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Review

Separation of proteases: old and new approaches

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Abstract

All methods of protein separations can be applied to proteases. Some emphasis is put in this review on a powerful technique specific to proteases purification: cyclic peptide antibiotics may be seen as general affinity ligands for proteases. Also, some examples of affinity chromatography of proteases on ligands with narrower specificity are given. The special interest of hydrophobic interaction chromatography for proteases purification is discussed. The merits of immobilized dye chromatography for proteases purification and the interest in empirically screening many immobilized dyes, as well as several eluents are discussed.

Keywords: Reviews; Proteases; Enzymes

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List of abbreviations

ACE	Angiotensin-converting enzyme
BPTI	Basic pancreatic trypsin inhibitor
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
EK	Enterokinase
EPPS	N-(2-Hydroxyethyl)piperazine-N′-(3-propanesufonic acid)
ETI	*Erythrina* trypsin inhibitor
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
MMP	Matrix metallo proteinase
MOPS	3-(N-Morpholino)propanesulfonic acid
PCI	Pop corn inhibitor
STI	Soybean trypsin inhibitor
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TPA	Tissue plasminogen activator

1. Introduction

Proteolysis plays a central role in all facets of life: the coagulation cascade [1] or complement activation [2] needs the proper functioning of several proteases; prohormones are processed by proteolysis to the active hormone [3]; endosomal proteolysis ensures processing of hormones [4]; presentation of antigenic proteins to the immune system needs prior proteolysis [4,5]; some receptors are activated through proteolysis [6]; proteases are present in the digestive tract and are necessary for hydrolysis of nutrients; apoptosis needs triggering of proteolytic events [7]; virulence of pathogens may be linked to the fact that they secrete proteases which attack host tissues [8]; etc. Hence, the purification and characterization of proteases are clearly of paramount importance.

All separation methods have been used for purification of proteases. The scope of this review is not to cover all procedures applicable to purification of proteins (and hence of proteases), instead emphasis will be put on procedures which are either specific to protease separation (chromatography on cyclic antibiotics), or are relatively seldom used in that field (chromatography on immobilized dyes which, by contrast, has been very often used for separation of dehydrogenases or kinases). Some examples of affinity chromatography of proteases on immobilized synthetic inhibitors and proteic ligands will be given and discussed. The importance of hydrophobic interaction chromatography for purification of proteases will be discussed. A short reminder of some procedures used for proteolytic activity evaluation will be given at the start.

2. Use of chromatography and electrophoresis for activity assay of proteases

Numerous activity assays for proteolytic activity have been described: Proteolysis of a dyed, biotinylated or radioactively labeled proteic substrate can be appreciated after separation of residual substrate and cleaved peptides: many variants of this general principle have been reported (e.g. [9]). Also, activity measurement of proteases is conveniently performed following hydrolysis in a spectrophotometer or fluorimeter of chromogenic or fluorogenic substrates when available [10].

Reversed-phase liquid chromatography can also be used to separate undigested substrate from product as long as any peptidic substrate is available [11,12]. As a rule, uncleaved substrate and product can be separated in isocratic mode. Automatic injection makes processing of a large number of samples easy.

Electrophoresis can also be used to detect protease

activity. Proteic substrate can be incorporated into the gel [13] or, the separating gel overlaid by a secondary substrate-containing "indicator" gel [14]. Protease is indicated by a cleared zone after coloration of the gel itself or of the indicator gel. Electrophoresis can also be used to detect specific enzymatic activities: e.g. viral proteases have been identified and assayed by separating cleaved products of the viral polyprotein substrate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [15]; or activity of mammalian collagenase is characterized by demonstrating collagen cleavage on SDS-PAGE according to a characteristic pattern [16].

3. Chromatography of proteases on immobilized antibiotics

3.1. Bacitracin and other cyclic peptide antibiotics as general affinity ligands for protease purification

Use of immobilized cyclic peptide antibiotics for purification of proteases (most often Bacitracin but also Gramicidin and Bacilliquin) began with a seminal report in 1976 [17]. Several reports [17–35] have demonstrated their usefulness for purification of proteases from all classes and from many different sources (Table 1).

The promoters of the technique noticed that the affinity for a variety of proteases demonstrated by cyclic antibiotics might be explained by the presence "of a relatively high content of hydrophobic amino acids in these peptides, i.e., of the residues known to match the specificity demands of numerous proteases and capable of binding to their primary and secondary binding sites" [17] (Fig. 1). It is worth noting that a direct inhibitory effect on Bacitracin for papain, leucine amino peptidase [36], subtilisin [36,37], thermitase, proteinase K [37], *Aspergillus niger* secreted proteases [25] and on a purified metallo protease from *Treponema denticola* [38] has been demonstrated. Bacitracin is often used as an additive to buffers or extraction media to suppress unwanted proteolysis (e.g. [39]).

The presence of D-amino acids, as well as the cyclic chain itself, might protect immobilized Bacitracin from digestion by bound proteases [17]: excellent stability of the support was repeatedly claimed.

Cyclic antibiotics have been immobilized by various chemical reactions on agarose, beaded cellulose or silica-based supports as summarized in Table 1. It must be noted that the immobilization technique might significantly influence the results since it has been shown, using several serine proteases and also pepsin, that linkage of Bacitracin by only one of its two amino groups led to loss of affinity for proteases [27].

Purification protocols for proteases on immobilized cyclic antibiotics (as summarized in Table 1) used equilibration buffers whose pH ranged from 4.0 to 8.5. Elution of protease might be obtained in some instances with increase of ionic strength [18,28] or with addition of EDTA (this might be linked to the fact that Bacitracin can bind zinc and/or to the mechanistic class of the enzyme: e.g. carboxypeptidase T. from *Thermoactinomyces* could be eluted with 20 m*M* EDTA in 1 *M* NaCl while a serine protease was retained on the column and eluted thereafter with isopropanol [18]). Often, elution of retained protease was obtained using 10 to 25% propanol in 1 to 2.5 *M* NaCl [18]. A protease from an extreme halophile was eluted with 10% ethanol in 4.5 *M* NaCl [26]. Use of continuous gradients might be used to ameliorate selectivity of desorption and eventually to separate several retained proteases [31]. Some described purification procedures were practically one-step procedures, a purification factor as high as 310 (with 60% yield) has been reported [23]. The fact that propanol in eluting buffer might suppress autolytic degradation of eluted protease is worth noting [23].

Some selectivity of the immobilized antibiotic itself is suggested by the fact that a protease from *Bacillus amyloliquefaciens* was retained on immobilized Gramicidin but passed through a Bacitracin column [18].

3.2. Use of other antibiotics or antibiotic derived substances for purification of proteases

3.2.1. Beta lactams

Several reports deal with the fact that beta lactams can inhibit proteases: derivatives of cephalosporin (a beta lactam antibiotic) have been screened to find inhibitors of leucocyte elastase and many inhibited the protease down to very low concentrations [40]. Most of the inhibitors acylated an active site residue

Table 1
Survey of published purification procedures using chromatography on immobilized cyclic peptide antibiotics

