The use of phenyl-Sepharose for the affinity purification of proteinases.

Mark Prescott[#], Keith Peek, Dallas P. Veitch and Roy M. Daniel[†]

[†]Thermophile Research Unit, University of Waikato, New Zealand.

[#] current address, Centre for Molecular biology and Medicine, Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia.

[†] Corresponding address and author.

Tel: +64 7 8563619

Fax: +64 7 8560115

Abstract

Phenyl-Sepharose is most often used as an adsorbent for hydrophobic interaction interaction chromatography (HIC). We report on its effective use for the affinity purification of some extracellular thermostable proteinases from bacterial sources. Proteinases belonging to the serine, aspartate and metallo mechanistic classes were effectively retained by the media. Purification factors in the range of 2.9 - 60 and enzyme activity yields in excess of 88% were obtained. In some cases homogeneous enzyme was obtained from culture supernatants in a single step. A number of other proteinases from mammalian sources were also retained. The specificity of the enzyme/support interaction was studied. Proteinases complexed with peptide inhibitors (pepstatin and chymostatin) showed reduced binding to phenyl Sepharose indicating interaction with the active site cleft whereas modification with low molecular weight active site directed inactivators such as PMSF and DAN did not, indicating that binding may not be dependent on sites immediately adjacent to the catalytic site. Pepsinogen and the pro-enzyme form of the serine proteinase from the thermophilic *Bacillus* sp., strain Ak.1 were not retained by the media and could be resolved in an efficient manner from their active counterparts.

Keywords

Abbreviations

Mes, 2-[N-morpholino]ethanesulphonic acid.

Tes, 1,1-bis(hydroxymethyl)ethyl]amino)ethanesulphonic acid.

Bis-tris, 2-bis[2-hydroxyethyl]amino-2-[hydroxymethyl]1,3-propanediol.

DAN, diazoacetylnorleucine methyl ester.

PMSF, phenylmethylsulfonyl fluoride.

Introduction

Affinity chromatography is a well established technique for the rapid and specific purification of proteinases. The technique helps to minimise autolysis, a property common to many proteinases, resulting in enzymes of higher specific activity than might otherwise have been obtained. The immobilisation of low molecular weight inhibitors and substrate analogues such as pepstatin, benzamidine [1], carboxy-D-phenylanine [2], phenylboronic acid [3] and lysine agarose [4] have been used with particular success with a number of proteinases.

However, we have found that some of the commonly used affinity supports to be less than satisfactory for the purification of a number thermostable proteinases under our investigation; for example, the serine proteinase from the *Thermus* strain Rt 41A [5] fails to adsorb to either benzamidine agarose or immobilised carboxy-D-phenylalanine and the acid proteinase from the *Bacillus* strain Wai 21a fails to asdsorb to pepstatin agarose (Prescott, unpublished observations).

We undertook to find a purification technique that was rapid, relatively specific for proteinases and suitable for both analytic and production purposes. While investigating the suitability of other chromatogaphic supports it became clear that phenyl-Sepharose was particularly effective for the purification of several proteinases in which we have an interest. Although there are reports describing the use of phenyl-Sepharose for the purification of proteinases [eg. 6] it is not apparent that the specificity of the interaction has been recognised.

This communication reports on the specific interaction of an adsorbent, usually employed for hydrophobic interaction chromatography of a wide variety of proteins, with a number proteinases from several mechanistic classes and its usefulness in their purification.

Materials and Methods

Materials

Phenyl Sepharose Fast Flow (Sepharose-OCH$_2$CH(OH)CH$_2$0-Phenyl; 20 μmol phenyl/ml of gel) was purchased from Pharmacia (Uppsala, Sweden). Chymostatin, pepstatin A, porcine pepsin and pepsinogen, *Aspergillus saitoi* acid proteinase, thermolysin, proteinase K, papain, subtilisin BPN', subtilisin carlsberg and alkyl agarose affinity chromatography media (agarose-NH(CH$_2$)$_n$CH$_3$) were purchased from Sigma Chemical Company, St. Louis, USA.

Methods

Proteinase assays

Thermostable serine proteinases, metallo-proteinases and the acid proteinase from *Bacillus* strain Wai21a were assayed as described previously [5, 7 and 8 respectively]. Exclusive assays for pepsin and pepsinogen were performed according to Rick and Fritsch [9] using haemoglobin as substrate.

Protein Estimations

Protein was estimated using the Lowry reaction [10] or by Coomassie blue binding [11] with bovine serum albumin as standard.

