was carried out at the lowest possible pH to increase the chance of breaking ionic interactions if any strong acidic group(s) were involved. Gel filtration was done using 0.02 mol/l HCl, pH 1.7 as eluent, because it is recommended that Sephadex gel not be exposed to more acidic conditions than this.

The 500 kDa aggregate (fractions 16 - 22) as shown in Fig. 3.5 was concentrated down to about 20 ml by rotary evaporation. When the pH of the sample was adjusted to 1.7 with HCl a precipitate formed at about pH 4.5. The precipitate was removed by centrifugation at 6,000 g (at r av 10 cm) for 10 minutes, and a part of the precipitate was taken and re-dissolved in 0.1 mol/l Tris-HCl buffer, pH 7.0. The pH of supernatant was re-adjusted to 7.0 with NaOH. Both the re-dissolved precipitate and the supernatant were tested for antibacterial activity against *E. coli* and were found to be active.

The precipitate was not dissolved by acid even at a pH as low as 1.0. Therefore, in order to study the precipitate at acidic pH, an attempt was made to dissolve the precipitate in various concentrations (10 - 50 %) of dimethylsulphoxide, a solvent known to dissolve some

precipitates formed from seminal plasma (P. Shannon, personal communication). However, the precipitate remained undissolved in all these concentrations.

A part of the supernatant (5 ml) was held at pH 1.7 at 4°C for 2 h, and then chromatographed through a column of Sephadex G-200F eluted with 0.02 mol/l HCl, pH 1.7. In order to prepare substantial amount of disaggregated material, the experiment was repeated four times. An identical elution and activity pattern was obtained each time. A typical result is shown in Fig. 3.10. Fractions 16 - 22 from all the repeated runs were pooled together, adjusted to pH 7.0 with NaOH and concentrated down to about 20 ml by rotary evaporation. The pH was found to be 7.0 after concentration. Fractions 38 - 51 were treated similarly. When testing the activity, no activity was detected in the high molecular weight peak (fractions 16 - 22 as in Fig. 3.10) and the activity was only present in the low molecular weight peak (fractions 38 - 51 as in Fig. 3.10).

Elution of all the activity in the low molecular weight region in Fig. 3.10 could either have been because of at least partial disaggregation at acidic pH or a result of acid hydrolysis of peptide bonds. To check the later possibility, the pH of a part of the concentrated sample of 500 kDa aggregate as shown in Fig. 3.5 was adjusted to 1.7 with HCl. In order to minimize any possible acid hydrolysis, it was quickly chromatographed through a column of Sephadex G-200F at pH 1.7. Again, an identical elution and activity profile to that shown in Fig. 3.10 was obtained. Because of the small amount of time the sample was kept at acidic pH, and the low temperature, it seemed very unlikely that the activity in the low molecular weight peak in Fig. 3.10 was due to acid hydrolysis. Instead, it seemed to be because of disaggregation at acidic pH. The high molecular weight peak (fractions 16 - 22, Fig. 3.10) could either have been an inactive carrier protein of active component(s), or a protein having nothing to do with the active aggregate, it just co-eluting with the aggregate.

To check this, a sample of about 0.5 ml of concentrated active low molecular weight peak (fractions 38 - 51, Fig. 3.10) was mixed with 4.5 ml 0.1 mol/l Tris-HCl buffer, pH 7.0, adjusted to pH 7.0 and incubated at 37°C for 2 h. It was then chromatographed through a column of

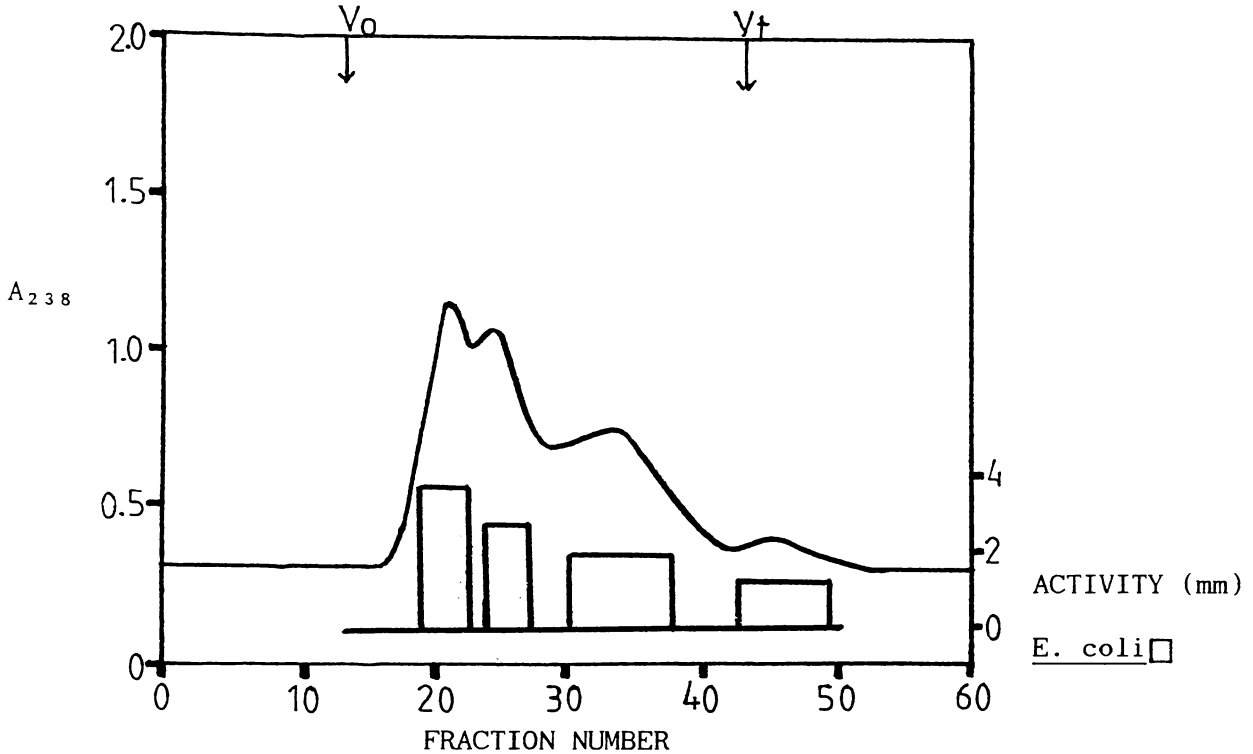


Fig. 3.9: Gel Filtration Chromatography of fractions 23-28 from Fig. 3.5
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris_HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 23-28 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

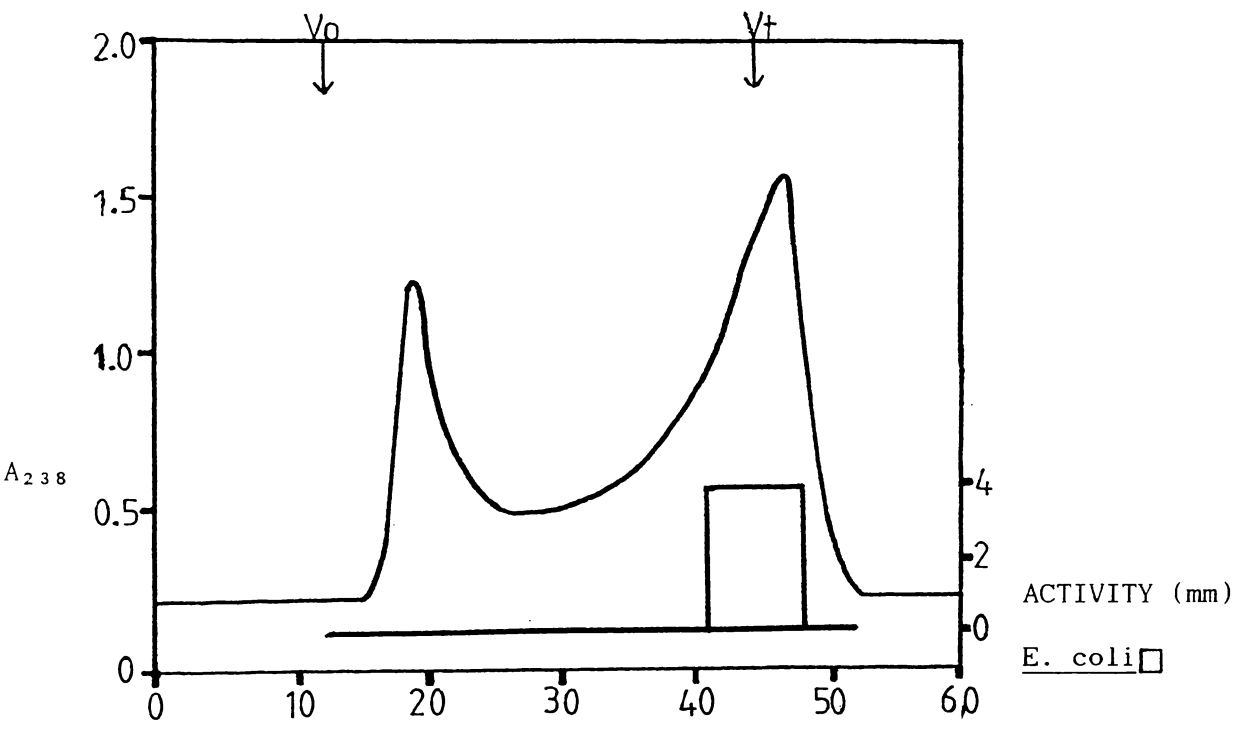


Fig. 3.10: Gel Filtration Chromatography of Fractions 16-22 from Fig. 3.5
Gel: Sephadex G-200F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 16-22 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. The results are shown in Fig. 3.11.

The experiment was repeated once. Identical results to those shown in Fig. 3.11 were obtained. The active low molecular weight peak (fractions 38 - 51) of Fig. 3.10 re-aggregated all the way up to the original position of the 500 kDa aggregate by itself at pH 7.0 (compare Fig. 3.11 with Fig. 3.5), therefore the active low molecular weight peak (fractions 38 - 51) of Fig. 3.10 must have contained all the component(s) required for the formation of active aggregates. Therefore, the inactive high molecular weight peak (fractions 16 - 22) of Fig. 3.10 was concluded to be a protein which just co-eluted with the active 500 kDa aggregate.

The elution of active peaks in the positions of 250 kDa aggregate and 20 kDa aggregate of Fig. 3.5 was another indication that the active 500 kDa, 250 kDa and 20 kDa peaks of Fig. 3.5 are the antibacterial aggregates made up of the same small component(s).

It was thought that some component(s) of the 500 kDa aggregate of Fig. 3.5 could have precipitated out with the pH 4.5 precipitate. To investigate this possibility, a small part of the precipitate was taken and re-dissolved in 5 ml 0.02 mol/l HCl, pH 1.7 and the solution held at 4°C for 2 h. It was then chromatographed through a column of Sephadex G-200F eluted with 0.02 mol/l HCl, pH 1.7. All the activity eluted at the low molecular weight region and the elution and activity profiles were identical to those shown in Fig. 3.10.

Similarly, when the active low molecular weight peak from this run was re-chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0 and identical elution and activity patterns to those shown in Fig. 3.11 were obtained.

As both the supernatant and the precipitate formed at pH 4.5 had activity and gave identical elution and activity profiles on gel filtration at pH 1.7 and pH 7.0, it was concluded that the supernatant and the precipitate had a similar composition and the precipitation could have been because of a concentration effect rather then any component precipitating out at pH 4.5. The supernatant was active and formed active aggregates at pH 7.0, thus it appeared to contain

component(s) required for formation of the high molecular weight antibacterial aggregates. Therefore, further work was done on the supernatant. It was decided when a model disaggregation method had been developed, both the precipitate and the supernatant would be disaggregated using the same method to confirm that they both contained same component(s).

In order to have a better idea about the size of the active low molecular weight peak shown in Fig. 3.10, it was decided to chromatograph it on a column of Sephadex G-25SF. Accordingly, about 0.5 ml of the concentrate of fraction 38 - 51 of Fig. 3.10 was diluted to 5 ml with distilled water. The pH of the sample was adjusted to 1.7 with HCl. The sample was held at 4°C for 2 h and then chromatographed through a column of Sephadex G-25SF eluted with 0.02 ml/l HCl, pH 1.7.

As can be seen in Fig. 3.12 the whole of the sample eluted on the void volume. The experiment was repeated once and an identical result was obtained. The component(s) had earlier passed through 3.5 kDa cut-off dialysing membrane, indicating the size of component(s) to be less than 3.5 kDa. Thus, the elution of all activity on the void volume on Sephadex G-25SF (suggesting a molecular weight of 5 kDa or over) meant that gel filtration at acidic pH had not caused complete disaggregation.

However, different results were obtained when chromatography was repeated without dilution of the sample. When the pH of 5 ml of concentrated fractions 38 - 51 from Fig. 3.10 was adjusted to 1.7 with HCl, the sample held at 4°C for 2 h, and then chromatographed through a column of Sephadex G-25SF at pH 1.7, more disaggregation was obtained. When the sample was 10 x more concentrated than before (Fig. 3.12), both active and inactive peaks inside the void volume were obtained (see Fig. 3.13), thus indicating more disaggregation. The experiment was repeated once and identical results to those shown in Fig. 3.13 were obtained.

Association of activity with the void volume peak on Sephadex G-25SF (molecular weight 5 kDa or over) indicated that complete disaggregation was again not achieved. It was, however, possible that the void volume peak of Fig. 3.13 was just a larger component of the 500 kDa aggregate, which would not have been present in the acid

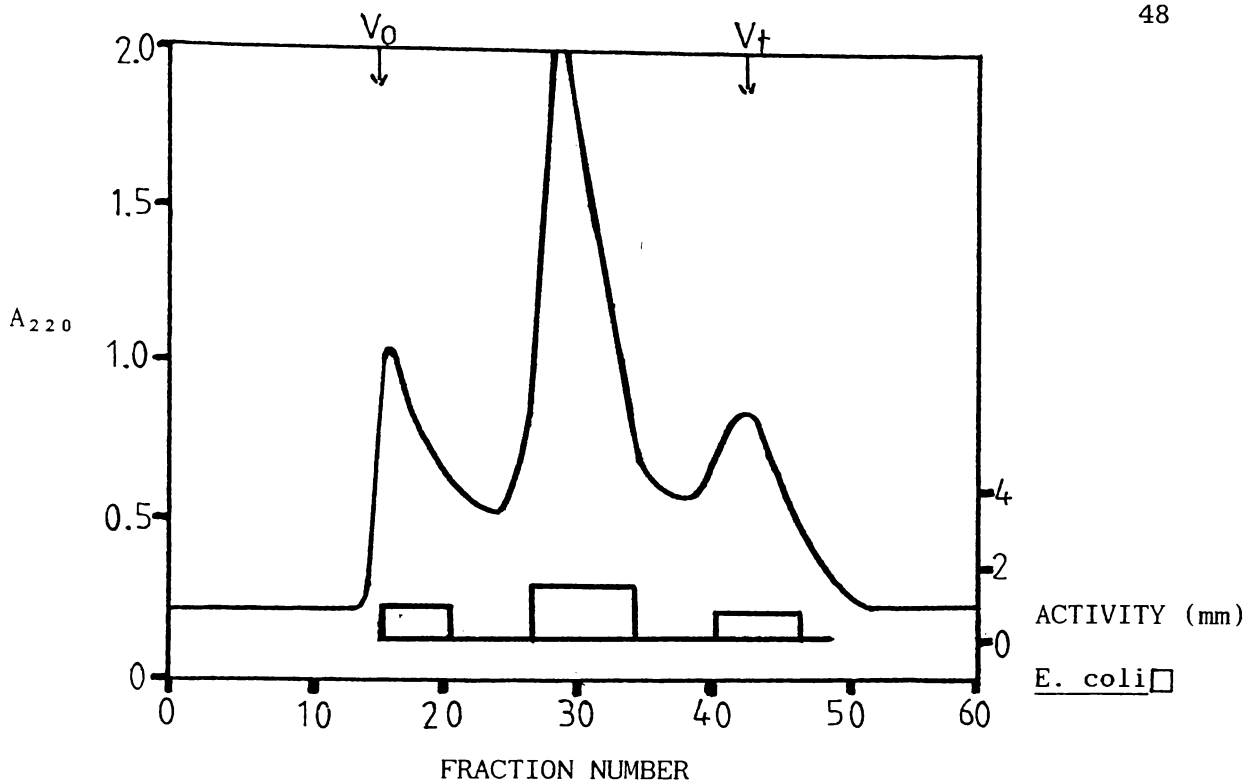


Fig. 3.11: Gel Filtration Chromatography of Fractions 38-51 from Fig. 3.10
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 38-51 from Fig. 3.10 (5 ml)
Fraction Volume: 4 ml

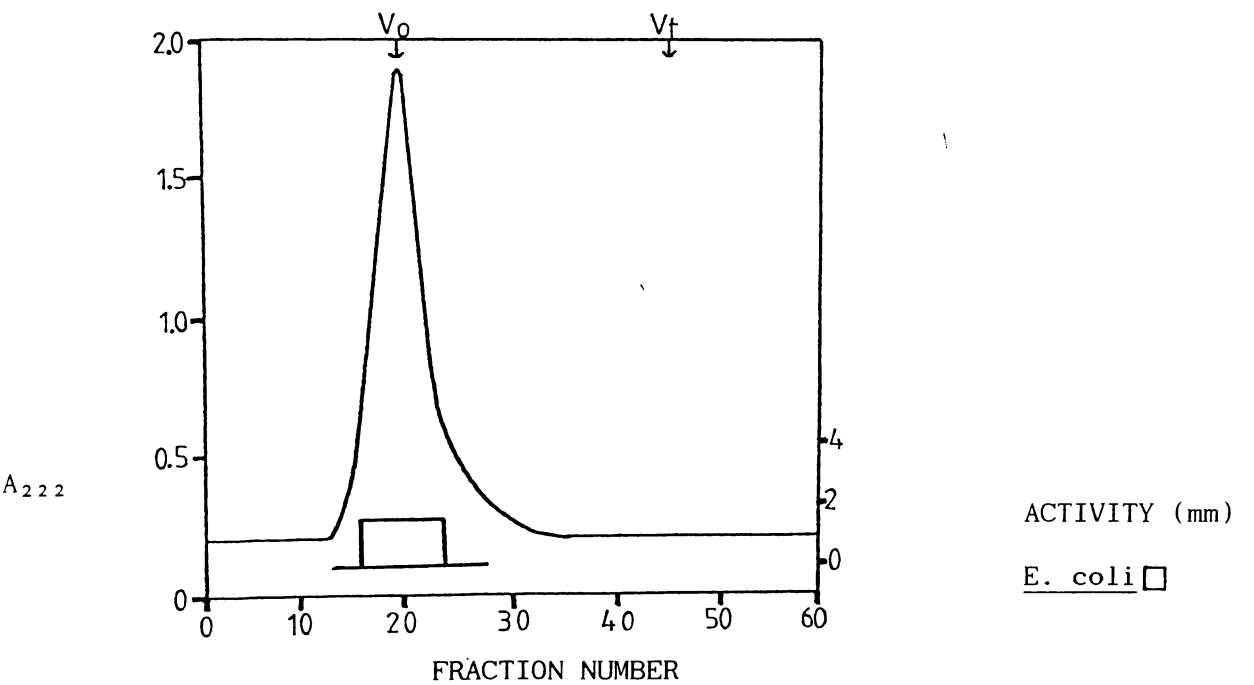


Fig. 3.12: Gel Filtration Chromatography of Fractions 38-51 from Fig. 3.10
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 38-51 from Fig.3.10 (5 ml)
Fraction Volume: 4 ml

diffusates. Therefore, to see if the low molecular weight component(s) (fractions 22 - 31, Fig. 3.13) would form the original 500 kDa aggregate without any larger component, these fractions were pooled, concentrated down to 5 ml by rotary evaporation and the pH of the sample was adjusted to 7.0 with NaOH. The sample was incubated at 37°C for 2 h, and then chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. As can be seen in Fig. 3.14, the sample re-aggregated up to about 500 kDa, indicating that the low molecular weight peaks of Fig. 3.13 contained at least the necessary component(s) of the active 500 kDa aggregate.

It was thought that the active void volume peak of Fig. 3.13 might be lysozyme and/or seminalplasmin bound to the aggregate, or just an undisaggregated aggregate. Thus, to find the molecular weight of the void volume peak shown in Fig. 3.13, it was concentrated down to 10 ml by rotary evaporation. Out of that, a 5 ml sample was then chromatographed through a column of Sephadex G-50SF eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.15. The molecular weights of the two active peaks obtained were estimated to be 30 kDa or over (fractions 11 - 17) and about 20 ($\pm$ 2) kDa (fractions 18 - 24).

Seminalplasmin is reported to have a molecular weight of 6.3 - 6.5 kDa (Theil and Scheit, 1983). No peak was obtained in this region, therefore, the activity in the void volume of Fig. 3.13 was not because of free seminalplasmin. The void volume peak of Fig. 3.15 was probably just an undisaggregated aggregate. The second peak (fractions 18 - 24) as shown in Fig. 3.15 could have been lysozyme or just an undisaggregated aggregate. To check this, a 5 ml lysozyme standard from egg-white was dissolved in 5 ml 0.02 mol/l HCl, pH 1.7 and chromatographed through the same column of Sephadex G-50SF at pH 1.7. The peak of egg-white lysozyme was eluted six fractions later than the second peak of Fig. 3.15. Eschenbruch (1980) found that lysozyme isolated from bovine seminal plasma apparently had a higher molecular weight than egg-white lysozyme. Thus, the later elution of lysozyme standard could have been because bovine lysozyme has a slightly larger size than egg-white lysozyme.

To further investigate this, the two peaks of Fig. 3.15 were assayed for lysozyme activity using the "Lyso-Plate" technique : agar plates containing *Micrococcus Lysodeikticus* were prepared

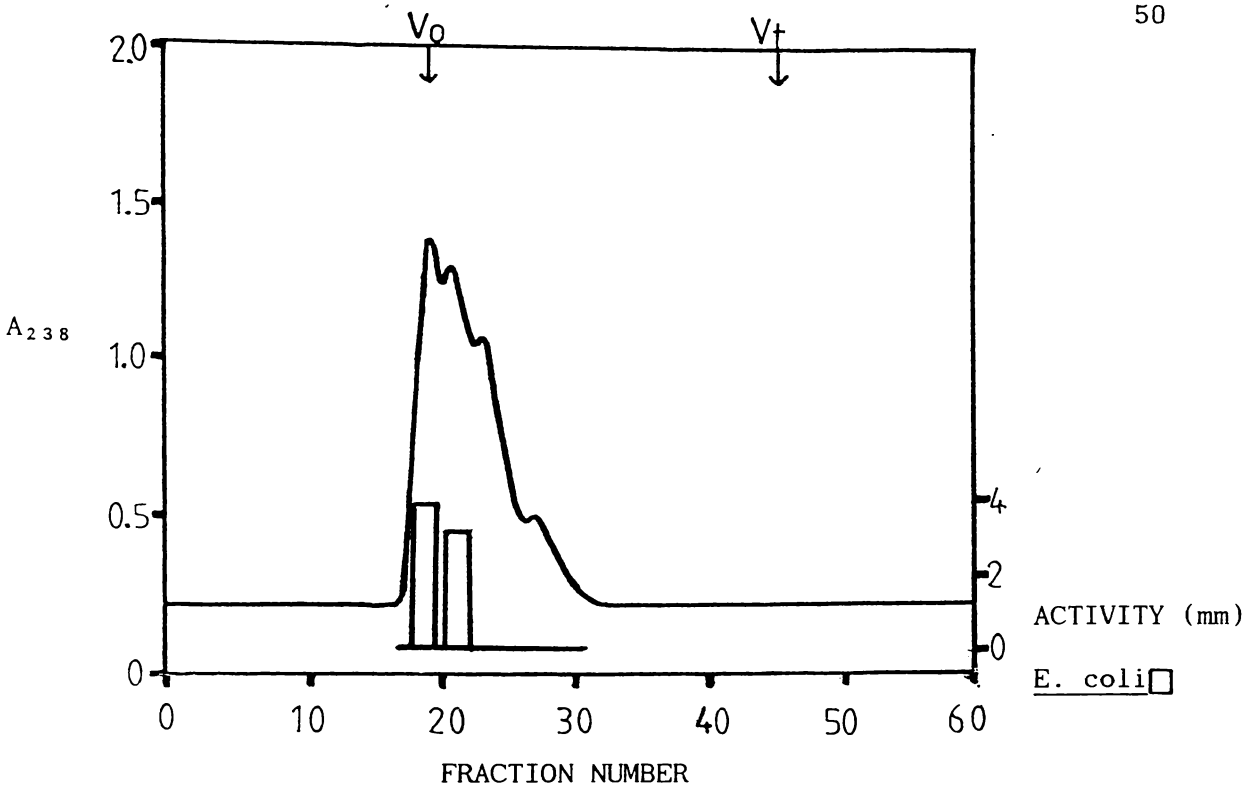


Fig. 3.13: Gel Filtration Chromatography of Fractions 38-51 from Fig. 3.10
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 38-51 from Fig. 3.10 (10 x concentrated sample than sample of Fig. 3.12)(5 ml)
Fraction Volume: 4 ml

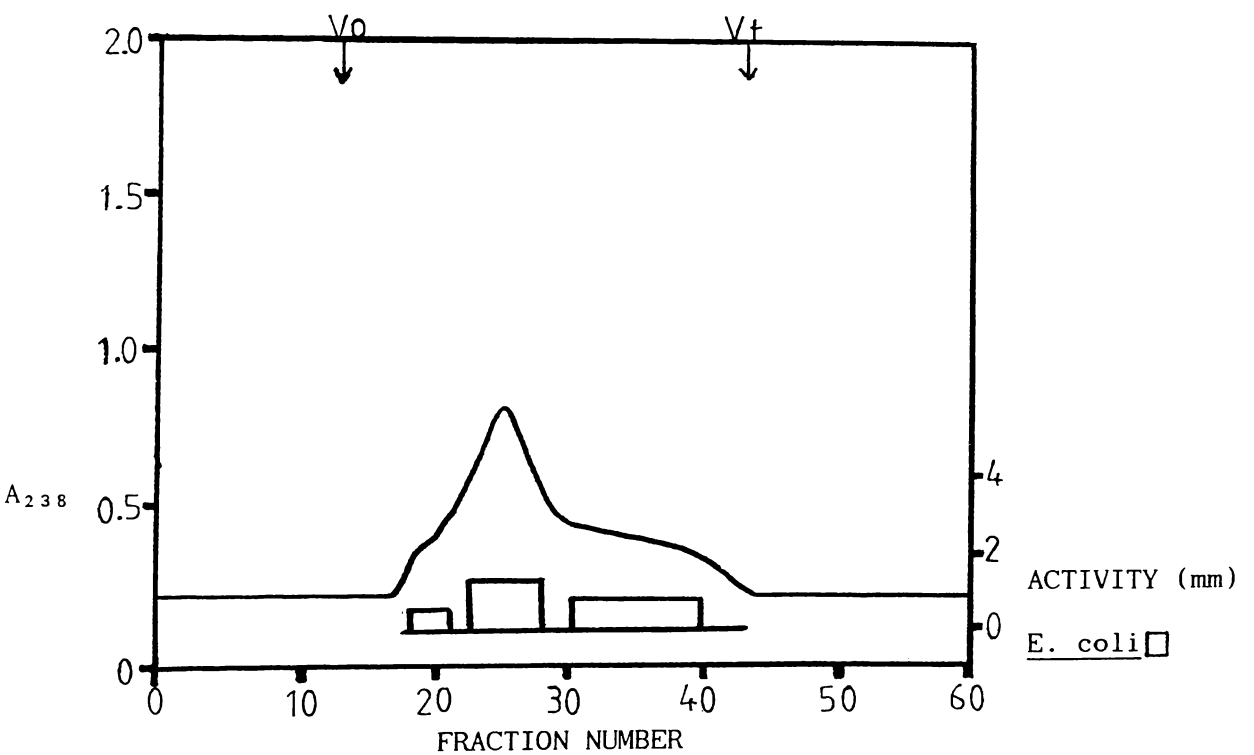


Fig. 3.14: Gel Filtration Chromatography of Fractions 22-31 from Fig. 3.13
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Concentrate of fractions 22-31 from Fig. 3.13 (5 ml)
Fraction Volume: 4 ml

and incubated as described in Chapter 2 and 0.1 ml samples of both the peaks were tested for their lytic activity. No lysozyme activity could be detected. As the size of the clearing obtained on "Lyso-Plates" with lysozyme standard was found to be much larger than the clearing on *E. coli* plates with lysozyme standard, it was concluded that the activity in the peaks was primarily due to a substance other than lysozyme. Thus, lysozyme does not seem to be involved in the formation of the 500 kDa aggregate. Therefore, it was concluded that the two active peaks of Fig. 3.15 were most probably just undissociated aggregates.

In order to have a better idea about the size of the low molecular weight component(s) dissociated from the 500 kDa aggregate (Fig. 3.10), about 2 ml sample from the concentrate of fractions 38 - 51 as shown in Fig. 3.10 was diluted to 5 ml by addition of 0.02 mol/l HCl, and its pH was adjusted to 1.7 with HCl. The sample was held at 4°C for 2 h and then chromatographed on a column of Sephadex G-50SF eluted with 0.02 mol/l HCl, pH 1.7. As can be seen in Fig. 3.16, a sharp peak was eluted inside the void volume. The column was calibrated by running Carbonic anhydrase (molecular weight 29 kDa), Cytochrome-C (molecular weight 12.4 kDa), Trypsin Inhibitor (molecular weight 6.5 kDa) and Insulin B chain (molecular weight 3.495 kDa) standards. The molecular weight estimated for the peak in Fig. 3.16 was 20 ($\pm$ 2) kDa.

Electrophoresis of Different Aggregates of Fig. 3.5 and the 20 kDa peak from Fig. 3.10

a) Cellulose acetate electrophoresis of the 20 kDa peak from Fig. 3.10

An attempt was made to find the net charge of the 20 kDa peak (fractions 38 - 51, Fig. 3.10) at neutral pH by cellulose acetate electrophoresis, so that the right type of polyacrylamide electrophoresis could be used to study the various aggregates.

To prepare the sample for electrophoresis, a 2 ml of the concentrate of fractions 38 - 51 of Fig. 3.1 was taken to dryness by rotary evaporation, and re-constituted in 1 ml 0.05 mol/l Tris-HCl buffer, pH 7.0. A cellulose acetate strip was soaked in 0.05 mol/l Tris-HCl buffer, pH 7.0 for 20 minutes and electrophoresis was carried out for 20 minutes as described in chapter 2.

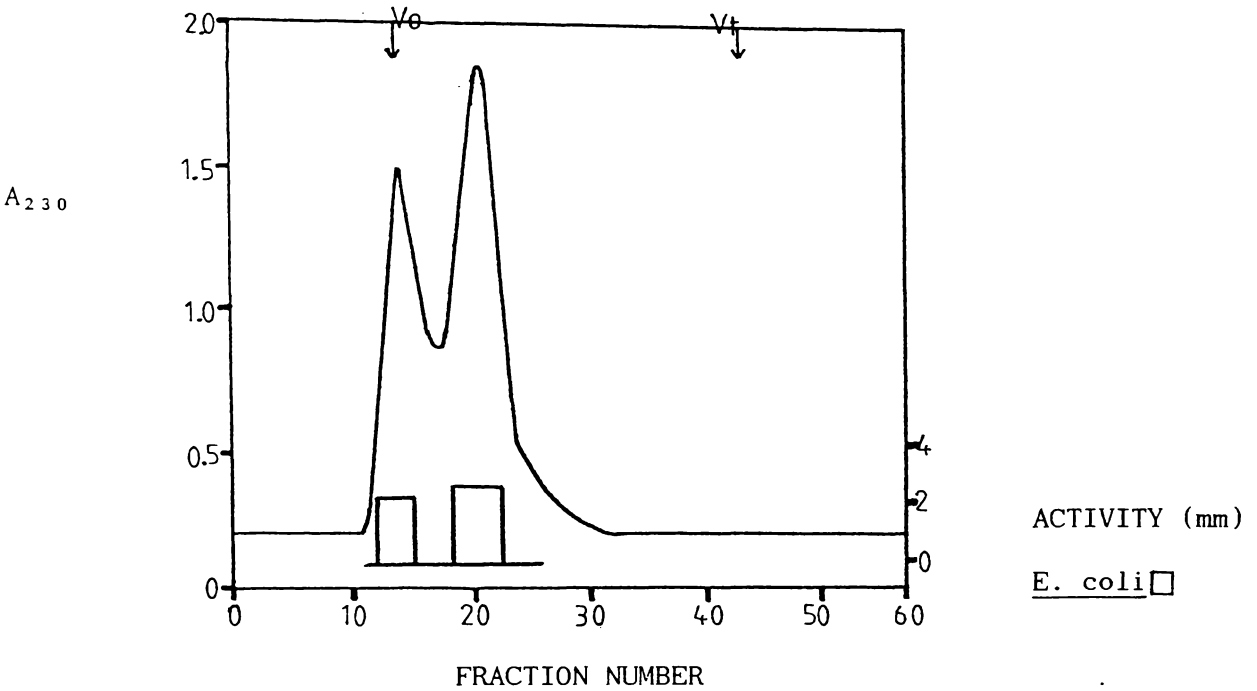


Fig. 3. 15: Gel Filtration Chromatography of Fractions 19-21 from Fig. 3.13
Gel: Sephadex G-50SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 19-21 from Fig. 3.13 (5 ml)
Fraction Volume: 4 ml

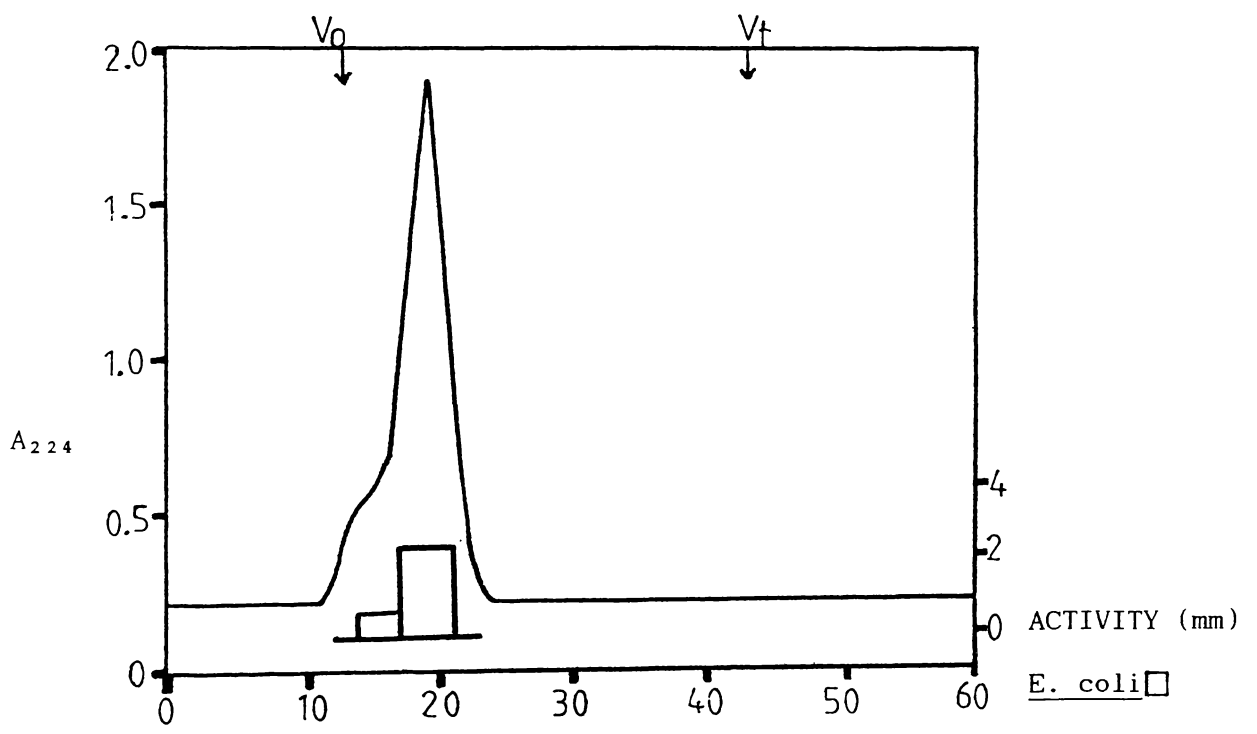


Fig. 3.16: Gel Filtration Chromatography of Fractions 38-51 from Fig. 3.10
Gel: Sephadex G-50SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 38-51 from Fig. 3.10 (5 ml)
Fraction Volume: 4 ml

Although most of the sample migrated as a streak towards the cathode, a small amount of sample also moved towards the anode. The lysozyme standard, being a highly basic protein, moved, as expected, towards the cathode. Thus, the 20 kDa molecule (fractions 38 - 51, Fig. 3.10) seemed to be predominantly basic in nature.

b) Cationic PAG electrophoresis of different aggregates of Fig. 3.5 and the active 20 kDa peak of Fig. 3.10

Since the 20 kDa peak of Fig. 3.10 behaved predominantly as a positively charged molecule, thus it was anticipated that different aggregates are positively charged. Therefore, cationic electrophoresis was used in order to compare the different active aggregates of Fig. 3.5 with each other and with the active 20 kDa peak (Fig. 3.10) which seemed to be made up entirely of the component(s) that make different active aggregates.

To prepare the samples for electrophoresis, about 2 ml of concentrate of each of the 500 kDa aggregate, 250 kDa aggregate, 20 kDa aggregate and the last peak of Fig. 3.5 and of the active 20 kDa peak (fractions 38 - 51) as shown in Fig. 3.10 were rotary evaporated to dryness and re-constituted in 1 ml of sample buffer. The samples were run on cationic discontinuous electrophoresis at pH 4.5 (Reisfeld *et* al., 1962). The results are shown in Plate 3.1.

Acidic pH seemed to have caused at least partial disaggregation as even the 20 kDa peak (obtained from the 500 kDa aggregate) gave several bands. There was only one faint band in the 500 kDa aggregate of Fig. 3.5. As part of the acid-disaggregated 20 kDa aggregate behaved as negatively charged at neutral pH on cellulose acetate electrophoresis, therefore, part of the 500 kDa aggregate could be negatively charged at pH 6.8 (pH of stacking gel) and thus, did not enter the gel. However, a more likely explanation is that the aggregate present in the 500 kDa peak in Fig. 3.5 was too large to enter the gel. The later possibility seemed more likely because of the large size of the aggregate.

However, the only band of the 500 kDa aggregate migrated similarly to the major band of the 250 kDa aggregate and the last peak of Fig. 3.5, and the 20 kDa peak of Fig. 3.10. This band migrated ahead of the lysozyme standard, thus indicating it was of smaller size and/or was

more basic than lysozyme. As can be seen in Plate 3.1 most of the bands of the 500 kDa , 250 kDa, 20 kDa aggregates and the last peak of Fig. 3.5 were common to each peak and to the acid-disaggregated active 20 kDa peak.

Thomas and Hodes (1981) suggested that better separation of cationic proteins could be achieved by using a new discontinuous buffer system at pH 6.8. Therefore, it was decided that the 500 kDa, 250 kDa, 20 kDa aggregates and the last peak of Fig. 3.5 and the 20 kDa peak of Fig. 3.10 should be run on cationic electrophoresis at pH 6.8. This would also allow the re-aggregation of the 20 kDa peak of Fig. 3.10 to be studied at near neutral pH. Accordingly, ~~a~~ 2 ml of the concentrate of the 500 kDa, 250 kDa, 20 kDa aggregates and the last peak as shown in Fig. 3.5 and of the 20 kDa peak (fractions 38 - 51) of Fig. 3.10 were separately rotary evaporated to dryness and re-constituted in 1 ml sample buffer. The samples were run on cationic discontinuous electrophoresis at pH 6.8 (Thomas and Hodes, 1981). A lysozyme standard was also run for reference. The results are shown in Plate 3.2.

Again, the 500 kDa aggregate of Fig. 3.5 gave no clear band which probably was because the 500 kDa aggregate was too large to enter the gel. The 500 kDa, 250 kDa, 20 kDa aggregates of Fig. 3.5 and the 20 kDa peak of Fig. 3.10 had a common major band, which migrated ahead of lysozyme standard, probably because of highly cationic nature of the aggregates. The 500 kDa, 250 kDa and 20 kDa aggregates and the last peak from Fig. 3.5 and the 20 kDa peak of Fig. 3.10 also had some other common bands, and some that were different.

Conclusions

The 500 kDa, 250 kDa and 20 kDa aggregates and the last peak (Fig. 3.5) had a major band and several other bands in common with the 20 kDa peak, thus indicating that the 500 kDa, 250 kDa, and 20 kDa aggregates shown in Fig. 3.5 are mainly antibacterial aggregates of the small and common component(s) which probably occur in small amounts as smaller aggregates or as free component(s) in the last peak of Fig. 3.5.

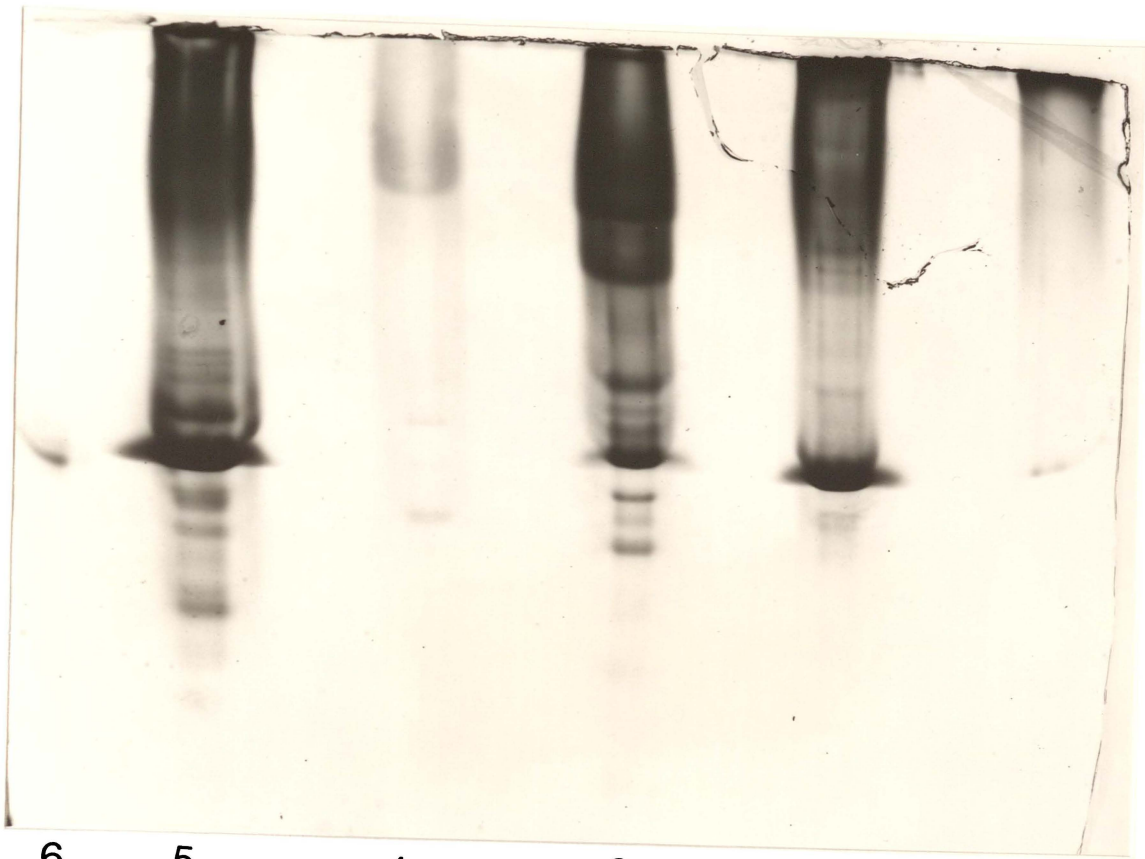
A concentrated sample of the 20 kDa peak from Fig. 3.10 gave several peaks (both active and inactive) on chromatography on Sephadex

Plate 3.1: Cationic Electrophoresis (pH 4.5)

1 - fractions 16 - 22 from Fig. 3.5 (500 kDa aggregate)
2 - fractions 23 - 28 from Fig. 3.5 (250 kDa aggregate)
3 - fractions 29 - 41 from Fig. 3.5 (20 kDa aggregate)
4 - fractions 42 - 53 from Fig. 3.5 (the last peak)
5 - fractions 38 - 51 from Fig. 3.10
6 - lysozyme standard

Plate 3.2: Cationic Electrophoresis (pH 6.8)

1 - fractions 16 - 22 from Fig. 3.5 (500 kDa aggregate)
2 - fractions 16 - 22 from Fig. 3.5 (500 kDa aggregate)
3 - fractions 23 - 28 from Fig. 3.5 (250 kDa aggregate)
4 - fractions 23 - 28 from Fig. 3.5 (250 kDa aggregate)
5 - fractions 29 - 41 from Fig. 3.5 (20 kDa aggregate)
6 - fractions 42 - 53 from Fig. 3.5 (the last peak)
7 - not relevant
8 - not relevant
9 - fractions 38 - 51 from Fig. 3.10
10 - fractions 38 - 51 from Fig. 3.10
11 - lysozyme standard


6
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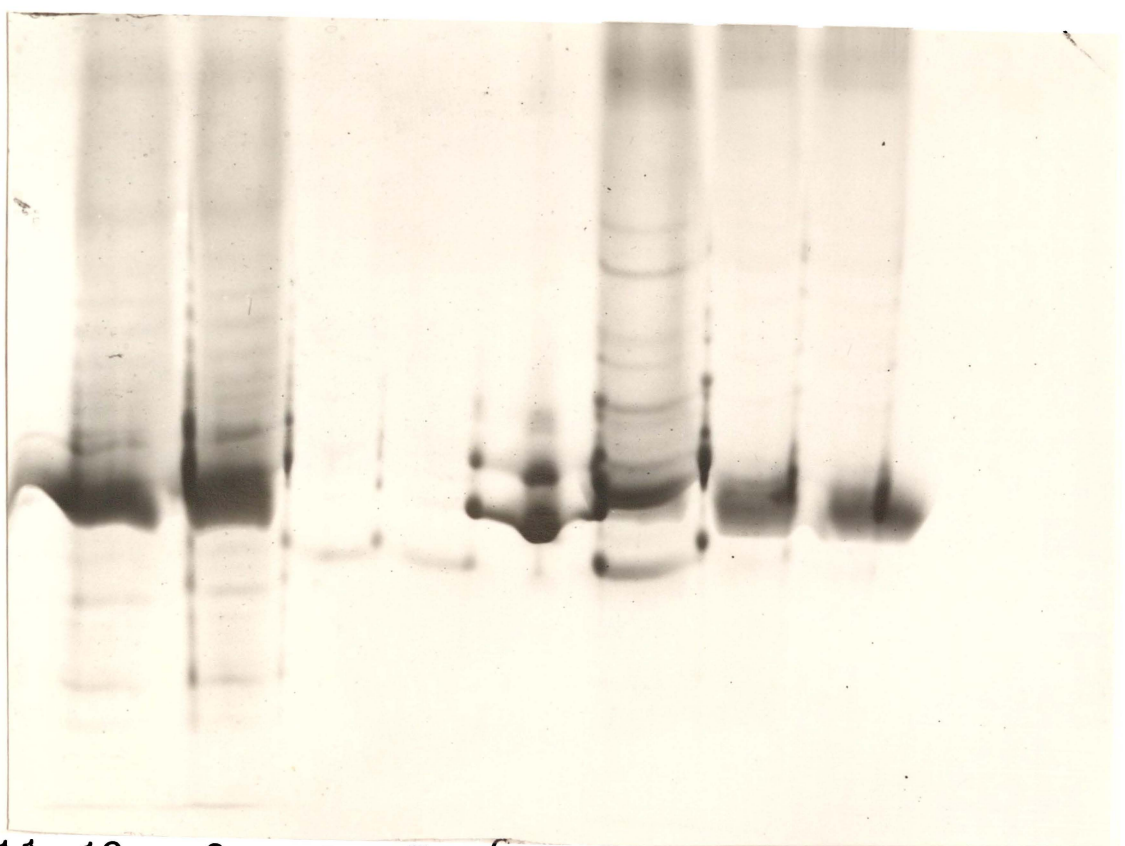
11
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G-25SF at pH 1.7 (Fig. 3.13), all of which were part of the aggregates as the 20 kDa peak came from the 500 kDa aggregate (Fig. 3.11), and the size of the component(s) of the aggregates was expected to be less than 3.5 kDa (as activity had been shown to pass across the 3.5 kDa cut-off dialysing membrane, but later re-aggregated at neutral pH [Figs. 3.1 - 3.3 and Fig. 3.7]). Also, the 20 kDa peak gave several bands on electrophoresis (Plates 3.1 and 3.2). All these results clearly indicated that the active 20 kDa peak as shown in Fig. 3.10 was still an aggregate rather a component. Thus, further attempts on disaggregation of the 20 kDa aggregate obtained from the 500 kDa aggregate of Fig. 3.5 were made to study the components.

An Attempt to Disaggregate the 20 kDa Aggregate Obtained from the 500 kDa Aggregate Using Immobilized Tris(carboxymethyl)ethylenediamine

In the presence of ethylenediaminetetraacetic acid (EDTA) antibacterial activity passes across 3.5 kDa cut-off dialysing membrane, thus indicating disaggregation, but EDTA binds to the component(s) and is difficult to separate afterwards (P. Molan, personal communication). To dissociate the 20 kDa aggregate (fractions 38 - 51, Fig. 3.10), an attempt was made to exploit the properties of EDTA as a disaggregating agent by using a column of immobilized Tris(carboxymethyl)ethylenediamine. It was hoped that this would work like EDTA but would not be present in any fractions released by disaggregation.

Accordingly, the pH of 5 ml concentrate of fractions 38 - 51 as shown in Fig. 3.10 was raised to 8.0 with NaOH (a pH of 8.0 was chosen because the aggregate was expected to be positively charged at pH 8.0, while the gel would have three negative charges at this pH). The sample was held at 4°C for 4 h and then loaded onto the column, followed by 0.01 mol/l Tris-HCl buffer, pH 8.0. There was a large peak (Pool A) which was not held by the column. To get the bound sample off the column 0.02 mol/l HCl, pH 1.7, was passed through the column. There was a small peak (Pool B) which was apparently held by the column. The results are shown in Fig. 3.17. The large peak which went straight through the column (Pool A, Fig. 3.17) could probably be because the capacity of the column was exceeded, rather than the possibility that most of the sample was negatively charged at pH 8.0.

The fractions making up each of the two pools were pooled, concentrated down to about 5 ml each by rotary evaporation, neutralised and checked for activity. Both were found to be active.

To see if the column had caused some disaggregation, the pH of the concentrates of both the pools as shown in Fig. 3.17 was adjusted to 1.7 with HCl. They were held at 4°C for 2 h and then chromatographed separately through a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. Both pools A and B of Fig. 3.17 were eluted on the void volume indicating a size of 5 kDa or over. As the component(s) were expected to have molecular weight of 3.5 kDa or lower if disaggregated, complete disaggregation, even of the sample bound to the column (Pool B, Fig. 3.17), was therefore not achieved with the column of Tris(carboxymethyl)ethylenediamine. Thus, it appeared that probably long exposure of sample to EDTA is required for disaggregation.

Further Attempts to Disaggregate the 20 kDa Aggregate Obtained from the 500 kDa Aggregate Using Cation Exchange Chromatography

When the concentrated sample of the 20 kDa aggregate from Fig. 3.10 was chromatographed through a column of Sephadex G-25SF at pH 1.7, several peaks were obtained including inactive ones (Fig. 3.13). As all these peaks came from an antibacterial aggregate, this indicated the presence of other component(s) besides the antibacterial peptide. Also, cationic electrophoresis of the 20 kDa aggregate gave several bands, both at pH 6.8 and pH 4.5, indicating the possibility of more than one component being present in the aggregates (Plates 3.1 and 3.2).

It was thought that if there are present more than one component in the antibacterial aggregates, then this fact could be exploited by using ion exchange chromatography as a disaggregating technique. A cation exchanger was used because the 20 kDa aggregate behaved predominantly as a positively charged molecule on cellulose acetate electrophoresis at pH 7.0. SP-Sephadex C-50 gel was used because it is recommended for medium sized proteins (with molecular weight of over 10 kDa).

Ion exchange chromatography was carried out with a starting buffer at pH 3.0, and the 20 kDa aggregate, being basic in nature, was

expected to bind to the gel at this pH. Different component(s) could then be eluted with a combination of pH and salt gradients, with anionic component(s) eluting earlier and cationic ones later. Antibacterial activity is reported to pass across the 3.5 kDa cut-off dialysing membrane in the presence of 0.1 mol/l citrate at pH 7.0, but does not diffuse through the membrane without citrate at pH 7.0 and also citrate is reported to bind to the cationic proteins of bovine seminal plasma (Shannon *et al.*, 1987). Thus, citrate seemed to be a good disaggregating agent, therefore citrate buffers were used as eluting buffers.

Accordingly, a 9 ml sample of the concentrate of the 20 kDa aggregate as shown in Fig. 3.10 was taken and in order to make 0.1 mol/l citrate strength of the sample 1 ml 1 mol/l citrate was added to it. The pH of the sample was adjusted to 3.0 with NaOH, and the sample was held at 4°C for 4 h. The sample was then applied to a column of SP-Sephadex C-50 ion exchanger, and followed with 0.1 mol/l citrate buffer, pH 3.0, until the absorbance at 230 nm of the effluent had returned to the baseline. The column was then eluted with a continuous salt gradient from 0 to 4.0 mol/l NaCl in 0.1 mol/l citrate buffer,pH 3.0. This was followed by a continuous pH gradient from 0.1 mol/l citrate buffer, pH 3.0, containing 4.0 mol/l NaCl to 0.1 mol/l citrate buffer, pH 8.0, containing 4.0 mol/l NaCl. Finally, a continuous pH gradient from 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl to 0.1 mol/l NaOH, pH 13.0 containing 4.0 mol/l NaCl was used. The antibacterial activity is unstable at high pH (P. Molan, personal communication). Thus, to avoid any loss of activity, the fractions eluted at pH 9.0 and over were neutralised immediately. The results are shown in Fig. 3.18.

Fractions making up the various peaks were pooled in separate pools (Pools A, B, C and D) as shown in Fig. 3.18. Two peaks were pooled together in Pool B because they were eluted with the same gradient. The antibacterial activity of the peaks could not be tested because of the presence of high concentrations of salt. As the component(s) of the aggregates seemed to be of small size, salt could not be removed by dialysis or ultrafiltration because of fear of losing any small component(s).