Protease or source for protease purification	Mechanistic class	Purification factor	Conditions for elution[a]	Immobilized antibiotic	Support[b]	Reference[c]
Bacteria and fungi						
Subtilisin BPN	Serine	1.5	n.i.[d]	Bacitracin	SI	[17][e]
Subtilisin 72	Serine	9	25% isopropanol in 1 *M* NaCl pH 6.5	Bacitracin	A	[18][e]
Thermoactinomyces vulgaris	Serine	90	n.i.	Bacitracin Bacilliquin	SI	[17][e]
Trichoderma koningii	Serine	43	20% isopropanol in 1 *M* NaCl pH 8.3	Bacitracin	SI	[17][e]
Subtilisin DY from *Bacillus subtilis*	Serine	n.i.	20% isopropanol in 1 *M* NaCl pH 8.3	Bacitracin	C	[19]
Protease from *Halobacterium mediterranei*	Serine	93	10% ethanol in 4.5 *M* NaCl pH 8.0	Bacitracin	A	[26]
Glutamic acid specific endopeptidase from *Bacillus licheniformis*	Serine	1.4	0.9 *M* NaCl pH 8.2	Bacitracin	A	[28]
Proteases from *Aspergillus terreus*	Serine and metallo proteases	25	25% isopropanol in 1 *M* NaCl pH 5.0	Bacitracin	SI	[29]
Trypsin from *Fusarium oxysporum*	Serine	n.i.	25% isopropanol in 1 *M* NaCl pH 7.0	Bacitracin	SIII	[31]
Recombinant serine protease from thermophilic bacillus species expressed in *E. coli*	Serine	n.i.	25% isopropanol in 0.5 *M* NaCl pH 8.0	Bacitracin	A	[33]
Elastinolytic extracellular protease from *Aspergillus fumigatus*	Metallo protease	5.3	25% isopropanol in 1 *M* NaCl pH 7.2	Bacitracin	A	[32]
Carboxypeptidase from *Thermoactinomyces* spp.	Metallo protease	38	20 m*M* EDTA in 0.1 *M* ammonium acetate pH 6.5[e]	Bacitracin	A	[18][e]
Bacillus neutral proteases (expressed in *Bacillus subtilis*)[f]	Metallo protease	1.8 (*B. subtilis* protease) 5.9 (*B. stearothermophilus* protease)	20% isopropanol in 2.5 *M* NaCl pH 5.0	Bacitracin	SII	[21][e]
Bacillus neutral proteases (expressed in *Bacillus subtilis*)[f]	Metallo protease	48 (*B. subtilis* protease) 310 (*B. stearothermophilus* protease)	20% isopropanol in 2.5 *M* NaCl pH 5.0	Bacitracin	SII	[23]
Trichoderma lignorum	Aspartic	13	20% isopropanol in 1 *M* NaCl pH 5.0	Gramicidin S	SI	[17][e]

Russula decolorans	Aspartic	24–40	15% isopropanol in 1 *M* NaCl pH 5.0	Bacitracin Bacilliquin Gramicidin S	SI	[17][c]
Recombinant protease from *Rhizomucor miehei*	Aspartic	8.1	25% n-propanol in 1 *M* NaCl pH 4.5	Bacitracin	SIII	[20][c]
Aspergillopepsin from *Aspergillus awamori*	Aspartic	n.i.	10% isopropanol in 1 *M* NaCl pH 4.5	Gramicidin	SI	[22]
Extracellular alkaline protease from *Bacillus subtilis*	n.i.	n.i.	20% isopropanol in 1 *M* NaCl pH 8.3	Bacitracin	C	[19]
Extracellular protease from *Enterococcus faecalis*	n.i.	193	25% isopropanol in 1 *M* NaCl pH 7.4	Gramicidin J	A	[24]
Proteases from *Aspergillus niger*	n.i.	n.i.	25% isopropanol in 1 *M* NaCl pH 7.8[g]	Bacitracin	A	[25]
Protozoa						
Serine protease from *Eimeria tenella* oocysts	Serine	36	1 *M* NaCl in 25% isopropanol pH 7.8	Bacitracin	A	[34]
Cysteine proteases from trichomonads	Cysteine	126	25% isopropanol in 1 *M* NaCl pH 7.0	Bacitracin	A	[30]
Animals[h]						
Hog pepsin	Aspartic	1.5 (Bacitracin) 1.5 (Bacilliquin) 3.5 (Gramicidin)	n.i.	Bacitracin Bacilliquin Gramicidin S	SI	[17][c]
Plants						
Papain	Cysteine	4	25% isopropanol in 1 *M* NaCl pH 7.0	Bacitracin	A	[18][c]
Bromelain	Cysteine	2	n.i.	Bacitracin	A	[18][c]
Ficin	Cysteine	3.9	n.i.	Bacitracin	A	[18][c]
Macluralisin from *Maclura pomifera* fruits	Serine	4.5 (Silica support) 1.6 (agarose)	25% isopropanol in 1 *M* NaCl pH 8.0	Bacitracin	SI and A	[35]

[a] Buffers used for pH adjustment are not indicated.

[b] Codes for immobilized antibiotics supports are as follows: antibiotics were immobilized on CNBr activated agarose (A); on aminosilica through benzoquinone activation (SI); on epoxy activated silica (SII); on a polyhydroxylated silica matrix through tresylchloride activation (SIII) on beaded cellulose through 2,4,6-trichlorotriazine activation (C).

[c] References in which full description of the immobilization technique is given. Two papers [17,18], which are clearly founding papers for the use of immobilized antibiotics for protease purification, contain several other examples of proteases purification on immobilized antibiotics, however, details are not indicated in each case and/or references are given to Russian published articles.

[d] (n.i.= not indicated).

[e] Serine protease retained on the column after elution of carboxypeptidase was eluted with 25% isopropanol in 1 *M* NaCl pH 6.5.

[f] Silica bead size used for Bacitracin immobilization was 100–150 μm in [21] and 40–60 μm in [23].

[g] Extract was loaded on the column equilibrated at pH 4.

[h] Examples of purification of proteases from animals are few. An article mostly devoted to evaluation of the influence on support capacity for proteases of two immobilization procedures (see text) indicates that human thrombin and bovine trypsin were retained on Bacitracin silica but description of chromatography conditions are lacking [27].

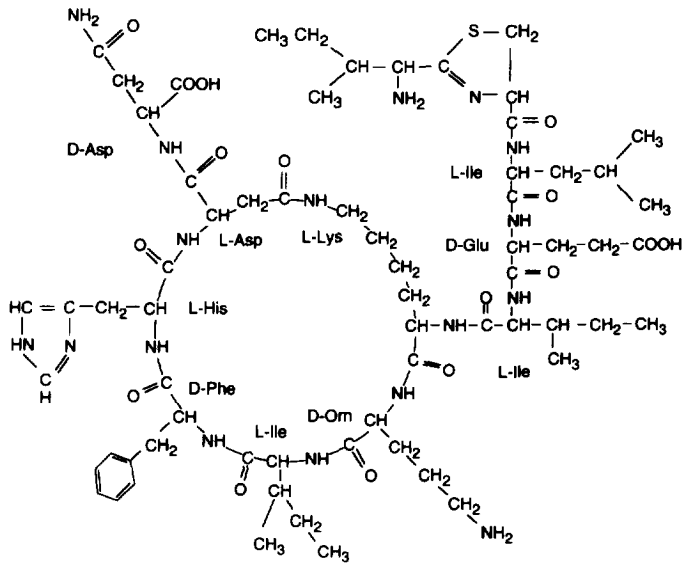


Fig. 1. Structure of Bacitracin A as described in [19].

but some of them were competitive, reversible inhibitors [40]. Penicillin G and other beta lactam antibiotics inhibit urokinase competitively while ampicillin and 6-amino penicillanic are without effect [41].

It seems that to date, immobilized beta lactam antibiotics have been used only for purification of bacterial carboxypeptidases which belong to the family of penicillin-binding proteins and are involved in cleavage of the peptide constituents of murein: a LD-carboxypeptidase was purified from *Escherichia coli* extract on immobilized nocarcidin [42]. Nocarcidin (a beta lactam antibiotic) inhibited this enzyme competitively while three other tested beta lactam antibiotics were without effect. Partially purified enzyme dialyzed in 10 m*M* phosphate buffer pH 7.2 was loaded on a column of immobilized nocarcidin equilibrated in the same buffer. Enzyme was retarded on the column and eluted during the washing with equilibration buffer. The purification factor was 7 and yield 30%.

Penicillin-binding protein 4 from *Escherichia coli* (PBP4, a DD-carboxypeptidase/endopeptidase) is inhibited by penicillin. Inhibition involves acylation of serine residue at the active site. 6-Amino penicillanic acid was coupled to N-hydroxysuccinimide ester activated CM Sepharose. Gel equilibrated in 50 m*M* Tris–HCl buffer pH 8, 1% Triton X 100 was incubated, in batch mode, for 1 h at 4°C, with bacterial extract prepared in the same buffer. After washing with 1 *M* NaCl, the gel was sedimented in a column and treated with 1 *M* hydroxylamine in pH 8 buffer at 30°C. This treatment led to elution of PBP4 and PBP6 (another carboxypeptidase); the two enzymes were separated and obtained in homogeneous form by a further chromatographic step on heparin agarose [43].