Purification of proteinases from culture supernatants

All column chromatography was performed at room temperature unless otherwise stated. The *Thermus* strain, Rt41A, was grown and the culture supernatant obtained as described previously [5]. The supernatant was clarified with a HFK-131 membrane (Koch Membrane Systems Inc.) and concentrated to 12 litres with a YM10 ultrafiltration membrane (Amicon Corp.). Activity was applied to a 500 ml column of phenyl Sepharose (9 cm x 7.8 cm) equilibrated in Tes buffer (10 mM Tes pH 7.0, 5 mM $CaCl_2$ and 0.01 % (v/v) Triton X-100) at a linear flow rate of 50 cm/hr. After washing first with equilibration buffer then equilibration buffer containing 20 % (v/v) ethanediol, proteinase activity was eluted with 50 % (v/v) ethanediol in equilibration buffer.

Culture supernatants containing either the serine proteinase from the *Bacillus* strain Ak.1 or strain of *Escherichia coli* containing and expressing the cloned Ak.1 proteinase gene, were desalted and concentrated using a YM10 ultrafiltration membrane. Cloned Ak.1 was expressed as a pro-enzyme and was converted to the active mature enzyme by incubating at 60 °C for 24 h before further purification [12]. Concentrates were applied to separate 50 ml (2.5 cm x 10 cm) phenyl Sepharose columns equilibrated in Tes buffer at a linear flow rate of 37 cm/h. The column was washed with Tes buffer containing 20 % (v/v) ethanediol and the proteinase activity eluted with 50 % (v/v) ethanediol.

Culture supernatant containing the acid proteinase from *Bacillus* strain Wai21a was obtained as described previously [8]. Enzyme eluted from chromatography on S-sepharose was applied directly to a column of phenyl Sepharose (2.5 cm x 10 cm) equilibrated in 20 mM citrate/NaOH pH 3.0 at a linear flow rate of 13 cm/h. The proteinase was eluted with a 100 ml linear gradient of 0-50% (v/v) ethanediol in equilibration buffer.

Culture supernatant from the *Bacillus* strain EA-1 containing the metallo proteinase was produced as described previously [7]. 500 ml of a desalted and 300 fold concentrated culture supernatant were applied to a column (4.5 cm x 60 cm) of phenyl Sepharose equilibrated in 20 mM Bis-tris pH 6.5, 5 mM $CaCl_2$ at a linear flow rate of 25 cm/h. Proteinase was eluted with equilibration buffer containing 1 M toluene-4-sulphonic acid at pH 6.5. The extracellular metallo proteinase from *Bacillus caldolyticus* was produced and purified in a similar manner.

Chromatography of inactivated proteinases

Pepsin was totally inactivated with DAN as described by Rajagopalan [13] and the pH of the incubation adjusted to 3.5 with dilute HCl after modifcation. Serine proteinase Rt41A (specific activity, 5887 U/mg) was inactivated with PMSF as already described [5] to give a preparation with a specific activity of 687 U/mg, equivalent to an inactivation of 88%.

500 μg and 150 μg of inactivated pepsin and Rt41A respectively were applied to separate 1 ml (0.4 cm x 2.5 cm) columns of phenyl Sepharose equilibrated with either 20 mM Na Formate pH 3.5 (pepsin) or Tes buffer (Rt41A). The columns were washed with 5 ml of equilibration buffer and bound protein eluted with 5 ml of equilibration buffer containing 50% (v/v) ethanediol.

Bound and unbound fractions were assayed for protein and enzyme activity.

Chromatography of pro-enzymes

When required pepsinogen and the pro-proteinase from *Bacillus* sp., strain Ak.1 were activated by incubation at pH 2.0 for 1h at 37 °C and pH 7.5 for 24 h at 60 °C respectively. 500 μg of pepsin, pepsinogen, proteinase Ak.1 or inactive pro-proteinase Ak.1 were applied to 1 ml columns of phenyl sepharose equilibrated in either 10 mM Na

formate, pH 4.0 for acid proteinases or Tes buffer for the serine proteinases. After washing with a further 5 ml of equilibration buffer bound protein was eluted with equilibration buffer containing 50 % (v/v) ethanediol. Proteinase activity was determined and expressed as a percentage of total retrieved from the chromatography.

Results

The interaction of a serine proteinase with HIC media.