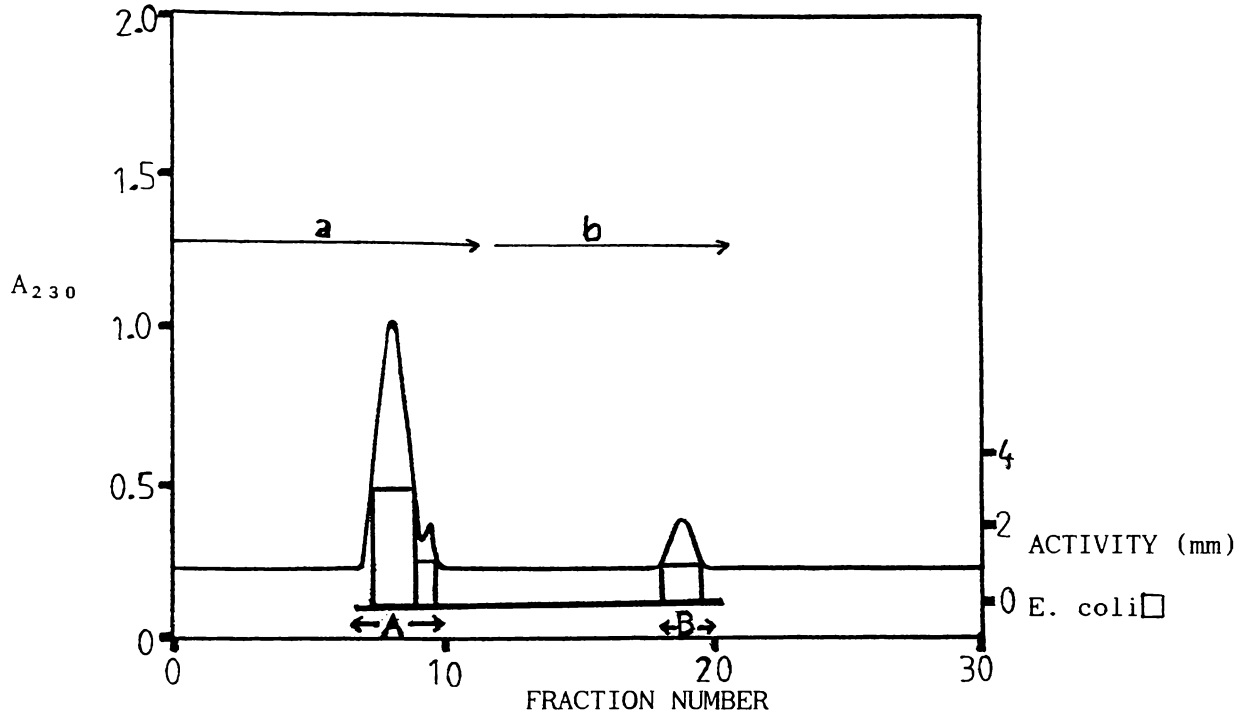


Fig. 3.17: Column Chromatography of Fractions 38-51 from Fig. 3.10 Using Tris(carboxymethyl)ethylenediamine column
Gel: Tris(carboxymethyl)ethylenediamine
Elution Buffer and Eluent: (a) 0.01 mol/l Tris-HCl buffer, pH 8.0; (b) 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 38-51 from Fig. 3.10 (5 ml)
Fraction Volume: 4 ml

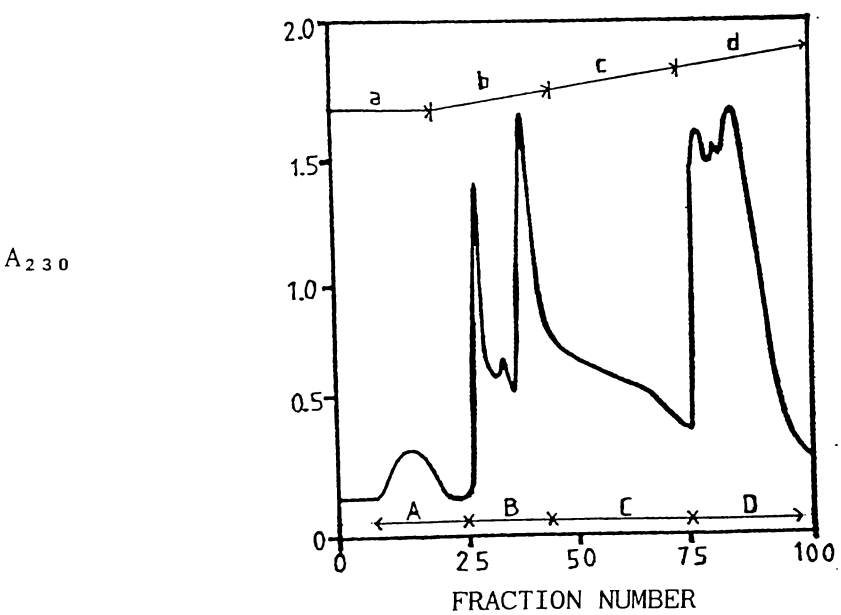


Fig. 3.18: Ion Exchange Chromatography of Fractions 38-51 from Fig. 3.10
Gel: SP-Sephadex C-50
Sample: Part of concentrate of fractions 38-51 from Fig. 3.10 (10 ml)
Elution Buffers: (a) 0.1 mol/l citrate buffer, pH 3.0; (b) salt gradient from 0 to 4.0 mol/l NaCl in 0.1 mol/l citrate buffer, pH 3.0; (c) pH gradient from 0.1 mol/l citrate buffer, pH 3.0 containing 4.0 mol/l NaCl to 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl; (d) pH gradient from 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl to 0.1 mol/l NaOH, pH 13.0 containing 4.0 mol/l NaCl
Fraction Volume: 4 ml

Gel filtration chromatography of the peaks from the cation exchange chromatography (Fig. 3.18) at acidic pH

All the pools, as shown in Fig. 3.18, were separately concentrated down to about 5 ml each by rotary evaporation. The crystals of salt formed were removed by centrifugation. The pH of the supernatants were lowered to 1.7 with HCl, then the pools were held at 4°C for 2 h. Each was then chromatographed separately through the column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7, to see whether cation exchange chromatography had caused disaggregation. The results are shown in Figs. 3.19 - 3.22.

The material which passed straight through the cation exchanger at pH 3.0 (Pool A, Fig. 3.18) was eluted as an inactive peak later than the void volume on Sephadex G-25SF at pH 1.7 (Fig. 3.19). The column of Sephadex G-25SF was calibrated by running standards of Trypsin Inhibitor (molecular weight 6.5 kDa), Insulin chain B (molecular weight 3.495 kDa), Insulin chain A (molecular weight 2.532 kDa) and Actinomycin C (molecular weight 1.280 kDa) through the column, and the molecular weight of the peak eluted in fractions 20 - 30 in Fig. 3.19 was estimated to be about 3 ($\pm$ 0.3) kDa. The large peak at the bed volume in Figs. 3.19 - 3.22 was assumed to be the citrate buffer from the ion exchange chromatography.

When the material which was eluted from the ion exchange column with the salt gradient at pH 3.0 (Pool B, Fig 3.18) was chromatographed through a column of Sephadex G-25SF at pH 1.7, again, a peak (fractions 20 - 30) with an estimated molecular weight of 3 ($\pm$ 0.3) kDa was obtained (Fig. 3.20). However, this peak was antibacterial in nature. There was another active peak of estimated molecular weight of 1.2 ($\pm$0.12) kDa (fractions 41 - 45, Fig. 3.20).

As can be seen in Fig. 3.21, the gel filtration chromatography of Pool C of Fig. 3.18 also gave an active peak (fractions 20 - 30) with an estimated molecular weight of about 3 ($\pm$ 0.3) kDa, and another active peak (fractions 41 - 45) of 1.2 (0.12) kDa.

Pool D of Fig. 3.18 (the material eluted with high pH) on the column of Sephadex G-25SF at pH 1.7 gave an active peak of about 2 (+ 0.2) kDa (fractions 25 - 35). The tailing of that peak on the end

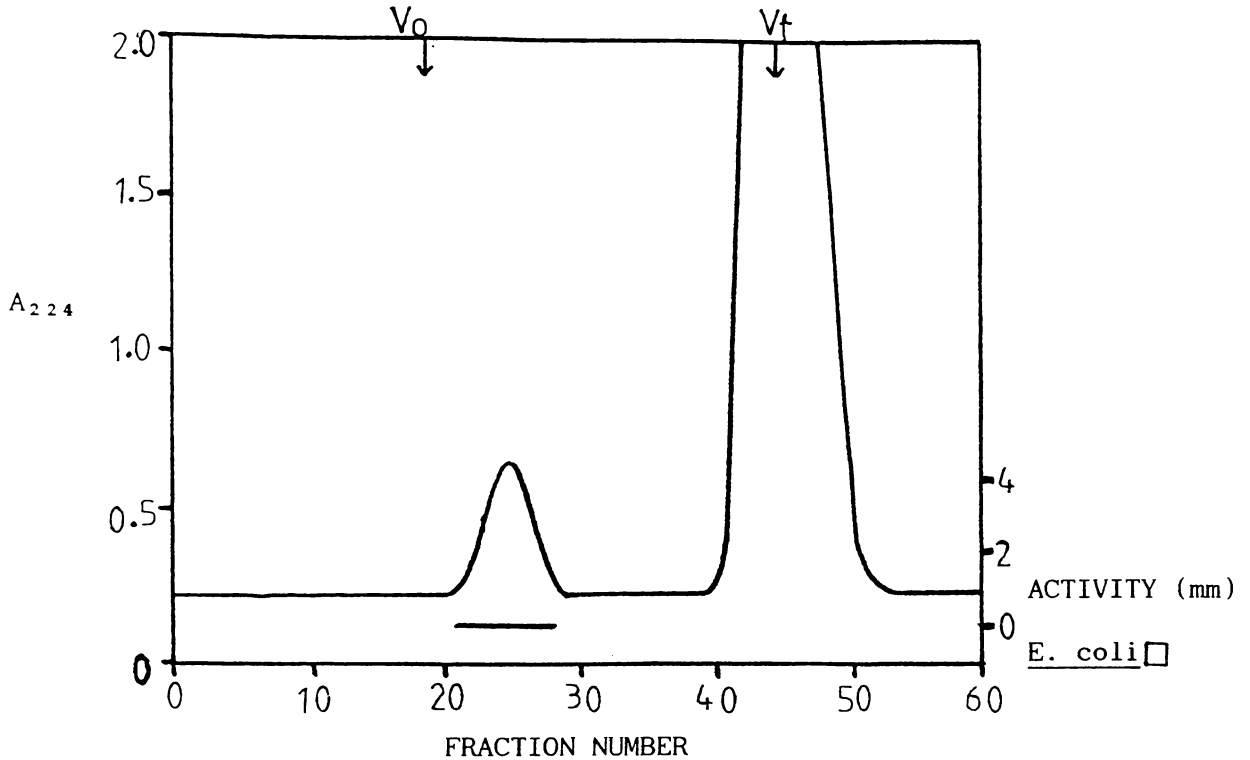


Fig. 3.19: Gel Filtration Chromatography of Pool A from Fig. 3.18
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool A from Fig. 3.18 (5 ml)
Fraction Volume: 4 ml

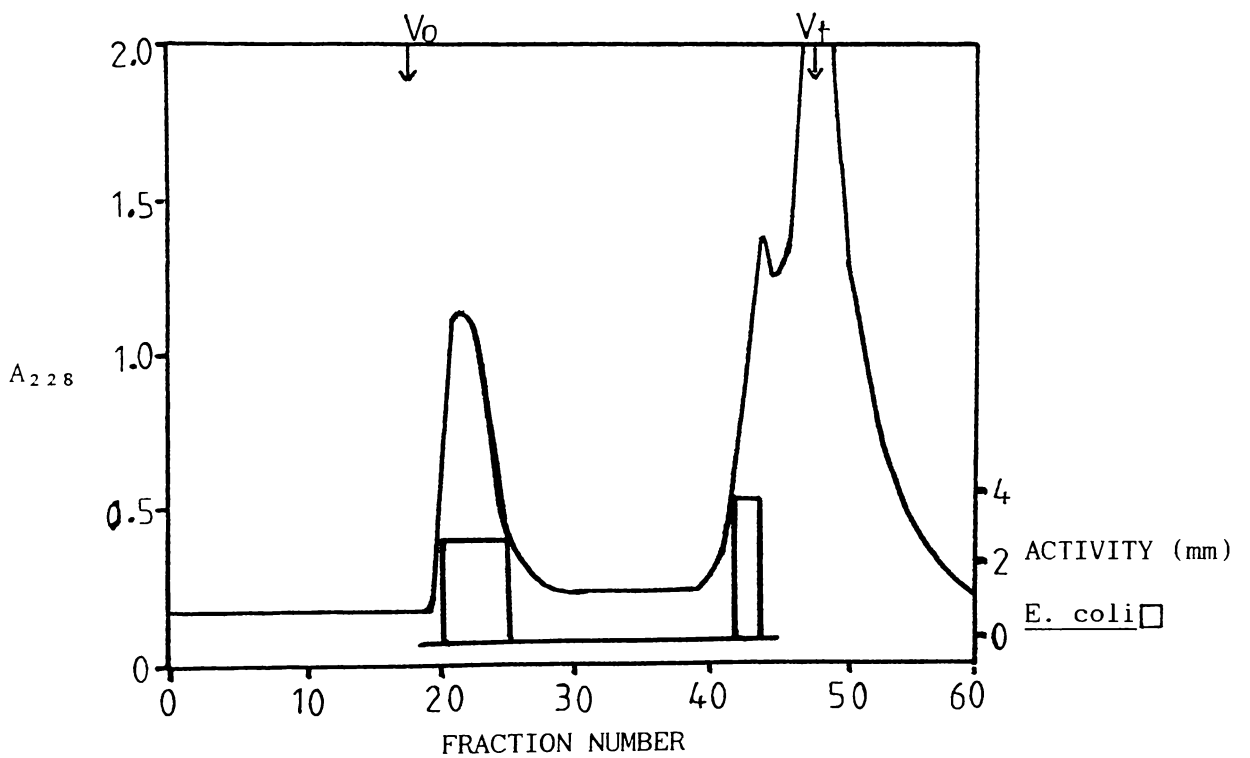


Fig. 3.20: Gel Filtration Chromatography of Pool B from Fig. 3.18
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool B from Fig. 3.18 (5 ml)
Fraction Volume: 4 ml

was possibly because of disaggregation of the peak as it ran through the column. There was also an active peak eluted in fractions 41 - 45, whose molecular weight was estimated to be 1.2 (± 0.12) kDa (see Fig. 3.22).

As the active peaks of 1.2 kDa were not very well separated from the large peak eluted at the bed volume in Figs. 3.20 - 3.23, it was decided to pool the 1.2 kDa and bed volume peaks of Figs. 3.20 - 3.22 and chromatograph them on a column of Sephadex G-15 at pH 1.7. This was also used to see whether there was a peak of any component hidden under the large bed volume peak, which was assumed to be predominantly citrate.

Accordingly, the 1.2 kDa and bed volume peak, as shown in Fig. 3.20, were pooled together (fractions 41 - 46) evaporated down to 5 ml by rotary evaporation. The excess citrate salt crystals came out of the solution and were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. The crystals were dried out by leaving at room temperature for a few hours and checked under a microscope. The sharp crystallinity seen indicated the presence of citrate salts rather than any material of protein nature. The pH of the clear supernatant was adjusted back to 1.7 with HCl, held at 4°C for 2 h and then chromatographed through a column of Sephadex G-15 eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.23.

The active peak (fractions 18 - 25) of Fig. 3.23 was probably the completely disaggregated active component. The peak eluted after the active peak was most probably citrate. However, the activity of the peaks after the active peak could not be tested because of salt. The column was calibrated by running Insulin Chain A (molecular weight 2.532 kDa), Actinomycin C (molecular weight 1.280 kDa), Bacitracin (molecular weight 1.450 kDa) and Bradykinin (molecular weight 1.060 kDa) standards. Molecular weight estimated for the active peak from the calibration curve was about 1.2 (± 0.12) kDa. This appeared to be the same active peak of 1.2 kDa (fractions 41 - 45) as shown in Fig. 3.20.

Since Pools C and D of Fig. 3.18 gave peaks in identical positions (1.2 kDa and bed volume, Figs. 3.21 and 3.22), the 1.2 kDa peaks and the bed volume peaks as shown in Figs. 3.21 and 3.22 were pooled

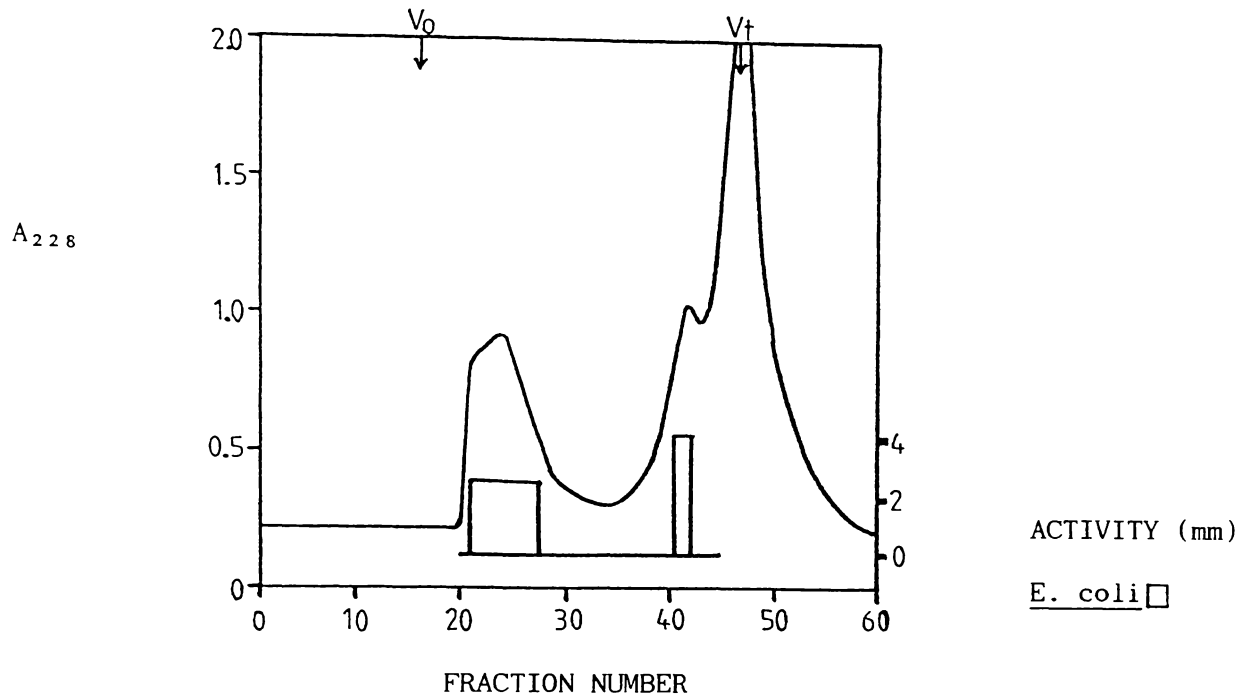


Fig. 3.21: Gel Filtration Chromatography of Pool C from Fig. 3.18
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool C from Fig. 3.18 (5 ml)
Fraction Volume: 4 ml

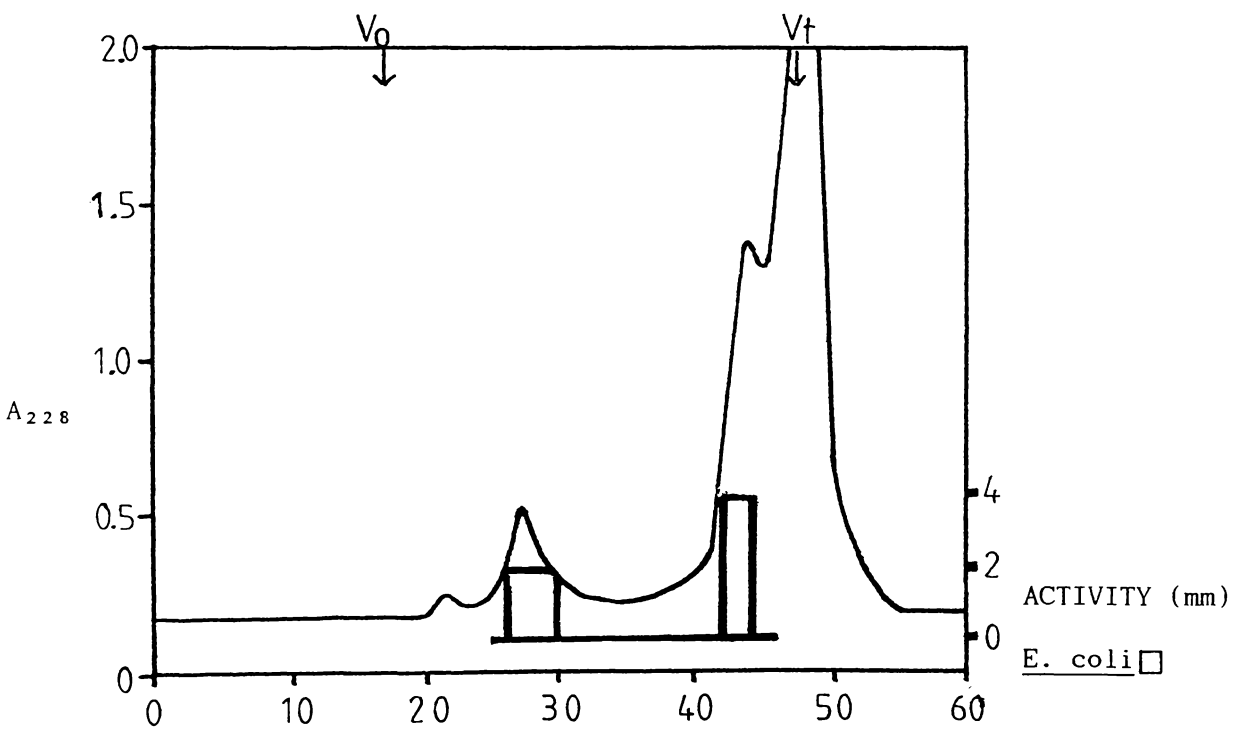


Fig. 3.22: Gel Filtration Chromatography of Pool D from Fig. 3.18
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool D from Fig. 3.18 (5 ml)
Fraction Volume: 4 ml

together (fractions 41 - 56) and concentrated down to about 5 ml by rotary evaporation. The crystals of excess citrate salt were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes, and the pH of the supernatant was adjusted to 1.7 with HCl. The sample was held at 4°C for 2 h and then chromatographed through a column of Sephadex G-15 eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.24.

Again, there was an active peak (fractions 18 - 25) with an estimated molecular weight of 1.2 (± 0.12) kDa, and there was also a large bed volume peak, which probably was citrate. The activity of this peak could not be tested because of salt.

Gel filtration chromatography at neutral pH to study the re-aggregation

The 3 kDa peak obtained from ion exchange chromatography (fractions 20 - 30 in Fig. 3.19) was inactive. To see whether this inactive peak played any part in the aggregation phenomenon, all fractions making up this peak were pooled and concentrated down to about 10 ml by rotary evaporation. The pH of a 5 ml sample was adjusted to 7.0 with NaOH. The sample was incubated at 37°C for 2 h, then chromatographed through a column of Sephadex G-200F at pH 7.0. As can be seen in Fig. 3.25, the sample re-aggregated. There was a small void volume peak which was of even higher molecular weight than the original 500 kDa aggregate of Fig. 3.5. The molecular weight estimated for the middle of the main peak (fractions 24 - 35) was about 80 kDa. An exact estimate of the molecular weight of the peak could not be made because of the spreading nature of the peak, which could possibly have been due to continuous aggregation and disaggregation as the sample passed through the column.

The re-aggregation was not achieved up to the size of the original aggregate (500 kDa) in Fig. 3.25. To see whether the incomplete aggregation was because of insufficient incubation, the pH of remainder sample (5 ml) of fractions 20 - 30 of Fig. 3.19 was adjusted to 7.0 with NaOH, and the sample was incubated at 37°C for 24 h. It was then chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. An identical elution profile to that shown in Fig. 3.25 was obtained.

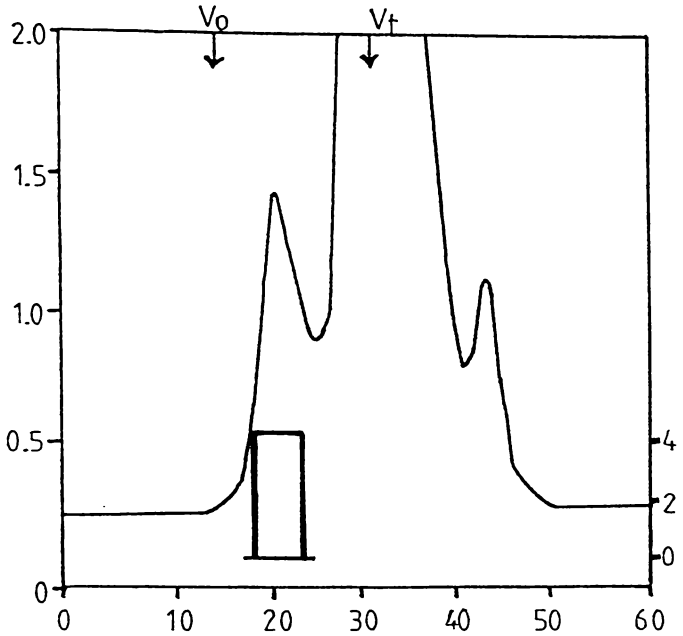


Fig. 3.23: Gel Filtration Chromatography of Fractions 41-56 from Fig. 3.20
Gel: Sephadex G-15
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 41-56 from Fig. 3.20 (5 ml)
Fraction Volume: 4 ml

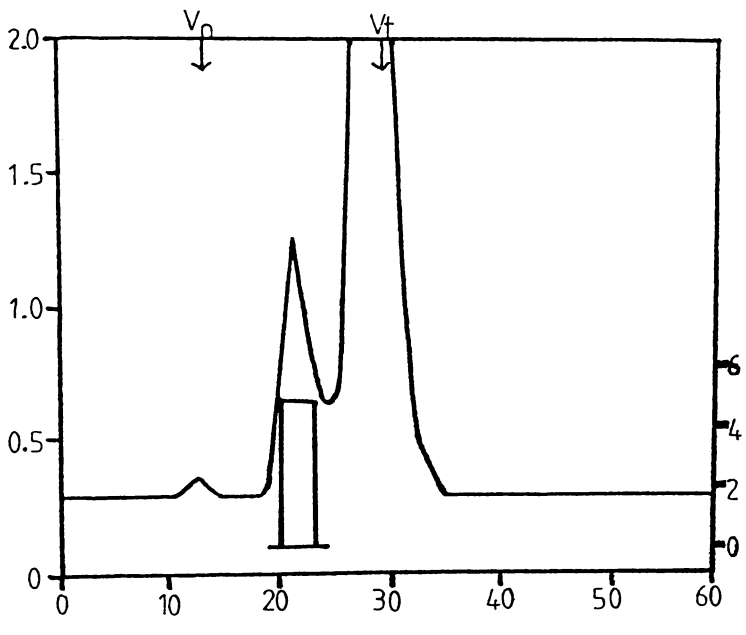


Fig. 3.24: Gel Filtration Chromatography of Fractions 41-56 from Figs. 3.21 and 3.22
Gel: Sephadex G-15
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 41-56 from Figs. 3.21 and 3.22 (5 ml)
Fraction Volume: 4 ml

However, more aggregation was obtained when the 3 kDa peak with antibacterial activity (fractions 20 - 30 as shown in Fig. 3.20) was evaporated down to 5 ml by rotary evaporation, incubated at 37°C for 2 h, and then chromatographed through a column of Sephadex G-200F at pH 7.0. As can be seen in Fig. 3.26, the sample re-aggregated up to a molecular weight estimated to be about 500 kDa (fractions 18 - 27, Fig. 3.26). Again, the broad nature of the peak could have been due to continuous aggregation and/or disaggregation of the sample as it ran through the column.

In order to see the re-aggregation of the active 3 kDa peak (fractions 20 - 30) as shown in Fig. 3.21, the pooled fractions were neutralised, concentrated down to about 5 ml by rotary evaporation, incubated at 37°C for 2 h, and then chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. A similar elution pattern to that shown in Fig. 3.26 was obtained (see Fig. 3.27).

In order to study the re-aggregation of the active 2 kDa peak shown in Fig. 3.22 (fractions 25 - 35), the fractions making up the peak were pooled and concentrated down to about 5 ml by rotary evaporation. The sample was incubated at 37°C for 2 h and then chromatographed through a column of Sephadex G-200F at pH 7.0. The results are shown in Fig. 3.28. The active 2 kDa peak of fig 3.22 re-aggregated up to the void volume on Sephadex G-200F indicating a molecular weight of 600 kDa or over. That aggregate was even larger than the original aggregate of 500 kDa (fractions 16 - 22, Fig. 3.5), that the sample came from originally.

In order to see the re-aggregation of the active 1.2 kDa peak shown in Fig. 3.23, fractions 18 - 25 (Fig. 3.23) were pooled and concentrated down to 10 ml by rotary evaporation. The pH of a 5 ml sample was adjusted to 7.0 with NaOH and incubated at 37°C for 2 h. The sample was then chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. The results are shown in Fig. 3.29. There was no aggregation seen, at least beyond the inclusion limit of Sephadex G-200F, as the sample eluted on the bed volume (indicating a molecular weight of 5 kDa or lower).

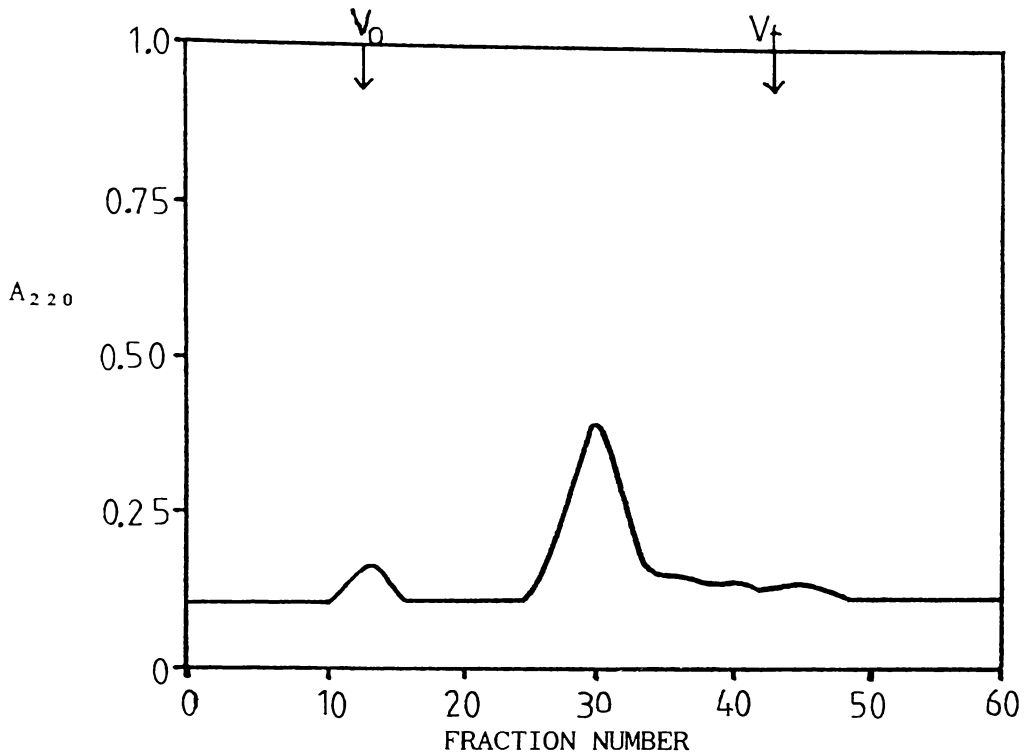


Fig. 3.25: Gel Filtration Chromatography of Fractions 20-30 from Fig. 3.19
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of the concentrate of fractions 20-30 from Fig. 3.19 (5 ml)
Fraction Volume: 4 ml

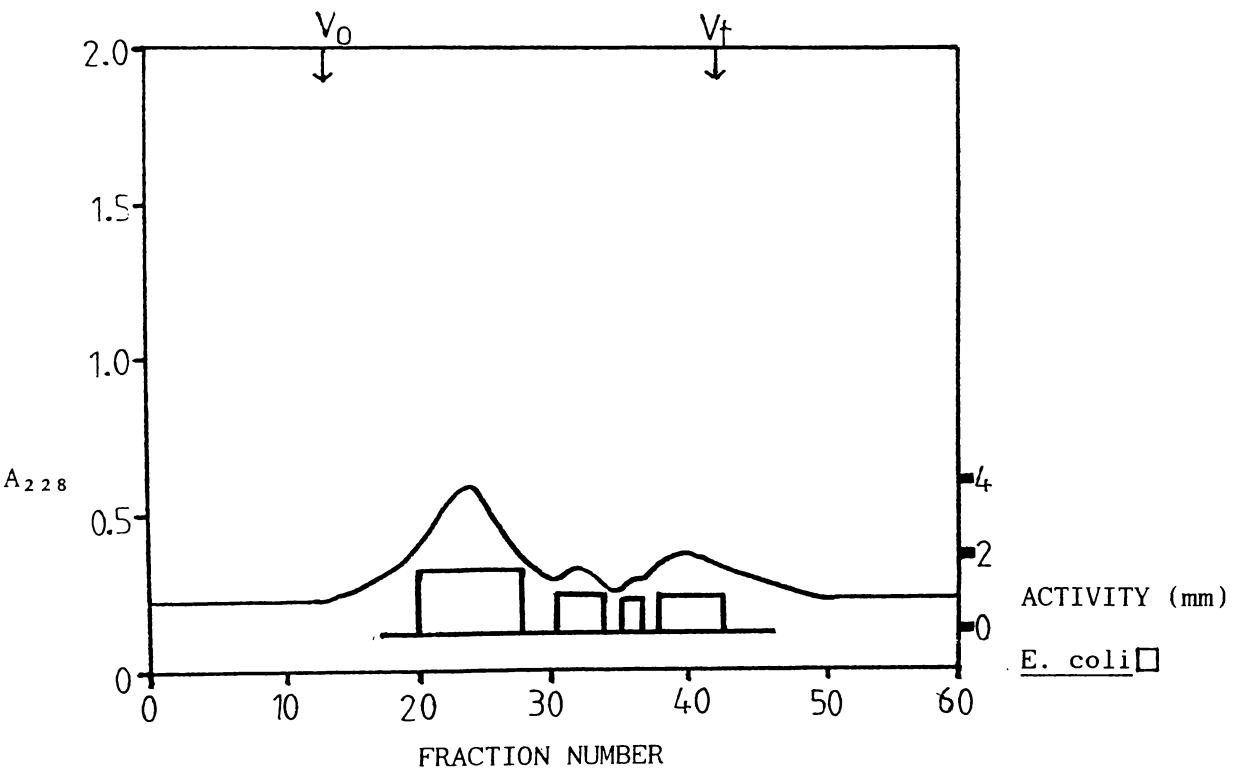


Fig. 3.26: Gel Filtration Chromatography of Fractions 20-30 from Fig. 3.20
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Concentrate of fractions 20-30 from Fig. 3.20 (5 ml)
Fraction Volume: 4 ml

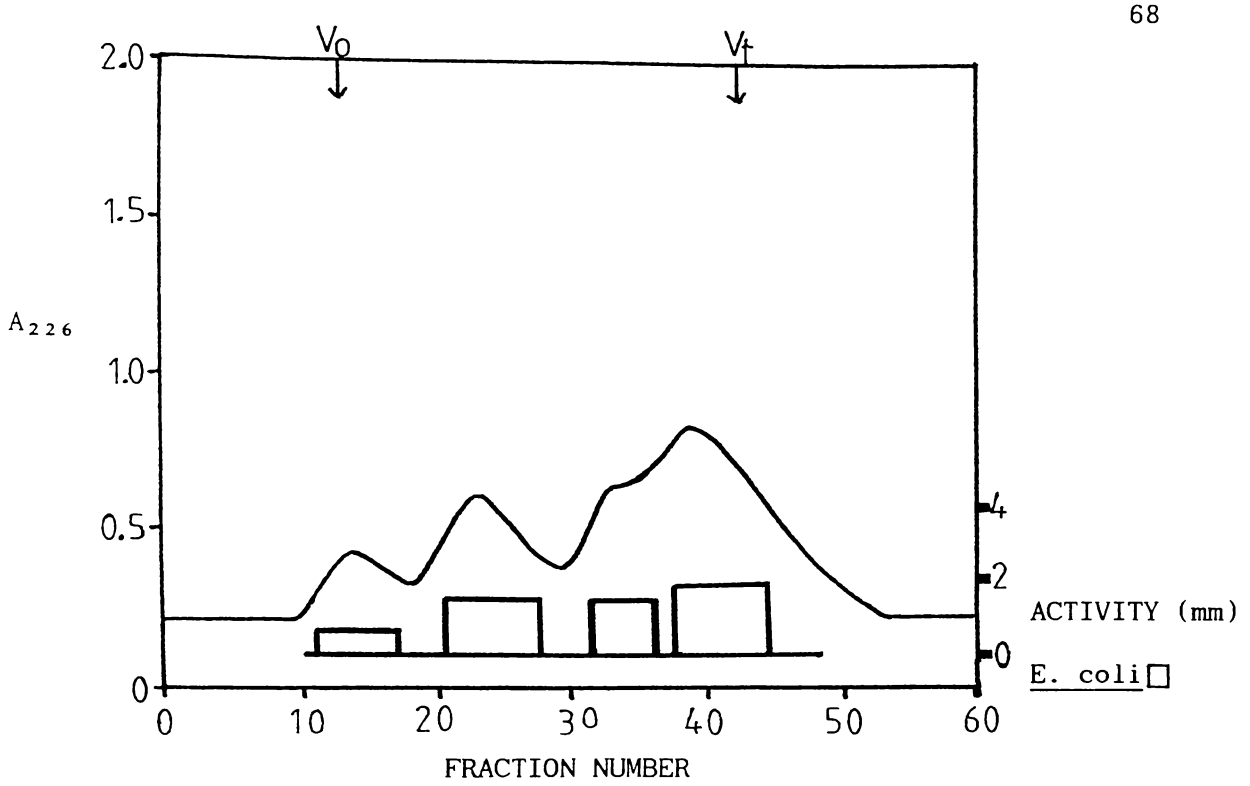


Fig. 3.27: Gel Filtration Chromatography of Fractions 20-30 from Fig. 3.21
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Concentrate of fractions 20-30 from Fig. 3.21 (5 ml)
Fraction Volume: 4 ml

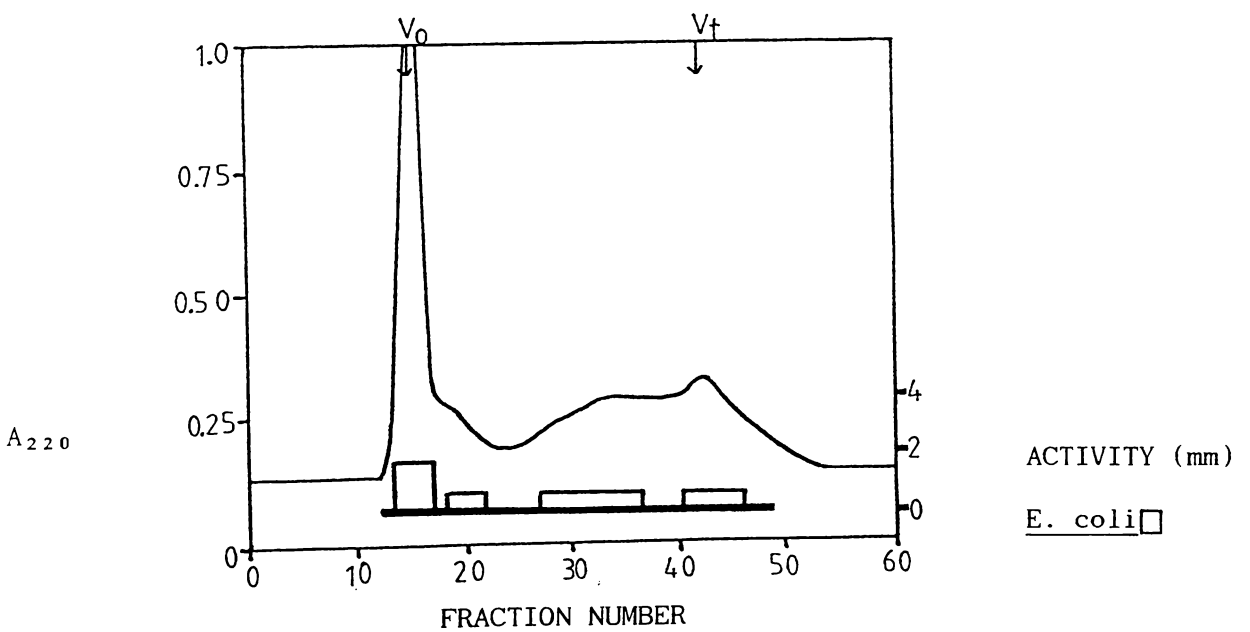


Fig. 3.28: Gel Filtration Chromatography of Fractions 25-38 from Fig. 3.22
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris_HCl, pH 7.0
Sample: Concentrate of fractions 25-38 from Fig. 3.22
(5 ml)
Fraction Volume: 4 ml

In order to see whether the active 1.2 kDa peak of Fig. 3.23 had formed a small aggregate, the pH of the remaining part of the concentrate was raised to 7.0 with NaOH, and then a sample of 200 μl was chromatographed on a column of Superose at pH 7.0. The sample was eluted at the bed volume, indicating a molecular weight of about 1 kDa or lower (Fig. 3.30).

The fractions making up the 1.2 kDa active peak (fractions 18 - 25) as shown in Fig. 3.24 were pooled and evaporated down to about 10 ml by rotary evaporation and the pH of a 5 ml sample was raised to 7.0 with NaOH. The sample was incubated at 37°C for 2 h, and then chromatographed through a column of Sephadex G-200F at pH 7.0. The sample, like the sample of the active 1.2 kDa peak shown in Fig. 3.29, was eluted at the bed volume. The pH of the remaining part of the concentrate was also adjusted to 7.0 with NaOH, incubated at 37°C for 2 h and then a sample of 200 μl was chromatographed through a column of Superose 12 at pH 7.0. Again, the sample was eluted at the bed volume, indicating a molecular weight of about 1 kDa or lower.

Conclusions

The cation exchange chromatography seemed to have caused at least partial disaggregation, as the sample before the ion exchange chromatography was of 20 kDa molecular weight, but after it no peak larger than 3 kDa was obtained. At least part of the 20 kDa aggregate completely disaggregated giving the active component. It was clearly evident from the results described in the preceding sections that the 500 kDa aggregate (from which the 20 kDa aggregate was obtained [Fig. 3.10]) was an aggregate made up of small component(s).

The active 1.2 kDa peaks eluted in fractions 18 - 25 in Figs. 3.23 and 3.24 appeared to be the same antibacterial component responsible for the formation of active aggregates. The active component seemed to be basic in nature because it was obtained in increasing amounts at the end of salt and pH gradient on a cation exchanger. The active component was readily dissolved in aqueous solutions at both neutral and acidic pH, thus indicating the hydrophilic nature of the component. But as the 1.2 kDa active peak did not form any aggregate at neutral pH (Figs. 3.29 and 3.30), it was anticipated that component(s) other than the active component would probably be involved in the aggregation.

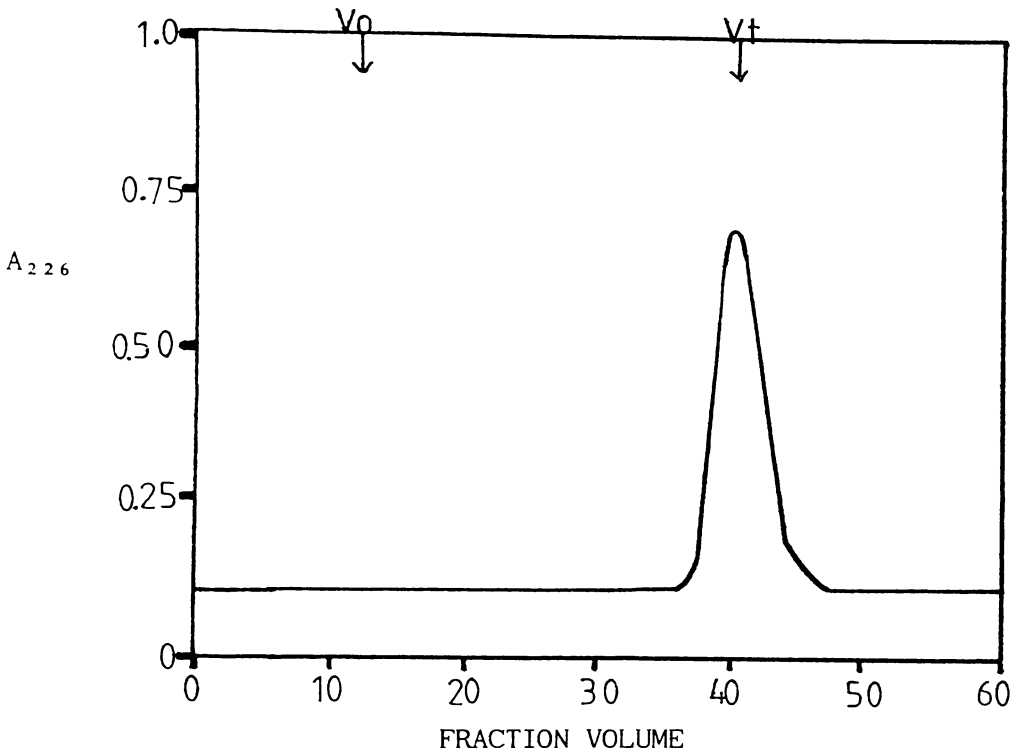


Fig. 3.29: Gel Filtration Chromatography of Fractions 18-25 from Fig. 3.23
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 18-25 from Fig. 3.23 (5 ml)
Fraction Volume: 4 ml

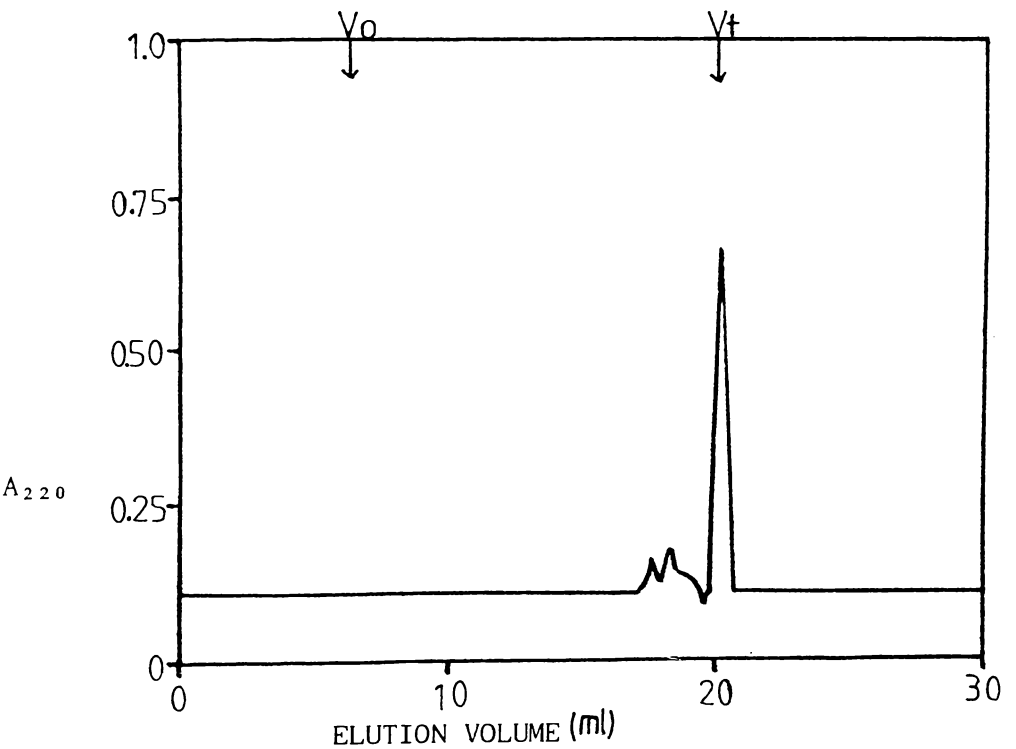


Fig. 3.30: FPLC of Fractions 18-25 from Fig. 3.23
Gel: Superose 12
Buffer: 0.1 mol/l phosphate buffer, pH 7.0
Sample: Part of concentrate of fractions 18-25 from Fig. 3.23 (200 µl)

The 3 kDa inactive peak (fractions 20 - 30, Fig. 3.19) formed aggregate at pH 7.0 (Fig. 3.25). But it was thought that the peak could either be an inactive aggregating component in homogenous form, or still a small aggregate with component(s) other than the aggregating component(s) present in it. The broad shape of the peak (fractions 20 - 30, Fig. 3.19) did not suggest that it was an inactive aggregating component in its homogenous form. Also, as active peaks capable of aggregation, with a molecular weight of 3 kDa were achieved from different peaks of ion exchanger (fractions 20 - 30, Figs. 3.20 and 3.21), and the size of the free active component was found to be 1.2 kDa (Figs. 3.23 and 3.24), therefore the inactive aggregating component(s) was expected to be of molecular weight less than 3 kDa. Thus, it was thought that the 3 kDa inactive aggregating peak (fractions 20 - 30) of Fig. 3.19 was probably still a small aggregate rather than the inactive aggregating component in its homogenous form. It possibly also contained the active component but in too small an amount to be picked up in the activity assay.

It was thought that the aggregating component was possibly hidden under the low molecular weight citrate salt peaks (fractions 26 - 45, Fig. 3.23 and fractions 26 - 40, Fig. 3.24), and attempts were made to isolate the aggregating component(s) from the salt peaks.

Attempts to Identify and Isolate the Inactive Component(s)

In order to see whether the aggregating component(s) was hidden under the salt peaks in Fig. 3.23, fractions making up the salt peak (fractions 25 - 40, Fig. 3.23) were pooled and concentrated down to 10 ml by rotary evaporation. Salt crystals were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. About 2 ml of the clear supernatant was diluted to 5 ml by addition of 0.1 mol/l Tris-HCl buffer, pH 7.0. The pH of the sample was raised to 7.0 with NaOH. The sample was incubated at 37°C for 2 h and then chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. The sample was eluted at the bed volume of Sephadex G-200F, showing no aggregation had occurred to a molecular weight greater than 5 kDa.

It was thought that the aggregating component(s) may require the active component to form aggregates. In order to check this

possibility, 2 ml each of the active component (fractions 18 - 25, Fig. 3.23) and supernatant of the salt peak (fractions 25 - 40, Fig. 3.23) were pooled together. The sample was diluted to 5 ml by the addition of 0.1 mol/l Tris-HCl buffer, pH 7.0, and its pH was adjusted to 7.0 with NaOH. After incubating the sample at 37°C for 24 h, it was passed through the column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer, pH 7.0. Again, the sample eluted on the bed volume, indicating no aggregation. Shannon *et al.* (1987) reported the binding of citrate with cationic peptides in seminal plasma, thus an explanation is that the binding of citrate with the aggregating component(s) prevented re-aggregation.

Attempts to Separate the Aggregating Component from the Citrate Peak

It was thought to be necessary to try to separate the aggregating component(s) from the citrate peak if it was hidden under it. A column of Sephadex G-10 was used to separate the aggregating component(s) from the citrate peak.

Accordingly, half of the pool making up the last two peaks (fractions 25 - 45) of Fig. 3.23 was concentrated down to about 10 ml by rotary evaporation and crystals of citrate were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. The pH of 5 ml of the clear sample was re-adjusted to 1.7 with HCl, and then chromatographed through a column of Sephadex G-10 eluted with 0.02 mol/l HCl, pH 1.7. As can be seen in Fig. 3.31, the sample was eluted mainly at the bed volume as a single peak. Therefore, the column of Sephadex G-10 seemed to be of no use for isolating the aggregating component(s) from the citrate peak.

A column of ion retardation resin was also used in another attempt to separate the aggregating component(s) from the citrate peak. The pH of the remaining 5 ml pool of fractions 25 - 45 in Fig. 3.23 was raised to 7.0 with NaOH. The sample was passed through a column of Dowex ion retardation resin-AG11A8 and eluted with distilled water. The results are shown in Fig. 3.32. The sample eluted as one peak. In order to find the elution volume of citrate from the column, about 5 ml 1 mol/l citrate buffer, pH 7.0, was also passed through the same column and eluted with distilled water. The citrate peak was eluted only one

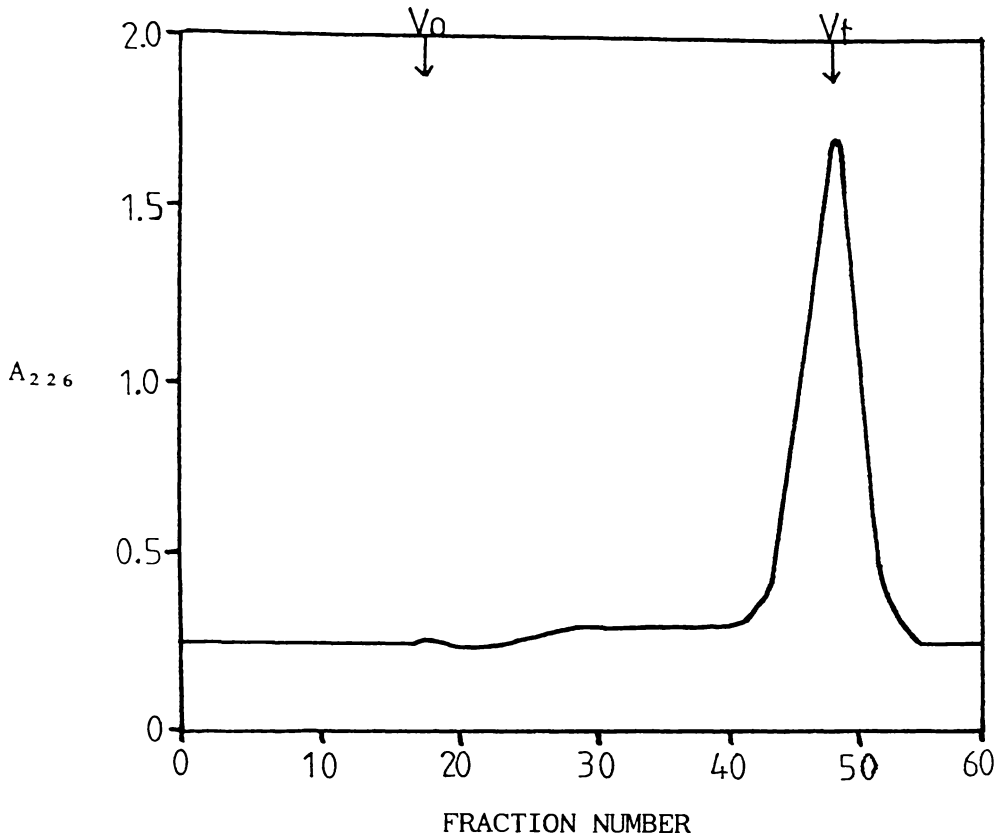


Fig. 3.31: Gel filtration Chromatography of Fractions 25-45 from Fig. 3.23
Gel: Sephadex G-10
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 25-45 from Fig. 3.23 (5 ml)
Fraction Volume: 4 ml

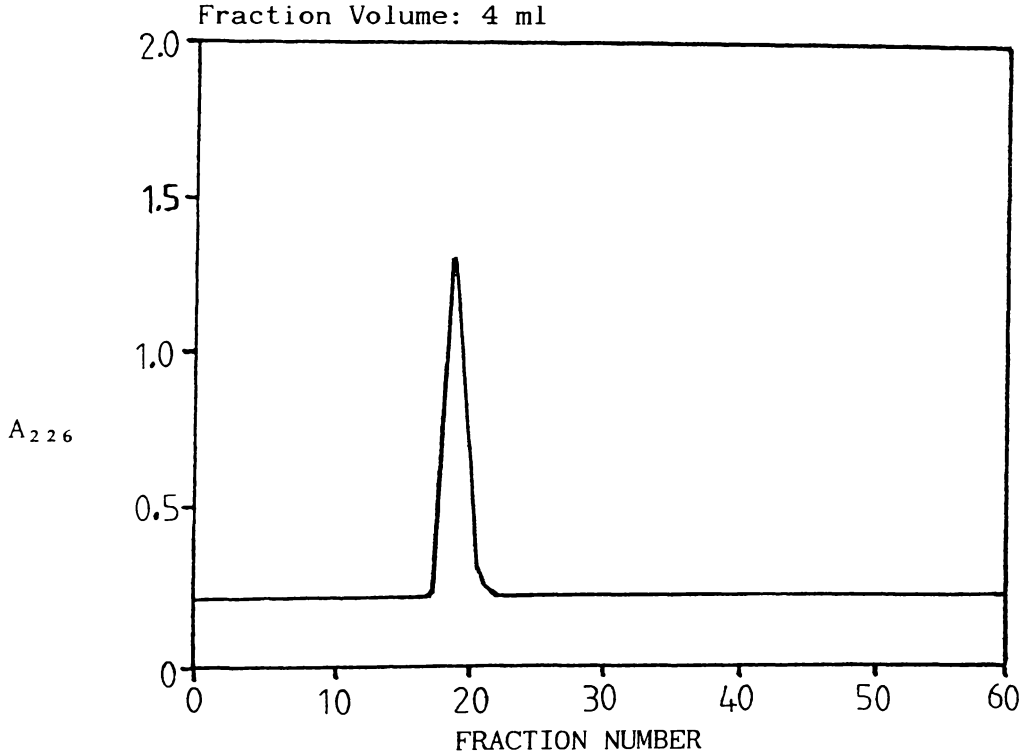


Fig. 3.32: Ion Retardation Chromatography of Fractions 25-40 from Fig. 3.23
Gel: Dowex Ion Retardation Resin AG11A8
Eluent: Distilled water
Sample: Part of concentrate of fractions 25-45 from Fig. 3.23 (5 ml)
Fraction Volume: 4 ml

fraction earlier than the sample of Fig. 3.32. This could either have been an experimental error or because of the fact that the aggregating component(s) was itself retarded by the resin. Thus, the column of ion retardation also appeared to be of no use for isolating the aggregating component(s) from the citrate peak.