3.2.2. Other antibiotics

Anthracyline antibiotics inhibit basement membrane degrading activity from a malignant tumor [44] and *Clostridium histolyticum* collagenase [45]. Tetracycline and derivatives thereof (eventually devoid of antibiotic activity) inhibit a gelatinase purified from malignant human tissue [46]. Puromycin inhibits the metallo enzyme dipeptidylaminopeptidase III [47]. A peptide antibiotic, actinomycin, inhibits subtilisin, thermitase and proteinase K [37]. Some structures of antibiotics cited above are shown in Fig. 2. Many structural variants exist and are produced on a large scale for therapeutic applications. To the best of our knowledge, no trials have been made using these molecules as ligands for purification of proteases

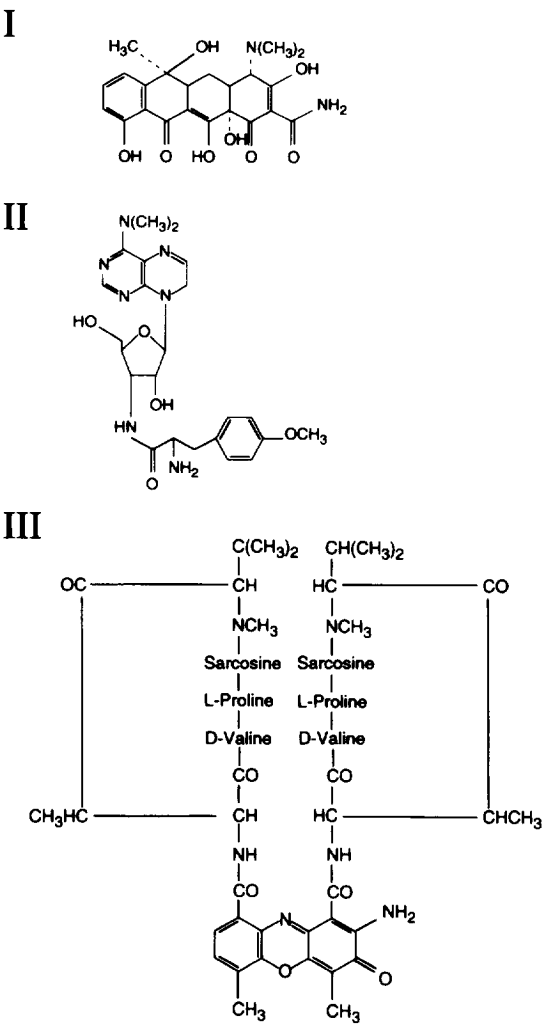


Fig. 2. Structures of tetracyclin (I), puromycin (II) and actinomycin (III).

although many of them might be easily immobilized on chromatography supports.

4. Use of immobilized dyes for protease purification

Use of immobilized reactive dyes for protein purification stems from the serendipitous observation that pyruvate kinase bound to the chromophore (Cibacron Blue F3GA) of soluble Blue Dextran used as a void volume marker in size exclusion chromatography [48]. Cibacron Blue F3GA is an anthraquinone reactive dye which was primarily designed for dying textile materials. Reactive dyes, produced by the ton by the dyes and pigments industry, contain reactive groups which provide for easy grafting on fabrics but also on chromatographic matrices. An enormous number of proteins and enzymes have been purified taking advantage of interactions of proteins with dyes but there are relatively few proteases among them.

Dyes used for purification procedures invariably are aromatic polycyclic molecules on which are grafted polar groups such as sulfonates, hydroxyls, amines or amides (Fig. 3). Retention of proteins on immobilized dye is thought to be due to cooperation, on the one hand, of hydrophobic interactions between hydrophobic pockets on the protein surface and the aromatic backbone of the dye, and on the other hand, of ionic interactions or other polar

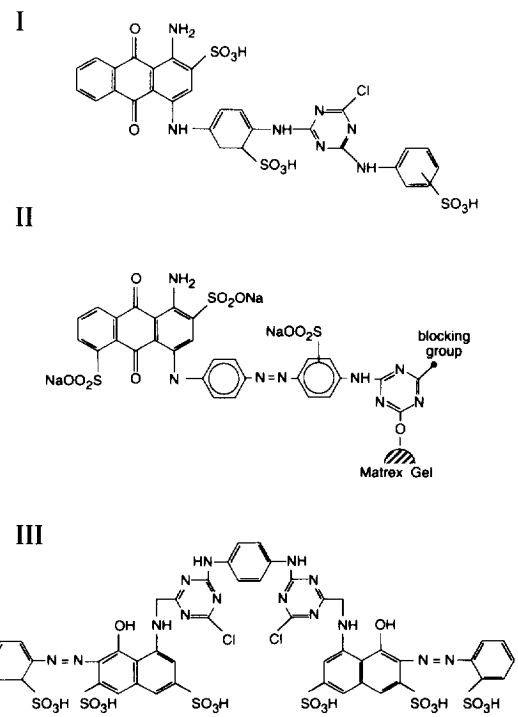


Fig. 3. Structures of Cibacron Blue F3GA (I), immobilized Dyematrex Green A as reported by the manufacturer [81] (II) and Reactive Red 120 (III).

interactions of amino acid side chains with dye substituents. It is clear from crystallographic data of dye–protein complexes [49–51] that a precise fit between one dye structure and an acceptor crevice on a protein surface can explain the strong affinity of a dye for one given protein and hence its selectivity.

It seems that, most often, dye for a purification procedure is chosen from among the few immobilized dyes which are readily commercially available. Probably also, previous publications reporting the use of a given commercial dye for purification of a similar protein, deter researchers from searching for better suited dyes. This probably explains in part, for example, the popularity of Cibacron Blue F3GA for purification of dehydrogenases and kinases.

However, it can be rewarding to screen many different immobilized dyes to find one or several of them which interact selectively with target proteins (or impurities contaminating target proteins). A comprehensive description of the benefits of screening many immobilized dyes, as well as practical hints, can be found in Hey and Dean [52] and Scopes's publications [53–55]. Variants of their screening strategies can be found elsewhere [56–64]. Some examples of dye screening for protease purification will be given below (Section 4.4.1) the interest in screening several eluting conditions once a dye is chosen for a purification procedure will be also discussed (Section 4.4.2).

To elute retained proteins from immobilized dyes, one can depress ionic interactions by increasing mobile phase pH (dyes are mostly often anionic) or increasing ionic strength. Addition of ethylene glycol, of solvents or, more rarely, lowering the ionic strength (see below) can be used to depress hydrophobic interactions [54.55]. Also, addition to mobile phases of substances able to compete with dye protein interactions, e.g. phosphorylated sugars, nucleotides etc., which, as the dyes are aromatic and bear charges, can be used to selectively elute target proteins [54–56,64–66].

Some dyes are metallized (metallization of dyes is used in the dyes and pigments industry to improve light and wet fastness or to produce specific shades [67]). Metals can also be deliberately added to mobile phases to modulate affinity of target proteins for the dye: e.g. carboxypeptidase G2 (which is not a protease but hydrolyses reduced folate) was bound to immobilized Procion Red H-8BN if the column equilibrating mobile phase (0.1 *M* Tris–HCl buffer pH 7.3) contained 0.2 m*M* zinc sulfate while without zinc ions, column capacity was dramatically lowered. Partially purified carboxypeptidase G2 was loaded on a dye column equilibrated with zinc-containing mobile phase and the column was then rinsed with 10 m*M* EDTA buffer pH 5.3. Lowered pH did not allow elution of retained enzyme, but it was eluted in pure form after application to the column of 0.1 *M* Tris–HCl buffer pH 7.3. Yield for this purification step was 83% and the purification factor was 26 [68].

Addition of metal interactions to the "usual" hydrophobic and ionic interactions of dyes with proteins gives other opportunities to selectively adsorb and/or elute target proteins, as demonstrated also in other reports [53,66,69–72].