The binding of a thermostable extracellular microbial serine proteinase, Rt 41A [5] by a range of alkyl substituted agarose supports was compared to that of phenyl Sepharose. The results in Table 1 show that although increasing the number of carbon atoms in the alkyl chain from 4-12 resulted in greater retention of proteinase activity, maximum retention was observed on phenyl Sepharose. Since phenyl Sepharose is often considered to have a hydrophobicity intermediate between straight-chain n-butyl and n-pentyl [14] and retention by phenyl containing media was greater than the considerably more hydrophobic dodecyl agarose, these findings indicate a degree of specificity for the phenyl ring of this support that would not appear to be governed by general considerations of hydrophobicity alone.

Chromatography of inactivated proteinases.

Chromatography of Rt41A proteinase (687 U/mg) inactivated with PMSF [15] did not result in an increase in the specific activity of the protein retained by the column. Since the homogeneous fully active proteinase had a specific activity of 5887 U/mg, the result indicates that both PMSF modified and active enzyme were being retained by the phenyl

Sepharose. Similarly, pepsin, inactivated with DAN [13], and serine proteinase Ak.1, a thermostable serine proteinase cloned from a *Bacillus* species and expressed in *Escherichia coli* as an inactive pro-form [12], inactivated with PMSF, could not be distinguished from their active forms by chromatography on phenyl Sepharose (results not shown).

The finding that the addition of nor-leucine to an aspartate residue at the active site pepsin or a phenylmethyl sulphonyl group to a serine residue at the active site of Rt41A or Ak.1 at the active sites of these enzymes does not effect their retention would suggest that if interaction is occurring within the active site cleft then sites at, or immediately adjacent to the catalytic centre of the proteinase (eg S_1 or S'_1 according to the nomenclature of Shecter and Berger [16]) may not be responsible for the retention of these proteinases by phenyl-Sepharose as thought to be the case with other affinity media containing benzene derivatives [1-4]. This does not, however , exclude the interaction of the phenyl group at other subsites responsible for substrate binding.

Chromatography of proteinase/inhibitor complexes

The results in Table 2 show that chromatography of pepsin in the presence of 100 μM pepstatin reduced the amount of pepsin protein bound by 38% compared to a control experiment containing no added pepstatin. The inclusion of 200 μM chymostatin in the batchwise adsorption of Rt 41a proteinase also altered the degree of adsorption but in this case was found to be time dependent such that a 65 % reduction in binding was observed after 30 mins but only a 5% after 1 h of incubation. .

The naturally occurring microbial inhibitors pepstatin and chymostatin are powerful inhibitors of aspartate and chymotrypsin-like proteinases respectively. The stoichiometric binding of pepstatin to the active site is sufficiently strong to be demonstrated

chromatographically and can be used as a active site titrant for pepsin. The K_i against

hydrolysis of Phe.Gly.His.Phe(NO_2).Phe.Ala.PheOMe is 97 pM [17]. The K_i for

chymostatin against Rt 41A for the hydrolysis of Suc.Ala.Ala.Pro.Phe.NH.Np is 37 nM

(Peek, unpublished observations). Since they are somewhat larger in size (tetrapeptide

and pentapeptide respectively) than adducts resulting from inactivation with PMSF and

DAN, it would be expected that they would occupy more of the sub-sites responsible for

determining the substrate specificity.

 The reduced binding in the presence of these inhibitors indicates that part of the active

site cleft of these enzymes is involved in the adsorption to the phenyl ring and that the

interaction appears to be sufficiently strong and specific to displace these inhibitors

Chromatography of pro-enzymes

When fully active pepsin is chromatographed at pH 4.0 all the pepsin remains bound and

was eluted with 50 % (v/v) ethanediol. Chromatography of pepsinogen under similar

conditions showed that 78 % of the pepsinogen did not bind. The remainder was found to

be bound to the column as pepsin and can be attributed to activation of the pepsinogen

since control experiments showed that over the duration of the experiment (45 min) the

conversion of pepsinogen to pepsin at this pH was limited to 23%. Below this pH 4.0 the

pepsinogen to pepsin conversion rate became excessive and at a higher pH pepsin was not

retained. Therefore, it maybe concluded that pepsinogen does not bind to phenyl

Sepharose under the conditions of the experiment and that at least part of the active site

cleft is involved in binding with phenyl Sepharose.

When the fully activated Ak.1 proteinase was subjected to chromatography on phenyl Sepharose, 96 % of the enzyme protein was retained and was eluted with 50% (v/v) ethanediol. The inactive pro-enzyme did not bind.