When these experiments of the column of Sephadex G-10 and Dowex ion retardation resin-AG11A8 were repeated with fractions 25 - 40 of Fig. 3.24. identical results to those shown in Figs. 3.31 and 3.32 were obtained.

Conclusions

Unfortunately, all attempts to isolate the aggregating component(s) from citrate peak using Sephadex G-10 and Dowex ion retardation resin-AG11A8 failed. Therefore, in order to obtain the aggregating component(s), another system, anion exchange chromatography, was used because the aggregating inactive 3 kDa peak (fractions 20 - 30, Fig. 3.19) which seemed to be a small aggregate made up at least predominantly of aggregating component(s) (as it was inactive in nature) had passed straight through the cation exchanger at pH 3.0, thus indicating the acidic nature of the inactive aggregating component(s).

Disaggregation of the 250 kDa Aggregate

Introduction

As described in the preceding sections, the combination of acidic gel filtration and the cation exchange chromatography disaggregated the 500 kDa aggregate. Therefore, to confirm that the 250 kDa aggregate of Fig. 3.5 is also an aggregate made up of the same small components which make the 500 kDa aggregate, and to study the components of the 250 kDa aggregate, the 250 kDa aggregate was also disaggregated using acidic gel filtration and cation exchange chromatography.

Disaggregation of the 250 kDa Aggregate Using Acidic Gel Filtration Chromatography

It was decided to chromatograph the 250 kDa aggregate through a column of Sephadex G-200F at pH 1.7. Accordingly, fractions 23 - 28 as shown in Fig. 3.5 from repeated runs were pooled together, and concentrated down to about 10 ml by rotary evaporation. The pH of the sample was lowered to 1.7 with HCl, ~~a~~ precipitate formed, like with the sample of the 500 kDa aggregate, at around pH 4.5. The precipitate was removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. A part of the precipitate was re-dissolved in 0.1 mol/l Tris-HCl buffer, pH 7.0. The pH of part of the supernatant was adjusted to 7.0 with NaOH. Both samples were tested for antibacterial activity and were found to be active.

A 5 ml sample of the supernatant was held at pH 1.7 at 4°C for 2 h, and then chromatographed through a column of Sephadex G-200F eluted with 0.02 mol/l HCl, pH 1.7. As can be seen in Fig. 3.33, all activity had disaggregated to about 20 kDa molecular weight. The experiment was repeated once and results similar to those shown in Fig. 3.33 were obtained.

The inactive peak (fractions 22 - 28) in Fig. 3.33 could either have been a carrier protein of antibacterial activity, or an inactive high molecular weight protein just co-eluting with the 250 kDa aggregate. In order to check this, fractions 38 - 51 as shown in Fig. 3.33, were concentrated down to about 10 ml by rotary evaporation. A 1 ml sample of this was diluted to 5 ml by addition of 0.1 mol/l Tris-HCl buffer, pH 7.0, and its pH was raised to 7.0 with NaOH. The sample was incubated at 37°C for 2 h, and then chromatographed through a column of Sephadex G-200F at pH 7.0. As can be seen in Fig. 3.34, the sample re-aggregated, indicating that inactive peak (fractions 22 - 28) of Fig. 3.33 was an inactive protein co-eluting with the 250 kDa aggregate rather than a carrier protein necessary for the formation of the aggregates.

These results were identical to those obtained with the 500 kDa aggregate earlier. This, however, was not surprising as the 500 kDa active peak formed the 250 kDa active peak, and the 250 kDa active peak formed the 500 kDa active peak, when each was re-chromatographed

through the column of Sephadex G-200F at pH 7.0. Also, both the peaks had shown similar electrophoresis patterns.

In order to study the precipitate formed at pH 4.5, a part of the precipitate was taken and re-dissolved in 5 ml 0.02 mol/l HCl, pH 1.7. The sample was held at 4°C for 2 h, and then chromatographed through a column of Sephadex G-200F eluted with 0.02 mol/l HCl, pH 1.7. Results identical to those shown in Fig. 3.33 were obtained. When the active low molecular weight peak was re-chromatographed through a column of Sephadex G-200F at pH 7.0, again, results identical to those shown in Fig. 3.34 were obtained.

Both the elution and activity profiles of the precipitate and the supernatant were identical and also both were active. Thus, it was concluded that both the supernatant and the precipitate contained the same components. The supernatant was active and acid-disaggregated peak (Fig. 3.33) reformed the active aggregates at pH 7.0 (Fig. 3.34), indicating that the supernatant had all the components required for the formation of active aggregates.

Therefore, further disaggregation on the 250 kDa aggregate was carried out with the supernatant. It was decided that when a model disaggregation method was developed, both the precipitate and the supernatant would be disaggregated using the same method to confirm that they both are made up of the same components.

Disaggregation of the 20 kDa Aggregate Obtained from the 250 kDa Aggregate Using Cation Exchange Chromatography

Since cation exchange chromatography disaggregated the 20 kDa aggregate from the 500 kDa aggregate, the 20 kDa aggregate from the 250 kDa aggregate was also submitted to cation exchange chromatography.

To make the sample 0.1 mol/l citrate, pH 3.0, 1 ml 1.0 mol/l citrate buffer, pH 3.0, was added to 9 ml concentrate of fractions 38 - 51 of Fig. 3.33 and the pH of the sample was adjusted to 3.0 with NaOH. After holding the sample at 4°C for 4 h, it was applied to the column of SP-Sephadex C-50 ion exchanger, and followed with 0.1 mol/l citrate buffer, pH 3.0, until the absorbance at 230 nm of the effluent had returned to the baseline. The next stage of the elution was achieved

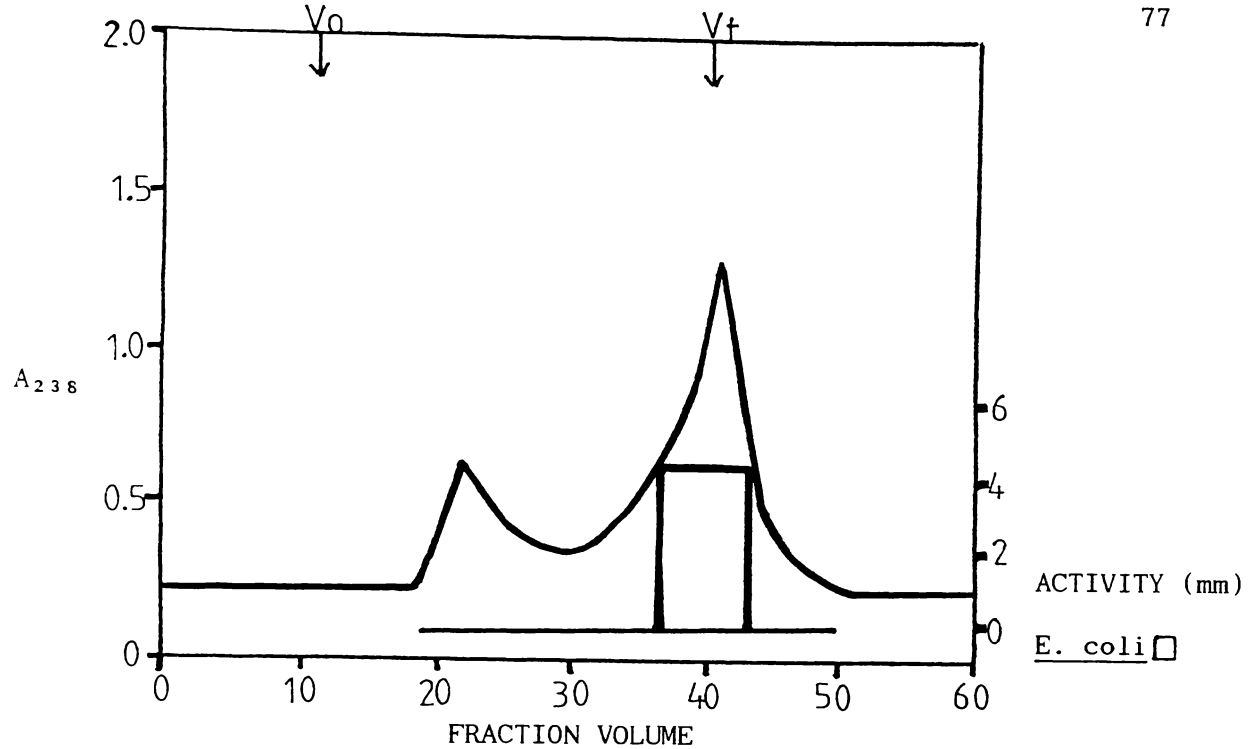


Fig. 3.33: Gel Filtration Chromatography of Fractions 23-28 from Fig. 3.5
Gel: sephadex G-200F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 23-28 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

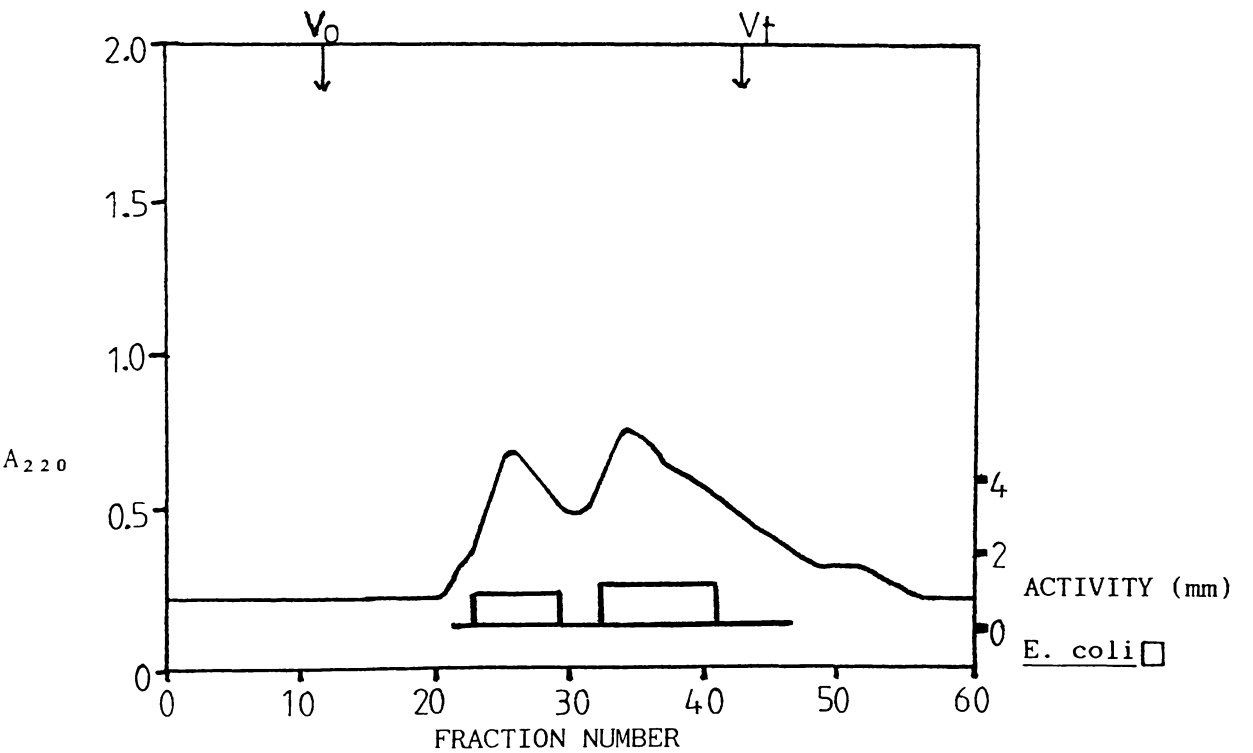


Fig. 3.34: Gel Filtration Chromatography of Fractions 38-51 from Fig. 3.33
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 38-51 from Fig. 3.33 (5 ml)
Fraction Volume: 4 ml

with a continuous salt gradient from 0 to 4.0 mol/l NaCl in 0.1 mol/l citrate buffer, pH 3.0. This was followed by a continuous pH gradient from 0.1 mol/l citrate buffer, pH 3.0, containing 4.0 mol/l NaCl to 0.1 mol/l citrate buffer, pH 8.0, containing 4.0 mol/l NaCl. Finally, a continuous pH gradient from 0.1 mol/l citrate buffer, pH 8.0, containing 4.0 mol/l NaCl to 0.1 mol/l NaOH, pH 13.0, containing 4.0 mol/l NaCl was used. To minimize any loss of activity due to high pH, the peaks eluting with high pH gradient at pH 9.0 and over were neutralised as soon as they eluted. As can be seen in Fig. 3.35, an elution profile similar to that shown in Fig. 3.18 was obtained.

Gel filtration chromatography of the peaks from the cation exchange chromatography (Fig. 3.35) at acidic pH

Fractions making up different peaks were pooled, as shown in Fig. 3.35. The antibacterial activity of different peaks could not be tested because of the high amount of salt present in these peaks. Pools A - D of Fig. 3.35 were separately evaporated down to about 5 ml each by rotary evaporation and the excess salt crystals were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. The pH of the clear supernatant solutions was lowered to 1.7 by the addition of HCl, samples were held at 4°C for 2 h and then, to see whether the cation exchange chromatography had caused any disaggregation, samples were chromatographed separately through a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Figs. 3.36 to 3.39.

The molecular weights of different peaks were estimated from the calibration curve of standards run earlier through the column. The material which passed straight through the cation exchanger at pH 3.0 (Pool A of Fig. 3.35) gave an inactive peak of about 3 ($\pm$ 0.3) kDa molecular weight (fractions 20 - 30) and a large peak at the bed volume which seemed to be a citrate salt peak (Fig. 3.36).

As can be seen in Fig. 3.37, the material eluted off the cation exchange column with the salt gradient at pH 3.0 (Pool B of Fig. 3.35), formed an active 3 ($\pm$ 0.3) kDa peak, an active peak of about 1.2 ($\pm$ 0.12) kDa and a citrate peak at the bed volume.

Pool C of Fig. 3.35 also formed active peaks of 3 ($\pm$ 0.3) kDa and

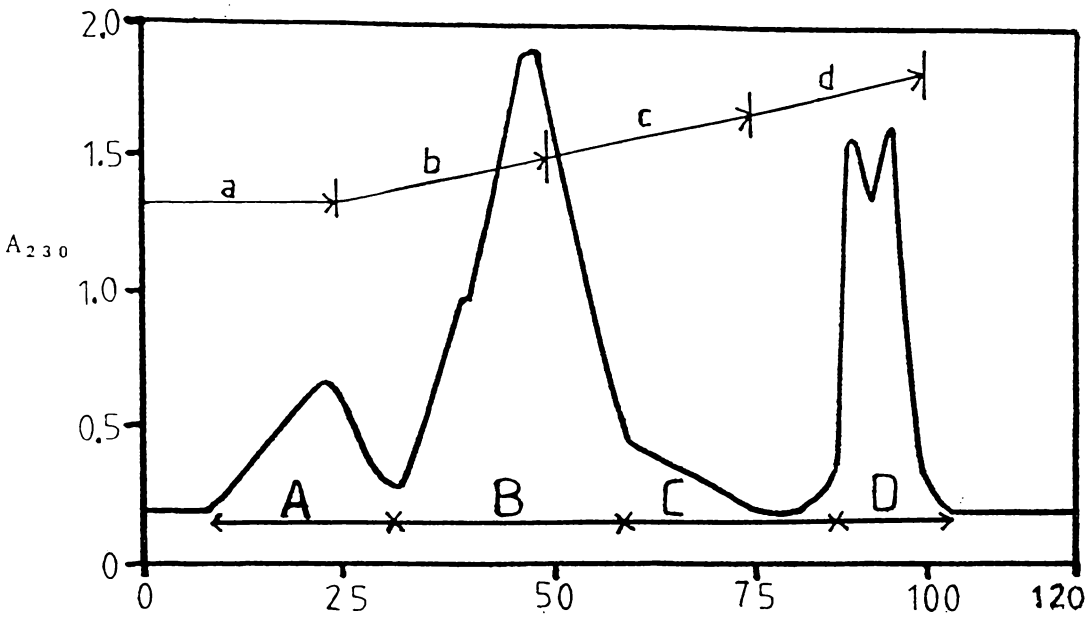


Fig. 3.35: Ion Exchange Chromatography of Fractions 38-51 from Fig. 3.33
Gel: SP-Sephadex C-50
Sample: Part of concentrate of fractions 38-51 from Fig. 3.33 (10 ml)
Elution Buffers: (a) 0.1 mol/l citrate buffer, pH 3.0; (b) salt gradient from 0 to 4.0 mol/l NaCl in citrate buffer, pH 3.0; (c) pH gradient from 0.1 mol/l citrate buffer, pH 3.0 containing 4.0 mol/l NaCl to 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl; (d) pH gradient from 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl to 0.1 mol/l NaOH, pH 13.0 containing 4.0 mol/l NaCl
Fraction Volume: 4 ml

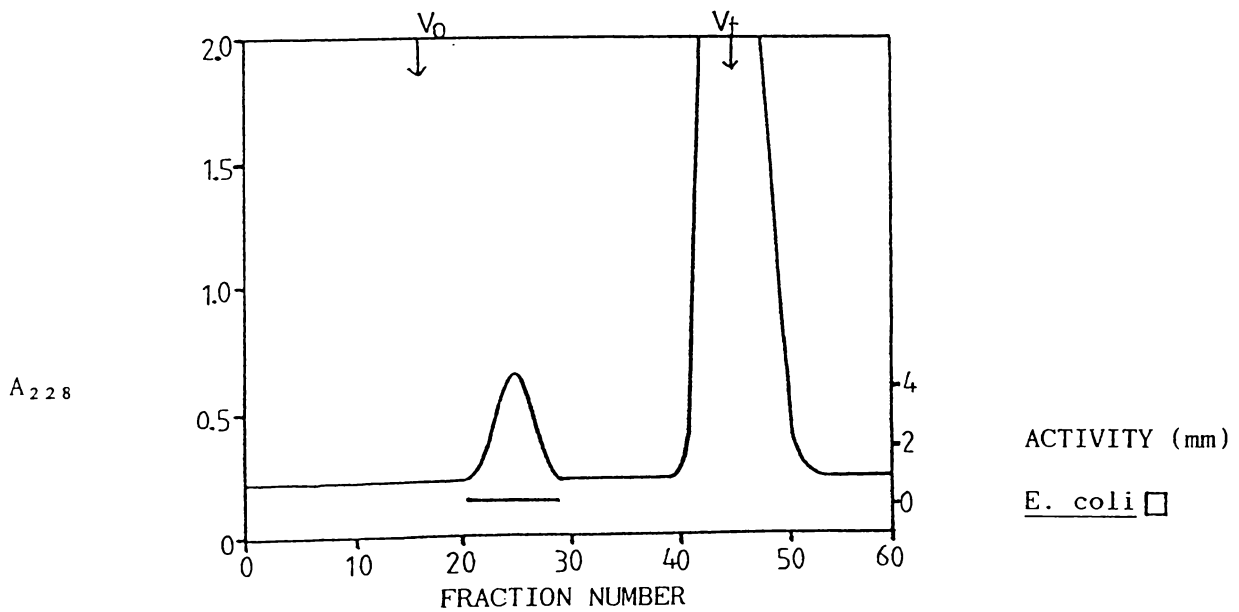


Fig. 3.36: Gel filtration Chromatography of Pool A from Fig. 3.35
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool A of Fig. 3.35 (5 ml)
Fraction Volume: 4 ml

1.2 (± 0.12)kDa and a large peak at the bed volume, which was probably citrate (Fig. 3.38).

Pool D of Fig. 3.35 formed active peaks of about 2 (± 0.2) kDa and 1.2 (± 0.12) kDa. There was also a large peak at the bed volume made up at least predominantly of citrate (Fig. 3.39).

In order to achieve better separation of the low molecular weight peaks of Fig. 3.36 to 3.39, it was decided to chromatograph these peaks through a column of Sephadex G-15 at pH 1.7. Accordingly, fractions 40 - 56 as shown in Fig. 3.36 (the material which passed straight through the cation exchanger at pH 3.0) were pooled together and evaporated down to about 5 ml by rotary evaporation. Salt crystals were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes and the pH of the sample was adjusted to 1.7 with NaOH. After holding the sample at 4°C for 2 h, it was chromatographed through a column of G-15 eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.40.

Similarly, fractions 40 - 56 from the material retained on the cation exchanger (Figs. 3.37 to 3.39) were pooled all together and concentrated to 5 ml by rotary evaporation. The salt crystals were removed by centrifugation at 6,000 g (r av.10 cm) and the pH of the supernatant was re-adjusted to 1.7 with NaOH. The sample was held at 4°C for 2 h and then chromatographed through a column of Sephadex G-15 eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.41.

The molecular weight of the peak eluted in the fractions 18 - 25 in Fig. 3.40 was estimated to be about 1.2 kDa from the calibration curve made by running standards through the column earlier. This peak was not active and came from the sample that passed straight through the cation exchanger at pH 3.0, indicating an acidic nature.

Gel filtration chromatography at neutral pH to study re-aggregation

In order to see whether this was the inactive aggregating component, fractions 18 - 25 of Fig. 3.40 were pooled and concentrated down to 10 ml by rotary evaporation. The pH of a 5 ml sample of this was raised to 7.0 with NaOH, the sample incubated at 37°C for 2 h, and then chromatographed through a column of Sephadex G-200F equilibrated

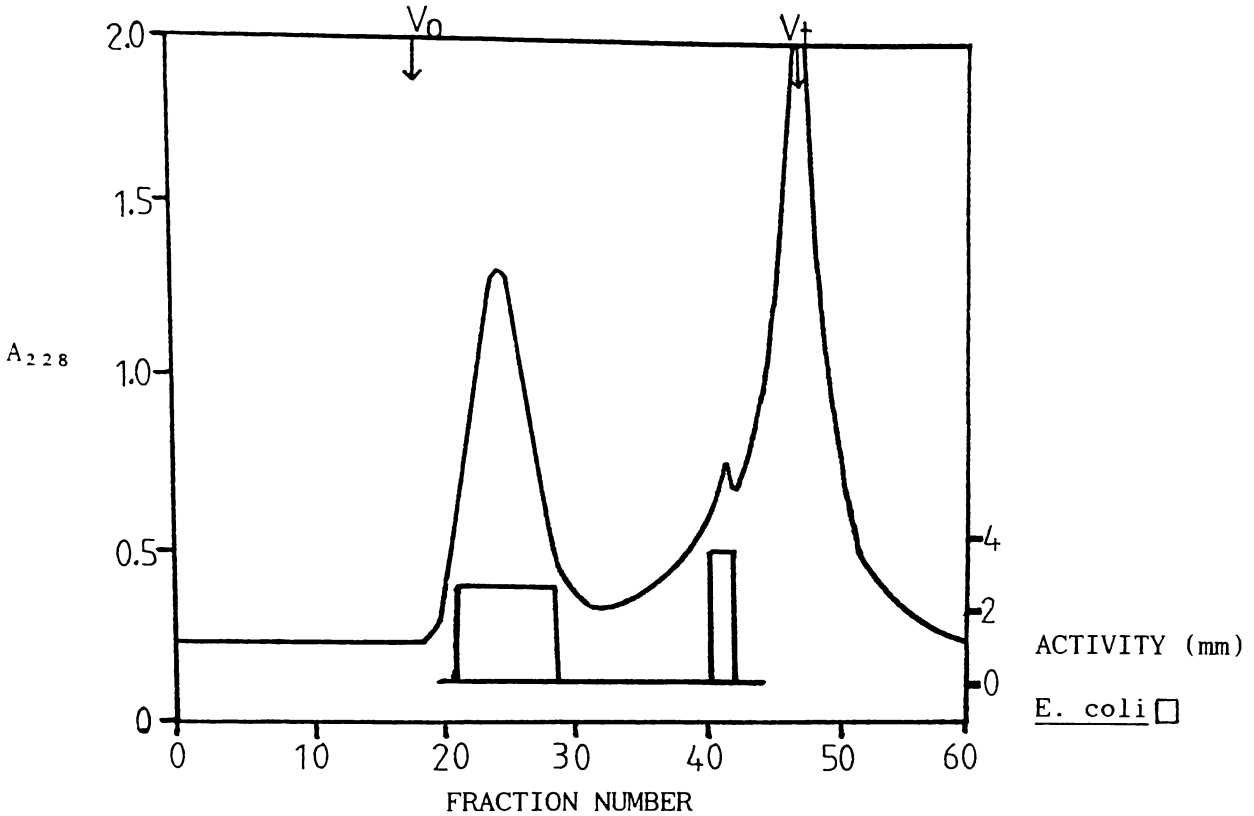


Fig. 3.37: Gel filtration Chromatography of Pool B from Fig. 3.35
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool B of Fig. 3.35 (5 ml)
Fraction Volume: 4 ml

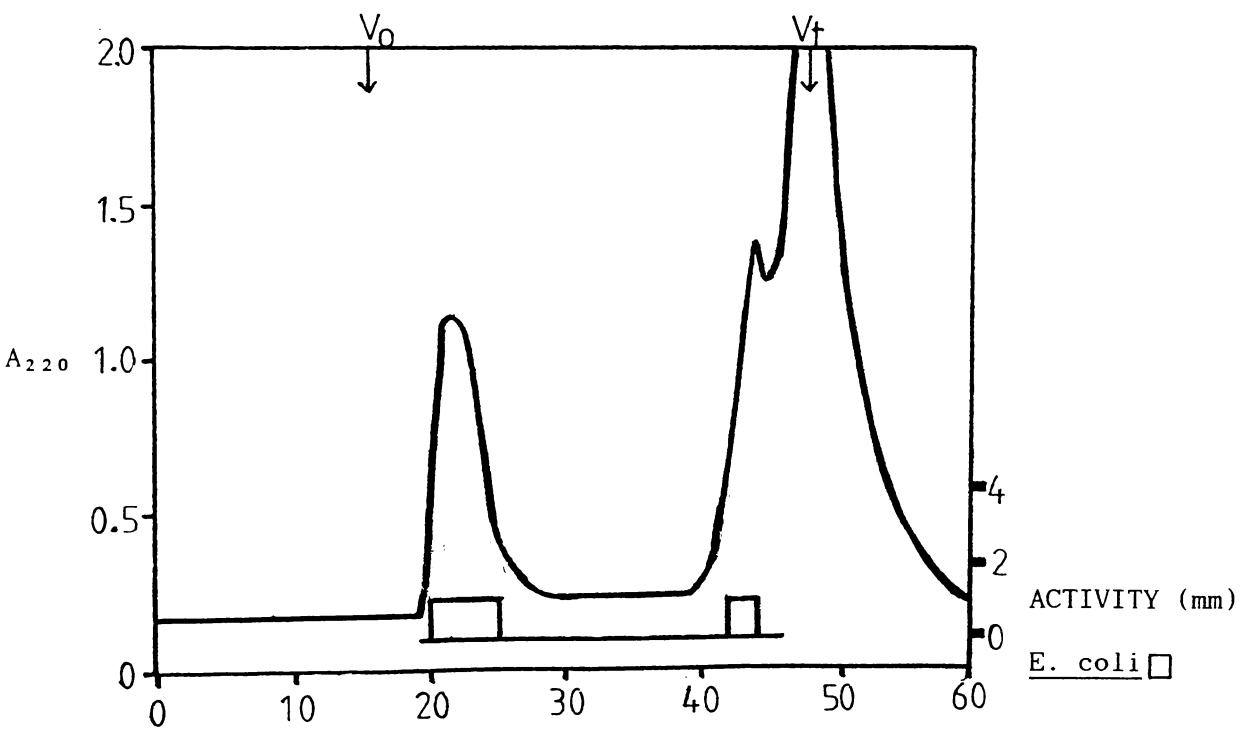


Fig. 3.38: Gel Filtration Chromatography of Pool C from Fig. 3.35
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool C of Fig. 3.35 (5 ml)
Fraction Volume: 4 ml

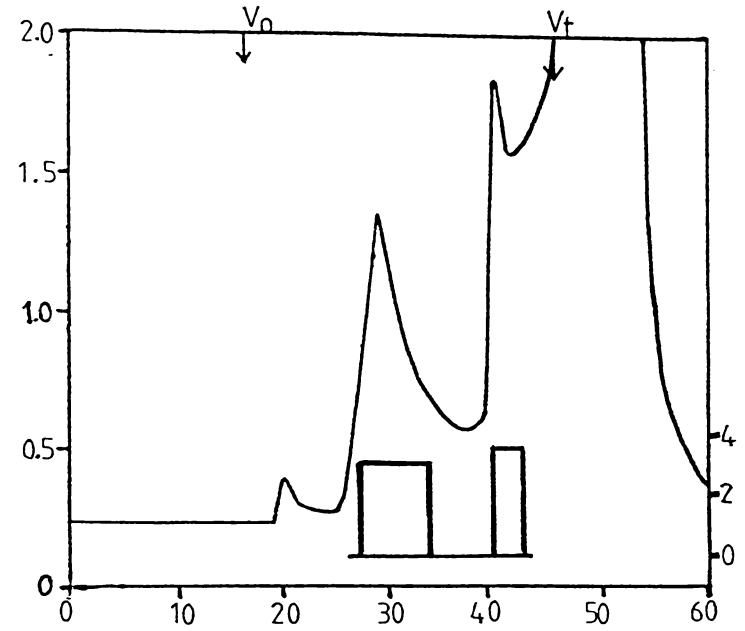


Fig. 3.39: Gel Filtration Chromatography of Pool D from Fig. 3.35
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool D of Fig. 3.35 (5 ml)
Fraction Volume: 4 ml

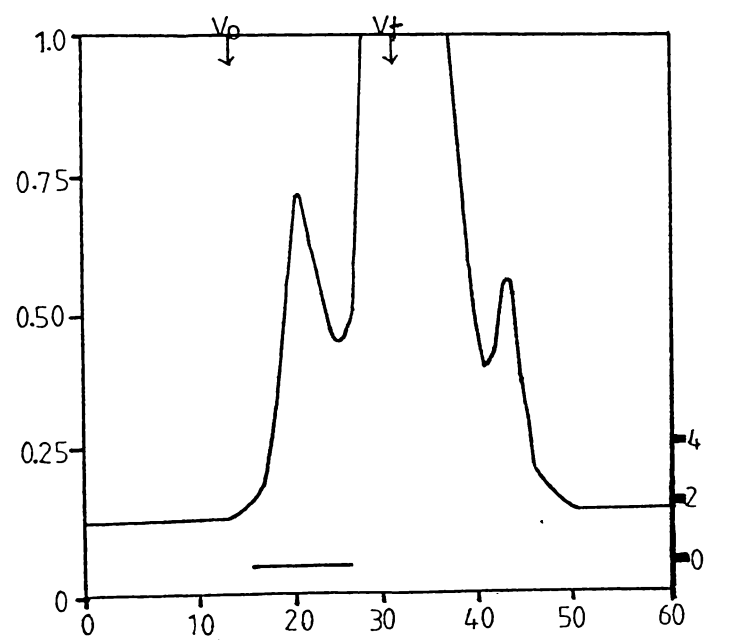


Fig. 3.40: Gel Filtration Chromatography of Fractions 40-56 from Fig. 3.36
Gel: Sephadex G-15
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 40-56 from Fig. 3.36 (5 ml)
Fraction Volume: 4 ml

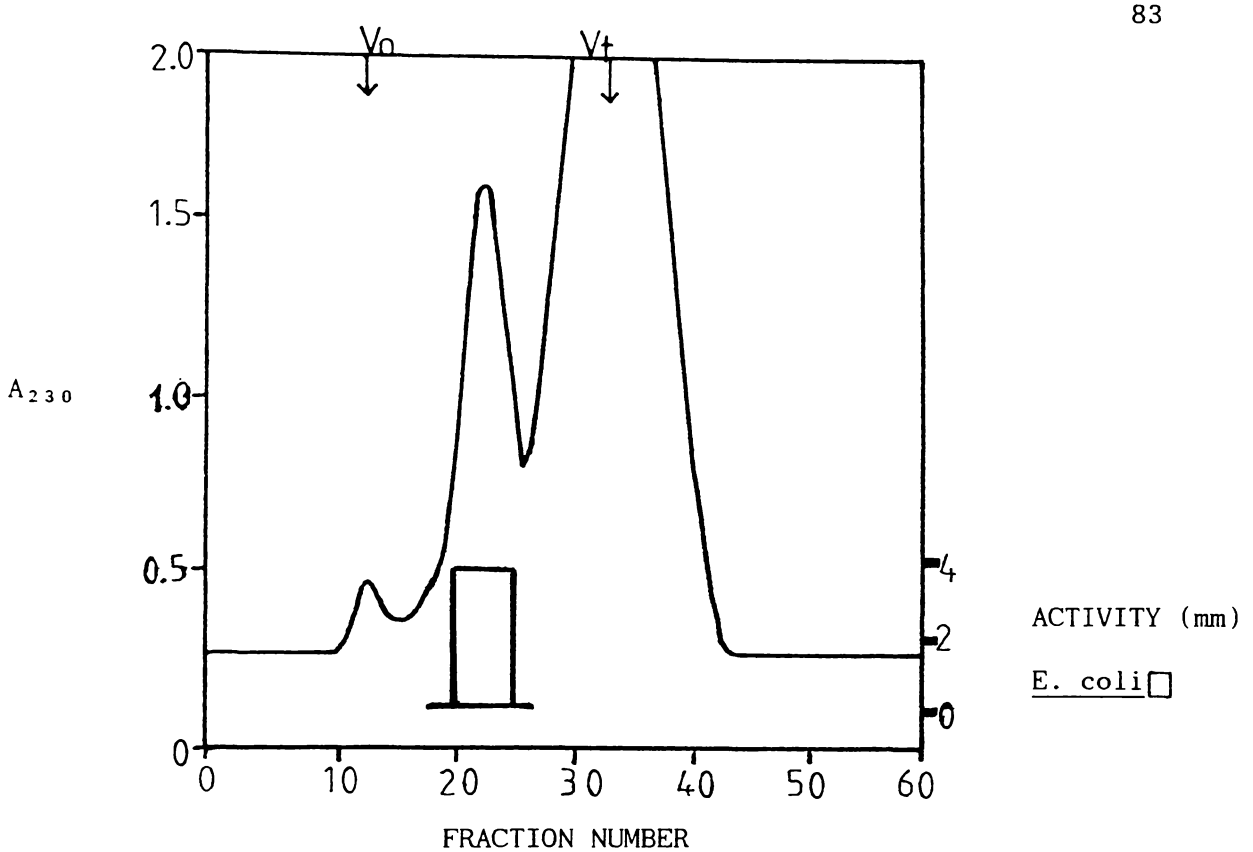


Fig. 3.41: Gel Filtration Chromatography of Fractions 40-56 from Figs. 3.37 to 3.39
Gel: Sephadex G-15
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 40-56 from Figs. 3.37 to 3.39 (5 ml)
Fraction Volume: 4 ml

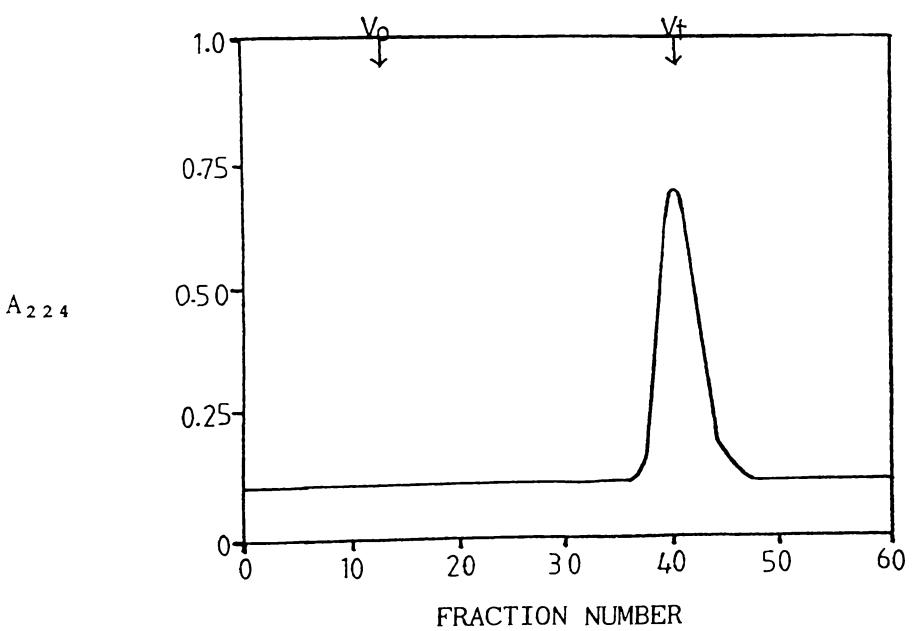


Fig. 3.42: Gel Filtration Chromatography of Fractions 18-25 from Fig. 3.40
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 18-25 from Fig. 3.40 (5 ml)
Fraction Volume: 4 ml

with 0.1 mol/l Tris-HCl buffer, pH 7.0. As can be seen in Fig. 3.42, the sample was eluted only at the bed volume on Sephadex G-200F, thus indicating a molecular weight of 5 kDa or lower, and thus no aggregation up to the high molecular weight.

In order to have a better estimate of the molecular weight of this peak, the pH of the remaining 5 ml sample of fractions 18 - 25 from Fig. 3.40 was adjusted to 7.0 with NaOH, and after incubation at 37°C for 2 h, a 200 µl sample from this was chromatographed through a column of Superose 12 with 0.1 mol/l phosphate buffer, pH 7.0. The results are shown in Fig. 3.43. There was one sharp peak obtained on the bed volume, indicating the molecular weight of the sample to be about 1 kDa and thus no aggregation. The peak eluted in fractions 18 - 25 in Fig. 3.40 was inactive, but of the same molecular weight as the active component (fractions 18 - 25, Figs. 3.23 and 3.24). Its inability to form any aggregate indicated that it was the active component, and the activity was not picked up because the sample was too dilute.

The antibacterial component behaves as if basic in nature (P. Molan, personal communication) and also the high amount of active component was achieved in the case of disaggregation of the 500 kDa aggregate with a high pH gradient on a cation exchanger (Fig. 3.22). Thus, the basic antibacterial component was expected to pass straight through the cation exchanger at pH 3.0. The elution of the basic active component straight through the cation exchanger at pH 3.0 could be explained by the possibility that the capacity of the column was exceeded. Alternatively, a small amount of active component could have been carried through the column by any anionic or less basic component, and they could later have been separated by the subsequent gel filtration chromatography the acidic component could be the aggregating component hidden under the citrate peak. In view of the low molecular weight nature and/or the possible retardation of the inactive aggregating component, the later possibility seemed more likely.

Similarly, fractions 18 - 25 as shown in Fig. 3.41 were pooled together and evaporated down to about 10 ml by rotary evaporation, and the pH of a 5 ml sample from this was adjusted to 7.0 with NaOH. The sample was incubated at 37°C for 2 h and then chromatographed through a column of Sephadex G-200F equilibrated with 0.1 mol/l Tris-HCl buffer,

pH 7.0. It was eluted, like the sample of Fig. 3.42, on the bed volume.

The pH of the remaining 5 ml of the same sample was also raised to 7.0 with NaOH, and the sample was incubated at 37°C for 2 h. A 200 μl sample from this was then chromatographed on a column of Superose 12 with 0.1 mol/l phosphate buffer, pH 7.0. A result identical to that shown in Fig. 3.43 was obtained, indicating a molecular weight of about 1 kDa or lower. Since the sample did not form any aggregate, it appeared to be the active component in a desegregated form.

In order to find the aggregating component(s) of the aggregates, fractions 26 - 40 as shown in Figs. 3.40 and 3.41 were together chromatographed through a column of Sephadex G-200F at pH 7.0, with and without fractions 18 - 25 from Figs. 3.40 and 3.41. No aggregation was achieved, and when the peak eluted with fractions 40 - 45 in Fig. 3.40 was incubated with the other two peaks of Figs. 3.40 and 3.41, again, no aggregation was obtained.

In order to investigate the peaks eluted in fractions 20 - 30 as shown in Figs. 3.36 to 3.38 and fractions 25 - 38 in Fig. 3.39, they were pooled and concentrated down separately to 5 ml by rotary evaporation. After incubating at 37°C for 2 h, they were chromatographed separately through a column of Sephadex G-200F at pH 7.0. They gave identical elution and activity profiles to those shown in Figs. 3.25 to 3.28, with the extent of the aggregation increasing from the samples of Fig. 3.36 to that of Fig. 3.39. This showed that the later the material eluted from the cation exchanger (or the more basic the material), the greater the extent of aggregation.

Conclusions

The 250 kDa aggregate (fractions 23 - 28, Fig. 3.5) behaved in an identical manner to the 500 kDa aggregate (fractions 16 - 22, Fig. 3.5) both on acidic gel filtration chromatography and cation exchange chromatography. This, however, was not surprising, as when each of these aggregates were re-chromatographed through the column of Sephadex G-200F at pH 7.0, it, besides forming the original aggregate, also formed the other aggregate (Figs. 3.8 and 3.9). Also, these two aggregates behaved similarly on cationic electrophoresis. Thus, it was

concluded that both the 500 kDa aggregate (fractions 16 - 22, Fig. 3.5) and the 250 kDa aggregate (fractions 23 - 38, Fig. 3.5) are antibacterial aggregates made up of the same small components.

But, unfortunately, the aggregating component(s) could not be isolated from the 250 kDa aggregate either. It was thought that the aggregating component(s) were either present under the citrate peaks on gel filtration chromatography (Figs. 3.40 and 3.41) or had been lost with the salt crystals after the cation exchange chromatography. Thus, anion exchange chromatography was used in an attempt to isolate the aggregating component(s).

Isolation of the Aggregating Component(s) Using Anion Exchange Chromatography at pH 12.0

Introduction

Since the aggregating component(s) could not be isolated by cation exchange chromatography because it was hidden under the citrate peak, anion exchange chromatography was used to obtain this aggregating component(s).

The 500 kDa and 250 kDa aggregates of Fig. 3.5 each formed the other aggregate on re-chromatography at pH 7.0, and both a common major band on cation electrophoresis. Also, both of these aggregates behaved identically on cation exchange chromatography, therefore indicating that both the 500 kDa and 250 kDa aggregates are made up of the same components. Thus, it was decided that to prepare the starting sample both of these aggregates could be pooled together.

Accordingly, two lots of 5 ml of seminal plasma were chromatographed through a column of Sephadex G-200F at pH 7.0. Results identical to those shown in Fig. 3.5 were obtained. The 500 kDa and 250 kDa aggregates were pooled together and evaporated down to 10 ml by rotary evaporation. The pH of the sample was lowered to 1.7 with HCl, and the precipitate formed at around pH 4.5 was removed by centrifugation at 6,000 g (r av.10 cm). After holding the sample at 4°C for 2 h, it was chromatographed in two lots through a column of Sephadex G-200F eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.44.

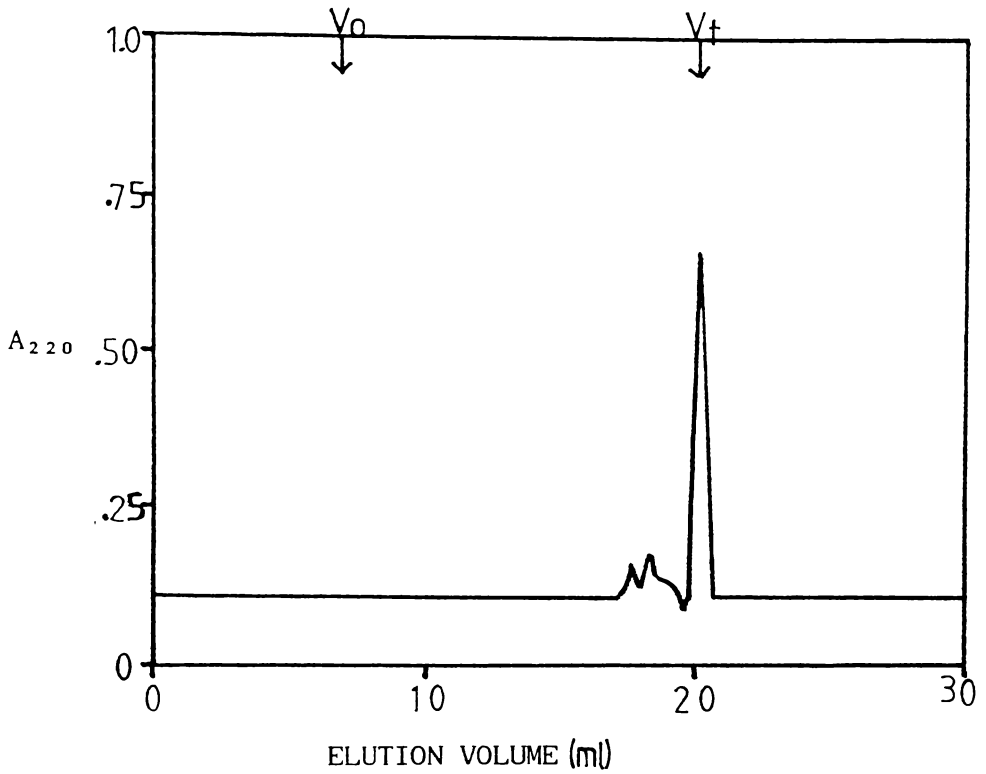


Fig. 3.43: FPLC of Fractions 18-25 from Fig. 3.40
Gel: Superose 12
Buffer: 0.1 mol/l phosphate buffer, pH 7.0
Sample: Part of concentrate of fractions 18-25 from Fig. 3.40 (200 µl)

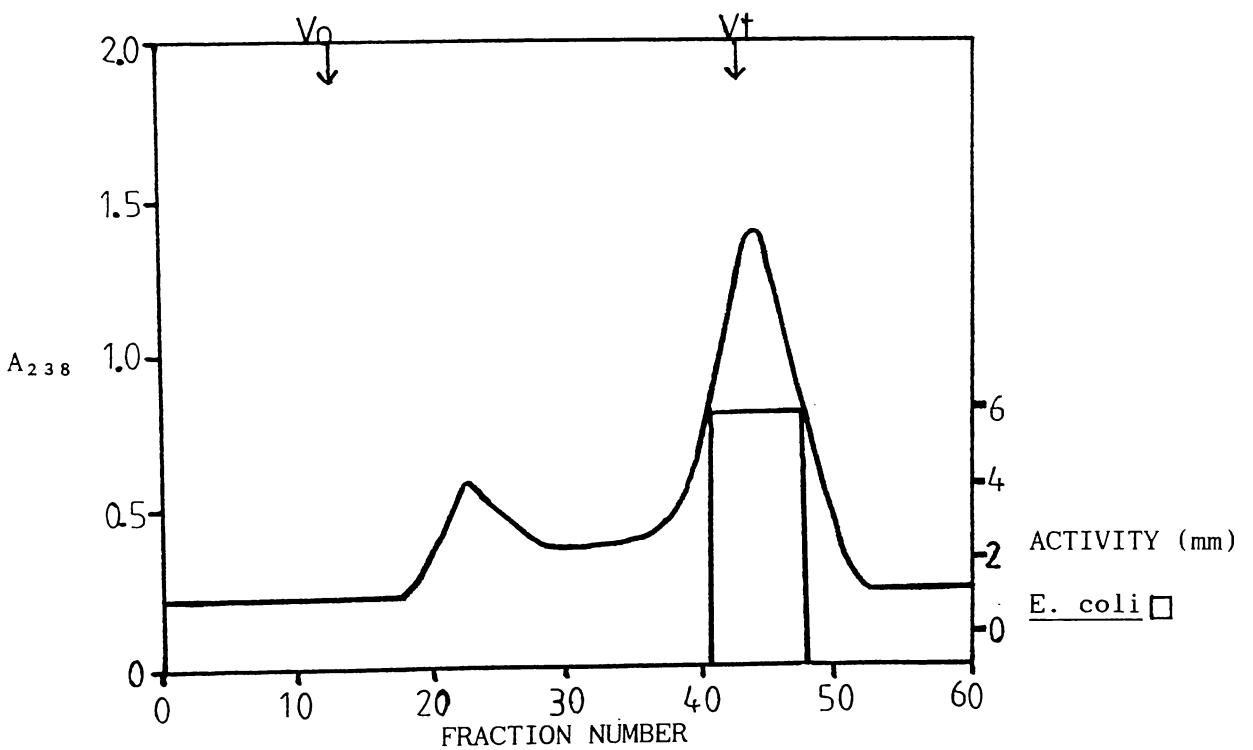


Fig. 3.44: Gel Filtration Chromatography of Fractions 16-28 as in Fig. 3.5
Gel: Sephadex G-200F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 16-28 as in Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

As expected, all the activity was dissociated to the 20 kDa aggregate. The inactive peak (fractions 16 - 38, Fig. 3.44) could have been proteins having nothing to do with the activity and just co-eluting with the active aggregates.

As the gel filtration chromatography at high pH had been reported to cause more disaggregation than at low pH (Eschenbruch, 1980), anion exchange chromatography was done at pH 12.0. It was hoped that since this pH was higher than the pK of amino groups of amino acids, it would at least partially disrupt any ionic interactions involved in the aggregations, and thus may form smaller aggregates upsetting the ratio of the components in these aggregates, and in turn favouring disaggregation. It was anticipated that the combination of high pH, salt gradient, and the competition for ionic interactions between the gel and the components and the components themselves might also result in the complete disaggregation of at least some of the unstable aggregates to their components. The components of the aggregates seemed to be of small size (smaller than 3.5 kDa), thus QAE-Sephadex A-25 gel, which is recommended for small molecules, was used.

It was hoped that since the active component is reported to be highly basic in nature, therefore at pH 12.0 it would either be still positively charged and pass straight through the anion exchanger, or weakly acidic and weakly bind to the gel. It was expected that the other component(s), which seemed to be acidic in nature from cation exchange chromatography, being highly acidic at this pH would strongly bind to the anion exchanger and different component(s) bound to the anion exchanger could be eluted at different stages of the salt gradient.

Since the antibacterial activity is known to be unstable at high pH (Eschenbruch, 1980), the pH of a part of the 500 kDa aggregate was adjusted to 12.0 with NaOH, the sample was held at 4°C for 6 h (approximate time of the anion exchange chromatography) and the activity of the sample was then tested against *E. coli* after neutralising it. By comparing the activity of the sample with that of a control, no loss of activity at high pH could be detected. Thus, the activity appeared to be stable at high pH for the time required for the anion exchange chromatography. Nevertheless, all fractions were neutralised as soon as they were eluted.

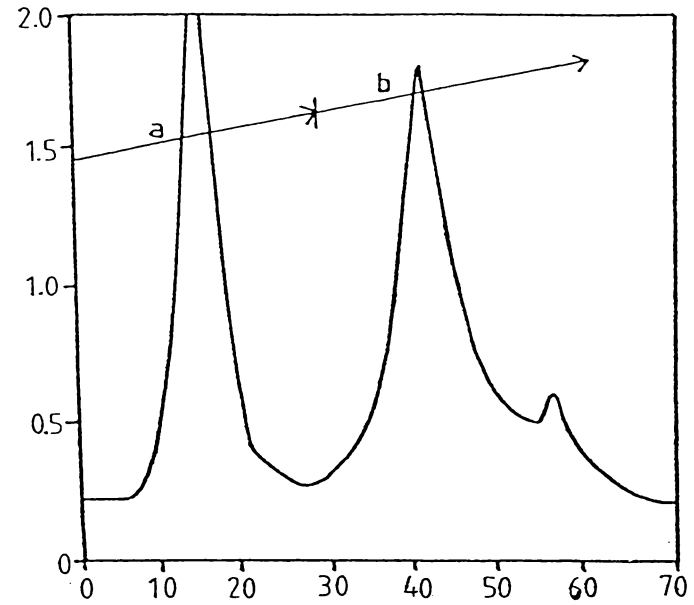
The 20 kDa aggregate (fractions 38 - 51 as shown in Fig. 3.44) was concentrated down to 10 ml by rotary evaporation. The pH of the sample was raised to 12.0 with NaOH, and after holding the sample at 4°C for 30 minutes it was run onto a column of QAE-Sephadex A-25 equilibrated with 0.01 mol/l NaOH, pH 12.0. The sample was followed with 0.01 mol/l NaOH, pH 12.0 until the peak reached the baseline and finally the column was eluted with a linear salt gradient from 0 to 4.0 mol/l NaCl in 0.01 mol/l NaOH, pH 12.0. The fractions were neutralised with HCl as soon as they were eluted. The results are shown in Fig. 3.45.