4.1. Purification of metallo proteases on Dyematrex Green A

Since a seminal work in 1985 [73], several reports on purification of matrix metallo proteases (MMP) from diverse starting materials with Dyematrex Reactive Green A have been published [74–80]. Dyematrex Reactive Green A is an azo-anthraquinone immobilized dye marketed by Amicon (Lexington, MA, USA) (Fig. 3) [81].

Retained proteases were eluted through ionic strength increase: e.g. Okada et al. [74] purified a matrix metallo protease (MMP3) and its proform (proMMP3) from culture supernatant of human rheumatoid synovial fibroblasts. Partially purified enzyme was loaded on a Green A column equilibrated in 50 m*M* Tris–HCl buffer, 0.15 *M* NaCl, 5 m*M* $CaCl_2$. The column was developed successively with 0.3 *M* NaCl, 0.05% Brij 35 in the same buffer (this eluted proMMP3) and with 2 *M* NaCl which eluted two other proteases, proMMP1 and proMMP2. It is worth noting that proMMP3, activated to MMP3 with phenyl mercury acetate, was no longer retained when reloaded on Green A. This underscores that immobilized dyes can be rather selective.

Reported yields and purification factors for these matrix metallo proteases were somewhat variable

depending on purification starting material but, for example, canine prostromelysin from cultured synoviocytes was purified to electrophoretic purity by only a DEAE Sepharose step followed by immobilized Green A [79].

4.2. Immobilized Cibacron Blue F3GA for purification of proteases

Cibacron Blue F3GA was the first immobilized dye used for protein purification [48]. It was used for separation of aspartic proteases from rennet by Subramanian [82]. The column was equilibrated in 25 m*M* citrate buffer pH 5.5. Pepsin present in rennet was not retained while chymosin was eluted from the column by either 1.7 *M* NaCl in the same buffer or by 50% ethylene glycol in 25 m*M* citrate buffer pH 6.2 (yield of clotting activity was 93%). The influence of ammonium sulfate addition to the column equilibrating mobile phase and to the loaded sample is worth noting: at 0.5 *M* ammonium sulfate concentration, neither chymosin nor pepsin were bound; at 0.75 *M* ammonium sulfate, pepsin was not bound (bound chymosin could be eluted with 1.7 *M* NaCl in 20 m*M* citrate pH 5.5); at 1.2 *M* ammonium sulfate, both enzymes were retained on the gel. These experiments demonstrate the interplay of electrostatic and hydrophobic interactions in affinity of dye for proteins and that affinity can be finely tuned by altering mobile phase composition.

Another aspartic protease secreted by *Candida albicans* was purified to electrophoretic homogeneity using as a first step chromatography on immobilized Cibacron Blue followed by size exclusion chromatography on Sephacryl S 200. Protease was not retained on the immobilized dye equilibrated in 10 m*M* citrate pH 6.5. The purification factor was 1.4 but yield was 90% [83].

Chromatography on immobilized Cibacron Blue was also used to purify IL-1β converting enzyme, a thiol protease (yield was 89% and purification factor 5) [84], a matrix metallo protease (yield was 100% and purification factor 6.5, the enzyme was further purified by chromatography on reactive Green A [73]) and recombinant mouse furin, a serine protease (yield was 57% and purification factor 3.2) [85]. In these three examples, proteases were retained on the immobilized dye at low ionic strength and eluted by an ionic strength increase.

4.3. Immobilized Reactive Red 120 for purification of proteases

Immobilized Reactive Red 120 (known also as Procion Red HE3B, Fig. 3) was the first immobilized dye used for protease purification in the early seventies [86]. Protocols for elution from this immobilized dye either used increasing ionic strength (supposed to counteract ionic interactions), decreasing ionic strength or ethylene glycol gradients which lower hydrophobic interactions (Table 2).

4.3.1. Elution from immobilized Reactive Red 120 through an ionic strength increase

In early studies immobilized Reactive Red 120 was used to extract plasminogen from human serum (plasminogen is the proenzyme of plasmin, the key serine protease involved in fibrinolysis). Serum was loaded at low ionic strength (50 m*M* phosphate buffer pH 7.4 and eluted with 0.2 *M* KCl in the same buffer) [86]. This procedure, even if it allows significant purification of plasminogen, does not outcompete lysine agarose chromatography which is practically a single-step purification procedure for plasminogen from human plasma [87].

Several matrix degrading metallo proteases have been purified using Reactive Red 120 as immobilized ligand [88–92] either from animal or human tissues, from cultured cell lines or from eukaryotic cells expressing recombinant enzymes [91,92]. Two representative examples will be given below.

Human neutrophil 57 kDa relative molecular mass collagenase was purified from human buffy coats using Reactive Red 120 chromatography followed by affinity chromatography on Sepharose CH-Pro-Leu-Gly-NHOH and a polishing step on gelatin agarose [88]. Five main proteolytic activities were present in the starting material. Buffy coat extract was loaded on the immobilized dye column equilibrated in 10 m*M* Tris–HCl buffer pH 7.5, 0.5 *M* NaCl, 0.05% Brij 35 and other additives for stabilization of the target enzyme. Elastase, most of gelatinase and cathepsin G activities and all aminopeptidase present in the crude extract were recovered in the flow

Table 2
Proteases purification procedures using immobilized Reactive Red 120. Information on other steps of the purification procedures are given in summarized form in the 2nd and 7th columns

Protease	Refs.	Source of starting material and purification steps performed before immobilized dye chromatography	Equilibrating buffer of the dye column	Eluting buffer	Yield/purification factor	Purification steps applied after immobilized dye chromatography (if any)
Elution by ionic strength increase						
Plasminogen	[86]	Human serum	50 m*M* Phosphate pH 7.4	0–2 *M* KCl gradient in equilibrating buffer	n.i.[a]	
Human neutrophil collagenase	[88]	Buffy coats extracts	10 m*M* Tris, 0.5 *M* NaCl, 0.05% (w/v) Brij 35, 20 μM $ZnSO_4$	same as equilibrating buffer but with 1 *M* NaCl	53 to 100%/13	Affinity chromatographies on Sepharose-CH-Pro-Leu-Gly-NHOH and gelatin Sepharose
Human neutrophil collagenase	[89]	Supernatant of stimulated neutrophils, immobilized zinc chelate and ion exchange chromatographies	50 m*M* Tris pH 7.5, 50 m*M* NaCl	50 m*M* to 2 *M* NaCl gradient in equilibrating buffer	n.i.	Ion-exchange, Sepharose-CH-Pro-Leu-Gly-NHOH and reversed-phase chromatographies
Transin	[91]	Culture supernatants of transformed rat liver cell line, ammonium sulfate fractionation and immobilized zinc chelate chromatography	20 m*M* Tris pH 7.5, 0.005% Brij 35	0.5 *M* NaCl in equilibrating buffer	n.i.	
Recombinant human stromelysin, collagenase and deletion mutants thereof	[92]	Supernatants of transfected mouse myeloma cells, ion-exchange and Dyematrex Green A	25 m*M* Tris pH 7.5, 10 m*M* $CaCl_2$, 0.05% Brij 35, 0.1 *M* NaCl	as equilibrating buffer but with 0.5 *M* NaCl	n.i.	Sephacryl S 200 chromatography
92 kD gelatinase	[93]	Supernatants of U 937 cells or of transfected pAHT2a cells expressing 92 kD gelatinase	20 m*M* Tris pH 7.5, 5 m*M* $CaCl_2$, 0.15 *M* NaCl	0–2 *M* NaCl gradient in equilibrating buffer	n.i.	Gelatin agarose chromatography
Collagenases from *Clostridium histolyticum*	[94]	Commercial collagenase preparations from various sources, hydroxyapatite, Sephacryl S 200 and Arginine Sepharose chromatographies	5 m*M* Tris pH 7.5, 5 m*M* $CaCl_2$	0–2 *M* NaCl gradient in equilibrating buffer	90% of applied collagenolytic activity recovered	Effluent pooled in four separate fractions from which six different enzymes were purified by one or two further ion-exchange steps
Endothelin converting enzyme	[95]	Detergent extract from membranes of cells in culture, chromatographies on ricin agarose ion-exchangers and immobilized metal chelate	20 m*M* sodium borate pH 8.0, 10% glycerol, 0.1% Tween	0–2 *M* NaCl gradient in equilibrating buffer	69%/6.4	Ion-exchange (concentration step)