 The conversion of pepsinogen to active pepsin involves the removal from the active site of part of a 44 amino acid N-terminal portion of the pro-enzyme through an intramolecular cleavage event. Activation of Ak.1 also involves the intramolecular cleavage and loss of a pro-peptide of 96 amino acids [12]. That phenyl Sepharose cannot retain a proteinase possesing a 'masked' active site provides further evidence that proteinases adsorb through binding to some aspect of the protein essential for substrtae recognition. Although the exact conformation of the peptide in the Ak.1 pro-enzyme has not been determined, it has been proposed that such pro-peptides associate closely with the active site cleft [18]. These results would support this proposal.

Purification of some thermostable proteinases.

The results in Table 3 show that we have successfully employed phenyl Sepharose for the purification from culture supernatants of a number of microbial thermostable extracellular proteinases from the aspartate, metallo and serine classes. Preparations of EA.1 proteinase were found to be homogeneous after a single chromatography step. Furthermore, preparations of native and cloned Ak.1 and Rt 41A were obtained that were found to be 86%, 82% and 90% pure respectively after a single step purification employing step elution from phenyl Sepharose. We have found that ethanediol concentrations above 30% (v/v) elute serine proteinases but 50% (v/v) was more effective and used routinely. Buffers of pH 4-10 did were ineffective for the elution of Rt 41A proteinase. However, pH values of 5.0 or above could be used for elution of the aspartate proteinase. The use of the ligand analogue, toluene-4-sulphonic acid (0.2 M), was found to be effective for the elution of metallo proteinases though higher concentrations (eg 1

M) resulted in enzymes eluting in a smaller volume. Ethanediol (50 % v/v) was also found to be effective.

The interaction of some other proteinases with phenyl sepharose

In order to establish whether our results with thermostable enzymes were more widely applicable, the interaction of a number of other proteinases with phenyl sepharose was investigated.

The results in Table 4 show that a number of proteinases could be adsorbed to phenyl sepharose although the degree of retention was somewhat variable. However, it should be noted that the results are from a single set of chromatographic conditions and that improved retention may be obtained if these conditions were optimised.

Discussion

HIC supports are often believed to interact with proteins in a manner dependent on the general surface hydrophobicity. However, there is evidence to suggest that HIC can be more specific than generally thought [19]. In addition, phenyl-Sepharose is often considered to possess similar hydrophobicity intermediate between butyl or pentyl containing media. However, it is not widely appreciated that its behaviour can be quite different due to aromatic interactions with phenyl and tosyl groups of proteins ($\pi-\pi$ orbital interactions)[13]. Such specific interactions have been exploited for example, in purification of β-amylase using phenyl Sepharose (Phenyl Sepharose application notes, Pharmacia).

The results presented here indicate that phenyl-Sepharose adsorbs proteinases through specific interaction with the active site cleft rather than by more generalised hyrophobic mechanism. However, the exact position of binding to the enzymes under investigation is unclear.

The enzymes that we have successfully purified with this support (Table 3) have been shown to preferentially hydrolyse peptide bonds belonging to amino acids with bulky or hydrophobic side groups in the β-chain of insulin [5 and unpublished data]. In these cases it would be reasonable to assume that the phenyl ring would be able to satisfy, at least to some extent, the requirements for interaction with subsites responsible for determining enzyme specificity and lead to retention of the enzyme and would therefore would behave in a similar manner to those affinity media already described containing a derivatised phenyl ring (eg.benzamidine, carboxy-D-phenylalanine or p-(ω-aminoethyl)phenylboronic acid). The fact that trypsin, which requires a basic amino acid residue adjacent to the scissile bond, did not interact with this media but is retained by benzamidine agarose [1] would support this suggestion.

Although the presence of PMSF or DAN adducts at the active site did not prevent binding to the ligand, it is possible, at least in the case of pepsin and Rt41A proteinase, that interaction is not occurring at those subsites immediately adjacent to the scissile bond. This is implied when the effects of pepstatin and chymostatin and the results with pro-enzymes are taken into consideration. For some proteinases interaction with the S_1 binding site is the primary determinant of binding (eg. trypsin) whereas interactions with other subsites on the enzyme are critical for substrate binding for other proteinases (eg. pepsin).

However, there remains the possibility that there may be sufficient space and flexibility to accomodate both the adduct and phenyl ring in the active site cleft of these enzymes. The fact that the more bulky immobilised carbobenzoxy-D-phenylalanine can gain access to the active site of chymotrypsin but binding is prevented by inactivation with PMSF lends support to this suggestion [2].