Gel filtration chromatography of the peaks from the anion exchange chromatography (Fig. 3.45) at acidic pH

In order to find the molecular weight of the peak which passed straight through the anion exchanger, fractions 9 - 24 as shown in Fig. 3.45 were pooled and evaporated down to about 15 ml by rotary evaporation. The pH of a 5 ml sample of this was adjusted to 1.7 with HCl and after holding the sample at 4°C for 2 h it was chromatographed on a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.46. A void volume peak and a peak just inside the void volume were obtained, and both of these peaks were found to be active. No active peak in the region of 1.2 kDa was obtained, indicating that no active component was present in its totally disaggregated form. Therefore, it appeared that not even a part of the aggregate which passed straight through the column had completely disaggregated.

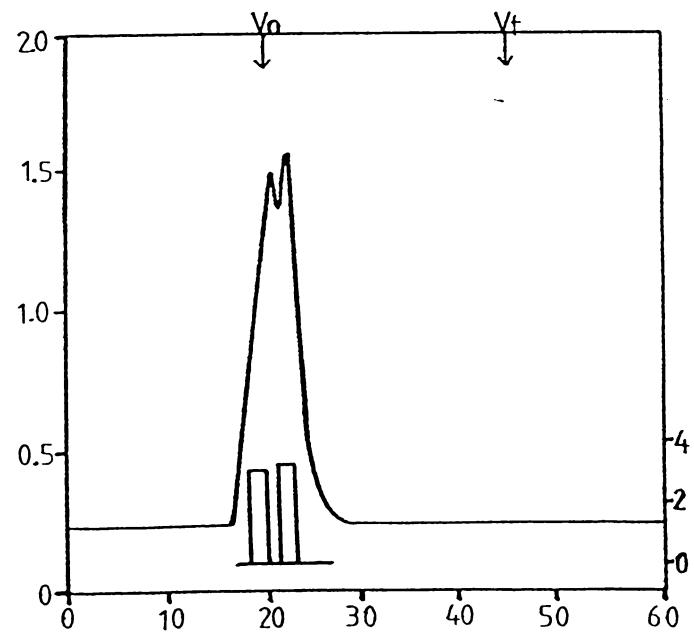
In order to check the aggregation capacity of the material which passed straight through the anion exchanger, a 5 ml sample of the concentrate of fractions 9 - 24, Fig. 3.45, was incubated at 37°C (at pH 7.0) for 2 h. After that it was chromatographed through a column of Sephadex G-50SF at pH 7.0. All the samples eluted as an active void volume peak indicating a molecular weight of 30 kDa or over, and thus re-aggregation. Therefore, all the material that passed straight through the anion exchanger at pH 12.0 seemed to be aggregates containing all the components required for the formation of large aggregates.

To find the molecular weight of the main peak eluted with salt gradient (fractions 32 - 54, Fig. 3.45), fractions 32 - 54 were pooled



A_{234}

Fig. 3.45: Ion Exchange Chromatography of Fractions 38-51 from Fig. 3.44
Gel: QAE-Sephadex A-25
Sample: Concentrate of fractions 38-51 from Fig. 3.44 (10 ml)
Elution Volumes: (a) 0.01 mol/l NaOH, pH 12.0; (b) salt gradient from 0 to 4.0 mol/l NaCl in 0.01 mol/l, pH 12.0
Fraction Volume: 4 ml



A_{232}

ACTIVITY (mm)

E. coli □

Fig. 3.46: Gel Filtration Chromatography of Fractions 9-24 from Fig. 3.45
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 9-24 from Fig. 3.45 (5 ml)
Fraction Volume: 4 ml

together and evaporated down to about 10 ml by rotary evaporation. The pH of a sample was adjusted to 1.7 with HCl, and after holding the sample at 4°C for 2 h it was chromatographed through a column of Sephadex G-25SF-G-10 mixture eluted with 0.02 mol/l HCl, pH 1.7. The mixture column of Sephadex G-25SF and G-10 (mixed in equal amounts) was used to separate the undisaggregated high molecular weight aggregates from the completely disaggregated components of low molecular weight. The results are shown in Fig. 3.47. There were two small peaks in the middle region of the column, and a large peak in the low molecular weight region. None of these peaks were active.

To see whether the peak eluted in fractions 32 - 54 of Fig. 3.45 had the component(s) to form the aggregates, a 5 ml sample of the concentrate of fractions 32 - 54 of Fig. 3.45 was incubated at 37°C for 2 h (at pH 7.0) and then chromatographed through a column of Sephadex G-50SF at pH 7.0. All the sample re-aggregated up to the void volume on Sephadex G-50SF, indicating that fractions 32 - 54 of Fig. 3.45 had all the components required for aggregation.

In order to examine which of the peaks of Fig. 3.47 is responsible for aggregation, it was decided to purify all the peaks of Fig. 3.47 by re-chromatography on the column of Sephadex G-25SF-G-10 mixture. Subsequently, the re-aggregation could be studied by gel filtration at pH 7.0.

Accordingly, the fractions making up the main peak (fractions 46 - 60, Fig. 3.47) were pooled together, evaporated down to about 10 ml by rotary evaporation, and out of that 5 ml was re-chromatographed through the column of Sephadex G-25SF-G-10 mixture at pH 1.7. As can be seen in Fig. 3.48, the sample re-aggregated.

Similarly, fractions 20 - 30 and 31 - 45 of Fig. 3.47 were pooled separately, concentrated down to 5 ml each by rotary evaporation, and separately re-chromatographed through the column of Sephadex G-25SF-G-10 mixture. Again, both the peaks eluted in fractions 20 - 30 and 31 - 45 in Fig. 3.47 re-aggregated and gave an identical elution profile to that shown in Fig. 3.48.

These results demonstrated that the peaks eluted in fractions 20 - 30 and 31 - 45 in Fig. 3.47 were the same component as the peak eluted

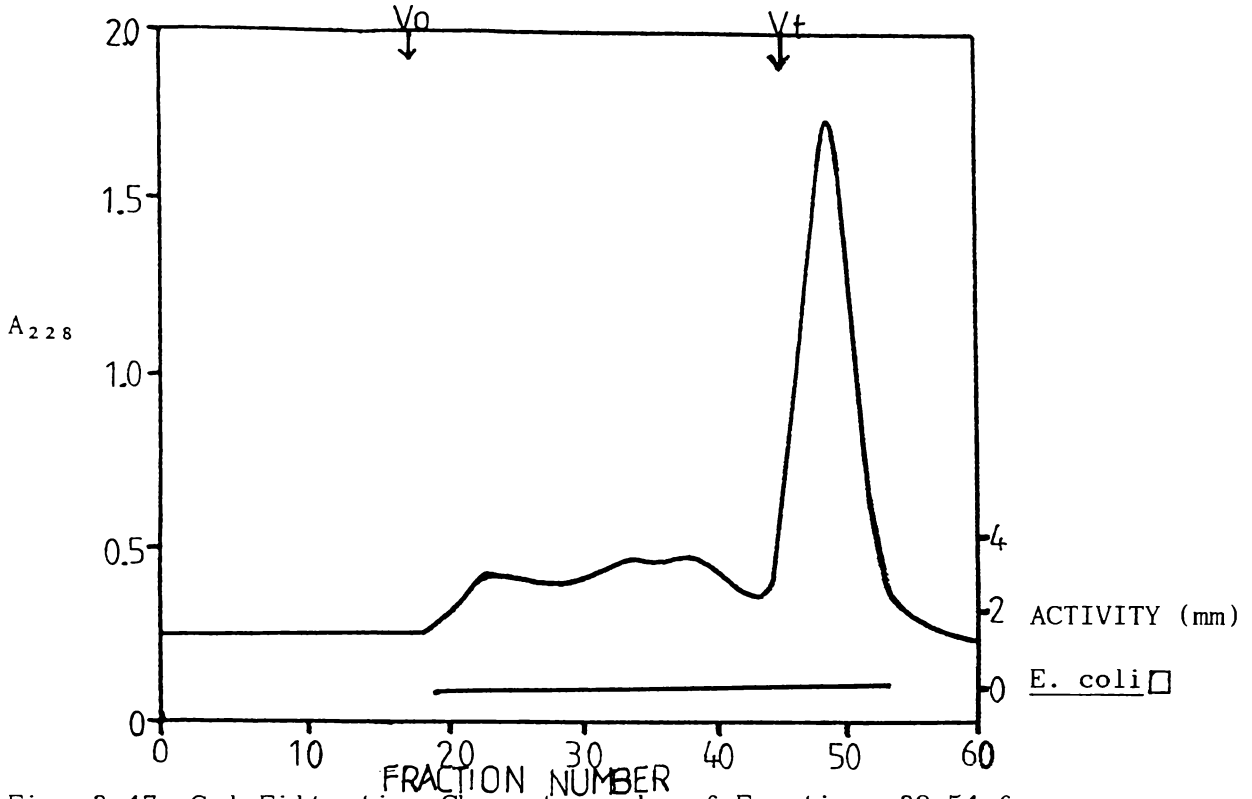


Fig. 3.47: Gel Filtration Chromatography of Fractions 32-54 from Fig. 3.45
Gel: Mixture of Sephadex G-25SF and Sephadex G-10 in equal proportions
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 32-54 from Fig. 3.45 (5 ml)
Fraction Volume: 4 ml

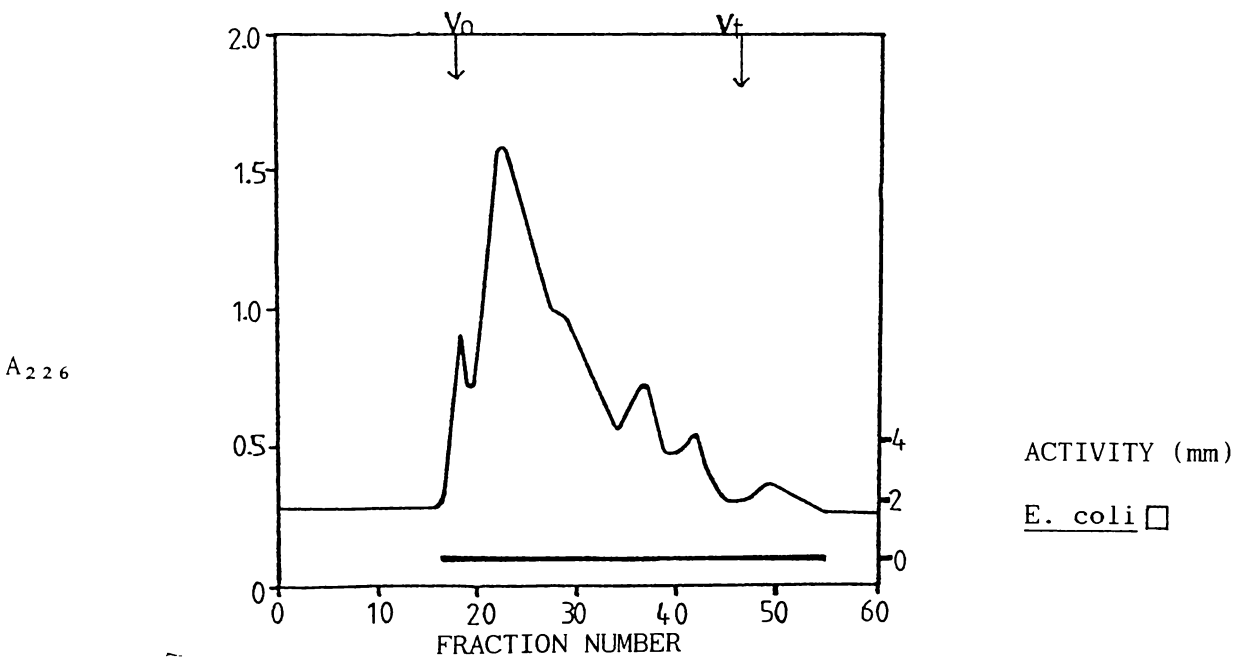


Fig. 3.48: Gel Filtration Chromatography of Fractions 46-60 from Fig. 3.47
Gel: Mixture of Sephadex G-25SF and Sephadex G-10 in equal proportions
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of the concentrate of fractions 46-60 from Fig. 3.47 (5 ml)
Fraction Volume: 4 ml

in fractions 46 - 60 in Fig. 3.47. The low molecular weight peak eluted in fractions 46 - 60 in Fig. 3.47 was probably the pure aggregating component in its unaggregated form and the two earlier peaks as shown in Fig. 3.47 were its small aggregates.

To see whether fractions 45 - 60 of Fig. 3.47 can re-aggregate, the pH of 1 ml of the sample was adjusted to 7.0 with NaOH. When neutralising, a small precipitate was formed at around pH 4.5. The precipitate was removed by centrifugation and remained undissolved at neutral as well as acidic pH (pH 1.7), but was partly dissolved in acetonitrile indicating the hydrophobic nature. Therefore, further work was carried out with the supernatent. The supernatent was incubated at 37°C for 2 h, and then a 100 μl sample of this was chromatographed through a Superose 12 column with 0.1 mol/l phosphate buffer, pH 7.0. As can be seen in Fig. 3.49, the sample re-aggregated up to the void volume of the column, indicating a molecular weight of 1 million or over. Thus, the main peak (fractions 46 - 60) of Fig. 3.47 could have been the aggregating component in its unaggregated form.

Investigations were then diverted to the question that if anion exchange chromatography had caused the complete disaggregation of at least a part of the aggregate to give the aggregating component (fractions 46 - 60, Fig. 3.47), then why did the component re-aggregate on re-chromatography under the same conditions (Fig. 3.48).

It was thought that this could be because of the fact that although anion exchange chromatography had caused disaggregation to give the aggregating component, the high amount of salt in the sample was necessary to keep the aggregating component in its unaggregated form. Salt could be acting by affecting the ionic interactions involved in the aggregation.

To see the elution volume of salt on the column of Sephadex G-25SF-G-10 mixture, 5 ml of 0.02 mol/l HCl, pH 1.7 saturated with NaCl was passed through the column of Sephadex G-25SF-G-10 mixture eluted with 0.02 mol/l HCl, pH 1.7. The elution volume of the salt was determined by measuring the conductivity of the fractions with a conductivity meter. The salt was found to be present in fractions 39 - 44. Since the salt eluted before the peak of aggregating component, on re-chromatography there was no salt to keep it in an unaggregated form.

To check this possibility, about 1 ml each of concentrated fractions 20 - 30, 31 - 45 and 46 - 60 were pooled together. The sample was diluted to 5 ml by addition of 0.02 mol/l HCl, pH 1.7 and NaCl was added to it to a concentration of 5 mol/l. After holding the sample at 4°C for 2 h it was then chromatographed on a column of Sephadex G-25SF-G-10 mixture eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.50. Apart from a small void volume peak which could have been some insoluble material, all the sample eluted in the region of low molecular weight. As can be seen in Fig. 3.50, the aggregating component was eluted earlier than it had before (Fig. 3.47).

However, in both cases it seemed to be retarded, being eluted after the salt. The deviation from the general elution behavior is known to be more prominent for small molecules on tightly cross-linked gels such as Sephadex G-10. Thus, it was thought that the retardation of the aggregating component may have been because of the Sephadex G-10 content of the column.

Therefore, in order to have a better idea about the molecular weight of the aggregating component, the pH of 5 ml of a concentrated sample of fractions 32 - 54 of Fig. 3.45 was lowered to 1.7 with HCl and after holding the sample at 4°C for 2 h it was chromatographed through a column of Sephadex G-25SF at pH 1.7. The results are shown in Fig. 3.51. The main peak of the aggregating component eluted on the bed volume indicating a molecular weight 1 kDa or lower, unless it was retarded even on Sephadex G-25SF.

In a further attempt to estimate the molecular weight of the aggregating component, the fractions making up the bed volume peak (fractions 45 - 55) in Fig. 3.51 were pooled, evaporated down to 5 ml by rotary evaporation and then chromatographed on a column of Sephadex G-15 at pH 1.7. Again, the main peak eluted at the bed volume, indicating the probable retardation of the sample (Fig. 3.52). The small void volume peak and peak just inside the void volume could be the insoluble material or small aggregates, and the broad shape of the bed volume peak was probably because of aggregation and disaggregation and/or adsorption of the sample as it ran through the column.

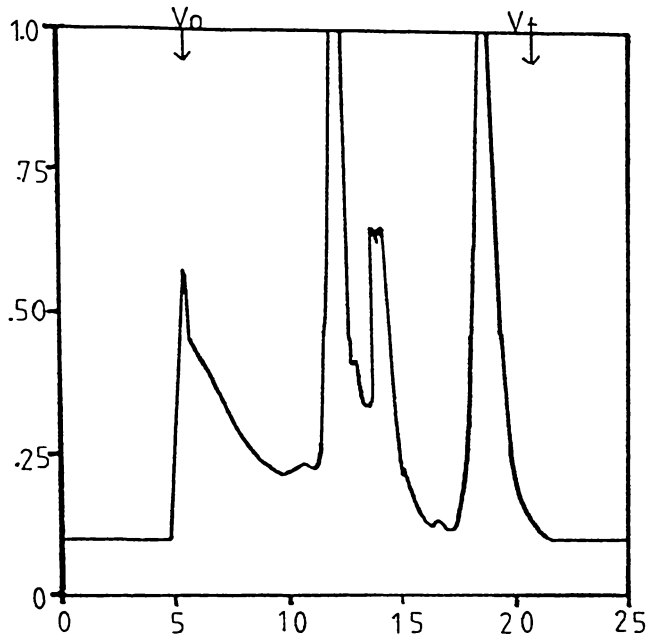


Fig. 3.49: FPLC of Fractions 46-60 from Fig. 3.47
Gel: Superose 12
Buffer: 0.1 mol/l phosphate, pH 7.0
Sample: Part of concentrate of fractions 46-60 from Fig. 3.47 (100 µl)

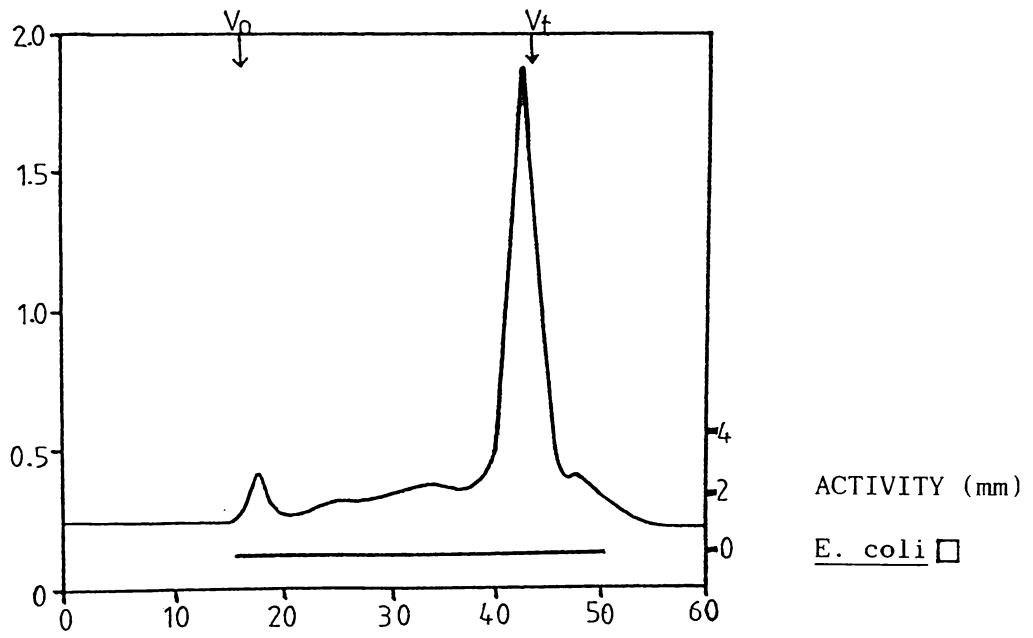


Fig. 3.50: Gel Filtration Chromatography of Fractions 30-60 from Fig.3.47
Gel: Mixture of Sephadex G-25SF and Sephadex G-10 in equal proportions
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 30-60 from Fig. 3.47 (5 ml)
Fraction Volume: 4 ml

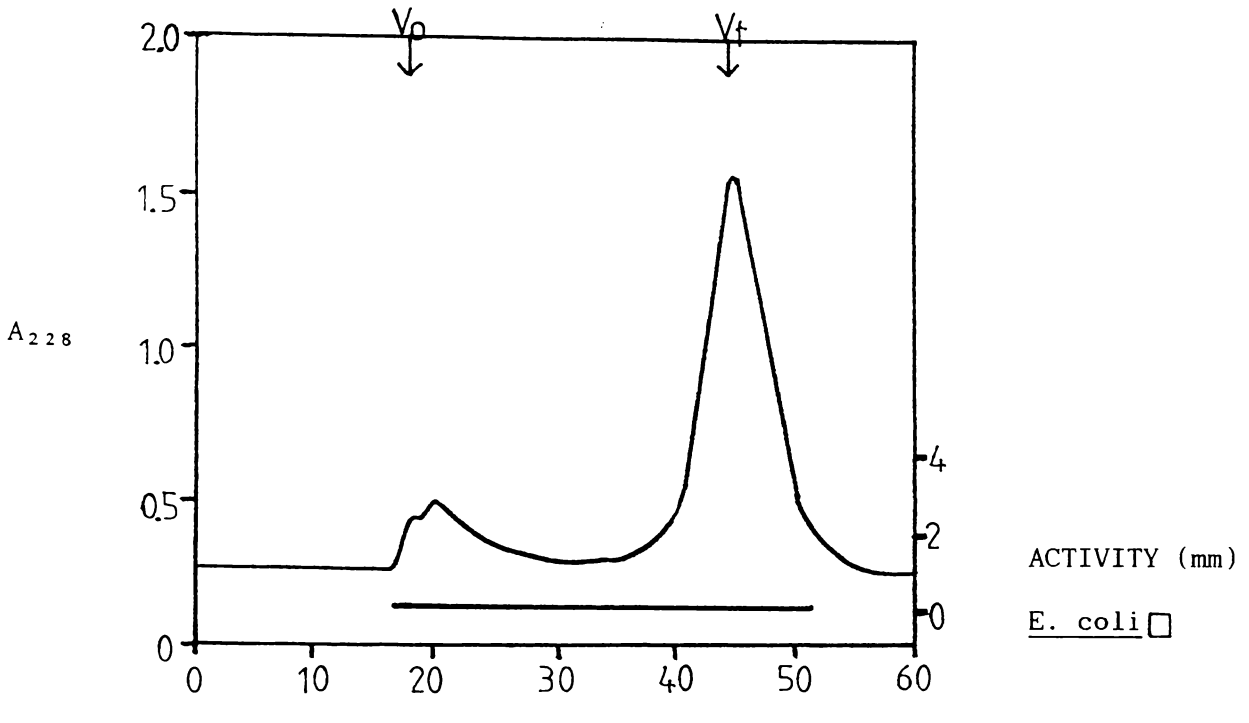


Fig. 3.51: Gel Filtration Chromatography of Fractions 32-54 from Fig. 3.45
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 32-54 from Fig. 3.45 (5 ml)
Fraction Volume: 4 ml

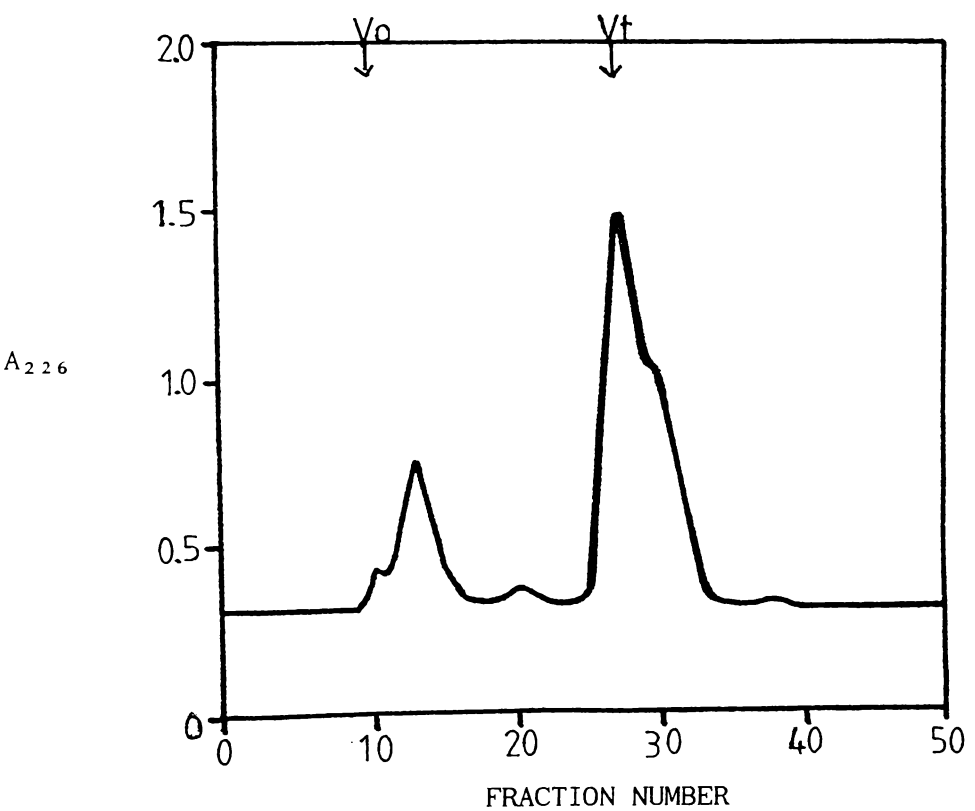


Fig. 3.52: Gel Filtration Chromatography of Fractions 45-60 from Fig. 3.51
Gel: Sephadex G-15
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 45-60 from Fig. 3.51 (5 ml)
Fraction Volume: 4 ml

As salt seemed to play an important role in disaggregation, it was decided to check whether anion exchange chromatography caused disaggregation or whether it was just the high amount of salt which caused disaggregation.

Accordingly, the 20 kDa aggregate was prepared again as shown in Fig. 3.44 (fractions 38 - 51, Fig. 3.44) and was concentrated down to 10 ml by rotary evaporation. Out of that, a sample of 5 ml was taken and NaCl was added to it to a concentration of 4 mol/l. Afterwards, the sample was chromatographed through a column of Sephadex G-25SF at pH 1.7. As can be seen in Fig. 3.53, the whole of the sample eluted on the void volume, indicating a molecular weight of 5 kDa or more, and in turn, no complete disaggregation of the sample.

The material which passed straight through the anion exchanger (fractions 9 - 24, Fig. 3.45) was not subjected to the salt gradient, and was found to be not completely disaggregated (Fig. 3.46). Therefore, to see if salt could disaggregate this material, NaCl was added to a 5 ml concentrated sample of fractions 9 - 24 of Fig. 3.45 to a concentration of 4 mol/l NaCl. The pH of the sample was adjusted to 1.7 with HCl and after holding the sample at 4°C for 2 h it was then chromatographed on a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. As can be seen in Fig. 3.54, the sample was eluted on the void volume, indicating a molecular weight of 5 kDa or over. Thus, complete disaggregation was not achieved, as the components were expected to be smaller than 3.5 kDa molecular weight. The slope of the peak could be because of a partial disaggregation and/or adsorption of the sample during chromatography.

In order to find the molecular weight of the peak eluted at the end of salt gradient (fractions 55 - 68, Fig. 3.45) the fractions making up the peak were pooled and concentrated down to about 5 ml by rotary evaporation. The pH of the sample was adjusted to 1.7 with HCl. A precipitate formed at around pH 4.5 which was removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. After holding the supernatant at 4°C for 2 h, it was chromatographed through a column of Sephadex G-25SF-G-10 mixture at pH 1.7. The results are shown in Fig. 3.55. There was a single peak which was eluted at the bed volume of the column, indicating retardation of the sample.

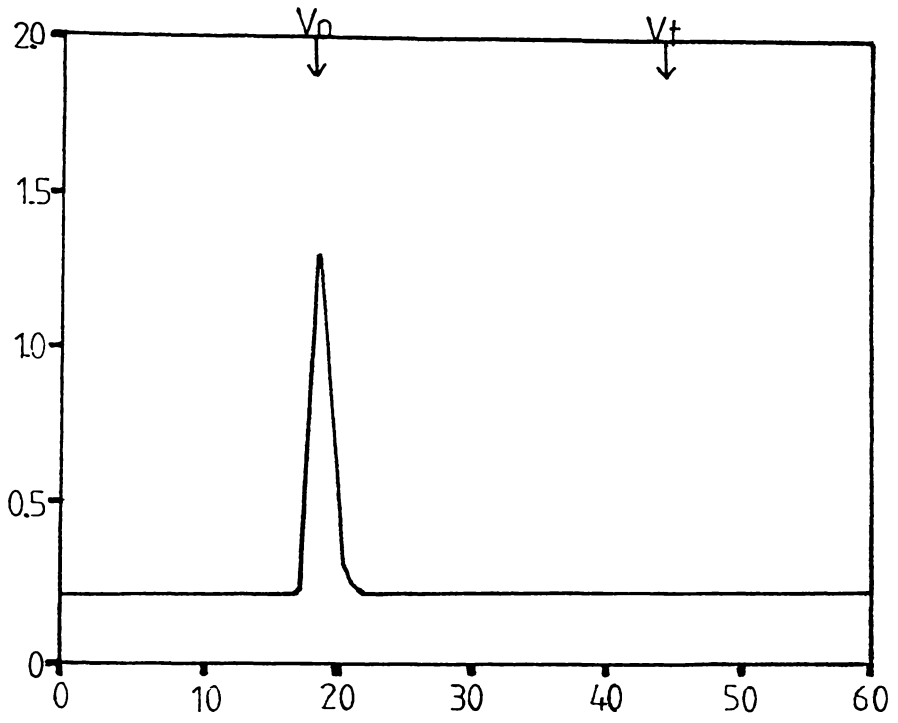


Fig. 3.53: Gel Filtration Chromatography of Fractions 38-51 from Fig. 3.44
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 38-51 containing 4 mol/l NaCl (5 ml)
Fraction Volume: 4 ml

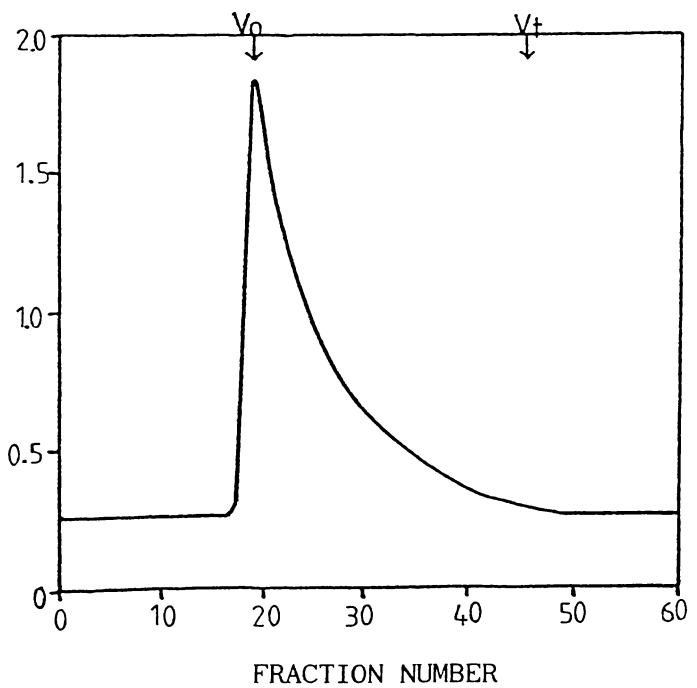


Fig. 3.54: Gel Filtration Chromatography of Fractions 9-24 from Fig. 3.45
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 9-24 from Fig. 3.45 containing 4 mol/l NaCl (5 ml)
Fraction Volume: 4 ml

To see if the peak of Fig. 3.55 could re-aggregate at neutral pH, fractions 42 - 58 of Fig. 3.55 were pooled, neutralised by addition of NaOH, and evaporated down to about 5 ml by rotary evaporation. The sample was incubated at 37°C at pH 7.0 for 2 h, and then 100 μl was chromatographed on a column of Superose 12 at pH 7.0. As can be seen in Fig. 3.56, the sample re-aggregated up to the void volume, indicating a molecular weight of up to 1 million or over.

Conclusions

The anion exchange chromatography successfully disaggregated at least part of the aggregate to give the aggregating component, which was eluted on different elution volumes, though always after bed volumes, thus indicating different levels of retardation. However, activity was still associated with the high molecular weight material which passed through the anion exchange column. If the guanidino groups of arginine (pK 12.5) are somehow involved in the aggregation then the partial dissociation obtained in anion exchange chromatography at pH 12.0 could have been because the guanidino groups were not suppressed at this pH. Also, at pH 13.0, all the forms of aggregates were expected to be negatively charged, and thus binding to the anion exchanger and given more chance for disaggregation.

Therefore, in an attempt to dissociate the active high molecular weight material which passed straight through the anion exchanger at pH 12.0, it was decided to subject it to anion exchange chromatography at pH 13.0.

Attempts to Disaggregate the Active Aggregate by Anion Exchange Chromatography at pH 13.0

The pH of 10 ml of concentrated fractions 9 - 24 as shown in Fig. 3.45 was raised to 13.0 with NaOH, and was held at 4°C for 30 minutes. The sample was then run onto a column of QAE-Sephadex A-25 equilibrated with 0.1 mol/l NaOH, pH 13.0, and followed with 0.1 mol/l NaOH, pH 13.0, until the absorbance returned to the baseline. A salt gradient from 0 to 4.0 mol/l NaCl in 0.1 mol/l NaOH, pH 13.0, was run through the column. In order to minimize any loss of activity due to high pH, fractions were neutralised as soon as they were eluted. As can be seen

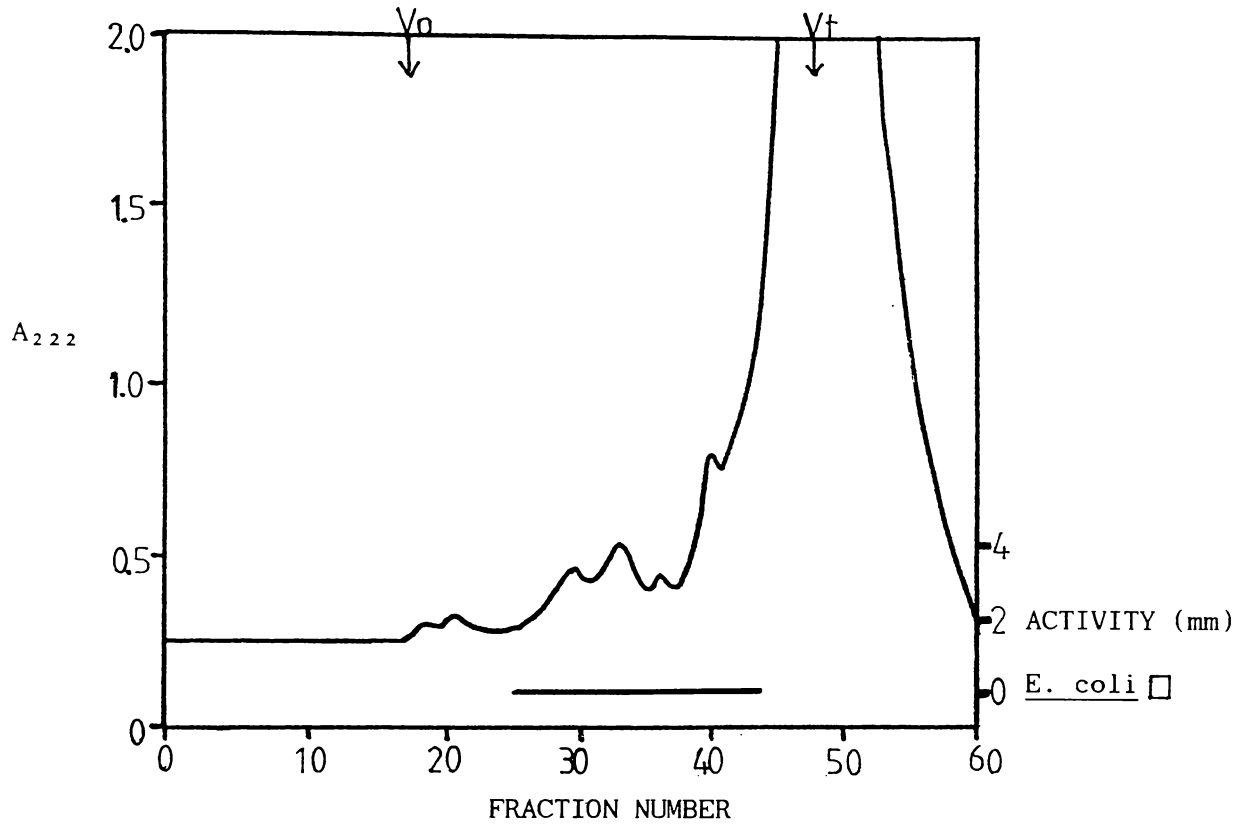


Fig. 3.55: Gel Filtration Chromatography of Fractions 55-68 from Fig. 3.45
Gel: Mixture of Sephadex G-25SF and Sephadex G-10 in equal proportions
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 55-68 from Fig. 3.45 (5 ml)
Fraction Volume: 4 ml

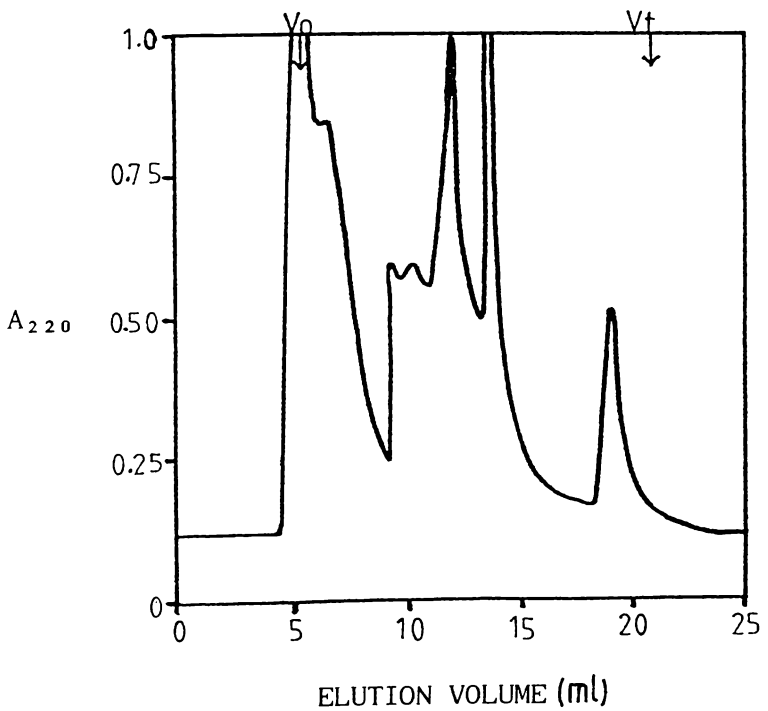


Fig. 3.56: FPLC of Fractions 42-57 from Fig. 3.55
Gel: Superose 12
Buffer: 0.1 mol/l phosphate buffer, pH 7.0
Sample: Part of concentrate of fractions 42-57 from Fig. 3.55 (100 µl)

in Fig. 3.57, an elution profile similar to that of anion exchange chromatography at pH 12.0 (Fig. 3.45) was obtained.

Gel filtration chromatography of the peaks from the anion exchange chromatography (Fig. 3.57) at acidic pH

Fractions 9 - 21 of Fig. 3.57 (the material which passed straight through the column) were pooled, rotary evaporated to 5 ml, and the pH of the sample was adjusted to 1.7 with HCl. After holding the sample at 4°C for 2 h, it was chromatographed on a column of Sephadex G-25SF at pH 1.7 to find the molecular weight of the peak. The results are shown in Fig. 3.58. The sample eluted on the void volume, indicating a molecular weight of 5 kDa or over, therefore it appeared that some aggregate passed straight through the anion exchanger, even at pH 13.0.

Because of the broad nature of the peak eluted in the middle of the salt gradient (fractions 22 - 43, Fig. 3.57) it was divided into two parts. To find the molecular weight of the earlier part of the peak, fractions 22 - 32 were pooled together and evaporated down to 5 ml by rotary evaporation. The pH of the sample was adjusted to 1.7 with HCl, and after holding the sample at 4°C for 2 h it was chromatographed through a column of Sephadex G-25SF-G-10 mixture at pH 1.7. As can be seen in Fig. 3.59, the sample was eluted over a wide range of molecular weight from very low molecular weight (bed volume of Sephadex G-10) to the void volume of Sephadex G-25SF indicating a molecular weight of 5 kDa or over. Because of the sizes and the shapes of the peaks, none of them looked like pure components. Instead, the peaks as shown in Fig. 3.59 seem to be small aggregates, and at least the one eluting at the last was probably retarded because of its elution on the bed volume of Sephadex G-10.

Similarly, fractions 33 - 43 from Fig. 3.57 were pooled and concentrated down to about 5 ml by rotary evaporation. The pH of the sample was adjusted to 1.7 with HCl, and the precipitate formed at pH 4.5 was removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. After holding the sample at 4°C for 2 h, it was chromatographed on a column of Sephadex G-25SF-G-10 mixture at pH 1.7. The results are shown in Fig. 3.60. Although there were three peaks of low molecular weight, because of their shapes and sizes they also

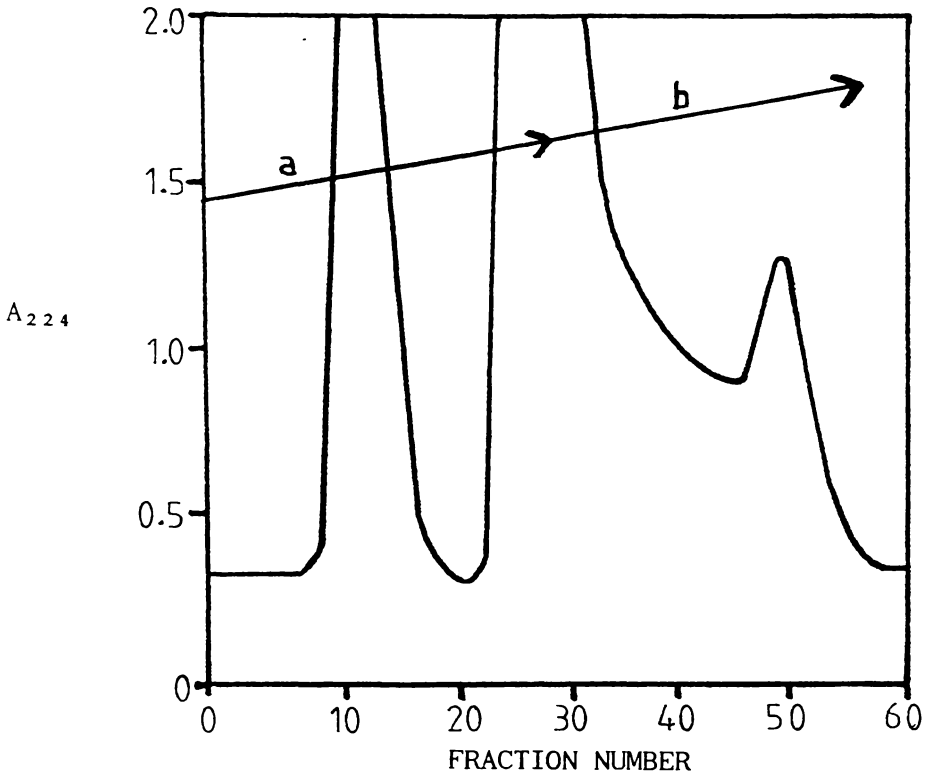


Fig. 3.57: Ion Exchange Chromatography of Fractions 9-24 from Fig. 3.45
Gel: QAE-Sephadex A-25
Sample: Concentrate of fractions 9-24 from Fig. 3.45 (10 ml)
Elution Eluents: (a) 0.1 mol/l NaOH, pH 13.0; (b) salt gradient from 0 to 4.0 mol/l NaCl in 0.1 mol/l NaOH, pH 13.0
Fraction Volume: 4 ml

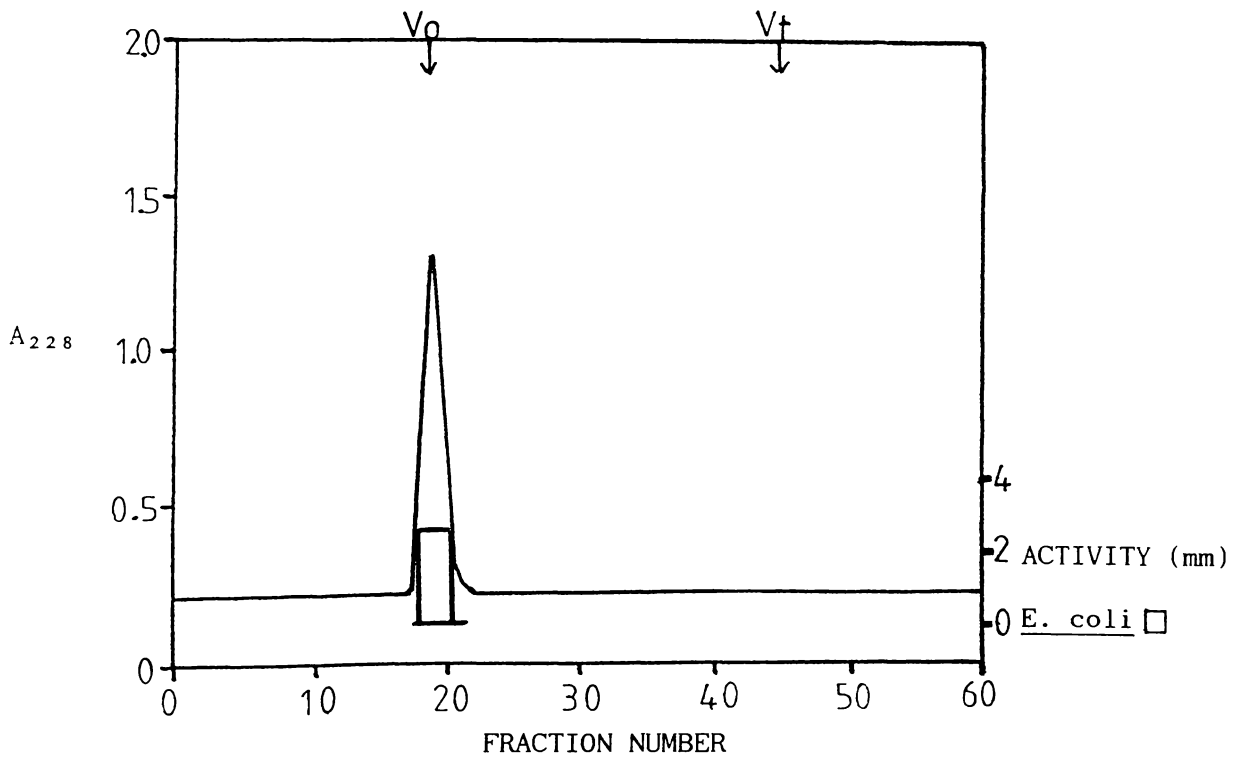


Fig. 3.58: Gel Filtration Chromatography of Fractions 9-21 from Fig. 3.57
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 9-21 from Fig. 3.57 (5 ml)
Fraction Volume: 4 ml

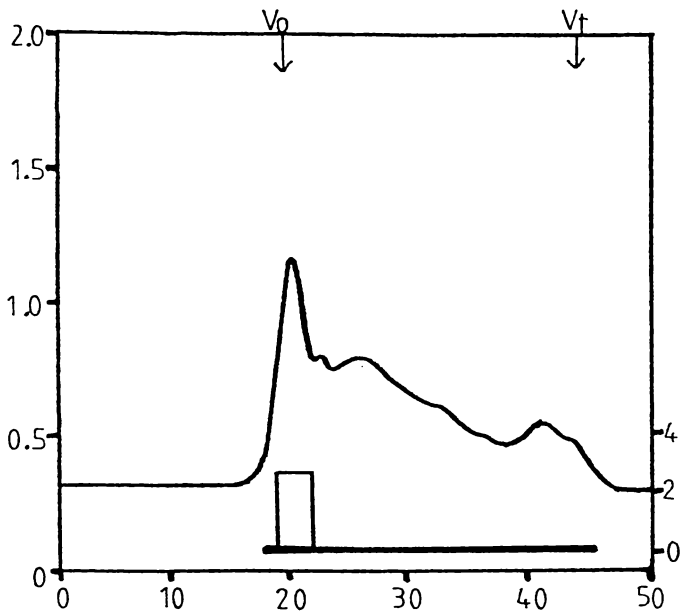


Fig. 3.59: Gel Filtration Chromatography of Fractions 22-32 from Fig. 3.57
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 22-32 from Fig. 3.57 (5 ml)
Fraction Volume: 4 ml

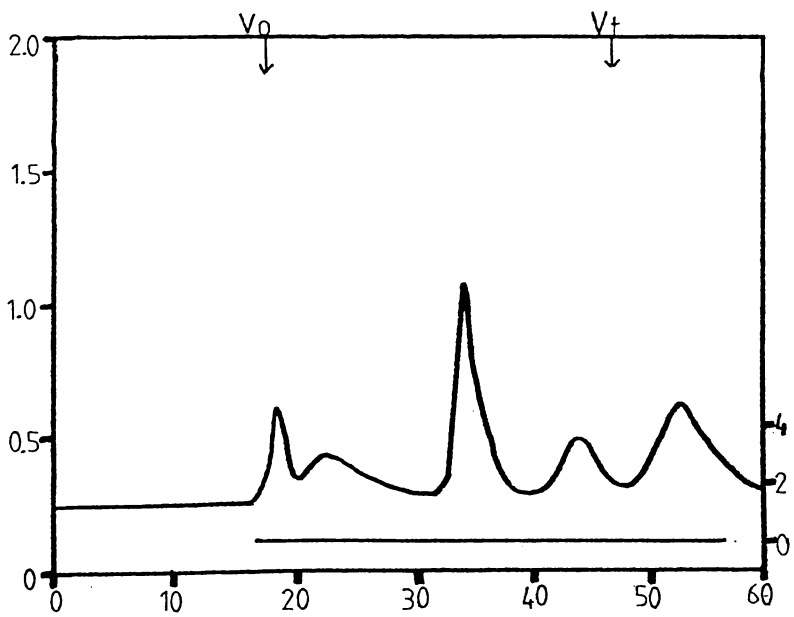


Fig. 3.60: Gel Filtration Chromatography of Fractions 33-43 from Fig. 3.57
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 33-43 from Fig. 3.57 (5 ml)
Fraction Volume: 4 ml

appeared to be small aggregates which seemed to be retarded on the Sephadex G-10.

In order to find the molecular weight of fractions 44 - 57 of Fig. 3.57, they were pooled and evaporated down to 5 ml by rotary evaporation. The pH of the sample was adjusted to 1.7 with HCl. The precipitate, formed at about pH 4.5, was removed by centrifuging at 6,000 g (r av.10 cm) for 10 minutes. The supernatant at 4°C for 2 h, and then chromatographed through a column of Sephadex G-25SF-G-10 mixture at pH 1.7. The results are shown in Fig. 3.61. There was a small void volume peak and broad peaks after the void volume (fractions 21 - 38) which probably were small aggregates. Alternatively, they were the result of some insoluble material re-dissolving as the sample passed through the column. There was a large peak near the bed volume (fractions 38 - 45, Fig. 3.61) and a large peak (fractions 55 - 67, Fig. 3.61) which was eluted very late after the bed volume of the column, and thus was very retarded on the Sephadex gel. This material eluted late from the Sephadex (fractions 55 - 67, Fig. 3.61) was even more retarded than the aggregating component isolated earlier (Figs. 3.47 and 3.55).

This retarded material could be a third component of the antibacterial aggregates besides the active and inactive components isolated earlier. Another possibility was that all the peaks eluted in earlier work were small aggregates, as were the peaks up to fraction 55 in Fig. 3.61. Further investigations on this retarding peak was done using HPLC and FPLC and by comparing it with the active and inactive components (to be described in a later section).

When neutralising, fractions 55-67 of Fig. 3.61 a small precipitate was formed at around pH 4.5. The precipitate was removed by centrifugation at 6,000 g (r av. 10 cm), and remained undissolved at neutral as well as acidic pH (pH 1.7). However, the precipitate was partially dissolved in acetonitrile indicating the hydrophobic nature of the component. Since the precipitate could not be dissolved further work was carried out with the supernatant and it was decided that when a suitable disaggregating method is developed the precipitate and the supernatent would both be disaggregated with the method to see if both contained the same component(s).

All of the fractions of Fig. 3.61, when tested for antibacterial activity against *E. coli*, were found inactive. This could either be because all these peaks were made up (at least predominantly) of the inactive aggregating components, or the activity of the active component was lost because of the exposure to pH 13.0. Shackell (1980) found that antibacterial activity was inhibited by salt at concentrations higher than 0.7 mol/l. But, the absence of activity in all peaks could not be attributed to the salt, as salt was expected to be eluted in fractions 39 - 44, but the active component with an estimated molecular weight of 1.2 kDa was expected to be eluted earlier than the region of salt elution.

In order to study the re-aggregation of the retarding component, fractions 55 - 67 as shown in Fig. 3.61 were pooled and evaporated down to about 10 ml by rotary evaporation. The pH of a 5 ml sample was raised to 7.0 with NaOH and it was incubated at 37°C for 2 h. Then, 100 μl was chromatographed through a column of Superose 12 at pH 7.0. As can be seen in Fig. 3.62, the sample re-aggregated up to the void volume on Superose 12, indicating the molecular weight of 1 million or over.

Conclusions

Anion exchange chromatography at pH 13.0 also failed to dissociate the aggregates completely. There was still some material which passed straight through the anion exchanger, which was active, was in the form of aggregates, and also formed larger aggregates, thus appeared to be undissociated aggregates. Interestingly, the material eluting from the anion exchanger at the end of the salt gradient gave a very retarded peak on Sephadex (fractions 55 - 67, Fig. 3.61), which formed very high molecular weight aggregates at pH 7.0 (Fig. 3.62). The re-aggregation of this retarding component (Fig. 3.62) was even more than what had earlier appeared to be the aggregating component (Fig. 3.49).