Table 2 (*continued*)

Protease	Refs.	Source of starting material and purification steps performed before immobilized dye chromatography	Equilibrating buffer of the dye column	Eluting buffer	Yield/purification factor	Purification steps applied after immobilized dye chromatography (if any)
Elution by ionic strength decrease or addition of ethylene glycol to mobile phase						
Calpain	[96]	Chicken gizzard, ammonium sulfate fractionation ion-exchange	20 m*M* MOPS pH 7.0, 1 m*M* EDTA, 1 m*M* EGTA, 1m *M* DTT, 0.5 *M* NaCl	equilibrating buffer but without NaCl	80%/7.3	
Ca^{2+} activated thiol protease	[97]	Rat liver, ion-exchange	Exact composition of buffer was not indicated; it contained 0.5 *M* NaCl	No NaCl	n.i.	
Ca^{2+} activated thiol protease	[98]	Drosophilia melanogaster eggs extract, ammonium sulfate fractionation, HIC	10 m*M* HEPES pH 7.5, 0.5 m*M* EDTA, 0.5 m*M* EGTA, 0.5 m*M* DTT, 0.5 *M* NaCl	equilibrating buffer but without NaCl	43%/7 (see text)	Ion-exchange step
Sea urchin hatching enzyme	[99]	Concentrated eggs supernatant (membrane filtration)	10 m*M* EPPS pH 8.0, 1 *M* NaCl, 10 m*M* $CaCl_2$, 0.5% CHAPS	0–60% ethylene glycol gradient in equilibrating buffer	69%/29.4	

[a] n.i.=not indicated

through while collagenase was eluted with 1 *M* NaCl in the same buffer. Yields ranged from 53 to 100% of applied collagenase depending on the amount of contaminating hemoglobin in the starting material. specific activity was increased by ten fold. It is worth noting the separation of unwanted proteases at the first step of the purification procedure and the fact that chromatography could be conducted at relatively high ionic strength, which is an advantage since low salt concentration promotes denaturation of target enzymes. Reactive Red 120 was chosen after screening five different immobilized dyes but no detail is given on the way the screening was performed [88].

A zinc chelate Sepharose chromatography followed by immobilized Reactive Red 120 allowed purification of Transin (rat stromelysin) from a transformed rat liver cell line supernatant. Enzyme was retained on the immobilized dye and eluted by ionic strength increase. The purification factor of the whole procedure was 21 and electrophoretic homogeneity was demonstrated [91].

Reactive Red 120 was also used for collagenase separations from a microbial source: six collagenases present in culture filtrate of *Clostridium histolyticum* have been purified to homogeneity by a multi-step procedure but "the key step in separation of the individual collagenases was achieved by use of the Reactive Red 120 resin" [94]. Collagenolytic activity (partially purified through, successively, hydroxyapatite, Sephacryl S 200 and Arginine Sepharose chromatographies) was loaded on a Reactive Red 120 column equilibrated in 5 m*M* Tris buffer pH 7.5, 5 m*M* $CaCl_2$. After long rinsing with equilibrating buffer, the column was developed with a shallow gradient from 0 to 2 *M* NaCl. The recovery of the applied collagenolytic activity was 90%. Further processing of four different fractions through DEAE cellulose or DEAE Cellulose and SP Sephadex chromatographies allowed the migration of the six collagenases as single bands on SDS gels to be obtained [95].

Endothelin converting enzyme (ECE), a membrane metallo protease was purified from a human endothelial cell line by a multi-step procedure with chromatography on immobilized Reactive Red 120 dye as the last step. Partially purified enzyme, dialyzed in 20 m*M* sodium borate pH 8.0 containing 10% glycerol and 0.1% Tween 20 was loaded on the column and after adequate washing, eluted through ionic strength increase (development of the column with the metallo protease inhibitor phosphoramidon failed to elute ECE). The purification factor of the immobilized dye chromatography was 6.4 and yield 69% [96].

4.3.2. Elution from immobilized Reactive Red 120 through ionic strength decrease or addition of ethylene glycol to the mobile phase

Immobilized Reactive Red 120 has been used for purification of calpain from chicken gizzard smooth muscle [96]. Partially purified protein was loaded on a column equilibrated in 20 m*M* MOPS buffer pH 7.2, 2 m*M* EGTA, 1 m*M* EDTA, 1 m*M* DTT, 0.5 *M* NaCl. After column washing with this buffer, electrophoretically pure protease was eluted with the same buffer but without NaCl. The yield for this step was 80% and purification factor was 7.3. A similar procedure was applied to purification of calpain from rat liver [97].

A Ca^{2+} activated thiol protease was purified from *Drosophila melanogaster* extract by chromatography on successively, phenyl Sepharose, Reactive Red 120 and an anion-exchanger. Partially purified enzyme was loaded onto the immobilized dye column equilibrated in 0.5 *M* NaCl, 10 m*M* HEPES–KOH buffer pH 7.5. Retained enzyme was eluted by developing the column with 10 m*M* HEPES–KOH buffer pH 7.5 with no NaCl added. Yields and purification factor were difficult to assess since the starting material probably contained more than one enzyme, but they were higher than 43% and 7, respectively [98].

Immobilized Reactive Red 120 was also used for purification of another calcium-activated protease, the sea urchin embryo hatching enzyme. Solubilized enzyme was concentrated by diafiltration and loaded on a column equilibrated in 10 m*M* EPPS pH 8.0 buffer containing 1 *M* NaCl, 0.5% CHAPS and 10 m*M* $CaCl_2$. Hatching enzyme was retained and after adequate column washing eluted in electrophoretically homogenous form with a 0–60% ethylene glycol gradient (a 30 kDa molecular mass protease passed unretained through the column, it seems to be a

degradation product of hatching enzyme devoid of hatching activity). Yield and purification factor of the immobilized dye chromatography step for hatching enzyme were 67% and 29.4, respectively [99].

The experiments summarized above demonstrate that hydrophobic interactions play an important role in affinity of dyes for proteins. Nevertheless, they do not sum up all possible interactions. It is worth noting, in this regard, that phenyl Sepharose was reported to be non satisfactory for purification of hatching enzyme while chromatography on immobilized Reactive Red 120 allowed pure enzyme to be obtained with a single chromatography step [99].

4.4. Screening procedures for purification of proteases by chromatography on immobilized dyes

4.4.1. Screening of several immobilized dyes

Different immobilized dyes were screened for purification of a collagenase from *Aspergillus fumigatus*, a fungal pathogen [11]. A commercial kit made of ten 3 cm×1 cm I.D. different immobilized dye columns (Affinity Chromatography, Isle of Man, UK) was used. A 3-ml volume of crude fungal extract (1 mg protein per ml) was loaded onto the columns equilibrated in 10 m*M* Tris acetate buffer pH 7.0. Columns were rinsed with 5 ml of the same buffer and developed with 3 ml 2 *M* NaCl in the same buffer. Total protein and proteolytic activity were assayed in flow through and wash fractions and in 2 *M* NaCl eluted fractions. One dye (Orange 3) retained 40.7% of applied proteins but not the target protease. Hence, this dye was chosen as a "negative column", i.e., a column "which binds as much protein as possible without binding the enzyme" [54]. From among seven other dyes of the kit which retained 100% of the applied proteolytic activity, Yellow 1 was chosen as a "positive column" since it retained "the enzyme and as little protein as possible" [54] (57.6% of total proteins in crude extract were retained). In order to find a suitable eluent for protease from the positive column, tests were carried out to see if 30 m*M* phosphate, 10 m*M* AMP, ADP, ATP, CMP, cAMP, 2,3-diphosphoglycerate and 6-phosphogluconate could elute the enzyme. All tested substances eluted the enzyme except cAMP but phosphate gave the highest specific activity and was retained as an eluent for the purification protocol. Preparative purification to homogeneity of *Aspergillus fumigatus* collagenase was performed by chromatography on the negative column which yielded 1.44-fold purification and 100% recovery, chromatography on positive column (yield for this step was 80% and purification factor 2.9) and two further chromatographic steps (on hydroxyapatite and hydrophobic chromatography on modified TSK HW 55 gel [11]). Another collagenase was purified from another fungal pathogen, *Trichophyton schoenleini*, using the same procedure [100].