Although the range of proteinases tested in this study is by no means exhaustive, it would appear that phenyl Sepharose is capable of adsorbing a wide variety of proteinases and therefore may be more generally applicable to the purification of proteinases than those media such as benzamidine agarose, designed for more specific tasks . We have found that the reduction in specificity observed with this media to be an advantage when attempting to purify novel or uncharacterised proteinases such as the Rt 41a serine proteinase, the pepstatin insensitive acid proteinase from the bacillus strain Wai 21a or the serine proteinase Ak.1. In this latter case the study of the activation phenomenon of pro-proteinases was facilitated by the capability of phenyl Sepharose to separate pro-forms and active forms of the proteinase.

Further advantages are to be gained from using such a commonly available medium. The need for time consuming syntheses or expensive ligands is avoided. In addition we have found that the high linear flow rate capabilities of these advanced media allow the rapid processing of large volumes of dilute proteinase solutions, as for example from culture supernatants, thus minimising bacterial contamination and possible autolysis.

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Table 1. Chromatography of Rt41A serine proteinase on HIC media

Alkyl chain length (number of carbons)	Activity bound (%)
4	7
5	14
6	22
8	35
10	48
12	72
Phenyl	93

1 ml (300 μg) of pure Rt41A serine proteinase was applied to a 1 ml (2.5 cm x 0.4 cm) column of affinity chromatography media equilibrated in Tes buffer at a flow rate of 30 ml/h. and washed with a further 10 ml of equilibration buffer. Unbound enzyme activity was determined and expressed as a % of the that applied to the column.

Table 2. Chromatography of enzyme/inhibitor complexes

Enzyme/Inhibitor	Enzyme protein bound (%)
Pepsin	95
Pepsin + pepstatin	61

Enzyme/Inhibitor	Enzyme activity bound (%) after incubation time:	
	30 min	60 min
Rt41A	87	86
Rt41A + chymostatin	22	81

500 μg portions of pure pepsin were preincubated in the presence or absence of 100 μm pepstatin A and applied to a 1ml column of phenyl Sepharose (2.5 cm x 0.4 cm) equilibrated with 20 mM Na formate buffer, pH 3.5 with or without added pepstatin (100 μM). Columns were washed with 5 ml of their respective equilibration buffers and bound protein eluted with 50% (v/v) ethanediol. Bound and unbound fractions were assayed for protein.

50 μg of pure Rt41A proteinase were preincubated either in the absence or presence of 200 μM chymostatin and added to 500 μl of a 50% slurry of phenyl sepharose equilibrated in Tes buffer with or without added chymostatin (200 μM). Supernatant samples were taken after 30 min and 60 min of continuous mixing, diluted to eliminate interference due to inhibitors and assayed for proteinase. Results are expressed as a percentage of the initial enzyme amount.

Table 3. Purification of some thermostable extracellular proteinases

Enzyme enzyme source	Mechanistic class	Specific Activity (U/mg)		Purification factor	Purity (%)
		Before	After		
EA.1	metallo	2,412	12,021	5.0	100
Rt41A	serine	1,089	5,887	5.4	90
Ak.1 (activated, cloned)	serine	3,364	22,200	6.6	86
Ak.1 (from native organism)	serine	6,179	17,300	2.8	82
Bacillus caldolyticus	metallo	95	5779	60	-
Bacillus sp.Wai21a	aspartate	1028	2976	2.9	28

Table 4. The retention of some proteinases by phenyl Sepharose.

Enzyme or enzyme source	Mechanistic class	Retention
A. saitoi	aspartate	+++
Proteinase K	serine	+++
Thermolysin	metallo	++
Papain	cysteine	++
Subtilisin carlsberg	serine	++
Subtilisin BPN'	serine	+
Chymotrypsin	serine	+
Trypsin	serine	-

1ml (75 µg) of each enzyme were applied to a 1ml column of phenyl Sepharose equilibrated in 10 mM Mes, pH 8.0 and 5 mM $CaCl_2$ except for the aspartate proteinase when the equilibration buffer was substituted for 10 mM Na formate, pH 3.5. After washing the column with 5ml of equilibration buffer bound protein was eluted with equilibration buffer containing 50 % (v/v) ethanediol. Proteinase assays were conducted at 45° C and pH 8.0 using 0.2 % (v/v) azocasein as substrate except aspartate enzyme which was conducted at pH 3.0 using 0.2 % (v/v) haemoglobin as substrate. The degree of retention is indicated as follows: +++, >90% ; ++, >50% ; +, <20% ; -, no binding.