This observation prompted the possibility of a component being present in the retarding peak, which helps the aggregating component in the aggregation, and its ratio probably determines the extent of aggregation. This component, which is retarded on the Sephadex gel, seemed to be acidic in nature because it was eluted at the end of salt

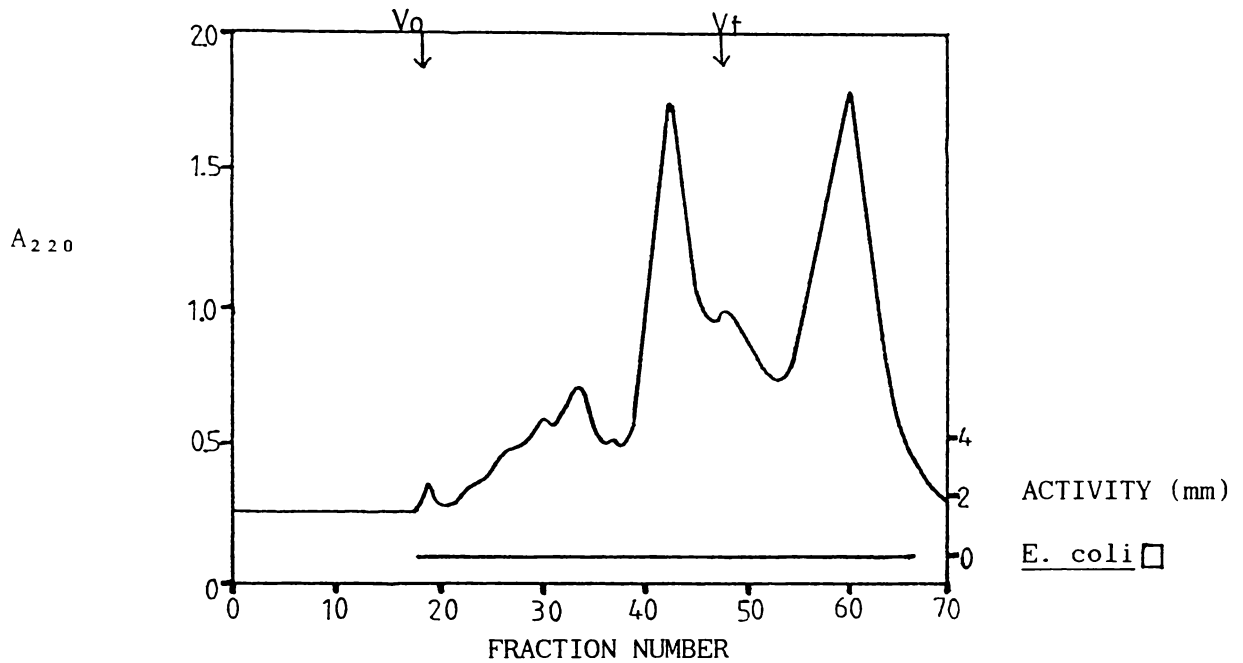


Fig. 3.61: Gel Filtration Chromatography of Fractions 44-57 from Fig. 3.57
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 44-57 from Fig. 3.57 (5 ml)
Fraction Volume: 4 ml

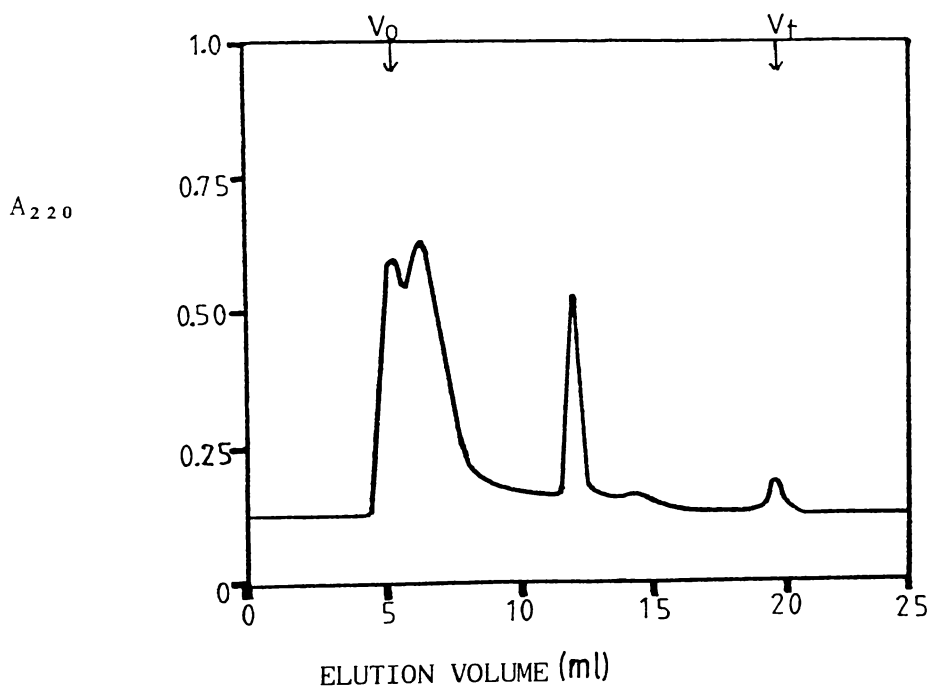


Fig. 3.62: FPLC of Fractions 55-67 from Fig. 3.61
Gel: Superose 12
Buffer: 0.1 mol/l phosphate buffer, pH 7.0
Sample: Part of concentrate of fractions 55-67 from Fig. 3.61 (100 µl)

gradient on anion exchange chromatography. Alternatively, the retarded peak was the inactive component in a totally disaggregated state.

Disaggregation of the Active 10 - 40 kDa Peak

Introduction

The middle of the active 20 kDa peak (fractions 29 - 41, Fig. 3.5) was estimated to be about 20 kDa from the calibration curve. However, this was a broad peak spreading over a range of estimated molecular weight of 40 kDa to 10 kDa (bed volume of Sephadex G-200F). Earlier in this investigation, the active 20 kDa peak of Fig. 3.5 had been referred to as the 20 kDa aggregate because of the possibility of the presence of the aggregate in this peak, however, later the 20 kDa aggregates were obtained on the disaggregation of the 500 kDa and 250 kDa aggregates.

Thus, to avoid any confusion, it was decided to refer to the active 20 kDa peak (fractions 29 - 41) of Fig. 3.5 as the 10 - 40 kDa peak rather than the 20 kDa aggregate. This was also more appropriate as the antibacterial activity of this peak appeared to be because of antibacterial compound(s) other than the aggregates.

An antibacterial protein, seminalplasmin, has been reported to be present in bovine seminal plasma with a molecular weight from 19.8 kDa to 6.3 kDa depending on the method used (Reddy and Bhargava, 1979 ; Thiel and Scheit, 1983). Eschenbruch (1980) also isolated lysozyme from bovine seminal plasma with an estimated molecular weight of about 14 kDa. These antibacterial compounds could be contributing to the antibacterial activity of this peak.

The spreading of the 10 - 40 kDa peak of Fig. 3.5 could have been due to the basic nature of the sample, its positive charges interacting with carboxyl groups of the dextran gel matrix, or to there being a series of aggregates of slightly different molecular weights, or due to the continuous aggregation/disaggregation of the aggregates as they ran through the column. Alternatively, this could also have been because of the presence of antibacterial compound(s) other than the antibacterial aggregates.

As shown in Fig. 3.5, the 10 - 40 kDa peak was found to be active against *M. lysodeikticus*. Since the 500 kDa and 250 kDa aggregates (Fig. 3.5) were not active against *M. lysodeikticus* indicating that the activity in the 10 - 40 kDa peak against *M. lysodeikticus* was probably due to an antibacterial compound other than the antibacterial aggregates. This antibacterial compound was most probably lysozyme as *M. lysodeikticus* is sensitive to lysozyme. But antibacterial aggregate of same small components which also make the aggregates of 500 kDa and 250 kDa, may also have been present in the 10 - 40 kDa peak of Fig. 3.5 as the acid diffusate on gel filtration at neutral pH formed an antibacterial peak of this size (Fig. 3.7).

Therefore, attempts were made to identify which antibacterial compound(s) are responsible for the antibacterial activity of the 10 - 40 kDa peak of Fig. 3.5.

Further gel filtration chromatography of the 10 - 40 kDa peak

It was thought that if an antibacterial aggregate made up of the same small components which also make the 500 kDa and 250 kDa aggregates is present in the 10 - 40 kDa peak, then it may re-aggregate on re-chromatography at pH 7.0.

Thus, fractions 29 - 41 from repeated runs as shown in Fig. 3.5 were pooled and evaporated down to 20 ml by rotary evaporation. Out of that a 2 ml sample was diluted to 5 ml by addition of 0.1 mol/l Tris-HCl buffer, pH 7.0, and then re-chromatographed through a column of Sephadex G-200F at pH 7.0. As can be seen in Fig. 3.63 the sample was eluted in the original position of the 10 - 40 kDa peak of Fig. 3.5. A small shoulder on the ascending side of the peak could have been either some re-aggregation indicating the presence of aggregating components or it was because of previous overlapping with the 250 kDa aggregate from Fig. 3.5. With the sample not re-aggregating to high molecular weights it thus appeared that the 10 - 40 kDa peak of Fig. 3.5 does not have a high proportion of the aggregating components.

It was thought that the 10 - 40 kDa peak present in seminal plasma at pH 7.0 (fractions 29 - 41, Fig. 3.5) was outside the optimum lower end of separating range of Sephadex G-200F. Thus, in order to have a

better idea about the composition of the 10 - 40 kDa peak, a 2.5 ml sample from the concentrate of this peak was mixed with an equal amount of 0.1 mol/l Tris-HCl buffer, pH 7.0 and chromatographed through a column of Sephadex G-50SF at pH 7.0. The results are shown in Fig. 3.64.

Since the molecules with estimated molecular weight of up to 40 kDa were present in the 10 - 40 kDa peak, the active void volume peak was probably an aggregate. The active 20 kDa peak (fractions 18 - 22, Fig. 3.64) could also be an aggregate of small components.

To check the presence of lysozyme, the fractions were also tested for lytic activity against pre-grown *M. lysodeikticus* cells. Some lytic activity was found in the void volume (fractions 13 - 17) and the 20 kDa peak (fractions 18 - 22), indicating a proportion of lysozyme was aggregated. Since lysozyme standard gave much higher activity against *M. lysodeikticus* than *E. coli*, the higher activity against *E. coli* than *M. lysodeikticus* in the void volume and the 20 kDa peak in Fig. 3.64 indicated the presence of an antibacterial compound other than lysozyme. This antibacterial compound was probably antibacterial aggregates.

However, the major lysozyme activity was present in the estimated region of 15 kDa (fractions 23 - 26, Fig. 3.64). This peak was most probably an unbound lysozyme.

When the pH of a 5 ml concentrated sample of the 10 - 40 kDa peak of Fig. 3.5 was adjusted to 1.7 with HCl to run it on Sephadex G-200F at pH 1.7, a precipitate was formed at around pH 4.5. This precipitation was identical to that observed earlier in the case of acidification of the 500 kDa and 250 kDa aggregates. This was an indication that an antibacterial aggregate of small components was present in the 20 kDa peak. The precipitate was removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes, and a part of the precipitate was re-dissolved in 0.1 mol/l Tris-HCl buffer, pH 7.0. The pH of 1 ml of concentrated supernatant was raised to 7.0 with NaOH. These solutions of both the supernatant and the precipitate were then tested for antibacterial activity against *E. coli* and were found to be active.

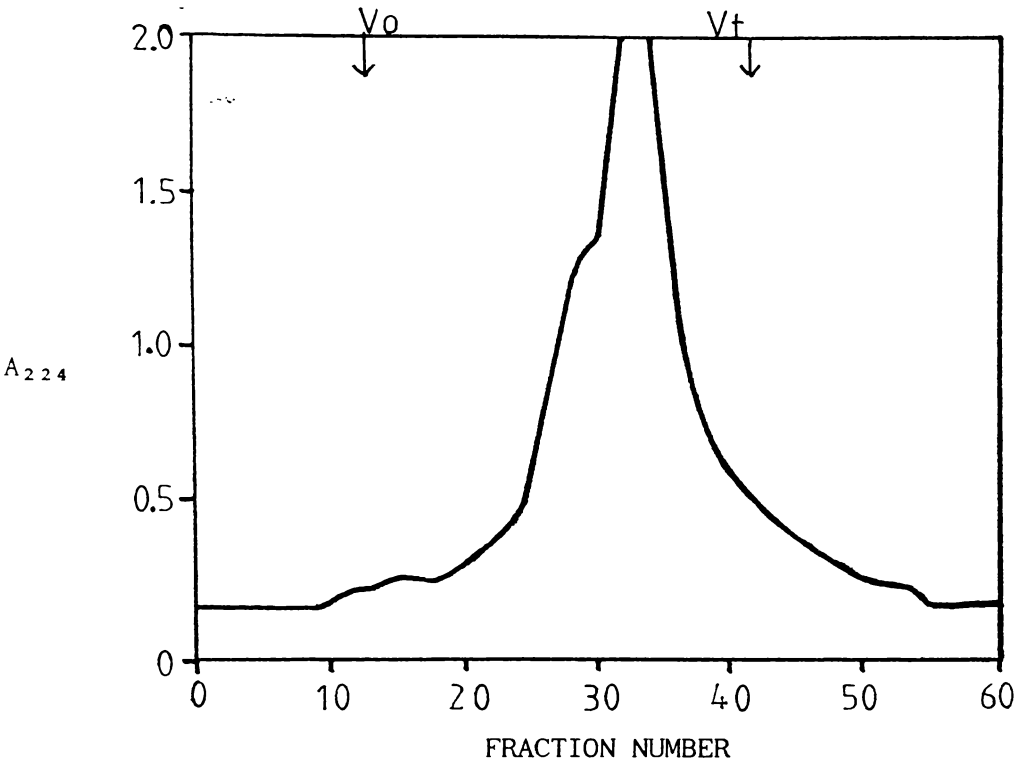


Fig. 3.63: Gel Filtration Chromatography of Fractions 29-41 from Fig. 3.5
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 29-41 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

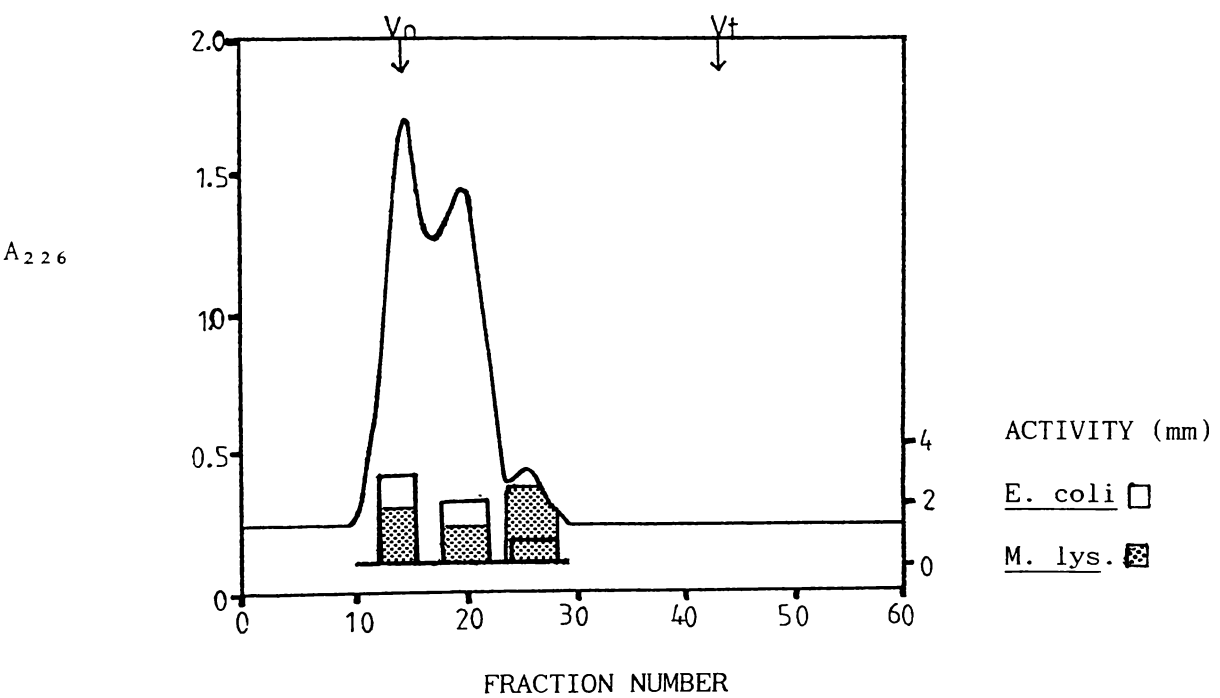


Fig. 3.64: Gel Filtration Chromatography of Fractions 28-41 from Fig. 3.5
Gel: Sephadex G-50SF
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 28-41 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

In order to see whether any antibacterial compound(s) and/or any components of the antibacterial aggregate were lost in the precipitate, it was decided to re-chromatograph both the supernatant and the precipitate through a column of Sephadex G-200F at pH 7.0 and then compare their elution profiles.

Accordingly, 1 ml of the supernatant of the concentrated sample was diluted to 5 ml by addition of 0.1 mol/l Tris-HCl buffer, pH 7.0 and its pH was adjusted to 7.0. The sample was incubated at 37°C for 2 h and then chromatographed through a column of Sephadex G-200F at pH 7.0. As can be seen in Fig. 3.65 the sample was eluted as a broad peak whose middle was estimated to be about 20 kDa molecular weight (original position of the 20 kDa peak in Fig. 3.5). A small shoulder (fractions 16 - 26, Fig. 3.65) could have been because of overlapping of the original 20 kDa peak with the 250 kDa aggregate from Fig. 3.5 rather than any re-aggregation.

Similarly, a part of pH 4.5 precipitate was re-dissolved in 5 ml 0.1 mol/l Tris-HCl buffer, pH 7.0, and after incubation at 37°C for 2 h chromatographed on a column of Sephadex G-200F at pH 7.0. An identical elution profile to that shown in Fig. 3.65 was obtained, indicating possibly a similar composition of the supernatant and the precipitate.

Further work was carried out with the supernatant, because the precipitate remained undissolved at acidic pH (even as low as pH 1.0), and thus could not be subjected to ion exchange chromatography and gel filtration at acidic pH. It was decided that when an effective disaggregation method for the aggregates was developed, the precipitate would be disaggregated to confirm that it contained the same components as those present in the supernatant.

It was decided to chromatograph the 10 - 40 kDa peak of Fig. 3.5 on Sephadex G-200F at pH 1.7 to compare its elution profiles with those of the 500 kDa and 250 kDa aggregates on Sephadex G-200F at pH 1.7. As the 10 - 40 estimated molecular weight ranging from 40 kDa to 10 kDa, it was thought that if it contained an antibacterial aggregate made up of the same components as the 500 kDa and 250 kDa aggregates, then the aggregate(s) on Sephadex G-200F at pH 1.7 would dissociate to give the active 20 kDa aggregate. Also, the acidic gel filtration may

dissociate lysozyme and/or seminalplasmin from the aggregates, if they are present in bound form with the aggregate.

Therefore, 1 ml supernatant from the concentrated sample of fractions 29 - 41 of Fig. 3.5 was diluted to 5 ml with 0.02 mol/l HCl. The pH of the sample was adjusted to 1.7 with HCl, and after holding the sample at 4°C for 2 h it was chromatographed through a column of Sephadex G-200F at pH 1.7. The results are shown in Fig. 3.66. The experiment was repeated once and gave an identical result. There was a broad peak whose middle was estimated to be about 40 kDa. The formation of a 40 kDa peak, instead of a 20 kDa peak as obtained from the 500 kDa and 250 kDa aggregates on Sephadex G-200F at pH 1.7, was probably because lysozyme or any other molecule was bound to the aggregate of small components. Alternatively, the aggregate and lysozyme were not combined but were not well separated because their molecular weights were outside the ideal separating range of Sephadex G-200F.

To check the later possibility, 2.5 ml of the concentrate of fractions 29 - 41 of Fig. 3.5 was diluted with an equal amount of 0.02 mol/l HCl, pH 1.7. The pH of the sample was adjusted to 1.7 with HCl and the sample was held at 4°C for 2 h. It was then chromatographed through a column of Sephadex G-50SF at pH 1.7. As can be seen in Fig. 3.67, all the activity, as also observed earlier with the 500 kDa and 250 kDa aggregates, disaggregated to the 20 kDa peak (fractions 16 - 24), indicating that at least part of the antibacterial activity in the 10 - 40 kDa peak of Fig. 3.5 was because of the aggregates of small components.

The 20 kDa peak of Fig. 3.67 had lytic activity, thus indicating possible binding of lysozyme with the aggregate and the peak with an estimated molecular weight of 15 ($\pm$ 1.5) kDa (fractions 24 -28) appeared to be free lysozyme because of its high lytic activity and low activity against *E. coli*. The inactive void volume peak (fractions 13 - 17, Fig. 3.67) was probably a protein having nothing to do with antibacterial activity, and just co-eluted with the 10 - 40 kDa peak of Fig. 3.5.

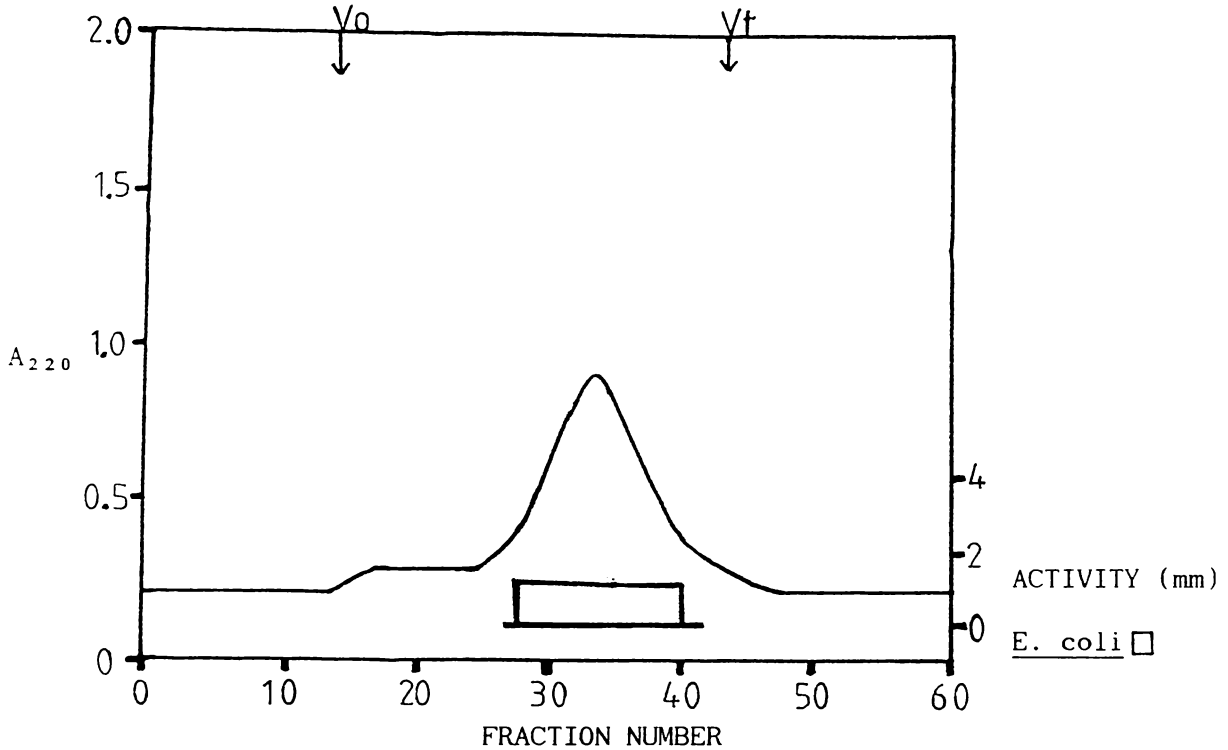


Fig. 3.65: Gel Filtration Chromatography of Fractions 28-41 from Fig. 3.5
Gel: Sephadex G-25SF
Buffer: 0.1 Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 28-41 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

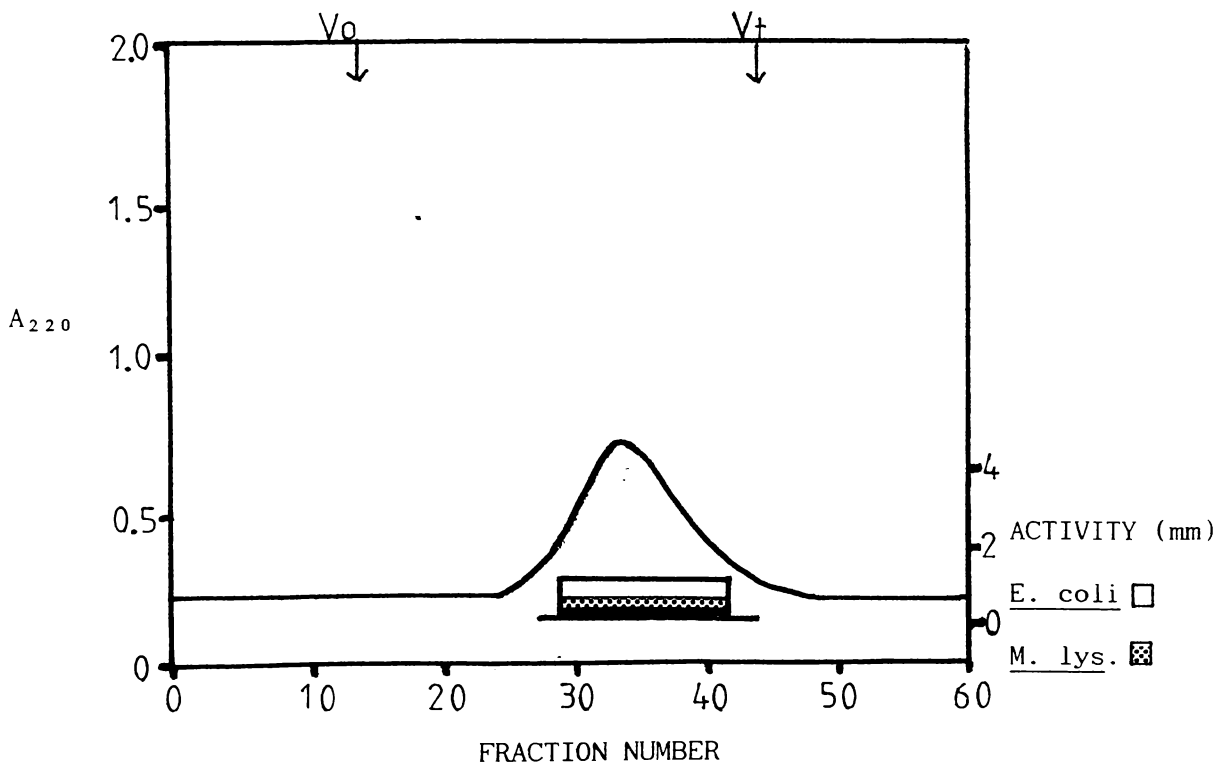


Fig. 3.66: Gel Filtration Chromatography of Fractions 29-41 from Fig. 3.5
Gel: Sephadex G-200F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 29-41 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

Attempts to Disaggregate the 10 - 40 kDa Peak Using Cation Exchange Chromatography

As described in the preceding sections, cation exchange chromatography disaggregated the 500 kDa and 250 kDa aggregates. Thus, in order to confirm that antibacterial activity of the 10 - 40 kDa peak is at least partly because of the aggregate of same small components which also form the 500 kDa and 250 kDa aggregates, this peak was subjected to cation exchange chromatography in the same way. Also, lysozyme or any other antibacterial compounds, if present in bound form to the aggregate, could be freed from the aggregate and identified later.

Accordingly, to 9 ml of the supernatant of the concentrate of 10 - 40 kDa peak (fractions 29 - 41, Fig. 3.5) was added 1 ml 1 mol/l citrate buffer, pH 3.0. The pH of the sample was adjusted to 3.0 with HCl, and after holding it at 4°C for 4 h, it was loaded onto a column of SP-Sephadex C-50. The sample was followed with 0.1 mol/l citrate buffer, pH 3.0 until absorbance at 230 nm of the effluent had returned to the baseline, and then was eluted with a continuous salt gradient of 0 to 4.0 mol/l NaCl in 0.1 mol/l citrate buffer, pH 3.0. This was followed by a continuous pH gradient of 0.1 mol/l citrate buffer, pH 3.0 containing 4.0 mol/l NaCl to 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl. Finally, the material still bound was eluted with the continuous pH gradient of 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl to 0.1 mol/l NaOH, pH 13.0 containing 4.0 mol/l NaCl. Fractions making up the various peaks were pooled in separate pools (Pools A, B, C and D) as shown in Fig. 3.68. The results are shown in Fig. 3.68. The activity of different peaks of Fig. 3.68 was not tested because of the presence of salt.

Gel filtration chromatography of the peaks from the cation exchange chromatography (Fig. 3.68) at acidic pH

In order to find the molecular weights of the material which passed straight through the cation exchanger (Pool A, Fig. 3.68), fractions making up the pool were pooled and evaporated down to about 5 ml by rotary evaporation. Because of the small size of the components if they had been disaggregated, salt could not be removed by dialysis or ultra-filtration. Salt crystals were removed by

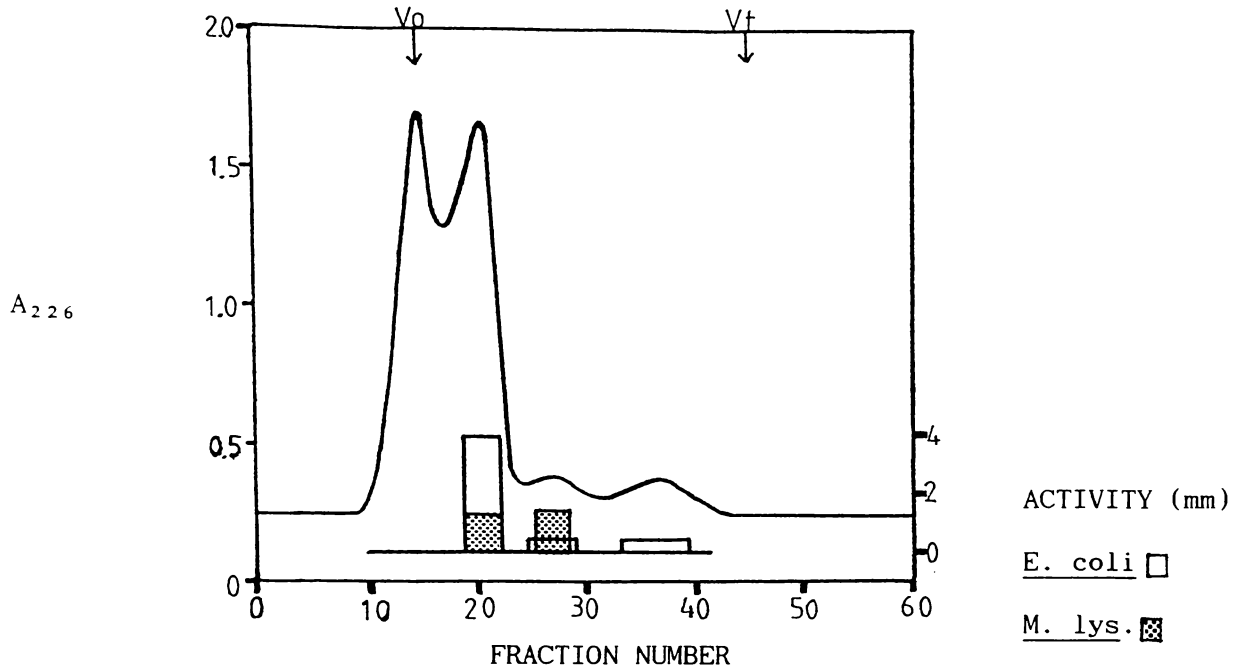


Fig. 3.67: Gel Filtration Chromatography of fractions 29-41 from Fig. 3.5
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 29-41 from Fig. 3.5 (5 ml)
Fraction Volume: 4 ml

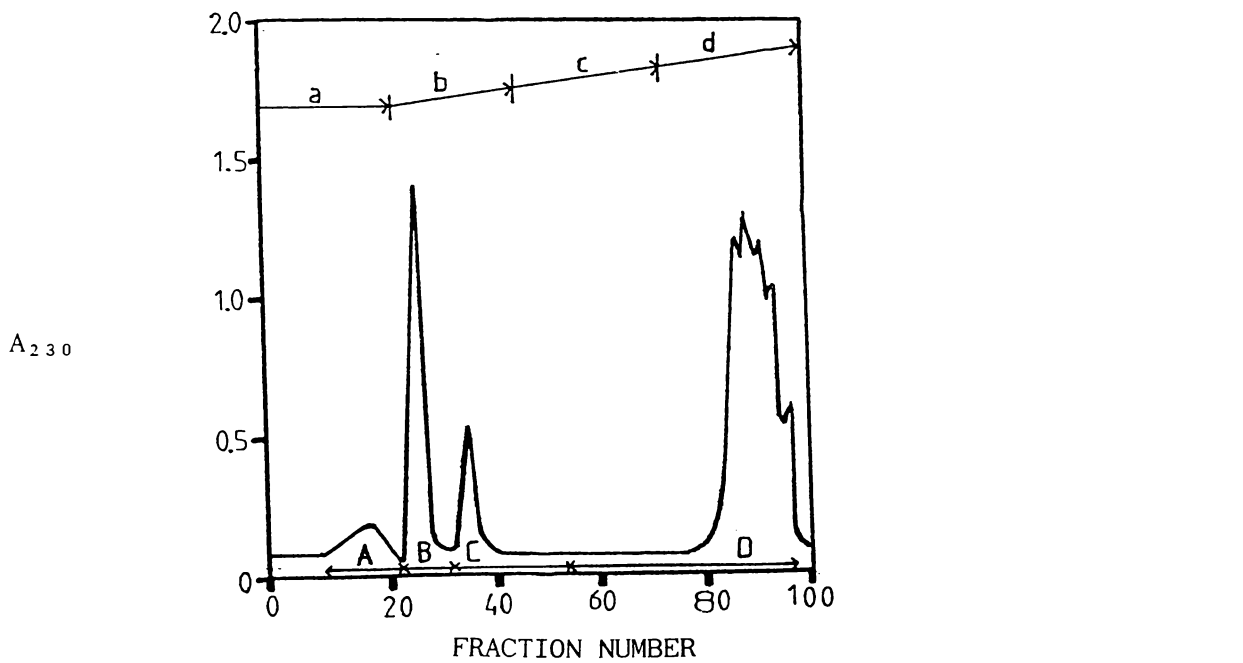


Fig. 3.68: Ion Exchange Chromatography of Fractions 29-41 from Fig. 3.5
Gel: SP-Sephadex C-50
Sample: Part of concentrate of fractions 29-41 from Fig. 3.5 (10 ml)
Elution Buffers and Eluents: (a) 0.1 mol/l citrate buffer, pH 3.0; (b) gradient from 0 to 4.0 mol/l NaCl in 0.1 mol/l citrate buffer, pH 3.0; (c) pH gradient from 0.1 mol/l citrate buffer, pH 3.0 containing 4.0 mol/l NaCl to 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl; (d) pH gradient from 0.1 mol/l citrate buffer, pH 8.0 containing 4.0 mol/l NaCl to 0.1 mol/l NaOH, pH 13.0 containing 4.0 mol/l NaCl
Fraction Volume: 4 ml

centrifugation at 6,000 g (r av.10 cm) for 10 minutes. The pH of the supernatant was adjusted to 1.7 with HCl and after holding at 4°C for 2 h it was chromatographed through a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.69. There was a void volume peak with a shoulder, both of which were of too high a molecular weight to be any free component. There was also a large bed volume peak which was possibly citrate. When fractions 18 - 25 were tested for activity, no activity was found. This was either because they were aggregates of the inactive aggregating component, or they were too dilute to show any activity. Alternatively, these peaks were proteins which were present in the 10 - 40 kDa peak but had nothing to do with antibacterial activity.

Similarly, fractions making up Pool B as shown in Fig. 3.68 were concentrated down to about 5 ml by rotary evaporation and salt crystals were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes. The pH of the supernatant was adjusted to 1.7 by addition of HCl, and after holding the sample at 4°C for 2 h it was chromatographed through a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. As can be seen in Fig. 3.70, the sample was again eluted on the void volume peak with a shoulder. However, this time the peaks were active. The active peak eluting in fractions 23 - 27 (Fig. 3.70), with an estimated molecular weight of about 4 kDa was too low a molecular weight to be seminalplasmin.

In order to have a better idea about the size of the active void volume peak (molecular weight of 5 kDa or over) of Fig. 3.70, fractions making up the void volume peak (fractions 19 - 23, Fig. 3.70) were pooled, and evaporated down to 5 ml by rotary evaporation. Then, the sample was chromatographed on a column of Sephadex G-50SF at pH 1.7. The results are shown in Fig. 3.71. There was a void volume peak (fractions 10 - 16, Fig. 3.71) and a 20 kDa peak (fractions 17 - 25, Fig. 3.71), both were active against *E. coli*. They both also lysed pre-grown *M. lysodeikticus*.

The molecular weight of lysozyme isolated from bovine seminal plasma had been reported to be about 14 kDa (Eschenbruch, 1980). To see the elution volume of egg-white lysozyme on Sephadex G-50SF at

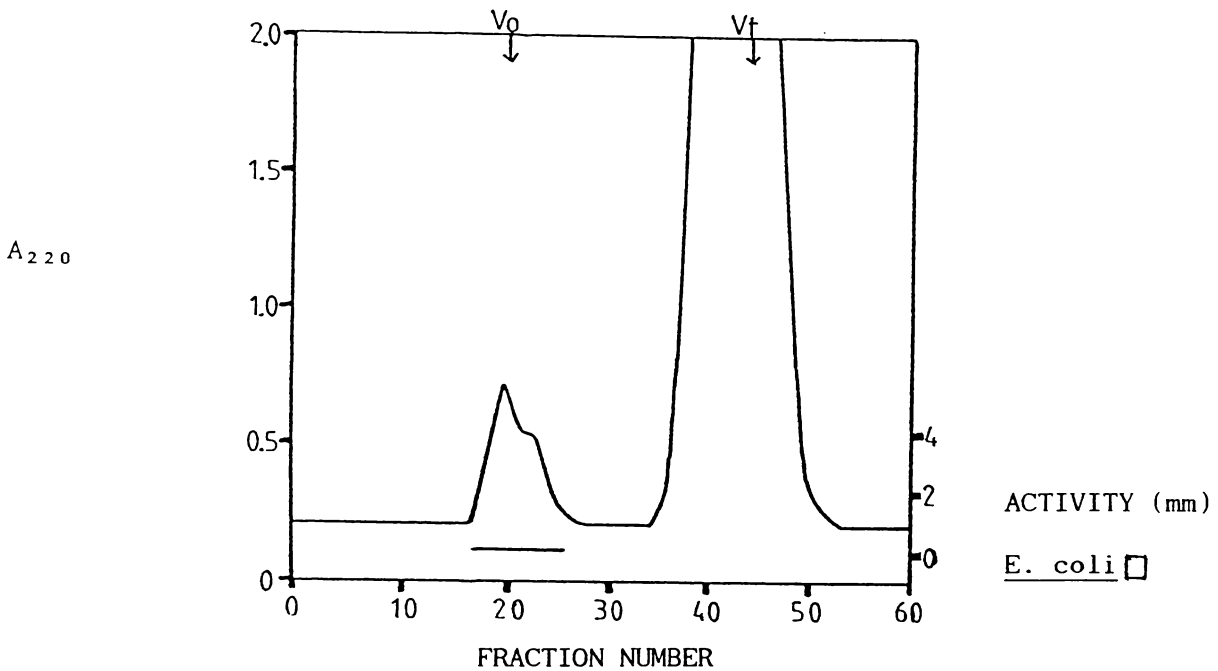


Fig. 3.69: Gel Filtration Chromatography of Pool A from Fig. 3.68
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool A from Fig. 3.68 (5 ml)
Fraction Volume: 4 ml

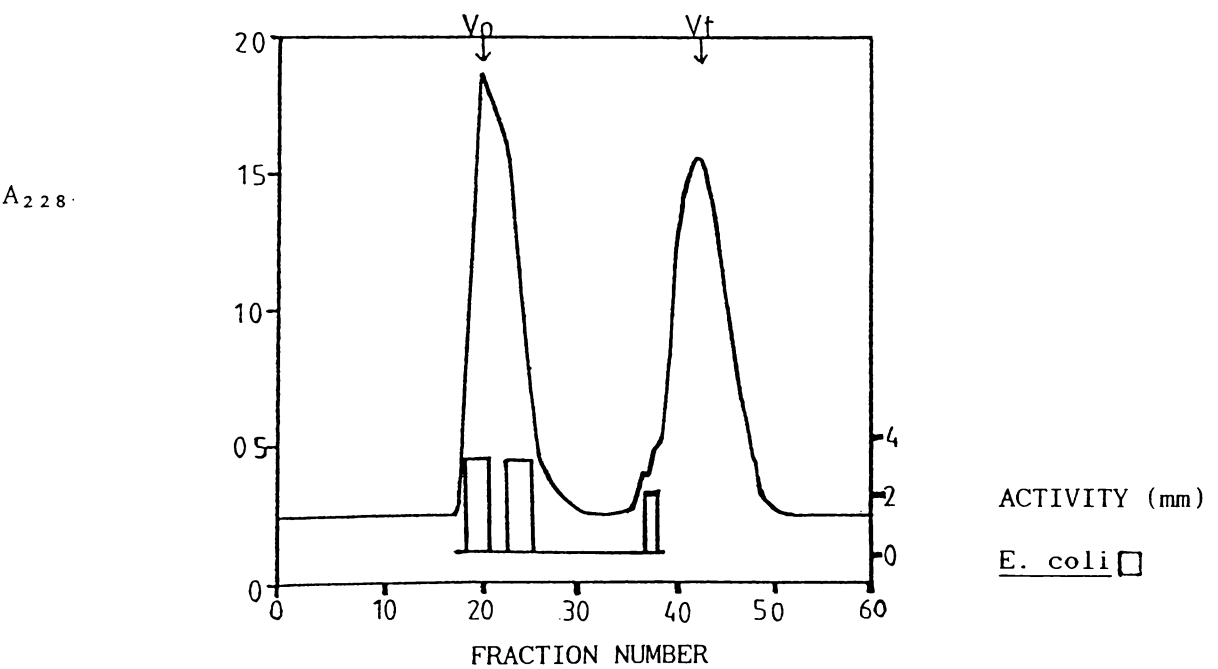


Fig. 3.70: Gel Filtration Chromatography of Pool B from Fig. 3.68
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool B from Fig. 3.68 (5 ml)
Fraction Volume: 4 ml

pH 1.7, a 2 mg lysozyme standard was dissolved in 5 ml 0.02 mol/l HCl, pH 1.7 and passed through a column of Sephadex G-50SF eluted with 0.02 mol/l HCl, pH 1.7. It was eluted 6 fractions later than the 20 kDa peak of Fig. 3.71.

Thus, the 20 kDa peak of Fig. 3.71 was probably lysozyme bound to some other protein. The presence of an antibacterial compound other than lysozyme in the void volume and 20 kDa peaks of Fig. 3.71 was supported by the different relative activities against *E. coli* and *M. lysodeikticus* in the two peaks. This antibacterial compound was most probably an antibacterial aggregate made up of the small components to which lysozyme was bound.

The void volume peak (fractions 10 - 16, Fig. 3.71) also had some lysozyme activity, which, however, was small compared with the activity of this peak against *E. coli*. This lysozyme activity on the void volume peak of Fig. 3.71 could either have been because lysozyme was present aggregated with some other protein, or because lysozyme was present as a dimer. Alternatively, the lysozyme activity could have been due simply to overlapping with the 20 kDa peak (fractions 17 - 25, Fig. 3.71), and the antibacterial activity of the void volume peak in Fig. 3.71 could have been due predominantly to aggregates which had not been disaggregated.

In an attempt to find the molecular weight of the void volume peak of Fig. 3.71, fractions 10 - 16 of Fig. 3.71 were pooled together and evaporated down to about 5 ml by rotary evaporation, and then chromatographed through a column of Sephadex G-200F at pH 1.7. As can be seen in Fig. 3.72, it was eluted as a broad peak, the middle of which was estimated from the calibration curve to have a molecular weight of about 30 kDa. The lower end of the peak was eluted on the bed volume indicating a molecular weight of about 10 kDa.

An explanation for the broad shape of the peak could be continuous aggregation and disaggregation of the sample as it ran through the column. The peak of Fig. 3.72 also showed activity against *S. aureus* indicating the presence of antibacterial aggregates, as *S. aureus* is not sensitive to lysozyme. The peak also had lysozyme activity. The molecular weight of the proteins in this peak, 30 kDa, was too high for it to contain any lysozyme co-eluting with the

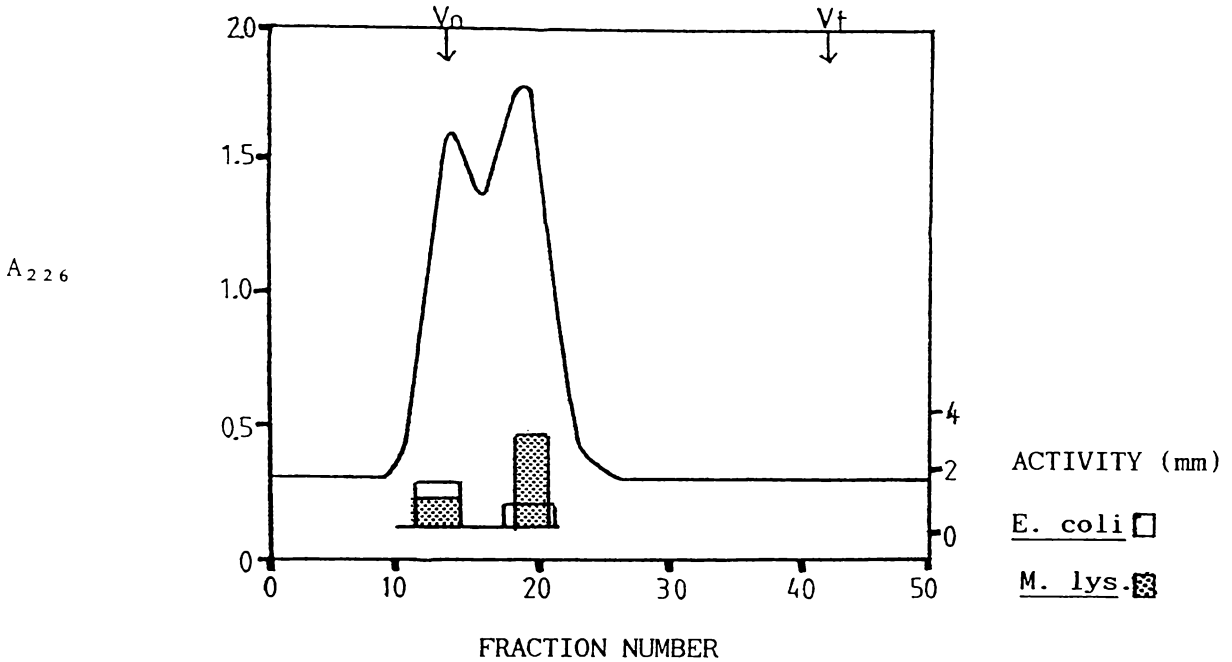


Fig. 3.71: Gel Filtration Chromatography of Fractions 19-23 from Fig. 3.70
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 19-23 from Fig. 3.70 (5 ml)
Fraction Volume: 4 ml

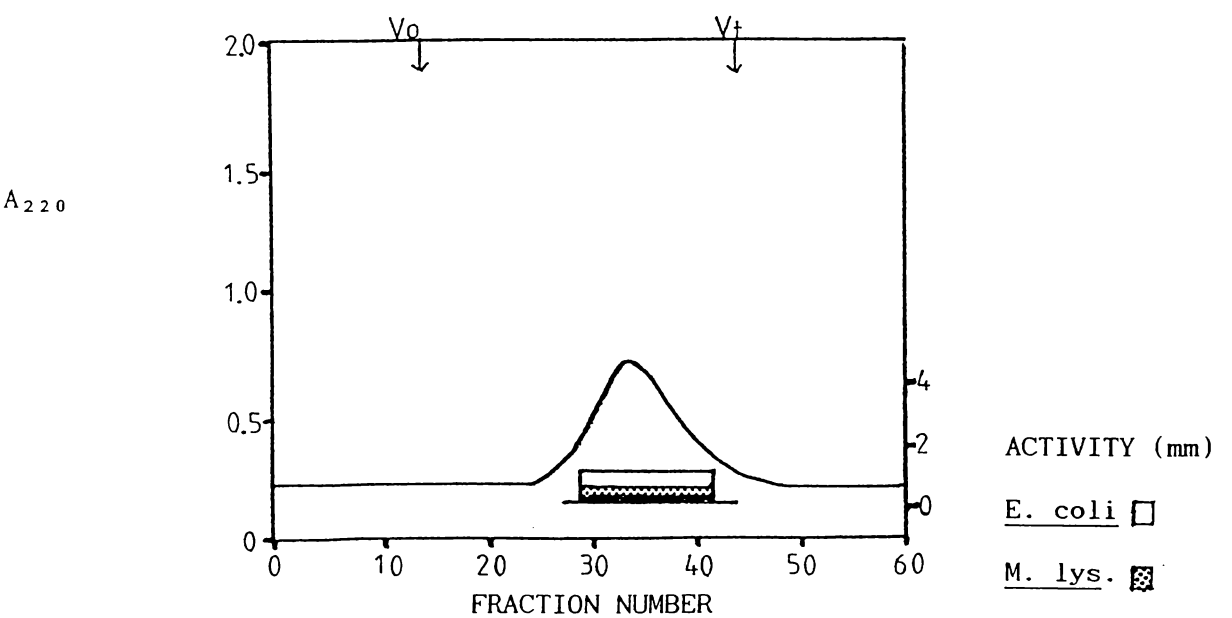


Fig. 3.72: Gel Filtration Chromatography of Fractions 10-16 from Fig. 3.71
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 10-16 from Fig. 3.72 (5 ml)
Fraction Volume: 4 ml

antibacterial aggregate. Thus, lysozyme appeared to be present in a bound form with a protein, probably to the antibacterial aggregate.

Further investigation of the lysozyme-containing peak of Fig. 3.72

In order to confirm whether the lysozyme in the peak of Fig. 3.72 is present as a dimer or is bound to the antibacterial aggregate or any other protein(s), HPLC and FPLC were used in the investigation of the peak of Fig. 3.72.

Fractions 28 - 42 as shown in Fig. 3.72 were pooled and half of the pool was concentrated down to dryness by rotary evaporation. The dried sample was re-constituted in 200 μl 0.1 % trifluoroacetic acid, and applied onto an analytical HPLC Brownlee RP C-18 reversed phase column, and eluted as shown in Fig. 3.73.

In order to compare the HPLC profile of the peak from Fig. 3.72 (Fig. 3.73) with that of the active aggregate, the 20 kDa aggregate was prepared as shown in Fig. 3.44, and a portion of the active 20 kDa aggregate was rotary-evaporated to dryness. The dried sample was re-constituted in 200 μl 0.1 % trifluoroacetic acid, and chromatographed in the same way. The results are shown in Fig. 3.74. The active 20 kDa aggregate which has all the components necessary to form antibacterial aggregates of various sizes, gave two peaks on HPLC eluted at 2.8 minutes and 3.1 minutes.

The peak of Fig. 3.72 when applied onto HPLC also gave two peaks at 2.8 minutes and 3.1 minutes (Fig. 3.73), indicating the presence of antibacterial aggregates made up of small components. This was not surprising as the peak was active against *S. aureus*. However, the peak from Fig. 3.72 gave one additional major peak (eluting at 14.0 minutes) on HPLC, which was probably lysozyme as the peak of Fig. 3.72 had lysozyme activity.

Since the molecular weights of the compound other than the antibacterial aggregate present in the peak of Fig. 3.72 could not be determined by HPLC, it was decided to chromatograph the peak of Fig. 3.72 on FPLC.

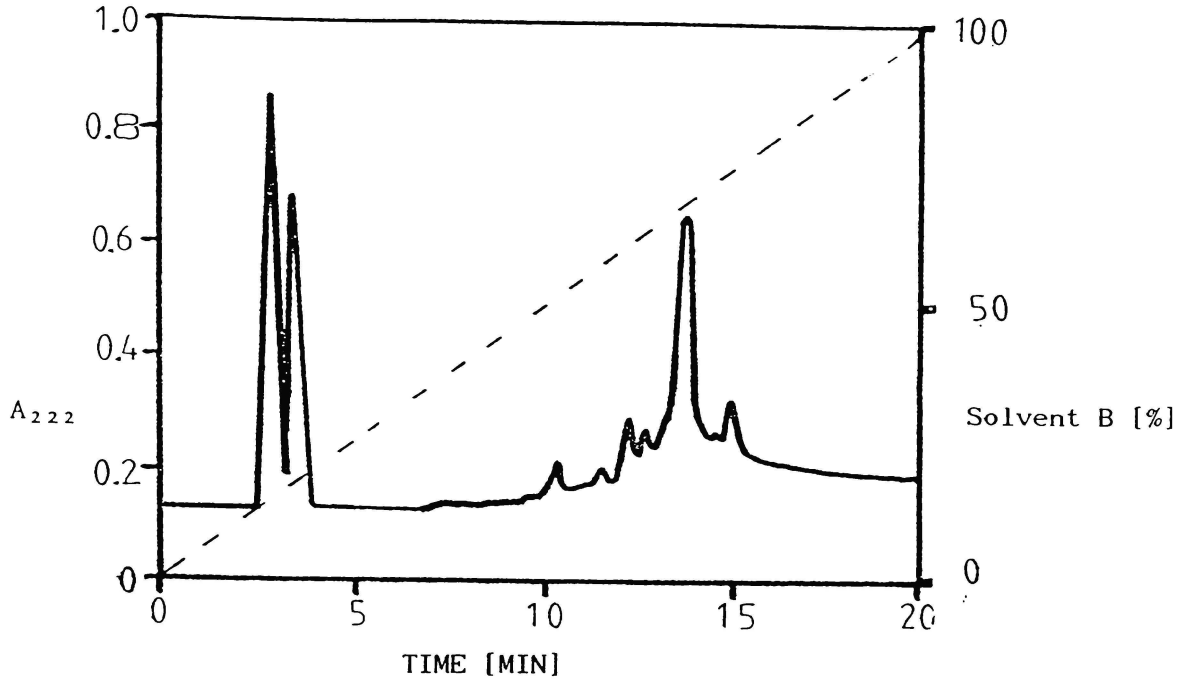


Fig. 3.73: HPLC of fractions 18-30 from Fig. 3.72
Column: Brownlee RP C-18
Sample: Part of concentrate of fractions 18-30 from Fig. 3.72 (200 µl)
Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B, 80% (v/v) acetonitrile in 0.1% trifluroacetic acid
Gradient: 0 to 100% Solvent B for 20 min

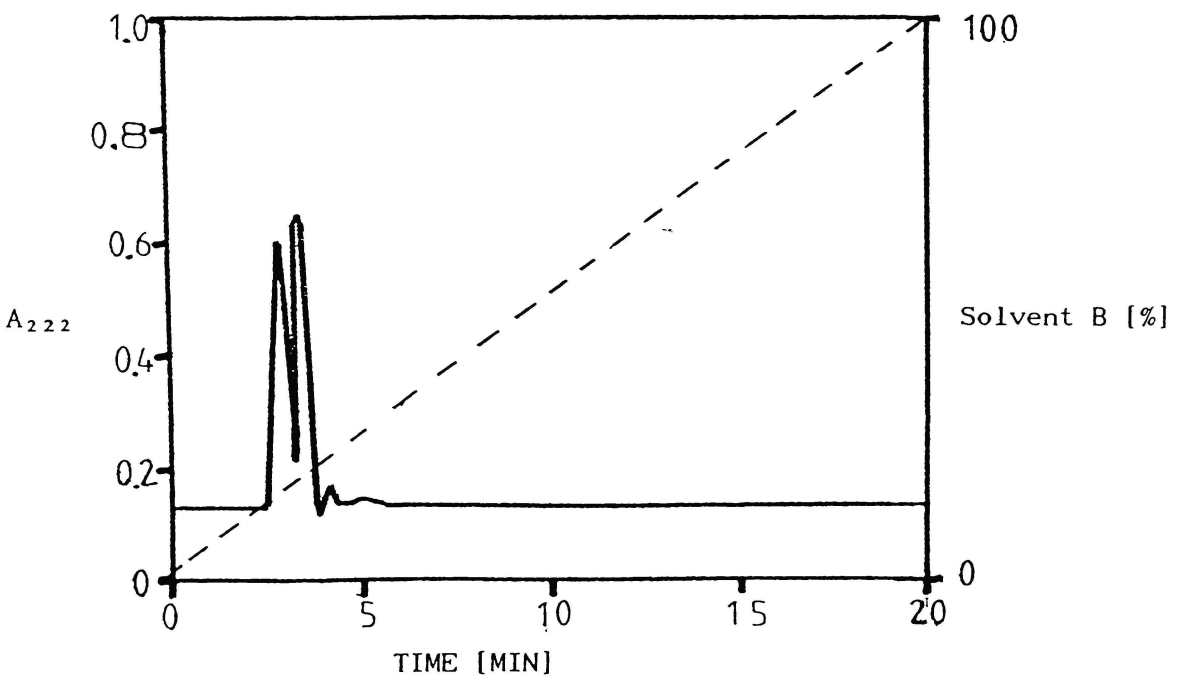


Fig. 3.74: HPLC of Fractions 38-51 from Fig. 3.44
Column: Brownlee RP C-18
Sample: Part of concentrate of fractions 38-51 from Fig. 3.44 (200 µl)
Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid
Gradient: 0 to 100% Solvent B for 20 min

Accordingly, the remaining half of the peak of Fig. 3.72 was evaporated down to dryness by rotary evaporation, and the sample was re-constituted in 1 ml 30 % (v/v) acetonitrile in 0.5 % trifluoroacetic acid. Then, a sample of 200 μl out of the re-constituted sample was loaded onto an analytical FPLC column of Superose 12. As can be seen in Fig. 3.75, two peaks eluted just before the bed volume, which were probably the low molecular weight components of the antibacterial aggregates.