Screening of nine dyes was performed to devise a purification procedure for PBP4 from an overproducing *Escherichia coli* strain. Dyes were immobilized on TSK HW 65 gel [60]. A 25-μl volume of immobilized dye was put in a microcentrifuge tube, equilibrated in 50 m*M* glycine-NaOH pH 10.5 and incubated for 10 min at 4°C with 0.1 ml of bacterial extract. Resin was washed with equilibrating buffer. Bound proteins were eluted with 1 *M* NaCl in equilibrating buffer and analyzed by SDS-PAGE. Seven out of the nine dyes exhibited affinity for the enzyme despite the rather high pH of equilibrating buffer. The dye displaying the greatest capacity, Cibacron Navy Blue 2GE, was retained for large scale purification. Large scale purification involved direct loading of 1.9 l of bacterial extract on a 19.5×1.1 cm I.D. column. Protein was eluted from the column after adequate washing with a gradient between 50 m*M* glycine–NaOH buffer pH 10.5 and the same buffer containing 2 *M* NaCl. A 24-fold purification, 93% purity and 71% yield were obtained with this single chromatography step. It is worth noting that several beta-lactams were tested for their ability to elute the protein without success and that preincubation of the enzyme with beta-lactams did not influence binding of the enzyme to the dye.

Penicillin binding protein 5 (PBP5, a DD-carboxy peptidase) was purified from an overproducing *Escherichia coli* strain by chromatography on Procion Rubine MX-B [61]. Screening was performed as above, except that 98 different immobilized dyes equilibrated in 10 m*M* Tris pH 7.0 were used and

that purified protein was used instead of crude extract for preliminary screening. Dyes showing affinity for the enzyme were thereafter tested with crude bacterial extract for their selectivity using SDS-PAGE of high salt eluted fractions. Preparative chromatography on immobilized PBP5 was performed by directly loading bacterial extract on immobilized dye equilibrated in the same buffer. PBP5 was eluted by a NaCl gradient. The essentially pure protein (as judged from the SDS gel pattern) was concentrated by chromatography on heparin Sepharose. PBP5 could not be eluted from the dye column by benzylpenicillin but pre-incubation of PBP5 with benzylpenicillin and addition of benzylpenicillin to all mobile phases precluded retention of PBP5 on the immobilized dye.

A fragment of Penicillin binding protein 2x from *Streptococcus pneumoniae* was expressed in *Escherichia coli* and purified by chromatography on Procion Blue HERD [62]. A screening of 98 dyes was performed (at pH 8.0). Crude bacterial extract was loaded on the immobilized dye, enzyme was eluted by ionic strength increase and electrophoretically homogenous enzyme was obtained by a polishing step on MonoQ anion-exchanger.

An *Escherichia coli* extract containing PBP4 was used to test the advantages of batch procedures for screening 98 immobilized dyes [62]. Protocol was essentially the same as above but instead of eluting retained proteins by ionic strength increase, immobilized dye pellets were directly extracted with SDS-PAGE loading buffer and the extract analyzed by electrophoresis. Such a procedure, obviously, is only applicable if the presence of target protein in SDS eluates can be readily detected on SDS-PAGE, hence, either is present in relatively large amounts or can be detected by Western blotting. Moreover, one may fail to detect dyes which would allow the target protein to be eluted using non-denaturing eluents, leaving behind, stuck to the column, uninteresting proteins. The influence of pH was studied using this modified procedure; it was shown that decreasing pH from 10.5 to 6 increased the capacity of the dye columns for proteins but that selectivity might be lost. Increasing ionic strength had similar effect to decreasing pH. Zn^{2+} addition to the mobile phase modified selectivity of immobilized dyes but the observed effects were influenced by pH [62].

4.4.2. Screening of mobile phases to elute proteases from immobilized dyes

If it can be worthwhile to screen several dyes to find the most appropriate for one given procedure, it is useful to screen mobile phase conditions to be able to elute selectively target protein from the positive column since, most often, positive columns retain both target protein and unwanted impurities. Examples are many in the field of dehydrogenases and kinases of satisfactory elution with substrates or cofactors. It has been shown in the case of *Aspergillus* metallo protease that several nucleotides could elute the enzyme (see above) [11]. In this regard, a serendipitous observation is worth reporting [59]. In order to test a new procedure to prepare mechanically resistant immobilized dyes for chromatography, purification of ribonuclease from pancreatic acetone powder extract was chosen as a model. In short, non-ionic polyoxyethylene-type detergents were modified by grafting reactive dyes at their hydrophilic end. Crude synthesis mixtures of the dyed detergents were pumped to reversed-phase columns. This treatment abolished the reversed-phase character of the packing material but detergent-grafted dye molecules were available for interaction with loaded proteins. A positive column was selected (Procion Navy HE-R150) after screening nine different dyes. RNAse, the target protein was easily eluted from the dye column with its ligand 5 m*M* CMP. Just one contaminant, identified as chymotrypsinogen, was obvious (the two proteins were thereafter easily separated by a classical reversed-phase chromatography step; see Fig. 4). The interesting point in the context of this review is that chymotrypsinogen was eluted by CMP, a substance which is not known to interact with it. It is further proof that nucleotides can be rather selective eluents for immobilized dye columns (only RNAse and chymotrypsinogen were eluted) and that useful eluting substances might be identified by systematic screening. Outside the field of protease purification, human immunoglobulins which are strongly retained by Drimarene rubine RK5BL cannot be eluted from this dye by conventional means other than by using 6 *M* urea containing buffers [58]. A systematic screening showed that quantitative elution was obtained with 10 m*M* imidazole plus 25 m*M* inositol hexaphosphate dissolved in 50 m*M* MES–NaOH buffer pH 6.0 [66].

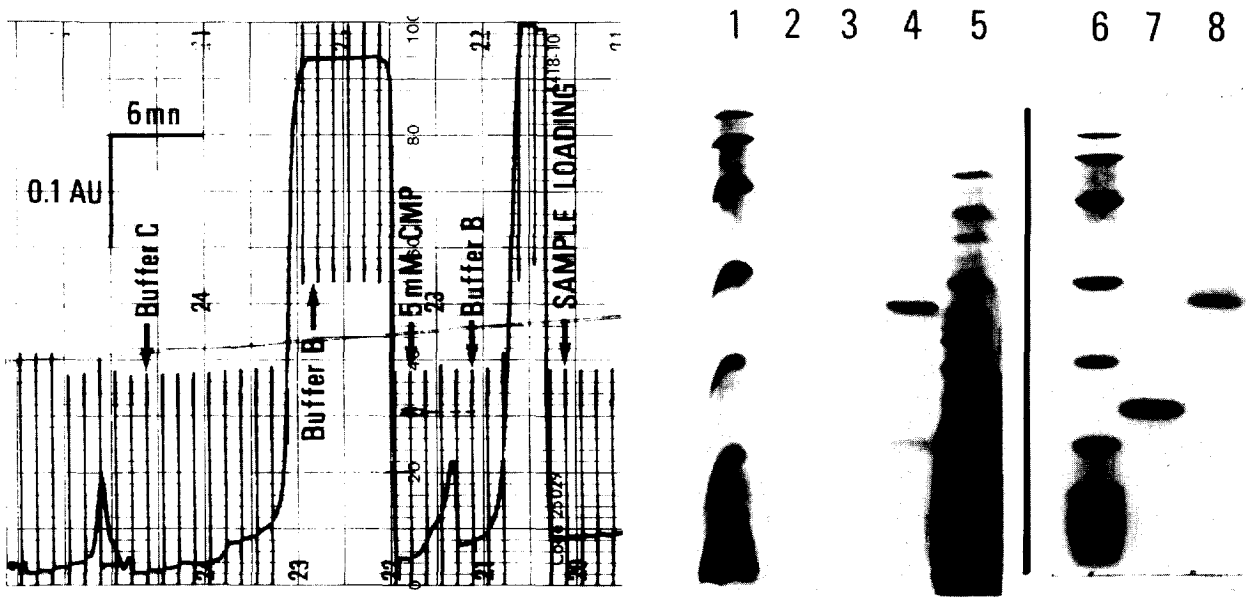
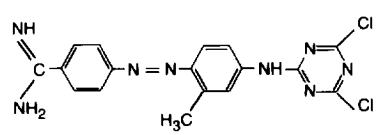


Fig. 4. Purification of RNAse and chymotrypsinogen from bovine pancreas acetone powder on immobilized dye. Left panel: chromatography of extract of bovine pancreas acetone powder on a dyed Brij 76 loaded reversed-phase column [59]. Surfactant grafted to Procion Navy HR-R150 was loaded as a crude synthesis mixture. Column: 2.5 cm ×1.5 cm I.D.. Flow-rate: 4 ml/min. Right panel: SDS-PAGE on a 15% polyacrylamide gel. Lanes 1 and 6 contain molecular mass standards (from top to bottom 94, 67, 43, 30, 20.1 14.4 and $6.6 \cdot 10^3$ relative molecular mass). Lane 5 contains bovine pancreas powder extract. Lanes 3 and 4 contain aliquots of the proteins eluted from the immobilized dye column by 5 m*M* CMP. Lanes 7 and 8 contain RNAse and chymotrypsinogen purified by subsequent reversed-phase chromatography.

4.5. Design of new dyes for protease purification

Work cited above used reactive dyes industrially produced for dying fabrics. Dyes have been specially designed for use as affinity ligands for proteases. The structural formula of one such dye is shown in Fig. 5. An amino benzamidine ring is present and probably primarily responsible for the affinity of this dye for trypsin, thrombin, carboxypeptidase B [101] and urokinase [102].

I



II

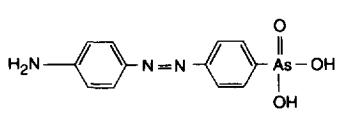


Fig. 5. One cationic reactive dye used after immobilization for purification of urokinase (I) [104] and 4-(4-aminophenylazo)phenylarsonic acid (II) used in immobilized form for subtilisin purification [106].

Cationic dye (Fig. 5) immobilized on Dynosphere XP-3507 was used to purify urokinase from a crude sample. Sample was loaded onto a column equilibrated in 20 m*M* MOPS–NaOH buffer pH 7.0, unwanted proteins were eluted with 1 *M* NaCl in the same buffer and 13-fold purified urokinase was eluted using 2 *M* NaSCN. Yield was 290% suggesting removal of inhibitors present in the starting material [102].

4-(4-Aminophenylazo)phenylarsonic acid (Fig. 5) is not a textile dye but inhibits subtilisin competitively [103]. It could be conveniently coupled on cyanogen bromide agarose through its amino group and used for purification of subtilisin from a crude industrial mixture [104]. Loading was performed on a column equilibrated in 20 m*M* acetate buffer pH

5.9 and elution obtained with 10 m*M* Tris–HCl buffer pH 9.0 containing 0.1 *M* NaCl. The purification factor was 210 and yield 95%.

5. Affinity chromatography of proteases on low-molecular-mass inhibitors

Affinity chromatography on synthetic or natural low-molecular-mass inhibitors for purification of proteolytic enzymes is a widely used technique and in fact was already described in the 1968 Cuatrecasas et al. paper which is often considered as one of the founding articles of affinity chromatography [105]. (This report describes chromatography of chymotrypsin and carboxypeptidase A on columns of immobilized D-Trp–OMe and L-Tyr–D-Trp, respectively). All classes of proteases have been purified using immobilized low-molecular-mass inhibitors. Space in this review does not allow even listing of all inhibitors which have been used for protease isolation by affinity chromatography. The reader is referred to a superb and reasonably recent monograph on protease inhibitors [106].

5.1. The example of benzamidine for purification of trypsin and trypsin-like enzymes

Benzamidine derivatives have been known for twenty five years as immobilized ligands for trypsin and trypsin-like enzyme purification [107,108]. Crystallographic data of trypsin from various species and of thrombin complexed with amidine derivative have been obtained [109,110]. These data demonstrated the existence of interactions between the guanidinium moiety of benzamidine and an aspartyl side chain situated at the bottom of the specificity pocket.

Elution of trypsin-like enzymes bound to immobilized aminobenzamidine can be obtained using competitive inhibitor added to mobile phase, e.g. trypsin and thrombin were eluted by 50 m*M* aminobenzamidine [108], plasmatic kallikrein was eluted from a benzamidine agarose column by a linear gradient from 25 m*M* to 75 m*M* guanidine in 50 m*M* imidazole buffer pH 6.0, 25 m*M* NaCl [111], while factor XIIa was eluted by a gradient between 0 and 1 *M* guanidine in 50 m*M* MES–NaOH buffer pH 6.0, 50 m*M* NaCl [112]. Trypsin was eluted from silica-based, *para*-aminobenzamidine containing support by adding arginine to mobile phase at concentrations ranging from 0.2 to 0.5 *M* varying with the nature of the support [113]. Tissue plasminogen activator (TPA) was bound to a *para*-aminobenzamidine column equilibrated in 1 *M* ammonium carbonate buffer pH 8.0. The column was developed with 0.1 *M* arginine in equilibration buffer, which eluted, in two separate peaks, the two glycosylation variants of one-chain TPA. Proteolyzed two-chain TPA, present in the starting material, was then eluted with 0.3 *M* arginine dissolved in 0.1 *M* sodium acetate, 1 *M* NaCl pH 4 [114]. Obviously, use of acid pH can induce elution of enzymes retained on aminobenzamidine supports [108,115].

Benzamidine has become a model for affinity interactions and has been used to evaluate the potential of new separation techniques: for example *para*-aminobenzamidine was incorporated into polymers which can be reversibly precipitated or solubilized by pH [116], temperature and/or ionic strength [117] variations. Also, immobilization of benzamidine was used to demonstrate the interest of dextran coated silica supports [113] or polymeric mechanically resistant supports [118] or to describe the merits of new activation techniques for affinity chromatography matrices [115].

Nevertheless, benzamidine-based supports should not be seen as endowed with an exclusive affinity for active trypsin-like enzymes at their active site since the ionic and hydrophobic character of benzamidine derivatives makes them prone to non-specific interactions. Possibly relevant to this latter observation is the fact that thrombin [119] or plasmin [120] were bound to immobilized amidines which were N,N′ substituted with alkyl substituents, although these amidine derivatives cannot, any more, accommodate protease specificity pockets. Also, noteworthy in this regard, are the observations that the proenzymes prekallikrein and Factor XII are retained on benzamidine supports and separated from the active proteases by the gradient elution schemes given above [111,112].

The fact that immobilized benzamidine may retain proteases by mechanisms which are not limited to true affinity interactions with enzyme active sites can nevertheless be very useful in practice, e.g., sepa-

ration of plasmin from glu and lys plasminogen (two different molecular forms of its zymogen) was performed on a column of Toyopearl HW65 immobilized benzamidine: the column was equilibrated in 50 m*M* sodium phosphate buffer pH 7.4, 0.1 *M* NaCl; Glu plasminogen was slightly retarded; Lys plasminogen was eluted by adding 20 m*M* 6-aminohexanoic acid (a ligand of plasminogen) to the mobile phase and lastly, plasmin was eluted with 20 m*M* 6-aminohexanoic acid and 3 *M* urea in phosphate buffer (a mixture of 20 m*M* 6-aminohexanoic and 1 m*M* leupeptin was also effective) [121]. Coupling to the chromatographic system of automated on line plasminogen activation and plasmin activity measurement devices, allowed the separation and quantitative assay of the three parent molecules even in complex protein mixtures like plasma [122].