The column of Superose 12 was calibrated by running Thyrogobulin (molecular weight 669 kDa), Beta-Amylase (molecular weight 200 kDa), Carbonic Anhydrase (molecular weight 29 kDa) and Suc-Ala-Ala-Ala-p-nitroanilid (molecular weight 0.471) standards. There was also a peak of about 15 ($\pm$ 3) kDa molecular weight estimated from the calibration curve eluted at 16 ml obtained in Fig. 3.75. This peak was probably lysozyme, as the peak of Fig. 3.72 was found to have lysozyme activity.

Gel filtration chromatography of the bed volume peak from Fig. 3.70

To see whether fractions 37 - 50 of Fig. 3.70 contained some disaggregated active component, these fractions were pooled and concentrated down to 5 ml by rotary evaporation. The excess salt was removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes, and the supernatant was chromatographed on a column of Sephadex G-15 eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.76. There was an active peak with an estimated molecular weight of about 1.2 kDa. There was also a low molecular weight peak which was probably citrate.

Gel filtration of Pools C and D of cation exchange chromatography

In order to find the molecular weight of Pool C of Fig. 3.68 the fractions making up the pool were pooled together and evaporated down to 5 ml by rotary evaporation. The pH of the sample was adjusted to 1.7 by addition of HCl, and after holding the sample at 4°C for 2 h, it was chromatographed on a column of Sephadex G-25SF at pH 1.7. As can be seen in Fig. 3.77, the sample was eluted as an active void volume peak, indicating a molecular weight of 5 kDa or over. There was also a bed volume peak, which was probably citrate.

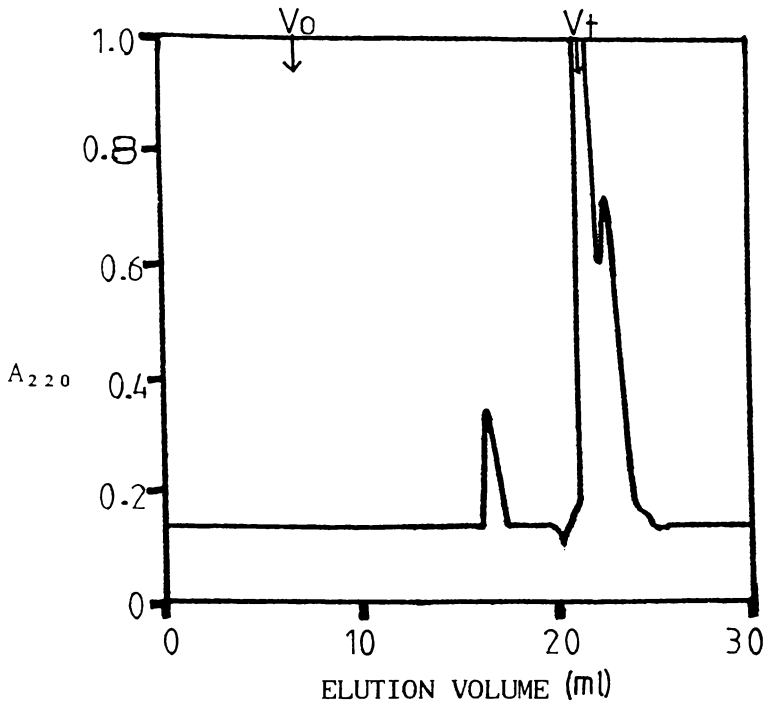


Fig. 3.75: FPLC of Fractions 18-30 from Fig. 3.72
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of fractions 18-30 from Fig. 3.72 (200 µl)

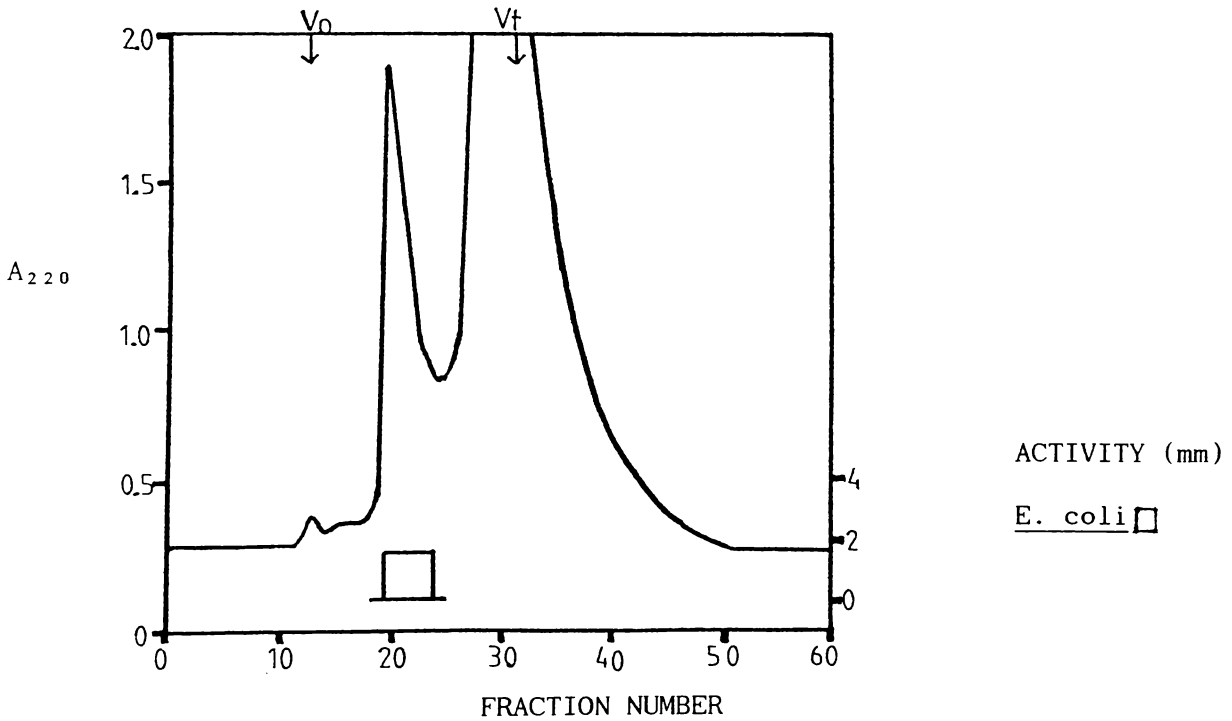


Fig. 3.76: Gel Filtration Chromatography of Fractions 37-50 from Fig. 3.70
Gel: Sephadex G-15
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 37-50 from Fig. 3.70 (5 ml)
Fraction Volume: 4 ml

To obtain a better estimate of the molecular weight of the void volume peak of Fig. 3.77, fractions 16 - 24 were pooled and concentrated down to 5 ml by rotary evaporation, and chromatographed through a column of Sephadex G-50SF eluted with 0.02 mol/l HCl, pH 1.7. The results are shown in Fig. 3.78. The sample was eluted mainly as a void volume peak on Sephadex G-50SF indicating a molecular weight of 30 kDa or over. When testing for activity, this peak was found to be active against both *E. coli* and pre-grown *M. lysodeikticus*. Thus, this peak, like the peak of Fig. 3.72, indicated the presence of lysozyme in a bound form to some protein, probably with the antibacterial aggregate.

To obtain an estimate of the molecular weight of the peak which was eluted with the gradient of high pH on cation exchange chromatography (Pool D, Fig. 3.68), fractions making up the pool were pooled together and evaporated down to about 5 ml by rotary evaporation. The salt crystals were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes, and the pH of the supernatant was adjusted to 1.7 with HCl. After holding the sample at 4°C for 2 h it was passed through a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. As can be seen in Fig. 3.79, it was eluted as a sharp active peak (fractions 39 - 41, Fig. 3.79), the molecular weight of which was estimated to be about 1.2 kDa from the calibration curve. This peak appeared to be the active component of the aggregates. There was also a large bed volume peak which was probably mainly citrate.

Conclusions

Antibacterial aggregates made up of the same small components which also form the 500 kDa and 250 kDa aggregates, and lysozyme, were both found to be responsible for the antibacterial activity of the 10 - 40 kDa peak (fractions 29 - 41, Fig. 3.5). Lysozyme was isolated from the peaks of up to about 30 kDa, and appeared to be present in bound form with a protein, probably with the antibacterial aggregate. However, no antibacterial peak of around 6 kDa was obtained on disaggregation of the 10 - 40 kDa peak, thus indicating seminalplasmin is probably not responsible for the antibacterial activity of the 10 - 40 kDa peak of Fig. 3.5.

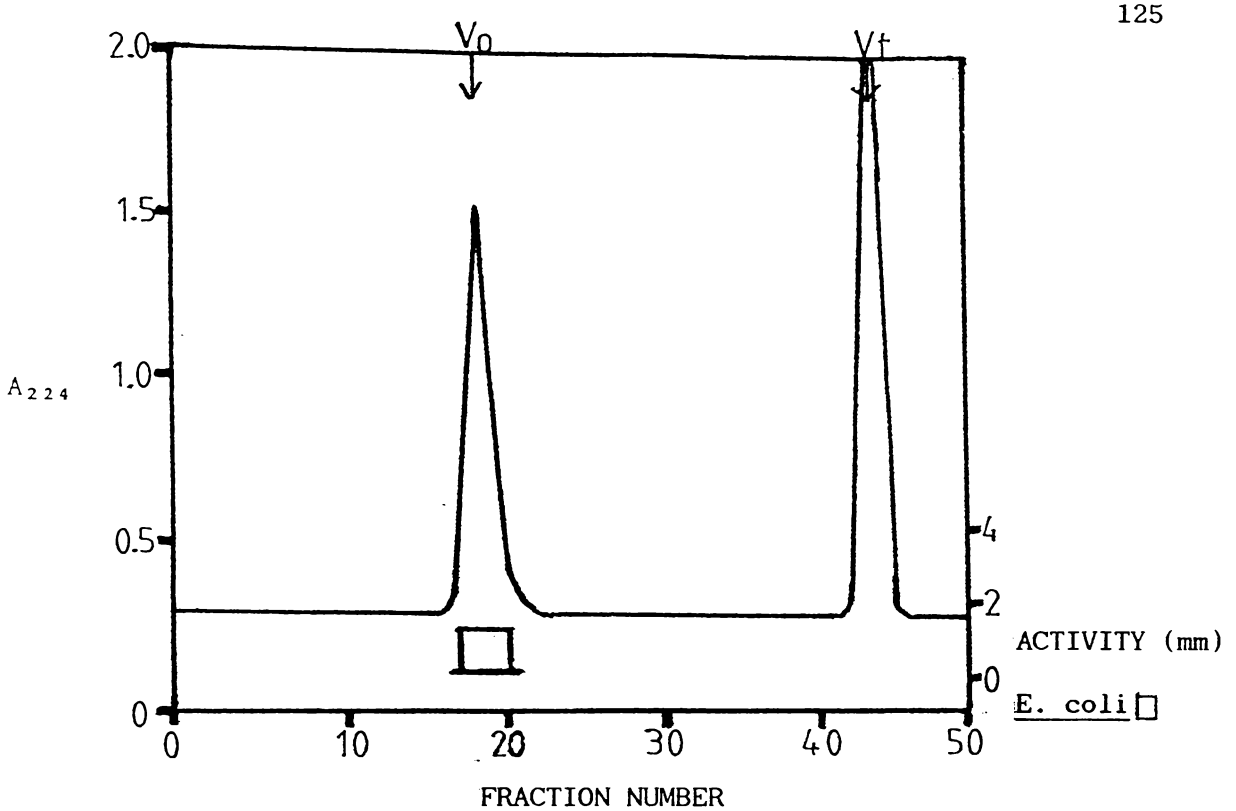


Fig. 3.77: Gel Filtration Chromatography of Pool C from Fig. 3.68
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool C from Fig. 3.68 (5 ml)
Fraction Volume: 4 ml

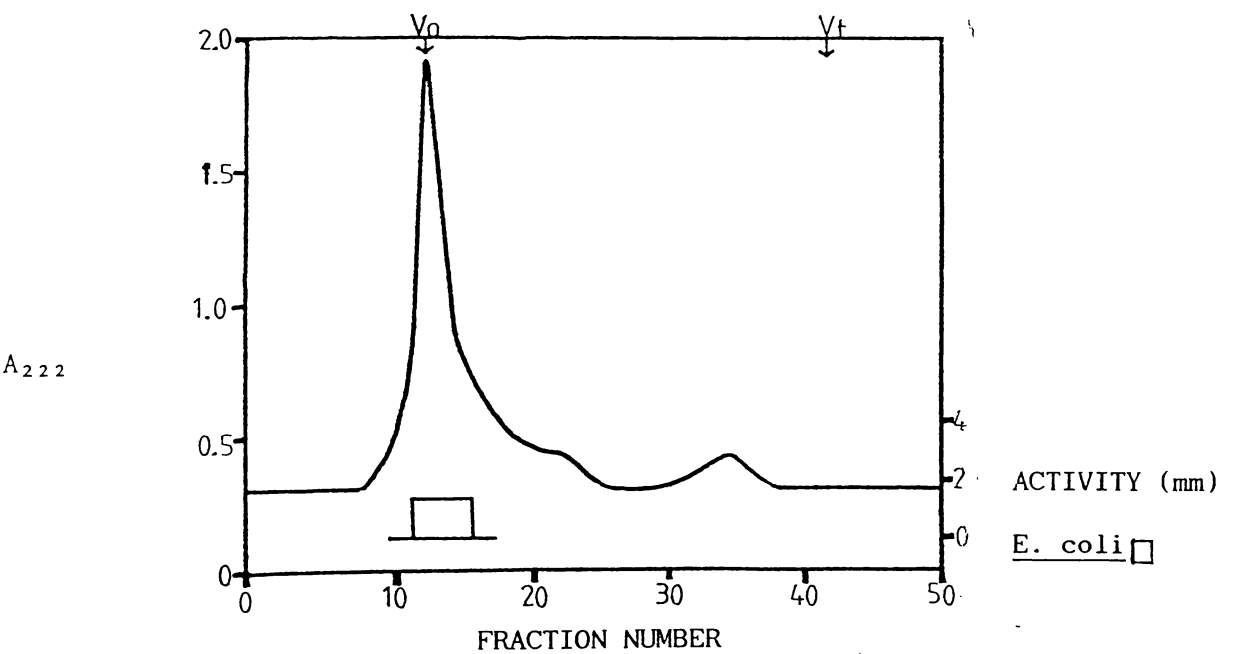


Fig. 3.78: Gel Filtration Chromatography of Fractions 16-24 from Fig. 3.77
Gel: Sephadex G-50SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of fractions 16-24 from Fig. 3.77 (5 ml)
Fraction Volume: 4 ml

Investigation of the Last Peak (of Lowest Molecular Weight) in Seminal Plasma on Sephadex G-200F at pH 7.0

Introduction

The last peak (of lowest molecular weight) obtained by gel filtration chromatography of seminal plasma on Sephadex G-200F (fractions 42 - 53, Fig. 3.5) was eluted just after the bed volume at pH 7.0. Therefore, the molecular weight of this peak could not be estimated from this chromatography.

As can be seen in Fig. 3.5, only the first half of the peak (fractions 42 - 46) which was eluted just after the bed volume was active against *E. coli*. *E. coli* is sensitive to all the antibacterial compounds known to exist in bovine seminal plasma, namely, antibacterial aggregates, lysozyme and seminalplasmin. Thus, the more retarded later half of the peak (fractions 47 - 53), being inactive, was of no interest in this study.

Activity was eluted until fraction 46 in the retarded peak of Fig. 3.5, and there was higher activity against *S. aureus* (which is not killed by lysozyme) in the last peak (fractions 42 - 46 of Fig. 3.5) than in the 10 - 40 kDa peak (fractions 29 - 41, Fig. 3.5), as shown in Fig. 3.5. Therefore, the antibacterial activity in the last peak could not have been because of overlapping with the 10 - 40 kDa peak. A significant amount of lysozyme activity was also found in the last peak (fractions 42 - 46, Fig. 3.5), which was considered to be too high to be because of overlapping with the 10 - 40 kDa peak of Fig. 3.5, therefore lysozyme also seemed to be eluted in the last peak (fractions 42 - 46) of Fig. 3.5.

Since seminalplasmin was not found in any other peak of Fig. 3.5, it also was expected to be present in the last peak of Fig. 3.5. Shackell (1984) demonstrated that polyamines are not present in a bound form with any high molecular weight protein fractions in bovine seminal plasma. Thus, if polyamines, spermine and/or spermidine is, in part, responsible for the antibacterial activity in bovine seminal plasma, it was expected to be present in the last peak of Fig. 3.5. Therefore, it was anticipated that more than one antibacterial compound(s) might be present in the last peak of Fig. 3.5.

It was thought that if the same small components which also form the 500 kDa and 250 kDa aggregates were present, free or in the form of a small aggregate, in the last peak of Fig. 3.5, then they might aggregate to larger size on re-chromatographing. Thus, the last peak (fractions 42 - 46, Fig. 3.5) from four repeated runs were concentrated down to about 10 ml by rotary evaporation, and a 5 ml sample was chromatographed through a column of Sephadex G-200F at pH 7.0. As can be seen in Fig. 3.80, the sample partly re-aggregated, possibly indicating the presence of the same small aggregating components which also form the 500 kDa, 250 kDa and 10 - 40 kDa aggregates (compare Fig. 3.5 with Fig. 3.80). The re-aggregated peak of Fig. 3.80 could not have been because of overlapping with the 10 - 40 kDa peak, as the overlapping could not have resulted in a larger high molecular weight peak (fractions 31 - 42) than the original peak (fractions 43 - 57).

In order to have a better idea about the composition of the last peak of Fig. 3.5, the pH of the other 5 ml of the concentrate of the peak was lowered to 1.7 with HCl, and after holding the sample at 4°C for 2 h it was then chromatographed on a column of Sephadex G-50SF at pH 1.7. The results are shown in Fig. 3.81. There was a small void volume peak indicating a molecular weight of 30 kDa or over, a shoulder with a molecular weight of about 20 ($\pm$ 2) kDa and a peak with a molecular weight of about 15 ($\pm$ 1.5) kDa. There was also a bed volume peak which was inactive against both *E. coli* and *M. lysodeikticus*, and because of its inactive nature it was of no interest in this study.

To study the nature of different antibacterial peaks of Fig. 3.81, the activity of egg-white was tested on pre-grown *M. lysodeikticus* cells and against *E. coli*, and it was observed that the lysozyme standard gave about 6 - 8 times more clearing on the pre-grown *M. lysodeikticus* plate than it gave on the *E. coli* plates.

Since the void volume peak (fractions 13 - 16) and the 20 kDa peak (fractions 17 - 24) had more activity against *E. coli* than on pre-grown *M. lysodeikticus*, these peaks were most probably antibacterial aggregates, with some lysozyme in a bound form.

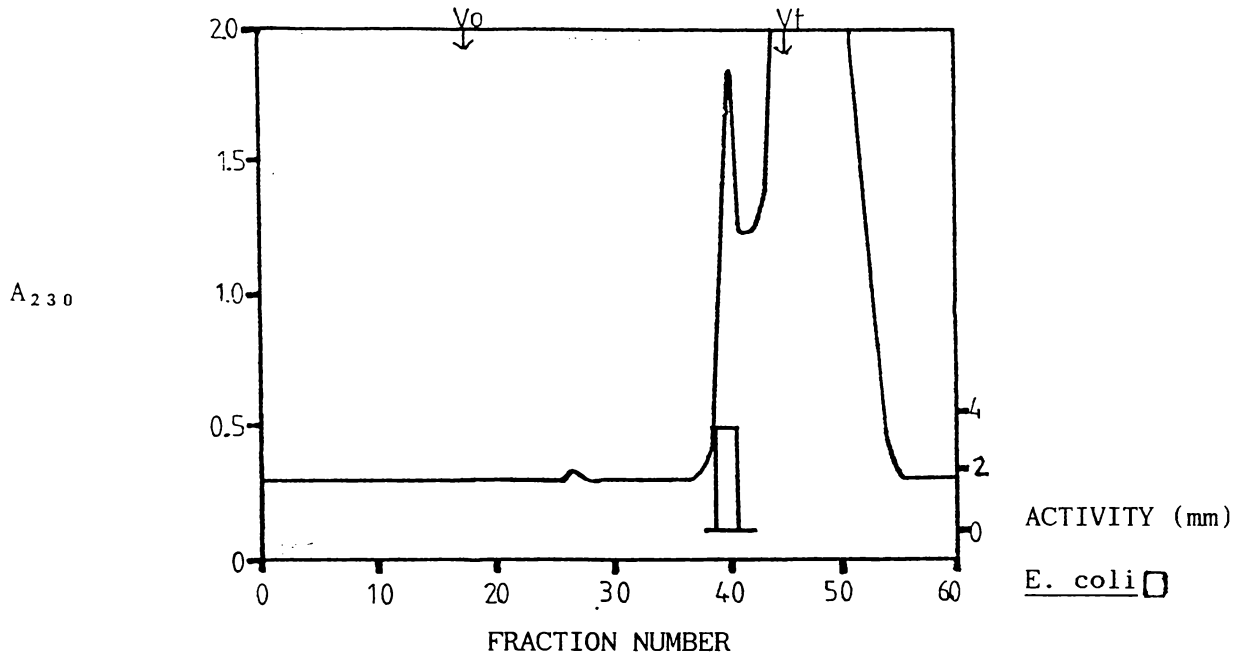


Fig. 3.79: Gel Filtration Chromatography of Pool D from Fig. 3.68
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Concentrate of Pool D from Fig. 3.68
(5 ml)
Fraction Volume: 4 ml

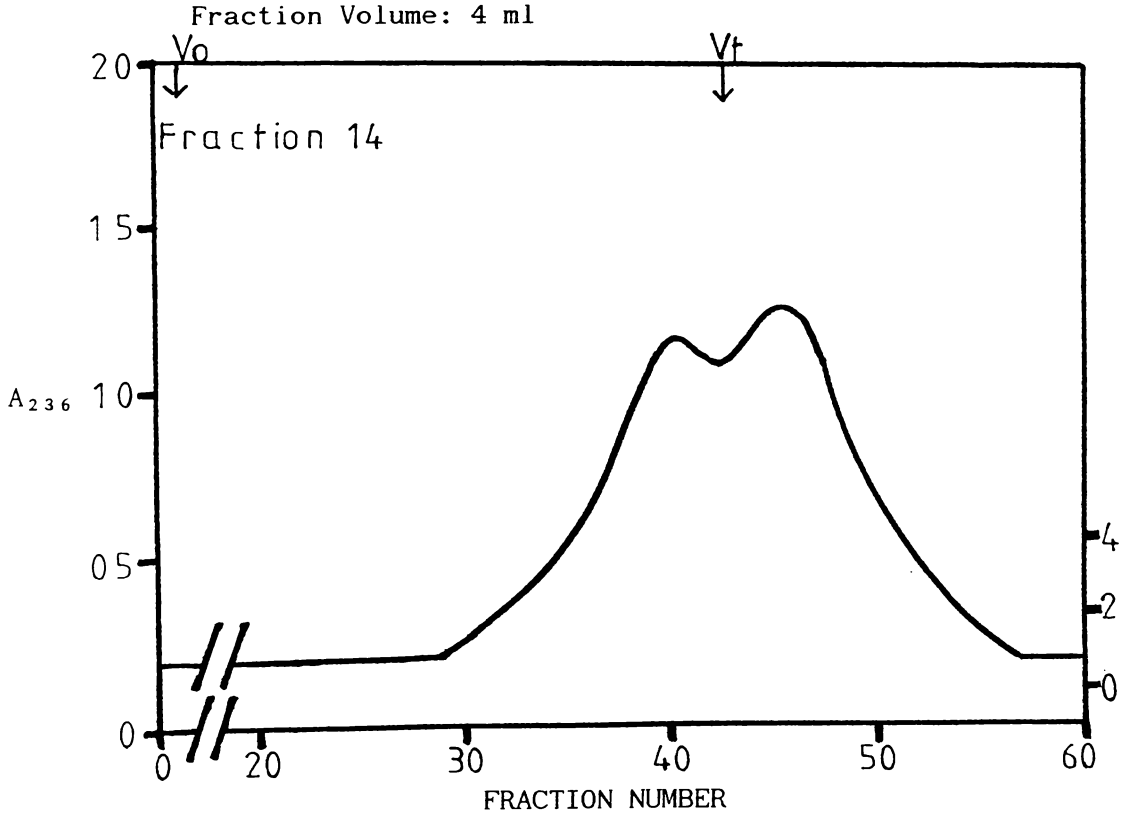


Fig. 3.80: Gel Filtration Chromatography of Fractions 42-53 from Fig. 3.5
Gel: Sephadex G-200F
Buffer: 0.1 mol/l Tris-HCl buffer, pH 7.0
Sample: Part of concentrate of fractions 42-53 from Fig. 3.5
(5 ml)
Faction Volume: 4 ml

The peak eluted in fractions 25 - 31 had very little activity against *E. coli*, but high activity against pre-grown *M. lysodeikticus* cells. Thus, this peak appeared to be free lysozyme. This possibility was further supported by its molecular weight being estimated to be about 15 kDa from the calibration curve, as Eschenbruch (1980) isolated lysozyme from bovine seminal plasma with an estimated molecular weight of about 14 kDa.

Conclusions

The last peak of seminal plasma on Sephadex G-200F at pH 7.0 (Fig. 3.5) was found to contain small antibacterial aggregates or the free aggregating components. These showed some further aggregation on re-chromatography at neutral pH. However, the major constituent of the last peak of Fig. 3.5 appeared to be free lysozyme.

Identification of the Type of Interactions Involved in the Formation of the 20 kDa Aggregate Obtained from the 500 kDa and 250 kDa Aggregates

As described in the preceding sections, the 500 kDa and 250 kDa aggregates dissociated on gel filtration at pH 1.7, apparently by suppressing the ionisation of negatively charged groups, to give the active 20 kDa aggregate. This indicates that the 20 kDa aggregate forms aggregates by ionic interactions.

However, as the components have been found to be of much smaller size, and also as they diffuse across a 3.5 kDa cut-off dialysing membrane, the 20 kDa molecule obtained on acidic gel filtration must have been still an aggregate.

Thus, it was decided to submit the 20 kDa aggregate to different disaggregating treatments, and it was hoped that the treatment(s) succeeding in breaking down the 20 kDa aggregate would indicate the type of interaction involved in the assembly of the 20 kDa aggregate.

Accordingly, two lots of 5 ml bovine seminal plasma was chromatographed on Sephadex G-200F at pH 7.0 and the 500 kDa and 250 kDa peaks (fractions 16 - 28, Fig. 3.5) were subsequently chromatographed on Sephadex G-200F at pH 1.7. The fractions making up

the active 20 kDa peak (fractions 38 - 51, Fig. 3.44) were pooled and evaporated down to about 30 ml by rotary evaporation.

Attempts to disaggregate the 20 kDa aggregate at high and low pH

Dawes (1964) and Shulmina *et al.* (1970) have described that the pH is an important factor in aggregation and disaggregation of proteins. It was thought that disaggregation of the 20 kDa aggregate by extremes of pH would indicate an ionic type of interaction.

As described in a preceding section, the 500 kDa and 250 kDa aggregates were not disaggregated below the 20 kDa aggregate (Fig. 3.16) at pH 1.7, thus to facilitate the breakdown of possible ionic interactions involved in the assembly of the 20 kDa aggregate, salt was used in combination with acidic pH as ions may compete for ionic binding sites which could be involved in the ionic interactions.

Thus, NaCl (2.5 mol/l) was added to a 5 ml sample of the 20 kDa aggregate and the pH of the sample was adjusted to 1.7 by addition of HCl. After holding at 4°c for 2 h, the sample was chromatographed on a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7. As can be seen in Fig. 3.82, the sample was eluted on the void volume indicating a molecular weight of 5 kDa or over. As the components of the antibacterial aggregates had been shown to be smaller than 3.5 kDa, this showed that complete disaggregation had not been achieved.

It was thought that a very strong acidic group might be involved in the assembly of the 20 kDa aggregate, the ionisation of which would not be suppressed at pH 1.7. To check this possibility, the pH of another 5 ml of the concentrate of the 20 kDa aggregate was adjusted to 1.0 with HCl. The sample was held at 4°C for 2 h and then chromatographed through a column of Sephadex G-25SF eluted with 0.1 mol/l HCl, pH 1.0. Again, the sample was eluted on the void volume, showing that disaggregation was not achieved.

Since acidic gel filtration did not disaggregate the 20 kDa aggregate it was decided to chromatograph the sample under highly alkaline conditions (pH 13.0) to suppress the ionisation of the basic

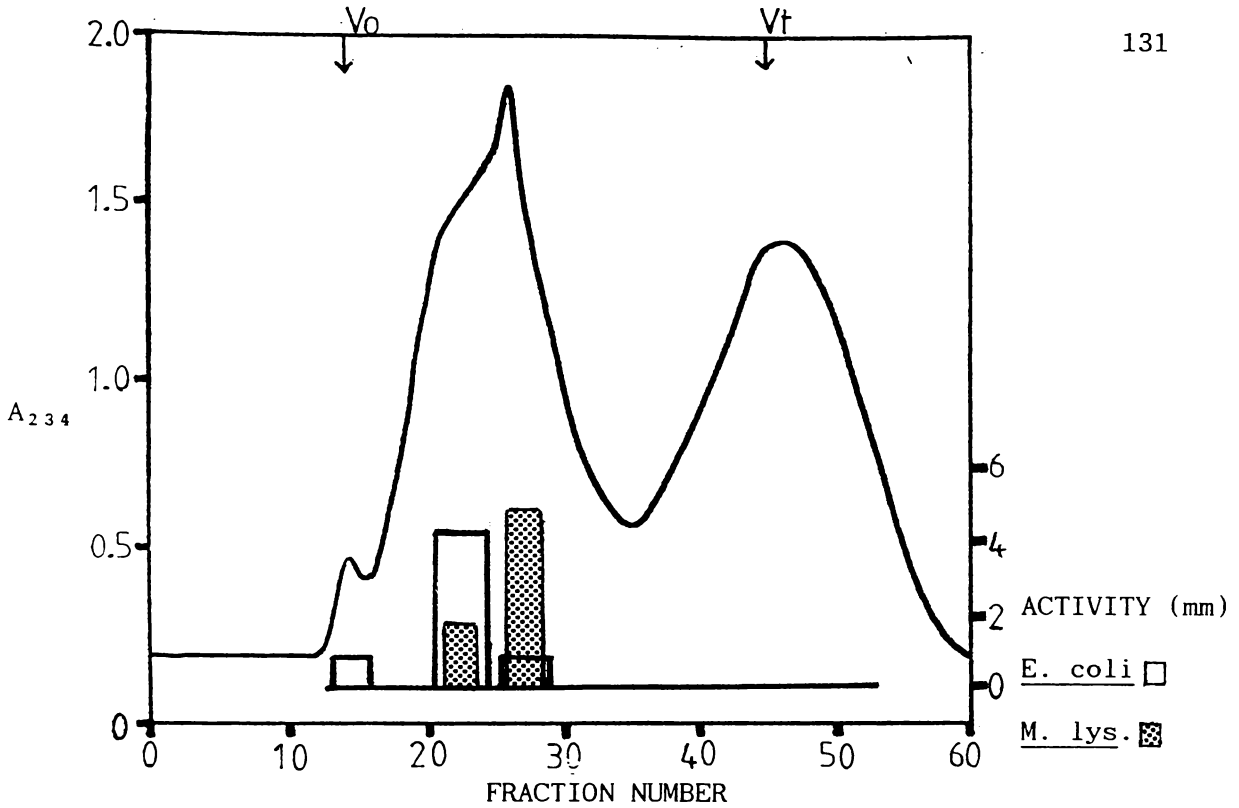


Fig. 3.81: Gel Filtration Chromatography of Fractions 42-53 from Fig. 3.5
Gel: Sephadex G-50SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fractions 42-53 from Fig.3.5 (5 ml)
Fraction Volume: 4 ml

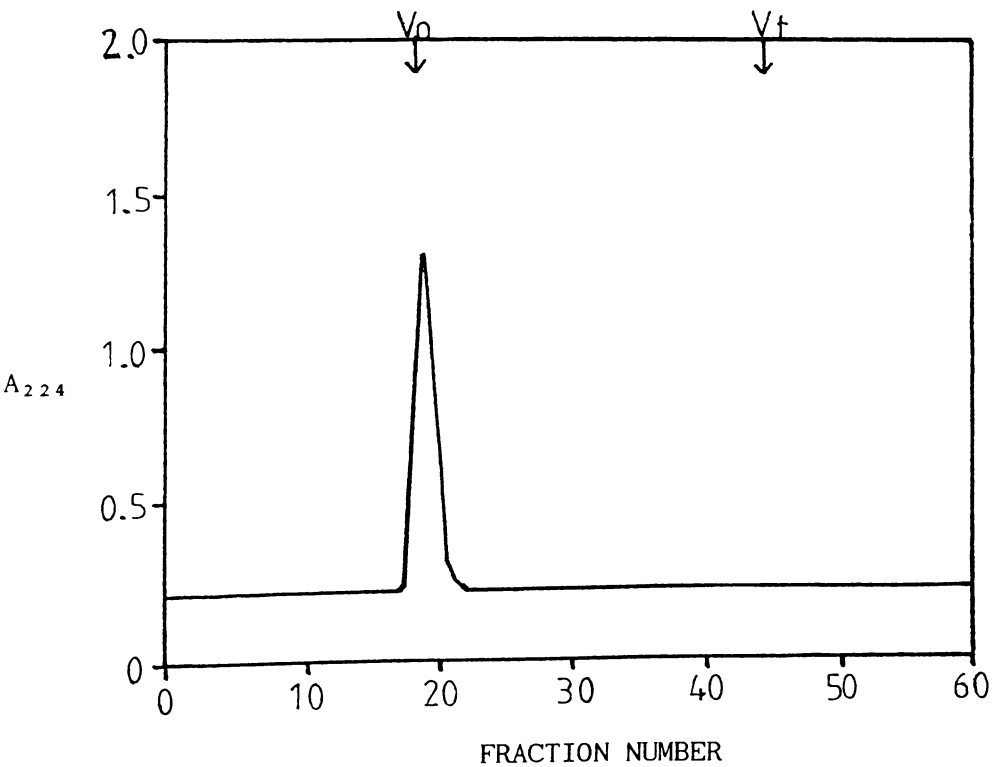


Fig. 3.82: Gel Filtration Chromatography of the 20 kDa Aggregate as Shown in Fig. 3.44 at Low pH With Salt
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Part of concentrate of fraction 38-53 from Fig. 3.44 containing 2.5 mol/l NaCl (5 ml)
Fraction Volume: 4 ml

groups. To minimize any denaturation of the sample, FPLC with an analytic column of Superose 12 was used because of its fast flow rate. Accordingly, the pH of a 1 ml sample of the concentrate of the 20 kDa aggregate was adjusted to 13.0 with NaOH, and after keeping the sample at room temperature for 20 minutes, a 100 μl sample was chromatographed through a Superose 12 column eluted with 0.1 mol/l NaOH, pH 13.0. The results are shown in Fig. 3.83. Although there was a small peak on the bed volume, the size of the major peak (15 ml, Fig. 3.83) was estimated to be about 20 ($\pm$ 2) kDa, thus indicating that the complete disaggregation of the 20 kDa aggregate was not obtained. The small bed volume could be because of partial disaggregation.

The results described in this section demonstrated that the 20 kDa aggregate is not assembled only with ionic interactions.

The effect of mercaptoethanol and urea in the disaggregation of the 20 kDa aggregate

It was thought hydrogen bonds and/or disulphide bonds could be involved in the aggregation to form the 20 kDa aggregate. Urea is widely used to dissociate proteins owing to its ability to break hydrogen bonds, and 2-mercaptoethanol, a reducing agent, is used to break disulphide bonds by reduction.

Thus, it was decided to use a combination of urea and 2-mercaptoethanol to see if they could disaggregate the 20 kDa aggregate.

Accordingly, first urea (to a concentration of 8 mol/l) and then 2-mercaptoethanol (1 %) were added to a 5 ml sample of the concentrate of the 20 kDa aggregate. The pH of the sample was lowered to 1.7 with HCl, and after holding the sample at 4°C for 4 h it was chromatographed on a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7 containing 8 mol/l urea. The results are shown in Fig. 3.84. There was a low molecular weight peak, which could either be a component of the aggregate, showing a partial disaggregation, or excess urea in the sample, or 2-mercaptoethanol.

To check the later possibility, urea was added to a 5 ml blank of 0.02 mol/l HCl, pH 1.7 in a concentration of 8 mol/l, then 1 % 2-mercaptoethanol was added to the blank, and finally the pH of the blank

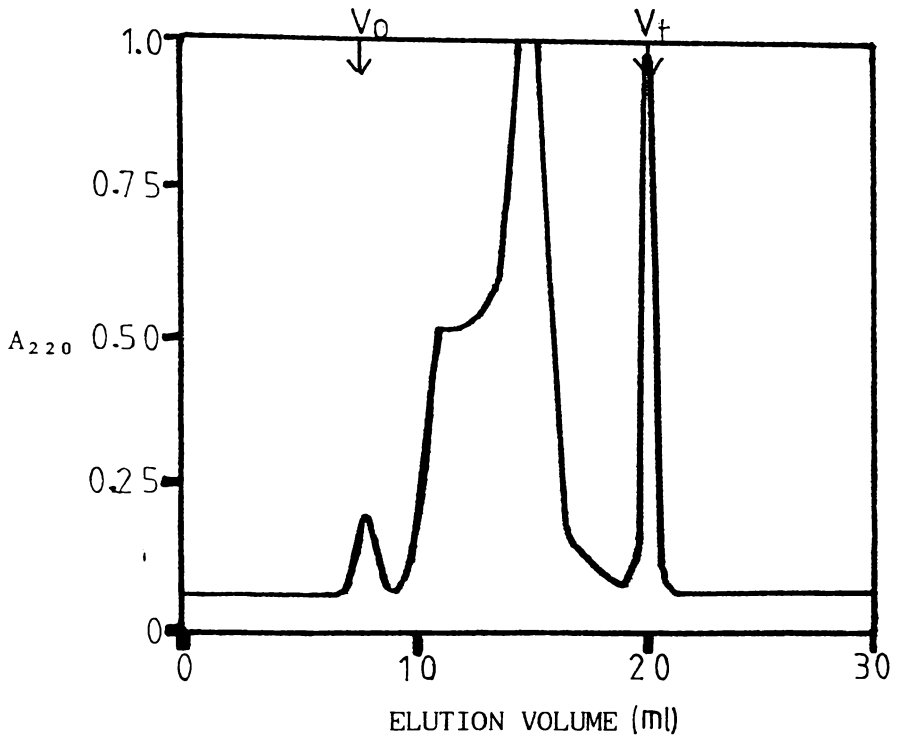


Fig. 3.83: FPLC of the 20 kDa aggregate as Shown in Fig. 3.44 at High pH
Gel: Superose 12
Eluent: 0.1 mol/l NaOH, pH 13.0
Sample: Part of concentrate of fractions 38-53 as shown in Fig. 3.44 (100 µl)

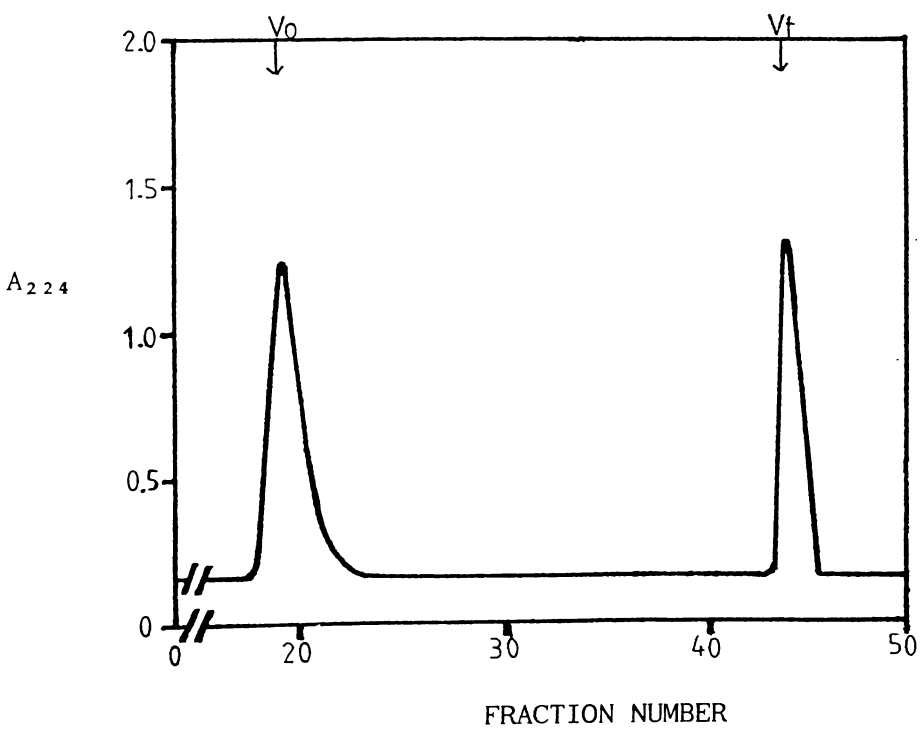


Fig. 3.84: Gel Filtration Chromatography of the 20 kDa Aggregate as Shown in Fig. 3.44 With Urea and Mercaptoehanol
Gel: Sephadex G-25SF
Eluent: 0.02 mol/l HCl, pH 1.7 containing 8 mol/l urea
Sample: Part of concentrate of fractions as shown in Fig. 3.44 (5 ml)
Fraction Volume: 4 ml

was adjusted to 1.7 with HCl. The blank was then passed through a column of Sephadex G-25SF eluted with 0.02 mol/l HCl, pH 1.7 containing 8 mol/l urea. The blank also gave a low molecular weight peak in the similar position and with a similar size to that of the low molecular weight peak shown in Fig. 3.84.

To further investigate the nature of the low molecular weight peak of Fig. 3.84, fractions making up the peak (fractions 43 - 46) were pooled and concentrated to approximately 5 ml by rotary evaporation. Urea crystals were removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes, the supernatant was chromatographed through a column of Sephadex G-15 eluted with 0.02 mol/l HCl, pH 1.7. Only the bed volume peak was obtained. Bearing in mind the elution profiles of the components isolated in the preceding sections (the active component eluted in fractions 18 - 25 on Sephadex G-15, Fig. 3.23 and the inactive component was retarded on Sephadex gel, Fig. 3.47), it was concluded that the bed volume peak of Fig. 3.84 was probably just 2-mercaptoethanol and/or excess urea of the sample.

Thus, it was concluded that hydrogen or disulphide bonds did not appear to be responsible for the formation of the 20 kDa aggregate.

The effect of EDTA on the disaggregation of the 20 kDa aggregate

Antibacterial activity diffuses across the 3.5 kDa cut-off dialysing membrane at neutral pH in the presence of 0.1 mol/l EDTA, a chelating agent, but not without it (P. Molan, personal communication). Therefore, it was thought that metal ion bridging may be involved in the formation of the 20 kDa aggregate.

To check this possibility, a 2 ml sample from the concentrate of the 20 kDa aggregate was analysed for the presence of various metal ions by atomic absorption spectrometry (these analyses were kindly performed by Ms. M.Watt of Department of Chemistry, University of Waikato, Hamilton). The results are shown in Table 3.2. Three divalent cations, namely Mg^{2+}, Fe^{2+} and Ca^{2+} were detected in reasonably high quantities in the sample, prompting the possibility of the involvement of some divalent cation(s) in the formation of the 20 kDa aggregate. Thus, it was decided to submit the 20 kDa aggregate to the effects of EDTA.

Table 3.2: Divalent metal ion analysis of the 20 kDa aggregate

Metal Ions	Amount Parts Per Million (ppm)
Zn++	0.29 ppm
Cu++	0.10 ppm
Ca++	0.51 ppm
Fe++	0.84 ppm
Mg++	1.98 ppm

Accordingly, EDTA at a concentration of 0.1 mol/l was added to a 1 ml sample of the 20 kDa aggregate, pH of the sample was re-adjusted to 7.0 with NaOH and after incubating the sample at 37°C for 24 h, a 100 μl sample was chromatographed through a column of Superose 12 eluted with 0.1 mol/l phosphate buffer, pH 7.0. The reason for incubating the sample for such a long time was to give EDTA sufficient time to bind ions that may have been bound to the components. As can be seen in Fig. 3.85, the sample gave a void volume peak with an estimated molecular weight of about 1 million or over (at 7 ml), and a peak with an estimated molecular weight of about 150 ($\pm$ 30) kDa (at 12 ml). Both of these peaks appeared to be the aggregates formed at neutral pH. A small amount of sample was also eluted on the bed volume (at 21 ml), thus possibly indicating a partial disaggregation. Alternatively, this could have been the EDTA peak.

It was thought that the disaggregation of the 20 kDa aggregate was not obtained in Fig. 3.85, possibly because enough EDTA was not available to chelate any metal ions involved in the aggregation.

Therefore, in an another attempt to disaggregate the 20 kDa aggregate with EDTA, EDTA was added to saturate a 5 ml sample of the concentrate of the 20 kDa aggregate, and the pH of the sample was adjusted to 7.0 by addition of NaOH. The sample was held at 4°C for 12 h and then chromatographed on a column of Sephadex G-25SF eluted with 0.1 mol/l Tris-HCl buffer, pH 7.0. Two peaks were obtained ; a large active void volume peak, indicating a molecular weight of 5 kDa or

over, and a bed volume peak, which was possibly only EDTA, as this absorbs strongly at the monitoring wavelength.

These results demonstrated, that the activity was still present in the aggregated form even when the samples were treated with saturated EDTA, thus EDTA failed to disaggregate the activity. Therefore, divalent cations did not appear to have been involved in the formation of the 20 kDa aggregate.

The effect of acetonitrile on the disaggregation of the 20 kDa aggregate

It was thought that the 20 kDa aggregate could be assembled with hydrophobic interactions. Acetonitrile is known to break hydrophobic interactions (Hodgkinson and Lowry, 1981). Therefore, in an attempt to break the possible hydrophobic interactions, it was decided to chromatograph the 20 kDa aggregate in the presence of acetronitrile.

Accordingly, a 5 ml concentrated sample of the 20 kDa aggregate was evaporated down to dryness by rotary evaporation, and then it was re-dissolved in 1 ml 30 % acetronitrile in 0.05 % trifluoroacetic acid. The solution was held at 4°C for 20 minutes and then a 200 μl sample was chromatographed through a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. As can be seen in Fig. 3.86, the major peak (eluted at 15 ml) still had a molecular weight estimated to be about 20 ($\pm$ 4) kDa. However, a bed volume peak (eluted at 21 ml) and a peak eluted just after the bed volume (at 23 ml) were also obtained, this indicated a partial disaggregation of the 20 kDa aggregate.

The partial disaggregation observed suggested the possibility that the 20 kDa aggregate is assembled by strong hydrophobic interactions and was not completely disaggregated because acetonitrile was not available in the concentration required for the complete disaggregation.

As the concentration of acetonitrile recommended for use with Superose 12 is 30 %, therefore 20 μl of the original sample of Fig. 3.86 was diluted to 200 μl by the addition of 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The 10 x dilution would have reduced

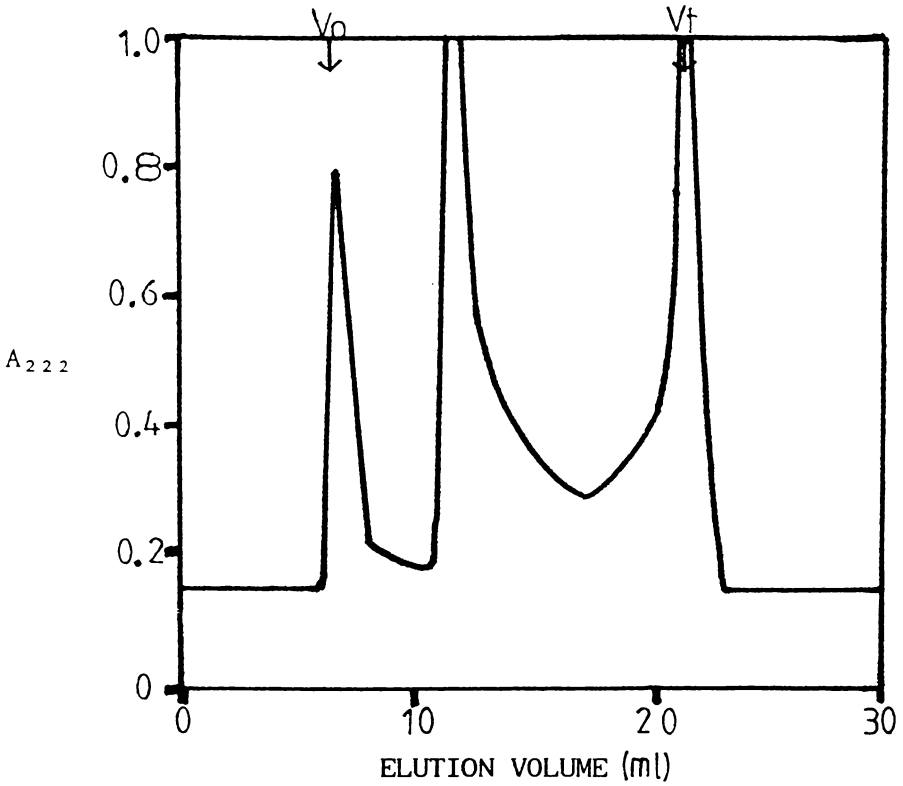


Fig. 3.85: FPLC of the 20 kDa Aggregate as Shown in Fig. 3.44 With EDTA
Gel: Superose 12
Buffer: 0.1 mol/l phosphate, pH 7.0
Sample: Part of concentrate of fractions 38-51 as shown in Fig. 3.44 containing 1 mol/l EDTA (100 µl)

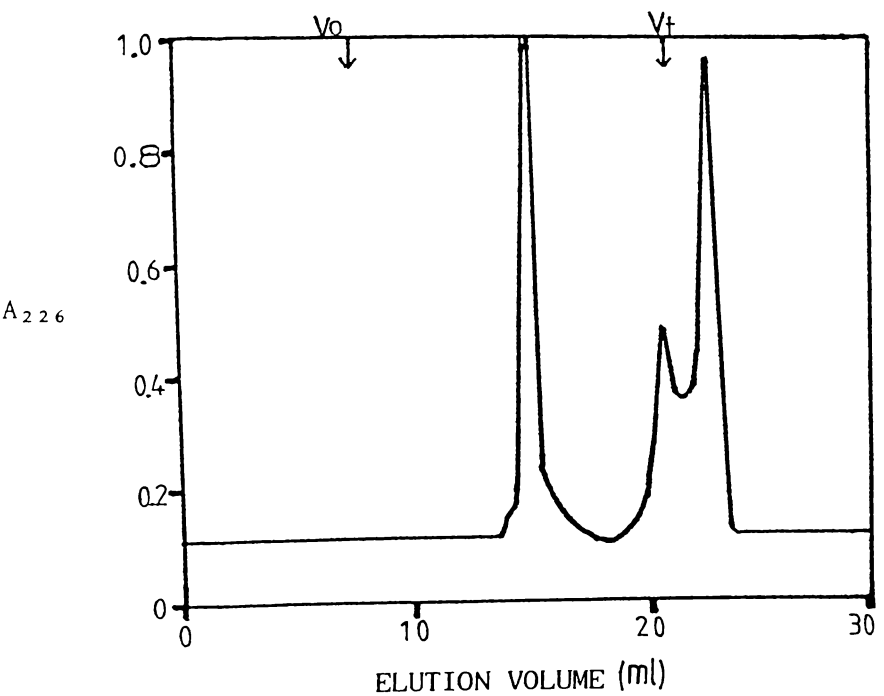


Fig. 3.86: FPLC of the 20 kDa Aggregate as Shown in Fig. 3.44 With Acetonitrile
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of fractions 38-51 as shown in Fig. 3.44 (200 µl)

the hydrophobic interactions between the components ten times. The sample was held at 4°C for 20 minutes and then chromatographed on a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The results are shown in Fig. 3.87. A smaller 20 kDa peak (eluted at 15 ml) and larger bed volume peak and the retarded peak (eluted at 21 ml and 23 ml respectively) resulted, indicating that the dilution of the sample had caused more disaggregation of the 20 kDa aggregate compared with that shown in Fig. 3.86.

To see the effect of further dilution on the disaggregation of the 20 kDa aggregate, a 10 μl of the original re-dissolved solution was diluted to 200 μl with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid (a 20 x dilution). The sample was held at 4°C for 20 minutes and then chromatographed through a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. As can be seen in Fig. 3.88, all the 20 kDa aggregate disaggregated to the two low molecular weight peaks ; a bed volume peak (eluted at 21 ml) and a retarded peak (eluted at 22 ml).

These two peaks were most probably the same two components of the aggregates isolated in earlier sections. One component was eluted on the bed volume (eluted at 21 ml), while the second component although still retarded (eluted at 22 ml), was eluted earlier than it was in the preceding runs (Figs. 3.86 and 3.87). This could have been because of different extents of adsorption of the component.

Conclusions

The results obtained in this section demonstrated that the 20 kDa aggregate was assembled by strong hydrophobic interactions. Although hydrogen and disulphide bonds were found not to be responsible in the formation of the 20 kDa aggregate, the possibility of the involvement of ionic interactions besides hydrophobic interactions could not be ruled out as disaggregation with acetonitrile was obtained at low pH (30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid, pH 1.3) which could have also broken down the ionic interactions.

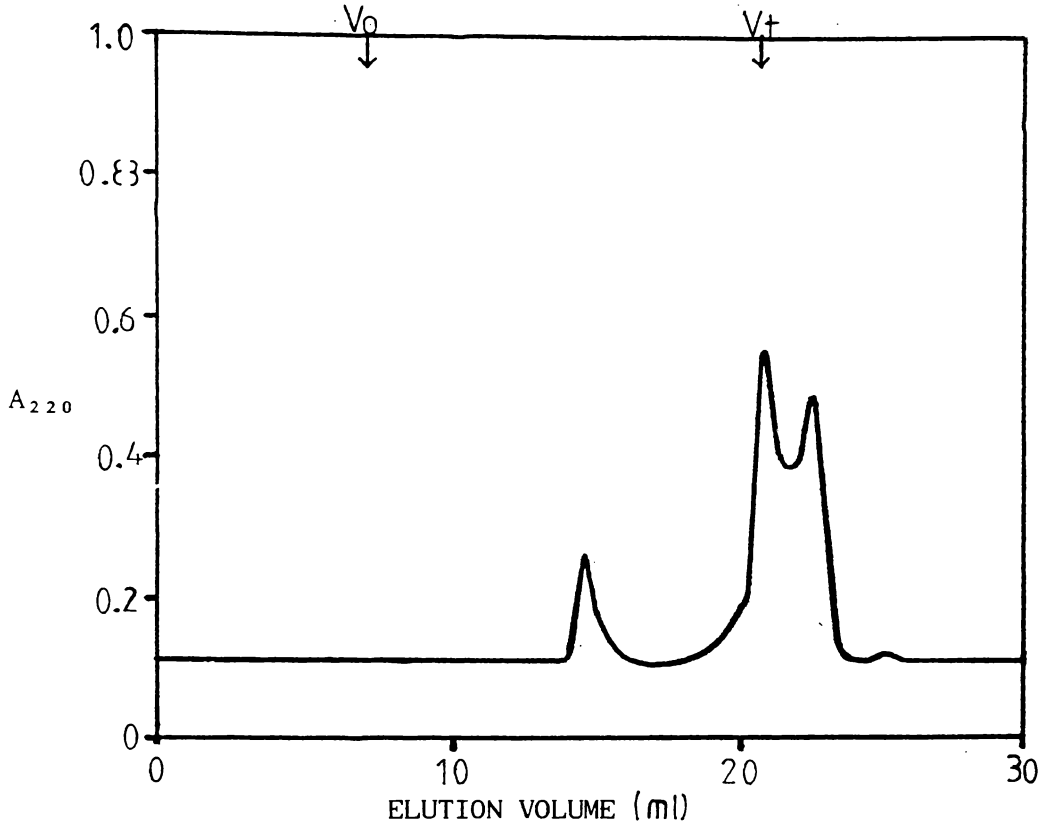


Fig. 3.87: FPLC of the 20 kDa Aggregate as Shown in Fig. 3.44 With Acetonitrile
Gel: Superose 12
Eluent: 30% acetonitrile (v/v) in trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of fractions 38-51 as shown in Fig. 3.44 (200 µl, 10 x diluted than the sample of Fig. 3.86)

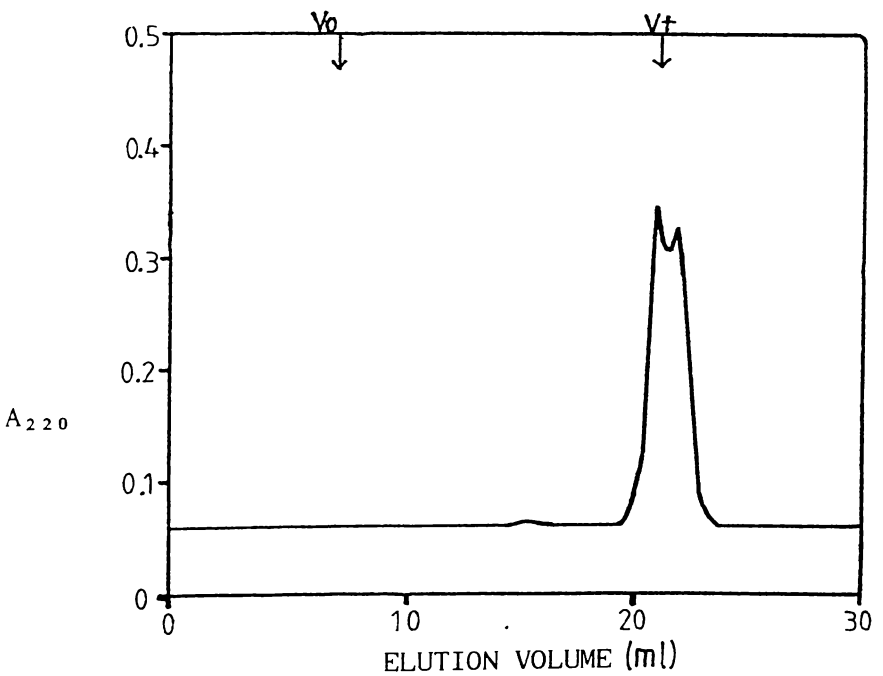


Fig. 3.88: FPLC of the 20 kDa Aggregate as Shown in Fig. 3.44 With Acetonitrile
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of fractions 38-51 as shown in Fig. 3.44 (20 x diluted than the sample of Fig. 3.86)

Disaggregation of the 500 kDa Aggregate with Acetonitrile and Trifluoroacetic Acid

As shown in Fig. 3.88, the 20 kDa aggregate, when diluted, was completely disaggregated by chromatographing through a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. Therefore, in order to see the composition of these aggregates, it was decided to submit the dilute samples of these aggregates to FPLC using acetonitrile and trifluoroacetic acid.

The 500 and 250 kDa aggregates were prepared as described in Fig. 3.5 and to avoid any overlapping of the peaks, the two fractions making up the middle of each peak were taken. The samples were reduced to 5 ml each by rotary evaporation. The pH of the samples was lowered to 1.7 with HCl. A precipitate formed at about pH 4.5, which was removed by centrifugation at 6,000 g (r av.10 cm) for 10 minutes, and then to prepare the 20 kDa aggregates the supernatant of the 500 kDa and 250 kDa aggregates were separately chromatographed through a column of Sephadex G-200F at pH 1.7 (as shown in Figs. 3.10 and 3.33).

The 20 kDa aggregate obtained from the 500 kDa aggregate was reduced to dryness by rotary evaporation, and then re-dissolved in 1 ml 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. To facilitate the complete disaggregation, a 20 μl sample was diluted to 200 μl by the addition of 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The sample was held at 4°C for 20 minutes and then chromatographed through a column of Superose 12. As can be seen in Fig. 3.89, the sample completely disaggregated. Two peaks were obtained ; a peak eluting on the bed volume (eluted at 21 ml), indicating a molecular weight of about 1 kDa kDa or lower and a peak eluting just after the bed volume (eluted at 22 ml), indicating retardation.

To see the composition of the precipitate of the 500 kDa aggregate formed at pH 4.5, a small proportion of the precipitate was taken and re-dissolved in 200 μl 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. After holding at 4°C for 20 minutes, the sample was chromatographed through a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. An identical result to that shown in Fig. 3.89 was obtained.

Disaggregation of the 250 kDa Aggregate with Acetonitrile and Trifluoroacetic Acid

The 20 kDa aggregate from the 250 kDa aggregate was also reduced to dryness by rotary evaporation, and then re-dissolved in 1 ml 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. To facilitate the complete disaggregation, a 20 μl sample was then diluted to 200 μl by the addition of 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The sample was held at 4°C for 20 minutes and then chromatographed on a column of Superose 12. As shown in Fig. 3.90, the sample was completely disaggregated, giving peaks in the identical positions obtained with the 20 kDa aggregate sample obtained from the 500 kDa aggregate (Fig. 3.89).

To see if the composition of the precipitate of the 250 kDa aggregate formed at pH 4.5 was also identical to that of the supernatant, a small proportion of the precipitate was taken and re-dissolved in 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The sample was held at 4°C for 20 minutes and then chromatographed through a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. An identical result to that shown in Fig. 3.90 was obtained.

Disaggregation of the 10 - 40 kDa Peak with Acetonitrile and Trifluoroacetic Acid

In order to investigate the composition of the active 10 - 40 kDa peak from Fig. 3.5, a 5 ml sample of the peak was reduced to dryness by rotary evaporation, and then re-constituted in 1 ml 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. For complete disaggregation, a 20 μl sample was diluted to 200 μl with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The sample was held at 4°C for 20 minutes and was then chromatographed on a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The results are shown in Fig. 3.91.

Although the antibacterial aggregates present in the 10 - 40 kDa peak shown in Fig. 3.5 seemed to have been completely disaggregated giving a bed volume peak (eluted at 21 ml, Fig. 3.91), indicating a

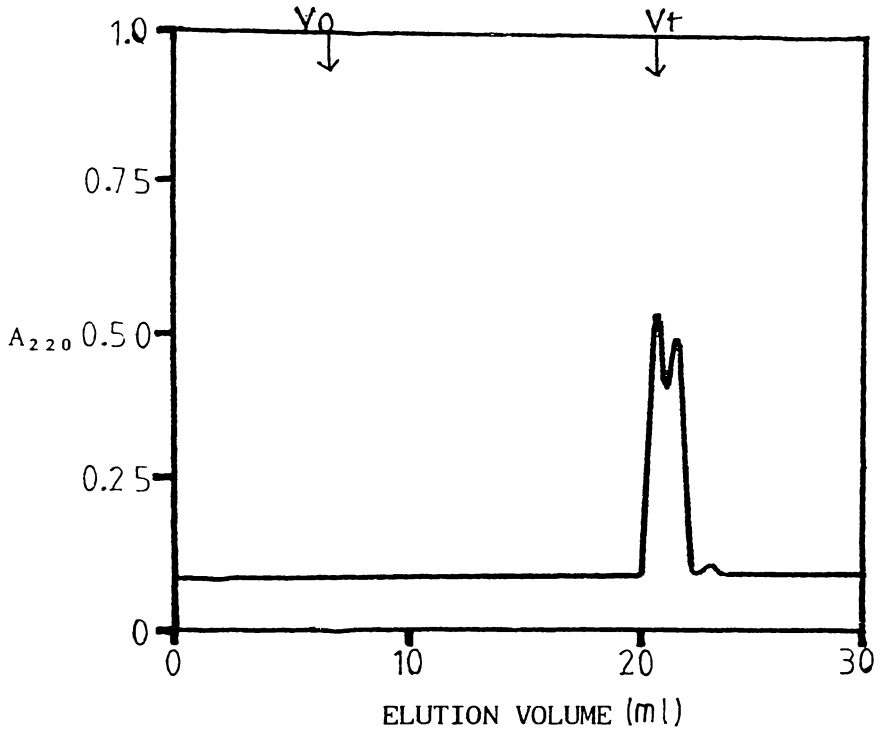


Fig. 3.89: FPLC of the 20 kDa Aggregate Obtained from the 500 kDa Aggregate
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of the 20 kDa aggregate obtained from the 500 kDa aggregate (as fractions 38-51 in Fig. 3.10) (200 µl)

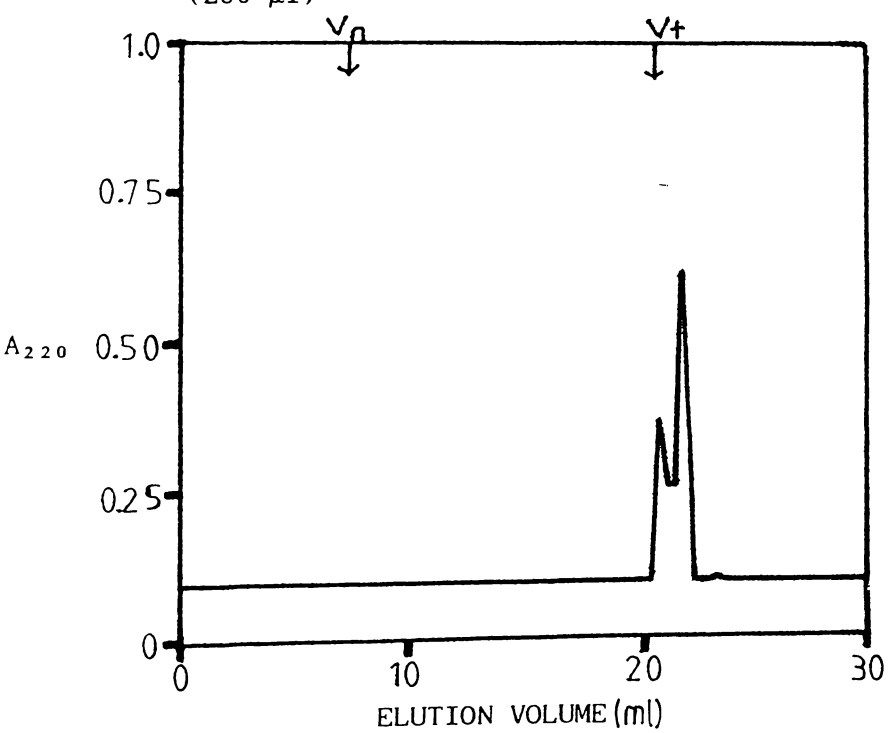


Fig. 3.90: FPLC of the 20 kDa Aggregate Obtained from the 250 kDa Aggregate
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of the 20 kDa aggregate obtained from the 500 kDa aggregate (as fractions 38-51 in Fig. 3.33) (200 µl)

molecular weight of about 1 kDa or lower and a retarding peak (eluted at 22 ml, Fig. 3.91), there was also a peak with an estimated molecular weight of about 15 (± 3) kDa (eluted at 16 ml, Fig. 3.91). This peak was most probably lysozyme, as lysozyme activity had been found in the 10 - 40 kDa peak of Fig. 3.5 as described in the preceding sections.

Conclusions

The supernatant and the precipitate of the 20 kDa aggregates obtained from 500 kDa and 250 kDa aggregates gave identical elution profiles (two low molecular weight peaks) when their diluted samples were applied on FPLC using acetonitrile and trifluoroacetic acid. This clearly demonstrated that these aggregates are made up of only two components and that no component was lost in precipitation at any stage. The precipitate and the supernatant of the 10 - 40 kDa peak gave the identical elution profiles on FPLC, indicating that no component was lost in the precipitation of this peak as well.

Characterisation of the Components of the Antibacterial Aggregates Obtained by Ion Exchange Chromatography

The 20 kDa aggregates obtained from the 500 kDa and 250 kDa aggregates gave positive Biuret test and Molisch test indicating the glycoprotein nature of the components.

Three components were isolated from the 20 kDa aggregates; an active component with a molecular weight of about 1.2 kDa (see Figs. 3.24, 3.41 and 3.76), which did not aggregate at neutral pH, an inactive aggregating component which was a little retarded on Sephadex (see Fig. 3.47) and an inactive component which was very retarded on Sephadex (see Fig. 3.61), and also re-aggregated at neutral pH.

However, FPLC of diluted samples of the 20 kDa aggregates from both the 500 kDa and 250 kDa aggregates using acetonitrile with trifluoroacetic acid as eluent gave only two peaks in the low molecular weight region, thus indicating that only two components are involved in the formation of the large antibacterial aggregates (see Figs. 3.89 and 3.90). All the components of the aggregates would have been present in the 20 kDa aggregates as they had antibacterial activity and also re-aggregated up to the sizes of the original aggregates. Also,

no components of the 500 kDa and 250 kDa aggregates could have been lost in the precipitate formed at pH 4.5, because both the supernatant and the precipitate were found to have activity and gave identical elution profiles on Sephadex both at neutral and acidic pH, and also on FPLC with acetonitrile and trifluoroacetic acid as eluent.

Therefore, to study the three components obtained by ion exchange chromatography, the active component (fractions 18 - 25, Fig. 3.24), the inactive aggregating component (fractions 46 - 60, Fig. 3.47), and the aggregating retarded component (fractions 55 - 67, Fig. 3.61) were submitted to cellulose acetate electrophoresis, FPLC and HPLC.

Attempts to Determine the Net Charge of the Components Using Cellulose Acetate Electrophoresis

Attempts were made to obtain an idea about the net charges on the components by cellulose acetate electrophoresis. It was hoped that the direction of migration of the components at neutral pH would indicate the net charge of the components.

Accordingly, parts of the samples of the three components were freeze-dried separately and each re-dissolved in about 100 μl 0.05 mol/l Tris-HCl buffer, pH 7.0. A cellulose acetate strip was soaked in 0.05 mol/l Tris-HCl buffer, pH 7.0 for 30 minutes. The samples and a solution of egg-white lysozyme in the same buffer were then applied, and electrophoresis was carried out for 20 minutes at 200 volts. Staining was carried out with 0.05 % Ponceu S in a 10 % aqueous solution of sulphosalicylic acid for 20 minutes. Excess dye was removed by washing repeatedly in 5 % acetic acid. Lysozyme, as expected, and a faint band of the active component migrated towards the cathode, indicating their basic nature. Faint bands of the inactive aggregating component and the retarding component were found near the anode, indicating the acidic nature of these.

In order to obtain more distinct bands, more concentrated samples and a shorter running time of electrophoresis (10 minutes) were tried separately, but, again faint bands of the samples migrating towards the electrodes as described above were obtained.

It was thought that the components, because of their small size, may have been washed off during staining or destaining. Therefore, in another attempt to obtain clear bands, the cellulose acetate strips were fixed with 25 % aqueous solution of glutaraldehyde for 10 minutes before staining, but again no clear bands were obtained.

In a further attempt to obtain distinct bands, the strip was sprayed with ninhydrin and then dried in an oven at about 60°C for 10 minutes. Unfortunately, again no distinct protein bands could be detected.

The failure to obtain distinct bands on cellulose acetate electrophoresis was most probably due to the small size of the components, as peptides below a molecular weight of 10 kDa have been reported by Righetti and Chillemi (1978) to be increasingly difficult to fix and stain, and are often washed out of the gel. Therefore, no further attempts to obtain the net charge or isoelectric point of the components by cellulose acetate electrophoresis or isoelectric focusing were made.

However, the cellulose acetate electrophoresis indicated that the active component is basic, while the inactive aggregating component and the retarding component are acidic in nature.

Investigations of the Components Using HPLC and FPLC

Since electrophoretic techniques could not be used for the reasons described earlier, HPLC and FPLC were used to investigate the homogeneity of the components obtained from different types of ion exchange chromatography.

Investigations of the active component

Investigations of the active component using HPLC

To investigate the active component, a proportion of the active component (fractions 18 - 25, Fig. 3.24) was concentrated down to dryness by rotary evaporation and then re-dissolved in 200 μl 0.1 % trifluoroacetic acid. It was then subjected to reversed phase HPLC on an RP C-18 column. Fig. 3.92 shows the gradient used and the elution

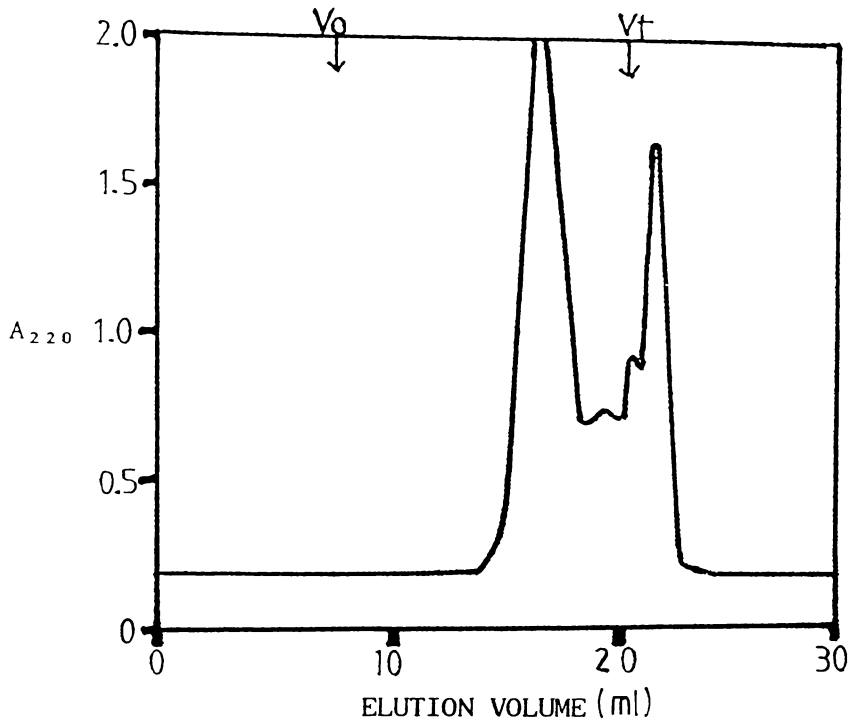


Fig. 3.91: FPLC of the 10-40 kDa Peak from Fig. 3.5
Gel: ~~Sephadex~~ Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate fractions 29-41 as shown in Fig. 3.5 (200 µl)

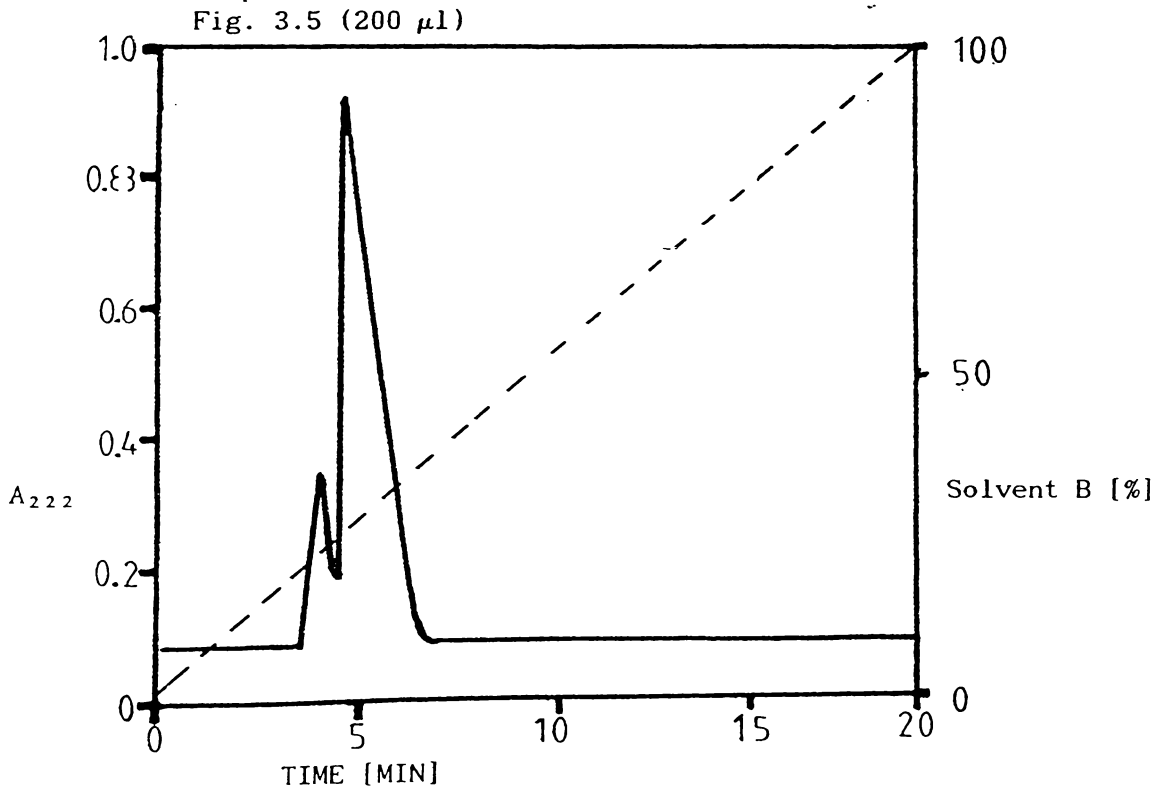


Fig. 3.92: HPLC of the Active Component
Column: Brownlee RP C-18
Sample: Part of concentrate of fractions 18-25 from Fig. 3.24 (200 µl)
Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B, 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid
Gradient: 0 to 80% Solvent B for 20 min

profile. Two peaks were obtained, a small peak eluted after 4.2 minutes and a major peak after 4.9 minutes.

This result suggested that either the preparation of the active component was a small aggregate which besides containing predominantly the active component also had a small amount of another component, or that the small peak was because of the overlapping of the active component with another component during gel filtration chromatography. This other component was probably the inactive aggregating component. Alternatively, the small peak could also be the active component, eluted earlier because the capacity of the column was exceeded. The peak eluted after 4.9 minutes showed tailing, indicating the possible presence of component(s) other than the active component.

Thus, to check these possibilities parts of the later half of the 4.2 minute peak and the first half of the 4.9 minute peak as shown in Fig. 3.92 were pooled together and reduced to about 100 μl by rotary evaporation and reloaded onto the RP C-18 HPLC column. Since the active component was eluted earlier in the gradient, to improve the resolution, a curved gradient, as shown in Fig. 3.93, was used giving more time at the lower concentrations of acetonitrile. As can be seen in Fig. 3.93, a single peak was eluted at 4.1 minutes, indicating that both the peaks shown in Fig. 3.92 were the same molecule, because if the peaks of Fig. 3.92 were different molecules then at least two peaks should have been obtained in Fig. 3.93, as the sample of Fig. 3.93 contained parts from both the peaks of Fig. 3.92.

Thus, it was evident from Fig. 3.93 that the sample of Fig. 3.92 contained only the active component, and the two peaks obtained in Fig. 3.92 were because the capacity of the column had been exceeded. Also, the shape of the peak in Fig. 3.93 indicated that the slope of the large peak in Fig. 3.92 was a characteristic of the active component, rather than because of the presence of more than one component in the peak. This was probably because of the interactions between the positive charges on the active component and exposed negative charges on the silica support. The later elution of the peaks in fig 3.92 than in Fig. 3.93 was probably because the sample of Fig. 3.92 was more concentrated, thus, causing high physical interactions between the molecules of the active component and the silica support.

FPLC of the two peaks obtained from HPLC of the active component

To further check the homogeneity of the two peaks shown in Fig. 3.92, and to estimate the molecular weight of the active component, it was decided to chromatograph the HPLC peaks of the active component on FPLC.

Accordingly, proportions of the two peaks as shown in Fig. 3.92 was separately reduced to dryness by rotary evaporation and then re-dissolved separately in 200 µl 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The samples were then chromatographed on a column of Superose 12. The results are shown in Figs. 3.94 and 3.95.

The almost identical elution profiles obtained (Figs. 3.94 and 3.95) were further evidence that both the peaks of Fig. 3.92 were the same active component. Both the samples gave peaks on the bed volume, indicating the molecular weight of the active component as about 1 kDa or lower. A small amount of material was eluted before the bed volume in Fig. 3.94, indicating a low degree of heterogeneity in the preparation.

Investigations of the active component using FPLC

A part of the active component (fractions 18 - 25, Fig. 3.24) was evaporated down to dryness and re-dissolved in 200 µl 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid, and to compare with the elution profiles of Figs. 3.94 and 3.95, was directly chromatographed through a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. As can be seen in Fig. 3.96, a similar elution pattern to those shown in Figs. 3.94 and 3.95 was obtained. Again, the active component was mainly eluted at the bed volume, indicating a molecular weight of about 1 kDa or lower. A small amount of material was eluted earlier, which indicated a small amount of heterogeneity in the preparation.

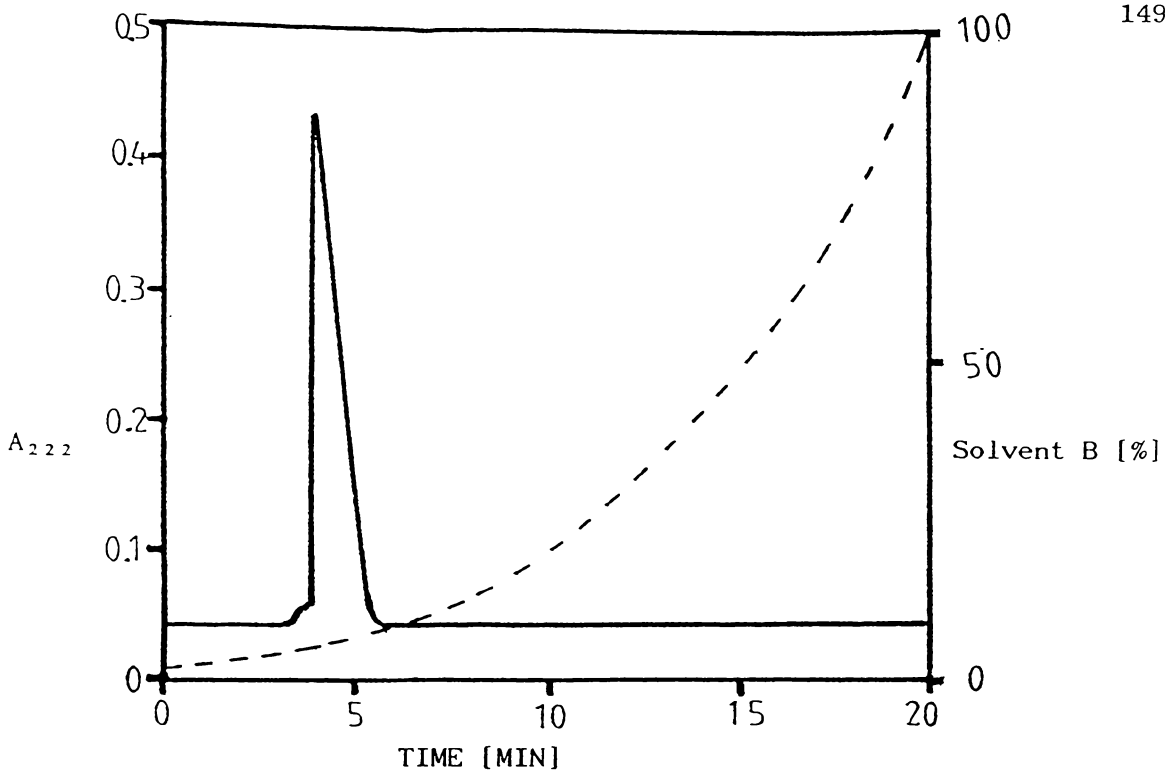


Fig. 3.93: HPLC of Later Half of 4.2 min Peak and First Half of 4.9 min peak of Fig. 3.92
Column: Brownlee RP C-18
Sample: Part of concentrates of later half of 4.2 min peak and first half of 4.9 min peak from Fig. 3.92 (100 µl) Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B, 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid, Gradient: 0 to 80% Solvent B for 20 min

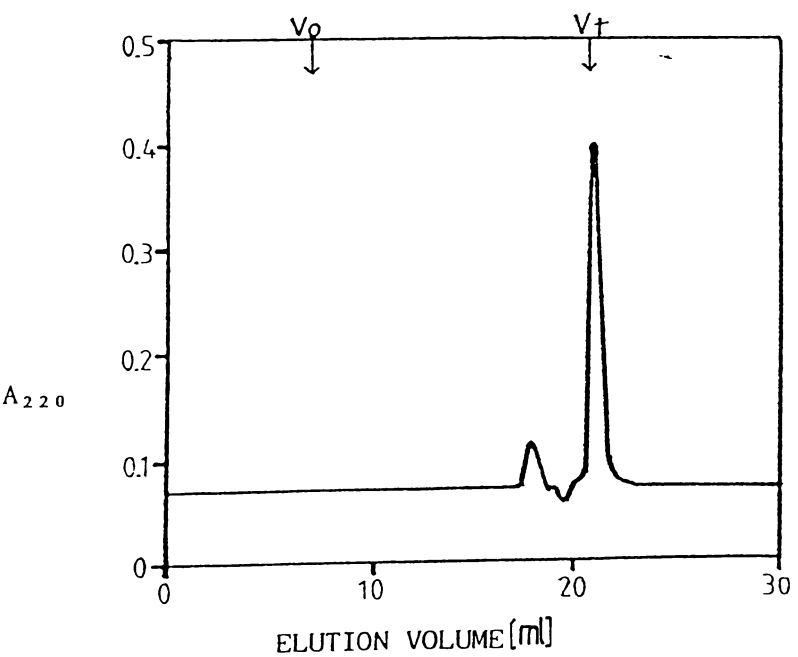


Fig. 3.94: FPLC of 4.2 Min Peak from Fig. 3.92
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of 4.2 min peak from Fig. 3.92 (200 µl)

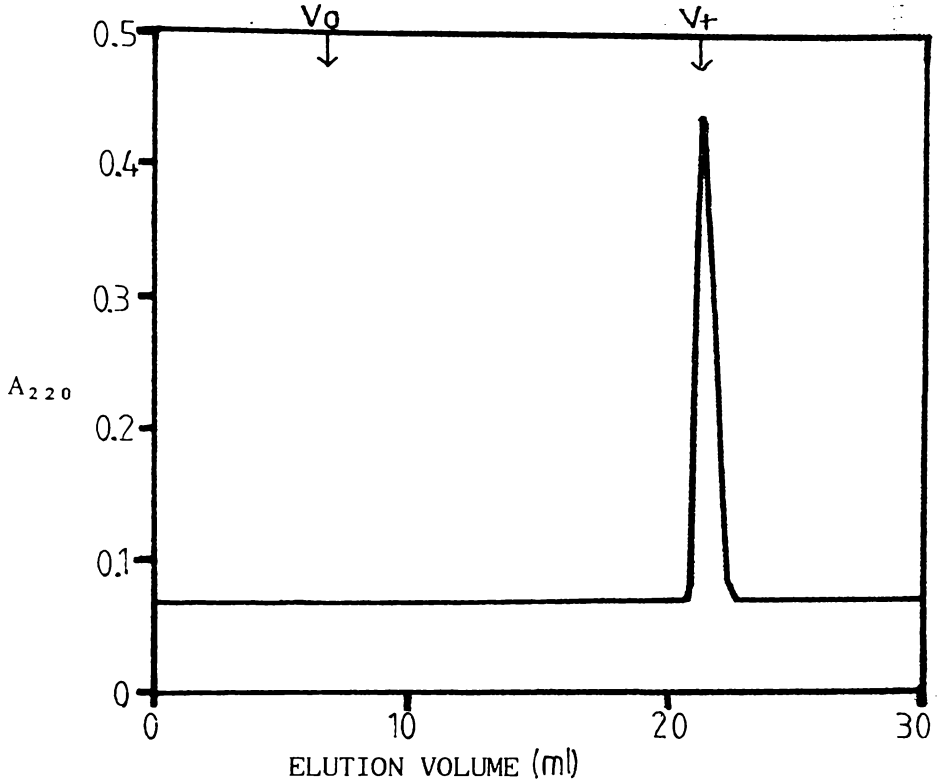


Fig. 3.95: FPLC of 4.9 Min Peak from Fig. 3.92
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of 4.6 min peak from Fig. 3.92 (200 µl)

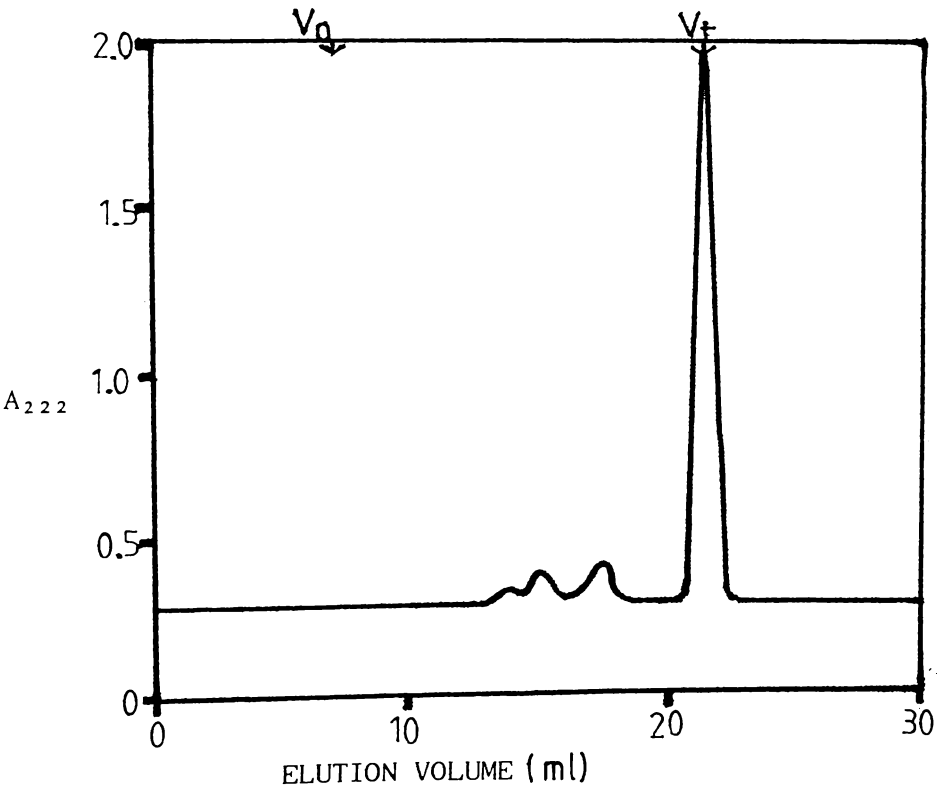


Fig. 3.96: FPLC of the Active Component
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of the concentrate of fractions 18-25 from Fig. 3.24 (200 µl)

Investigations of the inactive component

Investigations of the inactive component using HPLC

To investigate the inactive aggregating component, a part of the pool of fractions 46 - 60 of Fig. 3.47 was reduced to dryness by rotary evaporation and re-dissolved in 500 μl 0.1 % trifluoroacetic acid. A sample of 200 μl was subjected to HPLC on a RP C-18 column as outlined in Fig. 3.97. Three peaks were obtained. These peaks indicated the presence of three components. Alternatively, they were just aggregates formed because of interactions of the sample, which was not unlikely as the inactive component had been found to aggregate in the preceding sections. Another possibility was that the capacity of the column was exceeded.

The sample was eluted early in the gradient. Thus, to improve the resolution, a 100 μl sample of the inactive component was chromatographed again, with a curved gradient as shown in Fig. 3.98, giving more time in the early part of the gradient. However, as shown in Fig. 3.98, the peaks were again poorly resolved. The peaks obtained in Figs. 3.97 and 3.98 could have been the aggregates of the inactive aggregating component or the peaks were because of the heterogeneous nature of the sample. Another possible explanation for more than one peak was that the capacity of the column could still have been exceeded.

It was thought that a smaller sample loading would decrease both the physical interactions possibly causing aggregation and at the same time the capacity of the column would not be exceeded.

Accordingly, a 50 μl sample from the re-constituted sample of the inactive component was chromatographed with the same curved gradient as before. The results are shown in Fig. 3.99. There was a major peak eluted at 2.8 minutes, and a small shoulder at 2.4 minutes. The 2.8 minute peak was most probably the inactive aggregating component, while the 2.4 minute peak was probably an aggregate with a component other than the inactive aggregating component. Alternatively, the 2.4 minute peak was a component other than the inactive aggregating component, and since the active component eluted at 4.1 minutes under the same

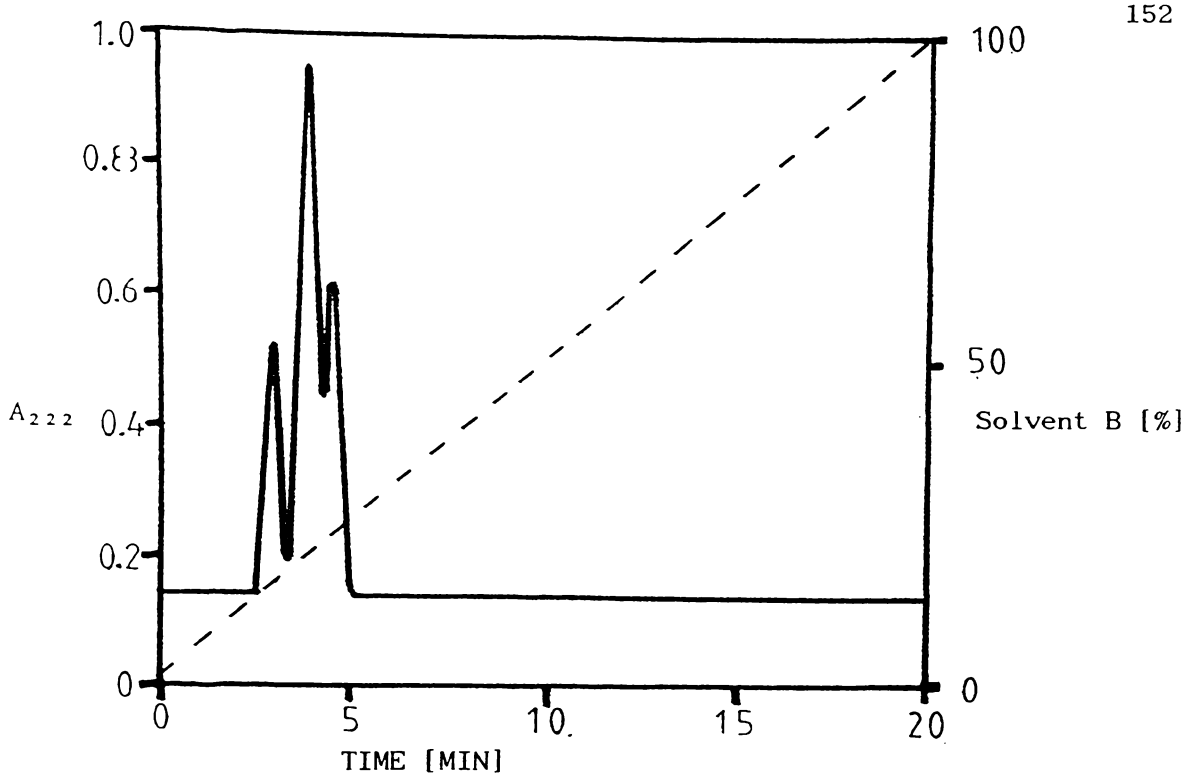


Fig. 3.97: HPLC of the Inactive Component
Column: Brownlee RP C-18
Sample: Part of concentrate of fractions 46-60 from Fig. 3.47 (200 µl)
Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B, 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid
Gradient: 0 to 100% Solvent B for 20 min

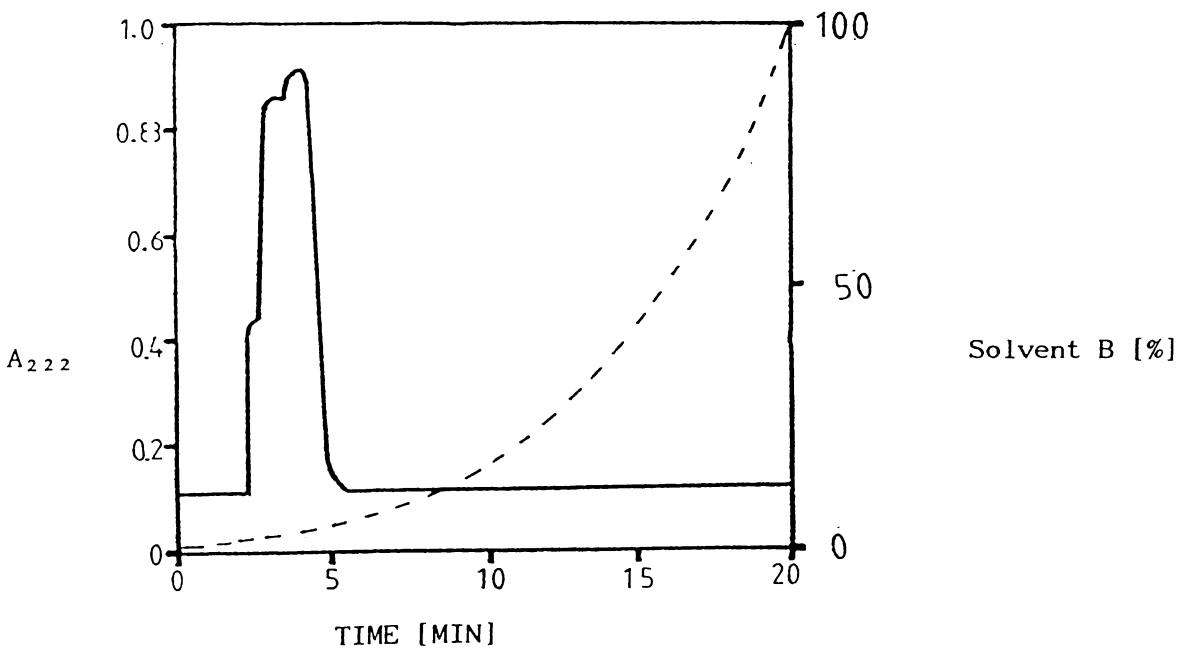


Fig. 3.98: HPLC of the Inactive Component
Column: Brownlee RP C-18
Sample: Part of concentrate of fractions 46-60 from Fig. 3.47 (100 µl)
Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B, 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid
Gradient: 0 to 100% Solvent B for 20 min

conditions (see Fig. 3.93), thus the 2.4 minute peak could have been the third component obtained from the ion exchange chromatography, the retarding component (see Fig. 3.61).

Investigations of the active component using FPLC

To further study the composition of the inactive aggregating component, a part of the pool of fractions 46 - 60 of Fig. 3.47 was reduced to dryness by rotary evaporation and then re-dissolved in about 250 μl 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. A 200 μl sample was chromatographed on a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The results are shown in Fig. 3.100. The major peak was eluted just after the bed volume (22 ml peak, Fig. 3.100), thus indicating retardation. There were, however, few peaks other than the inactive component. These peaks could either have been other components or just small aggregates.

It was found that in the preceding sections that a diluted sample on FPLC with acetonitrile and trifluoroacetic acid caused complete disaggregation, thus, in order to see whether the peaks before the bed volume in Fig. 3.100 were aggregates or other components, it was decided to run a diluted sample of the inactive component onto FPLC.

Accordingly, a 20 μl sample from the concentrate of the inactive component re-dissolved in 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid was diluted to 200 μl with the same solvent. The sample was chromatographed on a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The results are shown in 3.101. This time the major peak, most probably the inactive component in its completely disaggregated form, was eluted on the bed volume, indicating a size of about 1 kDa or lower. There was also a shoulder on the peak, and since the diluted sample was expected to have caused complete disaggregation, the shoulder was probably a component other than the inactive component. This component could either be the active component or the retarding component.

As can be seen in Fig. 3.101, there was a big dip in the elution trace just before the eluted sample. It was feared that some component(s) could have been obscured by the dip. The dip was thought to be because of a slight difference in the composition of the sample

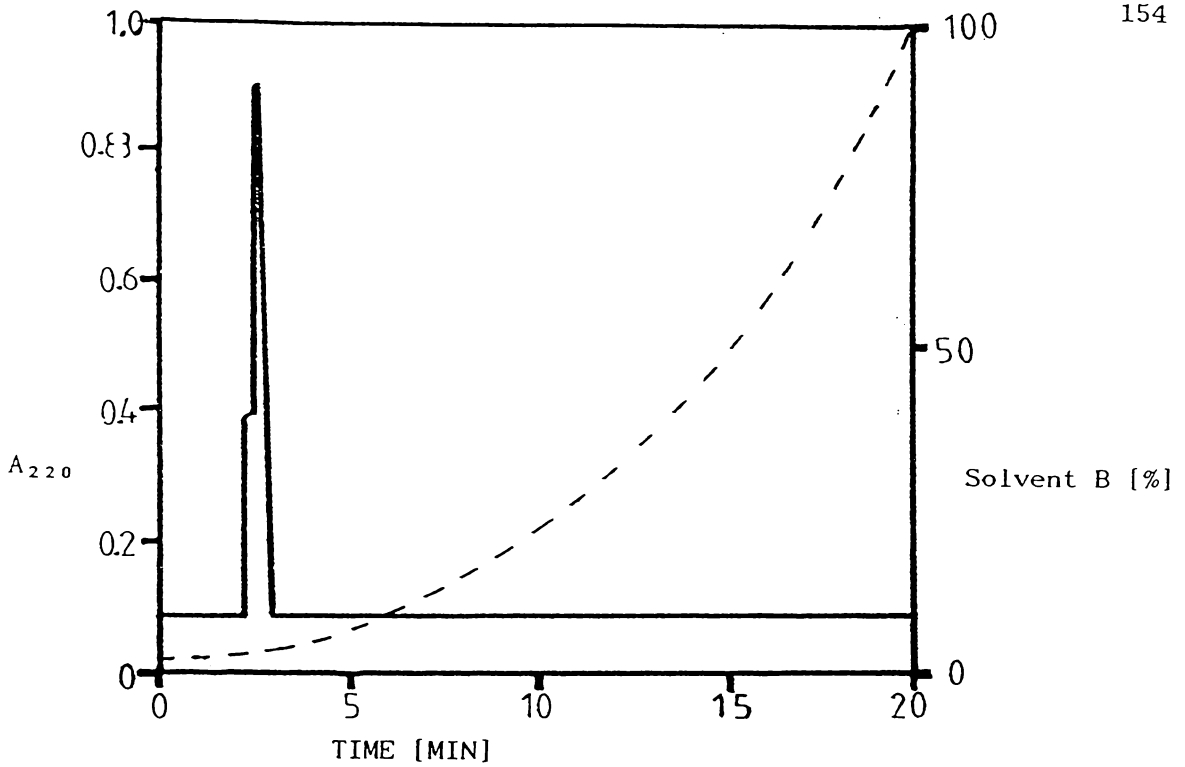


Fig. 3.99: HPLC of the Inactive Component
Column: Brownlee RP C-18
Sample: Part of concentrate of fractions 46-60 from Fig. 3.47 (50 µl)
Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B, 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid
Gradient: 0 to 100% Solvent B for 20 min

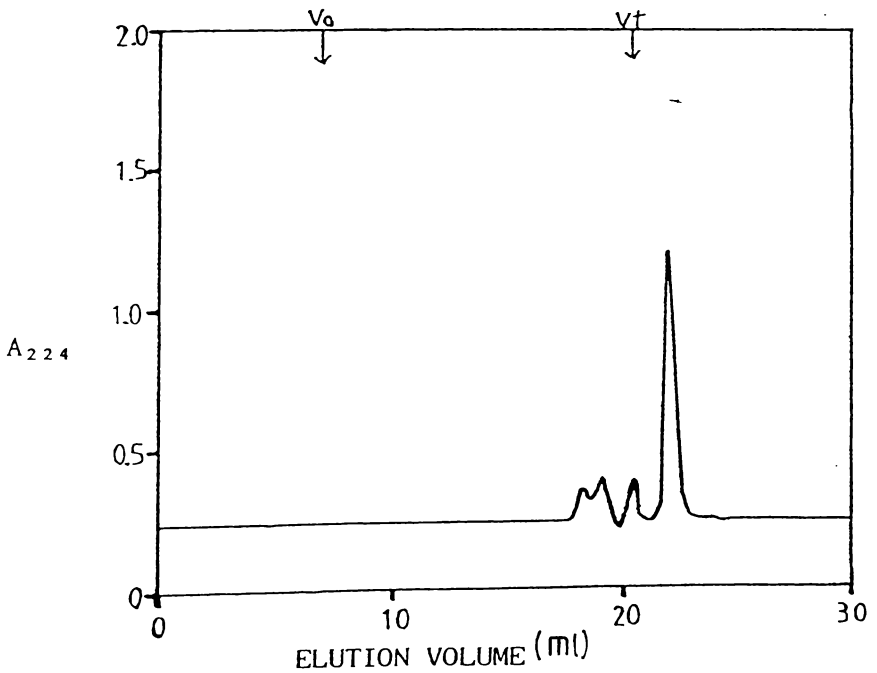


Fig. 3.100: FPLC of the Inactive Component
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of fractions 46-60 from Fig. 3.47 (200 µl)

solvent and that of the eluent. Thus, to get rid of the dip, a small part of the inactive component (fractions 46 - 60, Fig. 3.47) was freeze-dried and then re-dissolved in 200 μl 30 % acetonitrile in 0.05 % trifluoroacetic acid. The sample was then chromatographed as before. An identical elution profile pattern to that shown in Fig. 3.101 was obtained, but with a much smaller dip. The dip in this experiment was considered to be too small to obscure any major peak.

The results shown in Fig. 3.101 demonstrated that the peaks before the inactive component obtained in Fig. 3.100 were predominantly small aggregates, probably because of the high concentration of the sample causing a high amount of physical interaction and thus resulting in the formation of small aggregates. However, the inactive component appeared to have a small amount of another component, which probably was due to overlapping in the preceding gel filtration chromatography.

Disaggregation of the precipitate of the inactive component

As described in a preceding section, when the pH was lowered, the inactive component (fractions 46 - 60, Fig. 3.47 and 45 - 60, Fig. 3.51) formed a precipitate at around pH 4.5. The precipitate remained undissolved even at pH 1.7. Further investigations at that stage were continued with the supernatant. Chromatographing a dilute sample on FPLC with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid had been found to cause complete disaggregation.

Thus, to see if the precipitate contained any component other than the inactive component present in the supernatant, it was decided to submit the dilute sample of the precipitate to FPLC with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid.

Accordingly, a small proportion of the precipitate was taken and re-dissolved in 200 μl 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid, and then chromatographed through a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. An identical elution profile to that shown in Fig. 3.101 was obtained, indicating that the precipitate contained the same components present in the supernatant.

Investigations of the retarding component

Investigations of the retarding component using HPLC

The 20 kDa aggregate obtained from the 500 kDa and 250 kDa aggregates was expected to have all the components of the high molecular weight antibacterial aggregates as it was active and re-aggregated at neutral pH. But a diluted sample of the 20 kDa aggregate gave only two peaks on FPLC with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid as eluent, indicating that only two components are involved in the formation of the antibacterial aggregates. As described in the preceding sections, two components of the antibacterial aggregates, an active component and an inactive component, had been isolated by different types of ion exchange chromatography. These behaved identically to the two peaks on FPLC from the diluted 20 kDa aggregate.

However, a peak which was retarded on the Sephadex was also obtained from the anion exchange chromatography at pH 13.0 (fractions 55 - 67, Fig. 3.61). Therefore, to see if the retarding component was just a small aggregate of the two components or a third component, it was decided to submit it to both HPLC and FPLC.

A part of the retarding component (fractions 55 - 67, Fig. 3.61) was evaporated down to dryness by rotary evaporation, and was re-constituted in about 100 μl with 0.1 % trifluoroacetic acid. The sample was then subjected to HPLC as outlined in Fig. 3.102. Two peaks were obtained, at 4.1 minutes and 2.8 minutes, which were eluted at identical elution volumes to the active and inactive components on the same column under similar conditions (see Figs. 3.93 and 3.99).

It was evident from this result that the retarding component (fractions 55 - 67, Fig. 3.67) was in fact a small aggregate rather than a third component.

Investigations of the retarding component using FPLC.

To further study the composition of the retarding component, a part of it (fractions 55 - 67, Fig. 3.61) was reduced to dryness by

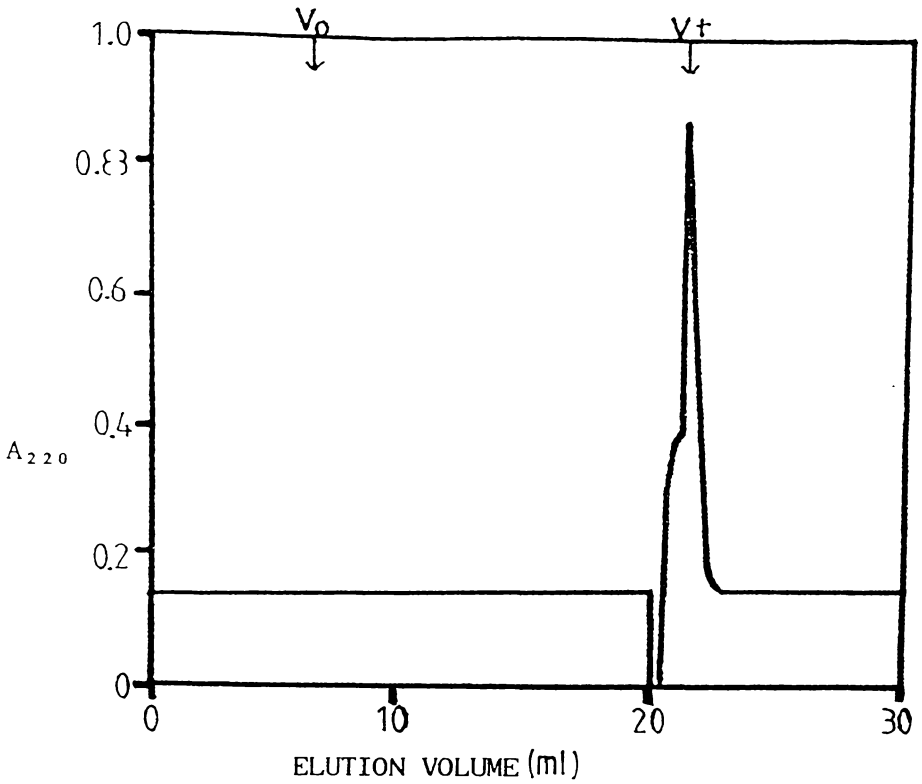


Fig. 3.101: FPLC of the Inactive Component
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of fractions 46-60 from Fig. 3.47 (200 µl, 10 x diluted sample than sample of Fig. 3.100)

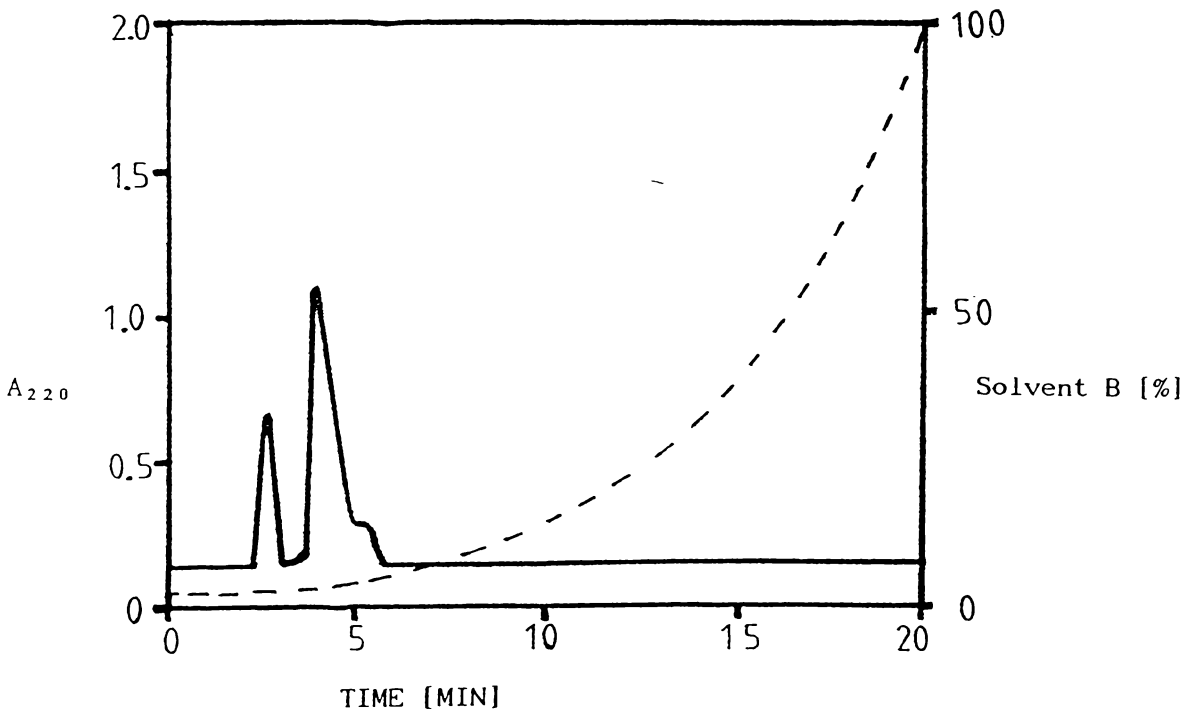


Fig. 3.102: HPLC of the Retarding Component
Column: Brownlee RP C-18
Sample: Part of concentrate of fractions 55-67 from Fig. 3.61 (100 µl)
Solvents: Solvent A, 0.1% trifluoroacetic acid; Solvent B, 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid
Gradient: 0 to 100% Solvent B for 20 min

rotary evaporation and re-constituted in about 200 μl 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. The sample was then chromatographed through a column of Superose 12. As can be seen in Fig. 3.103, two major peaks (eluting at 21 ml and 22 ml) were eluted at identical elution volumes to those of the active and inactive components on the same column under the identical conditions (see Figs. 3.96 and 3.100). However, a small peak (eluting at 18 ml, Fig. 3.103) also eluted before the two peaks, which could have been just a small aggregate, rather than a third component. This was supported by the fact that the molecular weight for the 18 ml peak was estimated to be about 10 (± 2) kDa from the calibration curve, which was too large to be a component, because the activity was earlier shown to pass across the 3.5 kDa cut-off dialysing membrane at acidic pH and later re-aggregate to large molecular weight aggregates at neutral pH, thus demonstrating that all the components of the antibacterial aggregates have a size of less than 3.5 kDa.

The result of the FPLC (Fig. 3.103) thus further demonstrated that the retarding peak (fractions 55 - 67, Fig. 3.61) was in fact a small aggregate, rather than a component, therefore no further investigation was done on the retarding peak.

Disaggregation of the precipitate of the retarding peak

As described in the preceding section, when the pH of the retarding peak (which was thought to be a component at that stage) was lowered, a precipitate formed at around 4.5, which did not dissolve even at pH 1.7. Further study was carried out with the supernatant. Since chromatography of a dilute sample on FPLC with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid had been found to cause complete disaggregation, therefore to see if the precipitate of fractions 55 - 67 of Fig. 3.61 contained any component other than the active and inactive components, a part of the precipitate was taken and re-dissolved in 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid.

The sample was then chromatographed on a column of Superose 12 eluted with 30 % (v/v) acetonitrile in 0.05 % trifluoroacetic acid. An identical elution pattern to that shown in Fig. 3.103 was obtained, indicating that the precipitate, like the supernatant, contained only the active and inactive components. A small peak eluted at 18 ml,

which most probably was only a small aggregate because with an estimated size of about 10 (± 2) kDa from the calibration curve it appeared to be too large to be a component.

Thus, it was clearly evident from this result that the precipitate of the retarding peak (fractions 55 - 67, Fig. 3.61) also did not contain any component other than the active and inactive ones.

Study of the Aggregation Capacity of the Components

It was shown in the preceding sections that the active component did not re-aggregate at neutral pH by itself (see Fig. 3.30). Although the inactive component re-aggregated at pH 7.0 (Figs. 3.49 and 3.56), it was later found to contain a small amount of the active component. Thus, it was decided to investigate the aggregating capacity of the pure inactive component.

Accordingly, the peak eluted at 22 ml in Fig. 3.100 (the pure inactive component) was reduced to dryness by rotary evaporation and re-dissolved in about 200 µl 0.1 mol/l phosphate buffer, pH 7.0. A sample of 50 µl was incubated at 37°C for 2 h and then chromatographed on a column of Superose 12 equilibrated with 0.1 mol/l phosphate buffer, pH 7.0. As can be seen in Fig. 3.104, the sample was eluted just after the bed volume (at 22 ml), because of retardation. The homogenous inactive component did not form any aggregate at neutral pH. The experiment was repeated again, and an identical result to that shown in Fig. 3.104 was obtained. These experiments clearly demonstrated that the inactive component does not aggregate by itself.

Similarly, to investigate the aggregating capacity of the active component, the peak of pure active component (21 ml peak, Fig. 3.96) was reduced to dryness by rotary evaporation. The sample was then re-dissolved in 200 µl 0.1 mol/l phosphate buffer, pH 7.0, and from that a 50 µl sample was incubated at 37°C for 2 h. The sample was then chromatographed on a column of Superose 12 equilibrated with 0.1 mol/l phosphate buffer, pH 7.0. As shown in Fig. 3.105, it was eluted on the bed volume, thus indicating a size of about 1 kDa or lower. It was clearly evident from this result that the active component does not aggregate by itself. This, however, was not surprising, as it had been

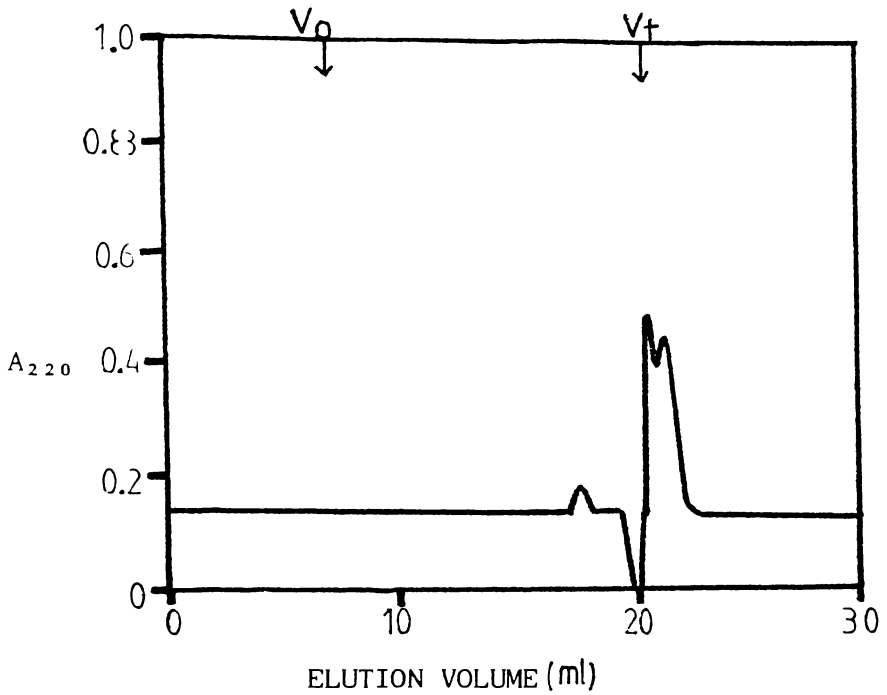


Fig. 3.103: FPLC of the Retarding Component
Gel: Superose 12
Eluent: 30% (v/v) acetonitrile in 0.05% trifluoroacetic acid, pH 1.3
Sample: Part of concentrate of fractions 55-67 from Fig. 3.61 (200 µl)

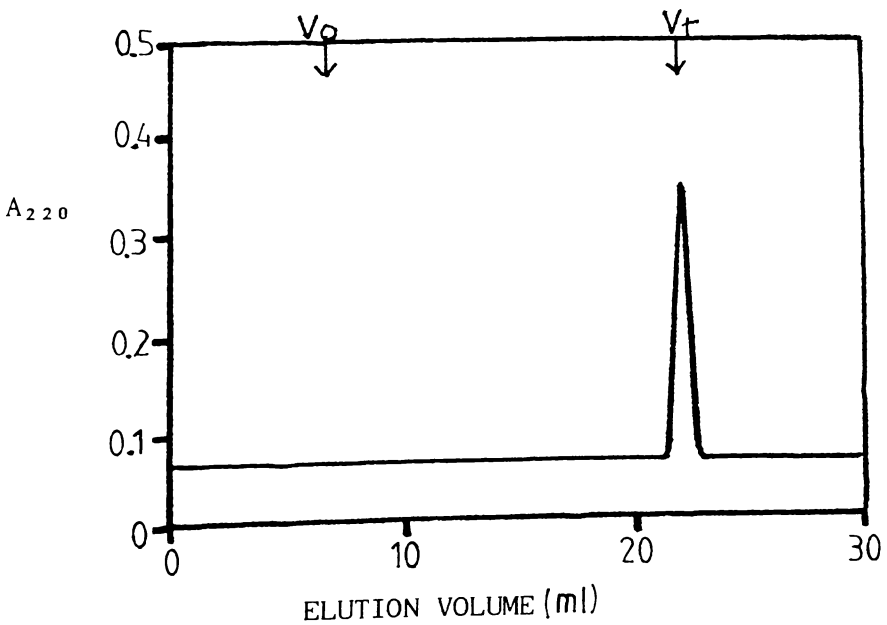


Fig. 3.104: FPLC of the Inactive Component
Gel: Superose 12
Buffer: 0.1 mol/l phosphate buffer, pH 7.0
Sample: Part of concentrate of 22 ml peak from Fig. 3.100 (50 µl)

found earlier that the active component did not re-aggregate at neutral pH (Figs. 3.30 and 3.43).

Since neither the active nor the inactive component re-aggregated at neutral pH, it was concluded that both the components would have to be together to form active aggregates. To confirm this, a 25 μl sample of the inactive component (from 22 ml peak, Fig. 3.100) was mixed with a 25 μl sample of the active component (from 21 ml peak, Fig. 3.96). The mixture was incubated at 37°C for 2 h, and then chromatographed through a column of Superose 12 equilibrated with 0.1 mol/l phosphate buffer, pH 7.0. As can be seen in Fig. 3.106, the mixture re-aggregated up to the void volume on Superose 12, indicating a molecular weight of about 1 million or over.

The major aggregate (12 ml peak, Fig. 3.106) was, however, estimated to have a molecular weight of about 150 ($\pm$ 30) kDa. The 500 kDa aggregate (the largest aggregate found in bovine seminal plasma) was probably not obtained because of the dilute sample, which would not have facilitated the aggregation physically. Alternatively, a particular ratio of the active and inactive components is required for the maximum aggregation, which was not present in the sample.

Conclusions

It was clearly evident from the results described in this section that neither the active nor the inactive component form aggregates by itself, and both the active and inactive components are required to be together for the aggregation.

Amino Acid Analysis

A concentrated sample of the active component (21 ml peak, Fig. 3.96) and a concentrated sample of the inactive component (22 ml peak, Fig. 3.100) were freeze-dried separately. Amino acid analysis of the samples were kindly carried out by Dr.D.Christie of the University of Auckland, Auckland. The samples were hydrolysed in 5.7 mol/l HCl at 110°C for 24 h under vacuum and the amino acids were separated as phenylisothiocyanate derivatives on reversed phase HPLC. The results are shown in Tables 3.3 and 3.4.

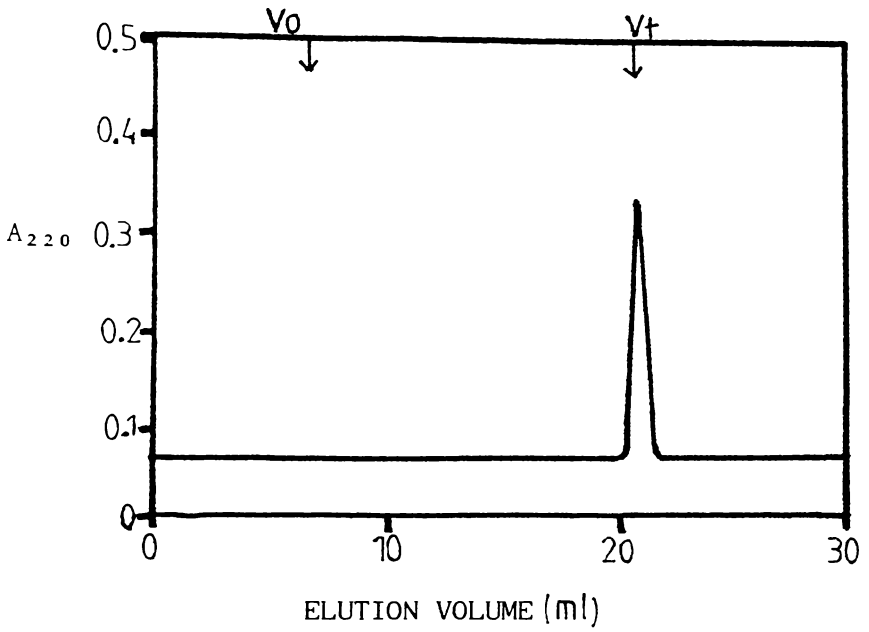


Fig. 3.105: FPLC of The Active Component
Gel: Superose 12
Buffer: 0.1 mol/l phosphate buffer, pH 7.0
Sample: Part of concentrate of 21 ml peak from Fig. 3.96 (50 μl)

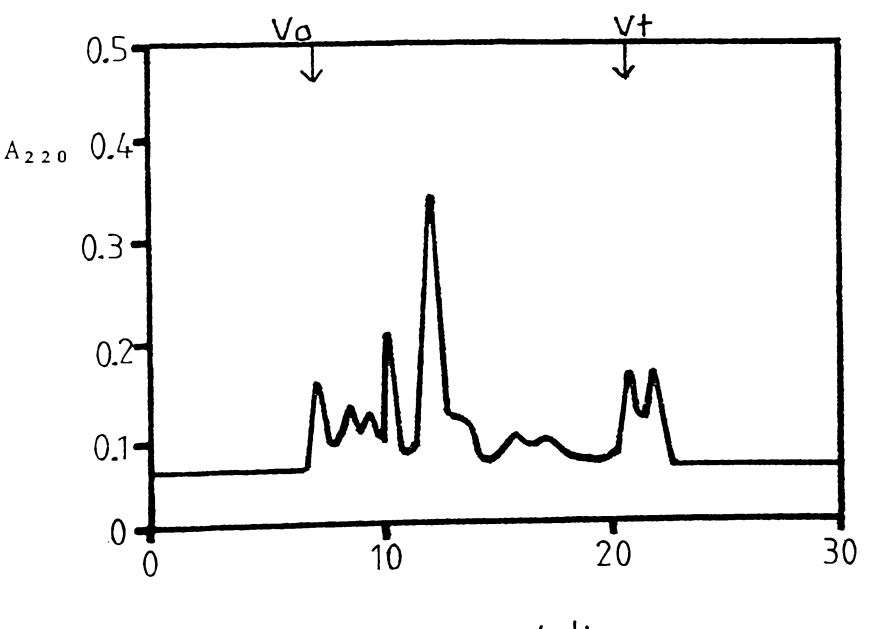


Fig. 3.106: FPLC of Mixture of the Inactive and Active Component
Gel: Superose 12
Buffer: 0.1 mol/l phosphate buffer, pH 7.0
Sample: Part of concentrates of the active (21 ml peak, Fig. 3.96) and inactive (22 ml peak, Fig. 3.100) components mixed in equal proportions (25 μl each)

Unfortunately, the results of amino acid analyses were complicated by the fact that the samples were not completely pure as they were purified on an analytical column of FPLC (only analytical column of FPLC was available). This was obvious by the observation that the amino acids which were present in large amounts in one component were also present in small amounts as impurity in other component. Thus, molecular weights of the components could not be calculated from the amino acid analyses.

The results were further complicated by the presence of an unknown peak in the sample of the inactive peptide (shown as * in the table 3.4). This unknown peak obscured the aspartate-asparagine peak in the sample of the inactive component. The unknown peak was probably carbohydrate as the parent sample (the 20 kDa aggregate) of the peptides was found to contain carbohydrates as described in an earlier section of this study.

As can be seen in the Tables 3.3 and 3.4, the inactive component appeared to be an acidic peptide, while the antibacterial component was a peptide, probably of basic nature, and the high amount of glutamate-glutamine in the antibacterial peptide was probably because of small overlapping with the inactive peptide as described earlier and/or it was present as glutamine in the antibacterial peptide.

These results were not surprising as the antibacterial peptide and the inactive peptide were found to behave as basic and acidic peptides respectively on ion exchange chromatography as described in earlier sections.

Table 3.3: Amino acid composition of the active component

Amino acid (nmoles)	No. of residues
Alanine	2.18
Arginine	1.57
Aspartate-asparagine	1.42
Glutamate-glutamine	3.16
Glysine	2.09
Histidine	0.65
Leucine	4.86
Lysine	1.64
Methionine	-
Phenylalanine	2.20
Proline	2.02
Serine	1.44
Threonine	1.44
Tyrosine	0.83
Valine	2.08

Table 3.4: Amino acid composition of the inactive component

Amino acid (nmoles)	No. of residues
Alanine	6.72
Arginine	0.15
Aspartate-asparagine	*
Glutamate-glutamine	16.40
Glycine	1.07
Histidine	0.70
Leucine	0.81
Lysine	-
Methionine	-
Phenylalanine	0.51
Proline	-
Serine	2.99
Threonine	2.96
Tyrosine	11.48
Valine	0.53

CHAPTER FOUR

DISCUSSION

This study showed that the antibacterial activity of bovine seminal plasma was in part due to two low molecular weight components which are present in different aggregated forms under physiological conditions. Amino acid analysis of the components showed that the components are peptides in nature. The high molecular weight proteins were found to be the major part of the protein constitution of bovine seminal plasma. About 80 % of the 500 kDa and 250 kDa peaks of seminal plasma present at pH 7.0 disaggregated to the 20 kDa aggregate at pH 1.7, indicating that it was present as high molecular weight antibacterial aggregates. The antibacterial aggregates were also found in the low molecular weight peaks of seminal plasma (see Fig. 3.5). Thus, the antibacterial aggregates appeared to be the major part of the protein constitution of bovine seminal plasma.

The three major antibacterial aggregates obtained were estimated to have molecular weights around 500 kDa, 250 kDa and 20 kDa. These aggregates were isolated by gel filtration chromatography at neutral pH. Aggregates with identical molecular weights were also obtained on gel filtration chromatography at physiological pH (pH 7.4) of bovine seminal plasma.

Antibacterial activity was also obtained in the last peak eluted just after the bed volume of Sephadex G-200F, so its molecular weight could not be estimated. However, re-chromatography of this peak on Sephadex G-50SF gave antibacterial peaks in the region of 20 - 30 kDa in a pattern similar to that obtained from the third peak from chromatography on Sephadex G-200F when it was re-chromatographed on Sephadex G-50SF. This suggested that the last peak obtained on Sephadex G-200F was the result of retardation of the same substances that were eluted in the third peak.

The aggregation of the antibacterial activity was initially demonstrated in this study by dialysis experiments. When seminal plasma was dialysed in 3.5 kDa cut-off dialysing membrane at pH 7.0, no antibacterial activity diffused into the diffusate. However, activity

dialysed across the membrane at pH 3.0. Later, the dialysed activity re-aggregated to form antibacterial aggregates of up to 500 kDa on gel filtration chromatography at pH 7.0. Similar results were also demonstrated by Shannon *et al.* (1987), who showed that the activity which passed across 10 kDa cut-off dialysing membrane at acidic pH was predominantly in the form of aggregates of up to at least 30 kDa on subsequent gel filtration chromatography at neutral pH. Thus, it was demonstrated by these results that antibacterial activity, at least in part, was due to small components which are present in aggregated forms in the physiological conditions of bovine seminal plasma. This was also confirmed by the observations that no antibacterial peak smaller than lysozyme (molecular weight 14 kDa) was obtained on gel filtration chromatography of seminal plasma at neutral pH.

Another observation made during acid dialysis was that antibacterial activity was slow to dialyse even at acidic pH. Both ionic as well as hydrophobic interactions appeared to be involved in the formation of the aggregates. Thus, the slow diffusion of the activity at acidic pH may be explained by the possibility that while the acidic pH broke down the ionic interactions, long exposure of the aggregates to low temperature (4°C) was required to break down what appeared to be strong hydrophobic interactions. Because of the slow disaggregation of the hydrophobic interactions an equilibrium probably existed between the large antibacterial aggregates and the small aggregates or the monomeric peptides at acidic pH. Most of the activity would have been present in the large antibacterial aggregates, and only a small amount of activity would have been disaggregated to the small aggregates or monomeric peptides. When the diffusate was replaced by a fresh solution, more high molecular weight activity would have disaggregated and then passed into the diffusate because of the concentration gradient.

Shannon *et al.* (1987) reported that the antibacterial activity of bovine seminal plasma passes across 10 kDa cut-off dialysing membrane at acidic pH and at neutral pH in the presence of citrate, but not without it. The activity also passes across the 10 kDa cut-off dialysing membrane at neutral pH in the presence of EDTA (P. Molan, personal communication). This could have been because of EDTA chelating metal ions required for aggregation. However, this did not

appear to be the case in the present study as even saturated EDTA did not cause complete disaggregation of the 20 kDa aggregates obtained from the 500 kDa and 250 kDa aggregates. The diffusion of activity in the presence of citrate and EDTA at neutral pH could more likely be explained by the possibility that citrate and EDTA by creating competition for ionic bonding break ionic interactions, the hydrophobic interactions then breaking on long exposure of the aggregates to low temperature (4°C). The combination of these two result in at least partial disaggregation and the activity passes across the dialysing membrane.

The electrophoretic analysis of the 500 kDa, 250 kDa and 20 kDa aggregates at pH 4.5 showed many common bands, indicating that these antibacterial aggregates are made up of the same components. The electrophoresis of the aggregates at pH 4.5 might have caused some disaggregation. Therefore, electrophoresis of the aggregates was also carried out at pH 6.8 to study the aggregates in their physiological forms. Again, the aggregates contained many common bands, indicating that they are made up of the same components.

Shannon *et al.* (1987) also, when studying the antibacterial preparations of bovine seminal plasma, demonstrated that different bands moved to each others positions on re-running of the bands on electrophoresis, thus indicating aggregation.

Broad shaped peaks were repeatedly obtained on gel filtration chromatography, particularly at neutral pH. This could have been due to the basic nature of the aggregates : positive charges might interact with carboxyl groups of dextran gel. But symmetrical broadness of the peaks was usually observed, thus the broadness of the peaks could not have been only due to the ionic interactions which would have given tailing only. The continuous aggregation and disaggregation of the aggregates could have also resulted in the formation of different aggregates, differing only slightly in their sizes, and thus resulting in the broad shaped peaks. Ease of re-aggregation observed on gel filtration chromatography at neutral pH when the aggregates were applied also supported this possibility. Since all the antibacterial aggregates gave several bands on electrophoresis both at pH 4.5 and pH 6.8, another possibility for the broad shaped peaks on gel filtration chromatography could be that a series of different aggregates, only

slightly different in their sizes, were present in the aggregates isolated from the gel filtration chromatography. Different proportions of the acidic and basic peptides in the aggregates could account for this.

Acidic gel filtration chromatography, cation exchange chromatography and FPLC of the 500 kDa, 250 kDa and 20 kDa aggregates also confirmed that these aggregates are made up of the same two peptides. Different high molecular weight antibacterial proteins found by Eschenbruch (1980), Thangavelu (1983), and Shackell (1984) appear from the findings in the present study, to be antibacterial aggregates of these peptides, as all the antibacterial activity larger than lysozyme (molecular weight 14 kDa) was found to be due to the aggregates.

When the 500 kDa and 250 kDa aggregates were chromatographed on Sephadex G-200F at pH 1.7, all the activity disaggregated to give the 20 kDa aggregate. This 20 kDa aggregate was found to be a good starting sample for the complete disaggregation of the aggregates as it was antibacterial, and re-aggregated to the original sizes of the aggregates at neutral pH, indicating the presence of all the components of the antibacterial aggregates, and it did not contain any contaminating protein.

The breakdown of the antibacterial activity of the 500 kDa and 250 kDa aggregates to the 20 kDa aggregate did not appear to be because of acid hydrolysis as hydrolysis is a time-dependent process, and the activity was found to dissociate to the 20 kDa aggregate even when the sample was applied on to gel filtration at pH 1.7 immediately after acidifying the sample. Also, if the breakdown of the activity to the 20 kDa aggregate was because of acid hydrolysis, then different hydrolysis products should have been obtained in the different runs depending on the extent of hydrolysis. Instead, always a 20 kDa aggregate was obtained on acidic chromatography of the 500 kDa and 250 kDa aggregates in this study. Also, acid hydrolysis usually causes denaturation of protein molecules leading to the loss of biological activity. But in this study, no loss of antibacterial activity was observed. Moreover, although activity does not pass through the 10 kDa cut-off dialysing membrane at neutral pH (without citrate or EDTA), it does pass through the membrane in the presence of citrate and EDTA

(Shannon *et al.*, 1987; P. Molan, personal communication) indicating that the breakdown of the activity is due to disaggregation rather than acid hydrolysis or proteolysis. Also, gel filtration chromatography was done at 4°C which would have reduced any acid hydrolysis or proteolysis. Furthermore, denaturation as a result of acid hydrolysis or proteolysis is an irreversible process in most cases. But the disaggregation of the antibacterial activity was found to be reversible in this study, as the 20 kDa aggregate always re-aggregated to give the original aggregates at neutral pH.

Bearing all these results and observations described above in mind, it could be safely concluded that the breakdown of the antibacterial activity of the 500 kDa and 250 kDa aggregates to the 20 kDa aggregate was because of spontaneous disaggregation on acidic gel filtration chromatography, which was due to the breakdown of ionic interactions rather than any kind of hydrolysis, proteolysis or denaturation. The same could be said about the further disaggregation of the 20 kDa aggregate obtained from the 500 kDa and 250 kDa aggregates.

Thus, the antibacterial aggregates appeared to be genuine aggregates which were made up of two peptides as complete disaggregation of the aggregates yielded two peptide peaks and no harsh treatment was used at any stage of disaggregation which could have caused hydrolysis, proteolysis or denaturation.

The disaggregation of the 500 kDa and 250 kDa aggregates to the 20 kDa aggregate on gel filtration chromatography at pH 1.7 was most probably a result of suppression of carboxyl group(s). Thus, the aggregation beyond the 20 kDa aggregate appeared to be through ionic interactions.

However, the 20 kDa aggregate did not appear to be held only by ionic interactions as chromatography of the aggregate at pH as high as 13.0 and as low as 1.0, which could have suppressed all possible groups involved in the ionic interactions, could not disaggregate the 20 kDa aggregate. However, the 20 kDa aggregate was found to be assembled by strong hydrophobic interactions, as the dilute sample of the 20 kDa aggregate was completely disaggregated to give two peptide peaks on FPLC with acetonitrile and trifluoroacetic acid. The complete

disaggregation of the dilute sample was probably obtained because low concentrations of the sample physically decreased the interactions of the molecules involved in the aggregation, thus causing complete disaggregation. However, involvement of ionic interactions along with hydrophobic interactions in the formation of the 20 kDa aggregate could not be ruled out as the FPLC was carried out at pH 1.3 which could have broken some ionic interactions as well. The possible involvement of some ionic interactions in the formation of the 20 kDa aggregate was also apparent by the observation that salt was found to be necessary to keep the partially pure inactive peptide in its completely disaggregated form during acidic gel filtration chromatography (Figs. 3.48 and 3.50).

Therefore, it was concluded that there are two major stages of aggregation : (1) the two peptides form the stable 20 kDa aggregate with strong hydrophobic interactions (and possibly with some ionic interactions as well); (2) the aggregation beyond the 20 kDa aggregate (in the formation of the 500 kDa and 250 kDa aggregates) is the result of only ionic interactions.

Interestingly, the finding of an antibacterial compound of about 20 kDa in bovine seminal plasma has been reported previously. Eschenbruch (1980) when trying to disaggregate the antibacterial activity in what appeared to be aggregates, almost always found a major antibacterial peak near the void volume on Sephadex G-50SF. Shackell (1984) also isolated an antibacterial peak near the void volume on Sephadex G-50SF. These peaks correspond to a molecular weight of about 20 - 25 kDa.

Similarly, Wheliham (1977) and Schollum *et al.* (1977) isolated antibacterial compounds from bovine seminal plasma with estimated molecular weights of about 15 kDa and 20 kDa respectively. Thangavelu (1983) when studying antibacterial compounds of bovine seminal plasma obtained two bands on SDS-electrophoresis with estimated molecular weights of 11.8 kDa and 17.5 kDa on SDS-electrophoresis and 19.9 kDa and 22.3 kDa on thin-layer gel filtration chromatography. Each of these bands moved to the other's position on re-running the electrophoresis indicating the similar nature of the compounds.

Although these antibacterial compounds were not characterised, it is very likely that the researchers isolated the 20 kDa aggregate isolated in this study.

Shackell (1980) found that SDS-electrophoresis is not a suitable method for the determination of the molecular weight of basic proteins. Waehneldt (1975) also demonstrated that cationic proteins behave anomalously when subjected to polyacrylamide gel electrophoresis in the presence of SDS. The error in determining histone molecular weights has been attributed to a lower net charge on the SDS complex due to a high positive charge on the histone chains (Noelken *et al.*, 1981; Panyim and Chalkley, 1971; Swank and Munkries, 1981). Hamana (1981) on the other hand concluded that anomalous behaviour of histones in SDS polyacrylamide gel electrophoresis is primarily due to the effective size of SDS - histone complex rather than the neutralisation of the negative SDS charge.

Lehtovaara (1978) gave three reasons why some proteins do not migrate according to their molecular weight on SDS-electrophoresis.
(a) Poor SDS binding to acidic proteins or proteins containing rigid S-S bands or carbohydrate,
(b) Neutralisation of the negative charge on SDS by basic proteins.
(c) Abnormal conformation of protein-SDS complexes.

Thus, the small discrepancy found in the estimated molecular weights of the 20 kDa aggregates could have been because SDS-electrophoresis was used by the researchers for the molecular weight determination.

The 20 kDa aggregate gave positive Biuret and Molisch tests, indicating the glycoprotein nature of the antibacterial activity. Unfortunately, the pure peptides were not obtained in large enough amounts (because only an analytical FPLC Superose 12 column was available) for them to be individually tested for the presence of carbohydrate. However, the inactive peptide was observed to give a deposit of carbon on acid hydrolysis for amino acid analysis, and it also gave an unknown peak during amino acid analysis, indicating its glycopeptide nature.

Isolation of two acidic glycoproteins with the estimated molecular weights between 15 kDa and 20 kDa from bovine seminal plasma has also been reported (Manjunath, 1984b; Manjunath and Sairam, 1985, 1987). These proteins are reported to be present in the disaggregated form (15 - 20 kDa) at acidic pH, but form aggregates at neutral pH and have gonadotropin-releasing activity.

The same group of workers has also reported basic proteins with similar characteristics (Sairam *et al.*, 1981; Manjunath, 1984a). The size, glycoprotein nature, disaggregation at acidic pH and aggregation at neutral pH are the characteristics of these gonadotropin-releasing proteins that are similar to those of the 20 kDa antibacterial aggregate isolated in this study.

Thus, it is likely that the antibacterial 20 kDa aggregate may also be responsible for the gonadotropin-releasing activity in bovine seminal plasma. The determination of different net charges on these proteins by the authors could be because they isolated the 20 kDa aggregate with slightly different ratios of the two component peptides present. Similarly, slightly different molecular weights reported for the 20 kDa protein could be explained by the difference in the molecular weights of the component peptides. Also, slightly different molecular weights reported for the aggregates could be due to a different ratio of the peptides being present, as the extent of aggregation was found to be dependent upon the ratio of two peptides in this study (to be discussed later).

In this study, the 20 kDa aggregate (obtained both from the 500 kDa and 250 KDa aggregates and from the 10 - 40 kDa peak of seminal plasma) was found to be a stable combination of multiple units of two different peptides, a basic antibacterial peptide and an acidic inactive peptide. The 20 kDa aggregate is possibly assembled in such a way that all the hydrophobic groups face towards the inside of the aggregate, with the ionic groups involved in the ionic interactions on the surface of the aggregate. This conformation would result in the formation of a stable aggregate with a definite size that depends on the size and the shape of the components. This could explain the aggregation beyond the 20 kDa aggregate (through ionic interactions) and the ease of disaggregation from high molecular weight aggregates to the 20 kDa aggregate by suppressing the ionic groups. This

conformation may also explain the failure of other workers to disaggregate the 20 kDa aggregate.

In the present study, the 20 kDa aggregate was disaggregated by a simple method of cation exchange chromatography. At least a part of the reason for successful disaggregation with cation exchange chromatography could have been the use of citrate buffer in the present study as Eschenbruch (1980) also attempted disaggregation of the aggregates by different types of ion exchange chromatography, but could not achieve complete disaggregation. The disaggregating effect of citrate is demonstrated by the fact that the antibacterial activity of bovine seminal plasma will pass into the diffusate at neutral pH in the presence of citrate but not without it (Shannon *et al.*, 1987). They found that citrate was less effective at lower pH. This suggests that it is the presence of multiple negative charges that is responsible for the disaggregating effect of citrate.

The disaggregation achieved on cation exchange chromatography could also be attributed to the possibility that the 20 kDa aggregate was strongly bound to the cation exchanger, and a combination of salt and pH gradient, and competition for ionic interactions between the gel and the components, and the components themselves would have broken down the ionic interactions, while the long exposure to low temperature would have disrupted the hydrophobic interactions. However, apart from the complete disaggregation of the part of the 20 kDa aggregate, small aggregates of 2 - 3 kDa molecular weight were also obtained. These stable aggregates appeared to contain both the peptides as they were active and could re-aggregate.

These results indicate that the antibacterial peptides of bovine seminal plasma reported by Shannon *et al.* (1975, 1975) and Eschenbruch (1980), with estimated molecular weights of 3.5 kDa and 2.2 kDa respectively, were most probably these stable small aggregates rather than the antibacterial peptide in its monomeric form as the antibacterial peptide was found to have a smaller molecular weight (1.2 kDa) in the present study.

A part of the 20 kDa aggregate was completely disaggregated on cation exchange chromatography giving a monomeric antibacterial peptide, which appeared to be basic in nature as it was the most

strongly retained component on cation exchange chromatography. The basic nature of the antibacterial peptide was also confirmed by cellulose acetate electrophoresis and amino acid analysis.

Different degree activity and extent of aggregation at pH 7.0 were observed in these aggregates. This could have been because they contained the two peptides in different ratios. It was presumed that the earlier the aggregates were eluted on cation exchange chromatography, the higher the ratio of the acidic inactive peptide to the basic antibacterial peptide. Similarly, it was presumed that the later the aggregates were eluted, the higher the ratio of the basic antibacterial peptide to the acidic inactive peptide. It was observed that the more basic the nature of these aggregates, the further the small aggregates aggregated at neutral pH. Thus, the extent of the aggregation appeared to depend upon the amount of basic antibacterial peptide in the aggregates.

This was also confirmed later as the 500 kDa aggregate was found to contain the highest proportion of the basic antibacterial peptide, while the 20 kDa aggregate (third peak of seminal plasma on Sephadex G-200F at pH 7.0) had the lowest proportion of the basic antibacterial peptide. Therefore, the extent of the aggregation (or the molecular weights of the aggregates) was found to depend on the ratio of the basic antibacterial peptide in the aggregates.

In the early part of this study, it was found that the antibacterial peptide did not re-aggregate at neutral pH, while a partially pure preparation of the inactive peptide did re-aggregate at neutral pH. Thus, the inactive peptide was referred to as the aggregating component, which was later found to be incorrect, as the preparation of the inactive peptide was found to contain some antibacterial peptide as well. It was found that neither the antibacterial peptide nor the inactive peptide form the aggregate. It was also found that both peptides had to be together to form aggregates, and the extent of the aggregation depends on the proportion of the basic peptide as described earlier.

When studying re-aggregation, it was often found that aggregates different from the original aggregates were obtained. This could have been because of the assembly of the two peptides in different ratios,

thus forming aggregates with different molecular weights. The concentration of the sample could also have affected the re-aggregation as a concentrated sample would physically facilitate aggregation, while a dilute sample would not.

The inactive peptide was found to be hidden under the citrate peak in cation exchange chromatography, therefore anion exchange chromatography was used to obtain the inactive peptide. Although a part of the sample was completely disaggregated with anion exchange chromatography, activity was still found to be associated with the high molecular weight fractions. This may be explained by the probability that the 20 kDa aggregate was still positively charged or weakly acidic at pH 12.0 and pH 13.0 (the pH at which anion exchange chromatography was carried out), thus it did not bind to the gel strongly, and therefore, was easily displaced mainly as an aggregate early in the gradient. Thus, enough time was not available for the hydrophobic aggregation to come apart.

The inactive peptide was eluted at the end of the anion exchange chromatography, indicating the acidic nature of the peptide. This was later confirmed by cellulose acetate electrophoresis and amino acid analysis.

All the antibacterial aggregates, namely the 500 kDa, 250 kDa and 20 kDa aggregates, formed a precipitate at around pH 4.5. This precipitate was also observed at the same pH when the acid diffusates were neutralised. Both the supernatant and the precipitate were always found to be active aggregates containing both the peptides of the aggregates as found on FPLC. Therefore, no component was lost in the precipitation at any stage of this study.

The precipitation was probably because at about pH 4.5 the acidic inactive peptide with the dissociation of carboxyl group would have acquired a net zero charge, thus allowing interactions of hydrophobic groups which would have then caused the molecules to precipitate out of the solution. The precipitate remained undissolved at acidic pH as low as 1.0. This was probably because below pH 4.5 aggregates were positively charged, repelling each other, and thus remaining out of the solution. It appeared that probably the maximum hydrophobic interaction (possibly also supported by some ionic interactions) both

within the same aggregate and the surrounding aggregate occurs at about pH 4.5, and thus the solubility of the aggregates at pH 4.5 was exceeded. This was supported by the fact that the precipitate and the supernatant had a similar composition and the precipitate was easily dissolved again.

The antibacterial peptide remained in the aqueous solutions at low as well as high pH, indicating the hydrophilic nature of the peptide. This was later confirmed by amino acid analysis. However, the partially pure preparation of the inactive peptide formed a precipitate in aqueous solutions at acidic pH, indicating the hydrophobic nature of the peptide which was later confirmed by the finding of a high amount of tyrosine in the inactive peptide in amino acid analysis. The inactive peptide remained partially insoluble in pH as low as 1.7. The hydrophobicity accounts for the insolubility of the inactive peptide below a pH of about 4.5 where the negative charges from the acidic groups would be lost.

The complete disaggregation of the precipitates obtained in all the stages was achieved by chromatographing diluted samples by FPLC with acetonitrile and trifluoroacetic acid. These results always showed two peaks corresponding to those of the antibacterial peptide and the inactive peptide obtained from different types of ion exchange chromatography. Thus, the high molecular weight antibacterial aggregates were made up of only two components, an antibacterial peptide and an inactive peptide, and no component other than these two was lost in the precipitation at any stage of the disaggregation.

A retarding peak was also obtained on anion exchange chromatography, which was considered to be a third component at that stage. However, it contained peaks identical to those of the antibacterial peptide and the inactive peptide obtained from different types of ion exchange chromatography, both on HPLC and FPLC under identical conditions. Thus, it appeared to be only a small stable aggregate rather than a component. This was confirmed by the fact that a dilute sample of the 20 kDa aggregate from the 500 kDa and 250 kDa aggregates gave only two peaks on FPLC, these corresponding to the antibacterial and inactive peptides. The retardation of this aggregate on Sephadex could be attributed to the presence of the high amount of the inactive peptide which was found to be retarded on Sephadex gel.

Difficulties were faced in this study when attempts were made to determine the net charges on the peptides by using cellulose acetate electrophoresis. Despite several attempts to detect the bands, only faint bands were obtained. However, these indicated the basic nature of the antibacterial peptide and the acidic nature of the inactive peptide.

Electrophoretic techniques could not be used in this study to determine the molecular weight, homogeneity or isoelectric point of the peptides because of their small sizes. Eschenbruch (1980) reported that the antibacterial peptide of about 2.2 kDa molecular weight was not fixed after electrophoresis and was washed off during staining or destaining. Righetti and Chillemi (1978) also reported that peptides below a molecular weight of 10 kDa are increasingly difficult to fix and stain. Thus, a combination of HPLC and FPLC was used to determine the homogeneity of the peptides.

Difficulties were also encountered when attempts were made to estimate the molecular weights of the peptides. Unfortunately, the molecular weights could not be estimated with SDS electrophoresis for the reasons mentioned above or by analytical ultracentrifugation because of their small sizes. Therefore, estimations of the molecular weights of the peptides were obtained using gel filtration chromatography and FPLC.

The antibacterial peptide was eluted just before the bed volume on Sephadex G-25SF and inside the void volume on Sephadex G-15, and its molecular weight was estimated to be about 1.2 kDa from the calibration curves. However, it was eluted on the bed volume on Superose 12 indicating a molecular weight of about 1 kDa or lower. Bearing in mind that separation in the lower range of Superose 12 is not ideal, the estimate of 1.2 kDa appears to be more correct.

The inactive peptide was obviously retarded on Sephadex gel, it being eluted after the bed volume on Sephadex. Small molecules are known to elute later than predicted for their size on tightly cross-linked gels such as Sephadex G-25SF and G-15. This is thought to occur because of ionic and aromatic interactions between gel-solute molecules. The high amount of tyrosine (a hydrophobic amino acid) may also account for the retardation. The inactive peptide was also found

to be eluted at different elution volumes after the bed volume on Superose 12, indicating different levels of retardation. Some times it was eluted on the bed volume, indicating a molecular weight of about 1 kDa.

The different levels of adsorption observed with the inactive peptide could have been because slight changes in factors such as the concentration and ionic strength of the sample, and the changes in the room temperature (FPLC was carried out at room temperature) could have affected the adsorption of the sample to the gel, resulting in a slightly different extent of retardation. Superose gel is known to have some hydrophobic adsorption properties.

The retardation of the last peak of seminal plasma on Sephadex G-200F at pH 7.0 (see Fig. 3.5) could also be attributed to the high ratio of the inactive component which was found to retard on Sephadex gel.

Thus, the molecular weights of the peptides, because of the possible retardation, particularly of the inactive peptide, could be slightly larger than estimated in this study. Similarly, the molecular weights of the aggregates could also be larger than estimated in this study.

In this study, free lysozyme was isolated from the active 10 - 40 kDa peak and the last peak (the retarded peak) of Sephadex G-200F at pH 7.0. However, lysozyme was also found to be present in the form of an aggregate with what was identified as the antibacterial aggregates on HPLC and FPLC. This was not surprising, as Eschenbruch (1980) also isolated lysozyme from bovine seminal plasma both in free form and in high molecular weight fractions, indicating aggregation.

Another antibacterial protein, seminalplasmin, with a molecular weight of 8 kDa - 19.8 kDa has been isolated from bovine seminal plasma by Reddy and Bhargava (1979). When these authors used different SDS-PAGE systems to determine the molecular weight of seminalplasmin, they got different molecular weight estimates (17 kDa and 10.6 kDa).

It is difficult to account for seminalplasmin in the present study as no distinct antibacterial peak was obtained in the region where

seminalplasmin was expected to be eluted. It is possible that Reddy and Bhargava (1979) isolated the stable 20 kDa aggregate found in the present study, rather than a homogenous protein. Different systems of SDS-PAGE could have caused different degrees of disaggregation of the aggregates, thus giving different results for molecular weights of the molecule.

Later, Theil and Scheit (1983a) reported the isolation of a pure seminalplasmin with a molecular weight of about 6.3 kDa. One of the steps used in the isolation of seminalplasmin was ion exchange chromatography, therefore it is possible that they had unknowingly achieved partial disaggregation of aggregates and isolated a smaller aggregate as was found by ion exchange chromatography in the present study.

Rao and Bhargava (1985) isolated an antiseminalplasmin from bovine seminal plasma. This was a protein with a minimum molecular weight of about 39 kDa, which inhibited the antimicrobial activity of seminalplasmin. Again, no protein with an inhibiting effect on the antibacterial aggregates was observed in the present study. However, this could be because no antibacterial aggregates were eluted in the region of the molecular weight where antiseminalplasmin was expected to be eluted.

But it is possible that seminalplasmin is a different entity from the antibacterial peptide and aggregates, and could not be detected in this study because its activity was inhibited by antiseminalplasmin. Another possibility is that seminalplasmin is resistant or less sensitive to the strains of microorganisms used for antibacterial assays in the present study (*E. coli* and *S. aureus*) and thus could not be identified in this study.

Difference in the molecular weights and amino acid analyses of seminalplasmin and the antibacterial peptide isolated in this study clearly indicate that seminalplasmin is a different entity from the antibacterial peptide.

Scheit (1986) reported the isolation of an antibacterial protein identical to seminalplasmin from bull seminal vesicle secretion. Thus, if these authors have been investigating a small aggregate made up of

the same peptides identified in this study, then it is tempting to predict that the peptides which form the antibacterial aggregate originate from the seminal vesicle. The seminal vesicle glands are known to contribute the largest part of the seminal plasma in bulls.

Beta-lysin is another antibacterial protein of low molecular weight (approximately 6.1 kDa) which has been reported to occur in mammalian tissues and secretions. The fact that beta-lysin is not reported in the literature as an aggregating protein, and also has a different molecular weight from the two peptides isolated in this study make it possible to speculate that the antibacterial aggregating system of two peptides discovered in this investigation is a different entity from beta-lysin.

In this study, no antibacterial activity of seminal plasma appeared to be due to lactoferrin (approximate molecular weight 77 kDa), Myeloperoxidase (approximate molecular weight 150 kDa) or lactoperoxidase (approximate molecular weight 78 kDa) as no antibacterial peak with the expected molecular weight of these antibacterial compounds was obtained in this study.

Thus, with the possible exception of small amounts of the above-mentioned proteins that were below the levels of detection, the antibacterial activity of bovine seminal plasma was found to be due to lysozyme and the aggregating system of small peptides.

The presence of lysozyme and antibacterial aggregates of two small peptides in different sizes raises the question : does the presence of the same compound in different antibacterial aggregates confer an advantage on the host? Size, charge and configuration are important determinants of the membrane-disrupting properties of cationic proteins (Ryser, 1967).

It is not unlikely, therefore, that different aggregates of differing sizes and charges possess different antibacterial spectra. The transformation of one aggregated form to another may be of value if one of the aggregated forms is effective against susceptible bacteria. The high concentration of citrate in bovine seminal plasma might favour the transition from one aggregated form to another. Secondly, large molecules (molecules larger than about 50 kDa) are usually not easily

excreted from the body, thus the antibacterial peptide in high molecular weight aggregated form probably escape excretion from the body. Also, the molecules present in aggregated form have been shown to have higher antibacterial activity than their monomeric form (Ikeda *et al.*, 1986). Also, the positively charged antibacterial peptide in the high molecular weight aggregated forms may not easily be inhibited by small negatively charged molecules.

This study demonstrated that aggregates, particularly the 20 kDa aggregate and the 2-3 kDa aggregates are held strongly together. Thus, it could be worthwhile to re-asses the various high molecular weight antibacterial proteins or polypeptides that have obtained from different sources and which did appear to be homogeneous. The disaggregation techniques used in this study, namely cation exchange chromatography with citrate buffer, anion exchange chromatography at high pH and FPLC with dilute samples in acetonitrile could be used to see if these preparations can be disaggregated. It may be found that some high molecular antibacterial proteins reported in literature are aggregates held together strongly rather than homogeneous proteins. It would be interesting to know if the antibacterial peptide and the inactive peptide isolated in the present study are components of any of these antibacterial preparations reported by other authors from various mammalian tissues and secretions.

Of particular interest would be the investigation of seminalplasmin isolated from bovine seminal plasma, as no antibacterial peak in the region of the molecular weight reported for seminalplasmin was obtained in this study. Seminalplasmin could be isolated by the method described by its discoverers (Reddy and Bhargava, 1979; Theil and Scheit, 1983). It could then be tested for disaggregation using the disaggregating techniques used in the present study. This would show if seminalplasmin is just a stable aggregate made of the two peptides isolated in this study or it is a different entity.

Two aggregating glycoprotein molecules of 15-20 kDa with gonadotropin-releasing activity isolated from bovine seminal plasma (Sairam *et al.*, 1981; Manjunath, 1984a, 1984b; Manjunath and Sairam, 1985, 1987) appear to be similar to the antibacterial 20 kDa aggregate isolated in the present study. Thus, another aspect of interest could be to determine whether the antibacterial 20 kDa aggregate found in the

present study has gonadotropin-releasing activity, and if so, then, whether either of the two peptides isolated in the present study are responsible for the activity or whether they both have to be together to have the gonadotropin-releasing activity.

Furthermore, research should be undertaken to gain a better understanding of the significance of the antibacterial peptide and the aggregating system discovered in this study in the non-specific defense system. It should include an investigation into the mechanism. This should be done by electron microscopy and by determination of released cell constituents after exposure of bacterial cells to both the antibacterial aggregates and the monomeric antibacterial peptide. This could explain the advantages of the aggregation of the antibacterial activity, and the antibacterial peptide with radioisotopes could be used to find if the aggregate or the peptide is transported across the bacterial membrane, and specific assays could be employed to determine if inhibition of cellular fractions occurs. The parts of the antibacterial peptide which are essential for the antibacterial activity could be determined by specific protein modification reactions.

The antibacterial spectrum of the different antibacterial aggregates isolated in this study should also be established. Different antibacterial spectra of the aggregates could explain at least one of the advantages of the existence of different antibacterial aggregates in bovine seminal plasma.

It would be of interest to find out how do pathogens of the bovine male reproductive tract compare with related non-pathogens in their sensitivity to the antibacterial aggregates isolated in this study. With resistant microorganisms, attempts could be made to find if there is any correlation with the nature of the cell wall and membrane. It would also be of interest to establish if the resistance is absolute, or is overcome by a higher concentration of the antibacterial aggregates.

In vivo studies should also be carried out in order to find out if the antibacterial aggregates in fact protect the host against experimental infections. Experiments with isolated tissues

could be used to show if and at which level the antibacterial aggregates are toxic to microorganisms.

Further studies should also include amino acid sequencing of the two peptides which form the antibacterial aggregates. As the molecular weights of both the peptides were found to be small in this study, it should not pose any problems. The correct values for the molecular weights could be calculated from the primary structure.

Finally, as the antibacterial peptide and the inactive peptide isolated in this study may or may not have the same origin, the origin of these two peptides should be determined. This could be done by studying the secretions separately from the various sex glands contributing to bovine seminal plasma.

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