5.2. Purification of angiotensin-converting enzyme using immobilized inhibitors

Purification of angiotensin-converting enzyme (ACE) from various tissues and species offers a perfect example of the power of affinity chromatographic techniques for protease purification. Angiotensin-converting enzyme releases a dipeptide from the carboxy terminus of a decapeptide, angiotensin I. The octapeptide produced, angiotensin II, is a potent vasopressor. ACE is either a membrane linked ectoenzyme or is present in body fluids after proteolysis of the membrane spanning carboxy terminal region [123]. Inhibitors of ACE are potent drugs used for treatment of hypertension. Two ACE inhibitors are shown in Fig. 6, captopril interacts with the catalytic Zn^{2+} atom at the enzyme active site through thiol and lisinopril with a carboxylic moiety.

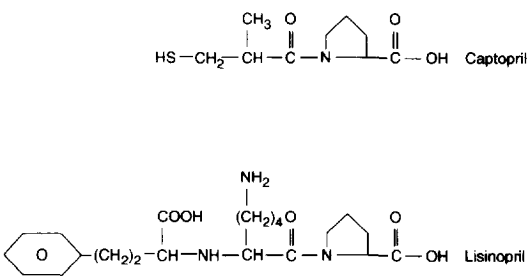


Fig. 6. Structure of two angiotensin-converting enzyme inhibitors.

In the first report, in 1982, on affinity purification of ACE on immobilized lisinopril, ligand was immobilized through the epsilon amine of lysine on butane diol diglycidylether activated agarose [124]. A clarified detergent extract from rabbit testicular tissue was loaded on the column equilibrated in HEPES buffer pH 7.5, 0.3 *M* KCl, 0.1 m*M* $ZnCl_2$. After column washing, electrophoretically pure ACE was eluted with 0.1 m*M* lisinopril. Yield was 26% and the purification factor 390.

ACE was purified from rabbit lung and human plasma on immobilized lisinopril prepared in the same way and operated with similar mobile phases. Purification factors obtained from these two sources were 607 and 110 000, respectively [125].

Use of lisinopril or other ACE potent inhibitors as eluents precludes immediate assay of enzymatic activity in the collected fractions. Protein has to be freed from inhibitors through extensive dialysis in EDTA-containing buffer and the enzyme thereafter re-activated by dialysis against zinc-containing buffer [125]. Alternatively, use of chloride-free buffer may speed up dialysis removal of inhibitors [126].

ACE inhibitors other than lisinopril have been used for ACE purification. The first affinity purification on immobilized ACE inhibitor was reported by Harris et al. [127]. The D-Cys-L-Pro dipeptide was immobilized on agarose bearing a diaminohexane spacer, through the carboxyl group of proline. ACE from human serum, partially purified by PEG precipitation steps, anion exchange and hydroxyapatite chromatographies, was loaded onto the column equilibrated in 100 m*M* Tris buffer pH 8.3. Enzyme was eluted with 1 *M* NaCl, 3 *M* urea dissolved in the same buffer. Yield for this step was 82% and the purification factor about 250. Enzyme was freed from residual contaminants by chromatography on immobilized Cibacron Blue F3GA which let the enzyme pass through. The overall purification factor was 130 000.

Pantoliano et al. [128] immobilized N-(1(S)-carboxy-5-aminopentyl]-L-phenylalanylglycine on agarose to purify ACE from rabbit lung. Several spacers have been used by these authors to link the ACE inhibitor to agarose with lengths ranging from about 10 to 28 nm. Increasing spacer length from 14 to 28 nm increased the capacity (measured by frontal chromatography) more than 350 fold. The 14-nm

long spacer was obtained by grafting inhibitor on butane diol diglycidyl ether activated agarose. The 28-nm long spacer was obtained by grafting 6-[N-(*p*-aminobenzoyl)amino]caproic acid to butane diol diglycidyl ether activated agarose, the carboxylic end of the spacer was thereafter activated through hydroxysuccinimide ester to graft ligand. ACE was solubilized from rabbit lung with detergent and fractionated through ammonium sulfate precipitation. The ACE containing pellet was dialyzed against 20 m*M* MES buffer pH 6, 0.5 *M* NaCl, 0.1 m*M* zinc acetate, 0.1% Nonidet-P40. It was loaded on the column (prepared with the long spacer) equilibrated in the same buffer. After washing of the column, ACE was eluted with 50 m*M* sodium borate pH 8.9. Yield was 70% and the purification factor 148. The same procedure was also successfully applied to ACE purification from rabbit testes. The better performance, in terms of increasing capacity, of the 28-nm spacer column has been attributed by the authors to better accessibility of inhibitor to ACE active sites. Nevertheless, hydrophobicity of this long spacer with an aromatic group is noteworthy. It is not completely unlikely that better capacity is explained by addition of hydrophobic interactions, due to the spacer, to biospecific interactions due to the ligand, even if hydrophobic interactions with the spacer alone cannot retain protein (ACE was not bound to a gel containing an unsubstituted spacer [130]). Scopes [55] has underscored that often hydrophobic or ionic interactions with matrix or spacer contributed to the overall result of affinity chromatography.

Whatever mechanism was operative, satisfactory results led many investigators to also adopt this 28-nm spacer for lisinopril immobilization [123,129,130].

A recent report on purification of ACE from *Drosophilia melanogaster* eggs [130] may be noted as relevant to the above discussion. Drosophilia eggs were homogenized in 10 m*M* Tris–HCl buffer pH 8.5 containing 0.2 *M* ammonium sulfate. Clarified extract was loaded on a lisinopril column (prepared with the long spacer) equilibrated with extraction buffer. Column was rinsed with extraction buffer and bound enzyme was then eluted with 10 m*M* Tris–HCl pH 8.5 without ammonium sulfate. This single chromatographic step produced pure enzyme (as judged from SDS-PAGE). The purification factor was 1000, demonstrating the efficiency of this affinity column. Mobile-phase compositions demonstrate the importance of hydrophobic interactions between ACE and immobilized lisinopril.

6. Affinity chromatography of proteases on immobilized proteic ligands

6.1. The example of chromatography on immobilized proteic protease inhibitors

6.1.1. Basic pancreatic trypsin inhibitor and soybean trypsin inhibitor

For 25 years, very effective purification procedures for serine proteases have been devised using immobilized proteic inhibitors [131,132]. Among them, the most popular are aprotinin, extracted from bovine pancreas or lung (BPTI) and soybean trypsin inhibitor (STI). These inhibitors are small proteins which are available at low cost and are very stable. They inhibit proteases by tight interaction at their active site through a peptidic loop which fits into the enzyme active site. In fact, the inhibitor peptidic loop may be cleaved by the protease but also the broken bond may be resynthesized. Proteases purified by chromatography on such immobilized inhibitors are applied at neutral pH and eluted through pH decrease.

Again, interaction between these proteic inhibitors and their target enzymes affords a good model for evaluating chromatographic supports [133] or new separation techniques (e.g. STI was grafted to an alginate polymer which could be precipitated with bound trypsin by addition of calcium ions. Interestingly, trypsin was eluted from the washed precipitate either at low pH or with 625 m*M* arginine [134]. STI was grafted to a polymer which was precipitated by raising the temperature to 45°C. This modified polymer was used to test the feasibility of affinity thermoprecipitation of trypsin [135]).

Two examples will show the usefulness of affinity chromatography on immobilized STI. A recombinant catalytic domain of bovine enterokinase (RtEK) was expressed in *Escherichia coli* as a fusion protein to the C-terminus of DsbA (an analog of thioredoxin) [136]. The linker between the two proteins contained

a hexa-His sequence and a cleavage site for enterokinase. Presence of DsbA ensured periplasmic export of the fusion protein. Fusion protein was purified on immobilized nickel chelate. RtEK liberated by autocatalytic cleavage was purified by chromatography on STI agarose equilibrated in a pH 8 buffer. Elution was obtained with 100 m*M* sodium formate pH 3, 0.1 *M* NaCl. Only active, correctly folded proteins were bound to the immobilized inhibitor.

A trypsin-like enzyme from a pathogenic nematode *Anisakis simplex* was purified from larval extract by adsorption on STI agarose following an anion-exchange chromatography step [137]. Active peak from the ion-exchange step (20 ml) was incubated for 10 h in batch mode with 1 ml of STI agarose equilibrated in 50 m*M* Tris pH 8.5, 100 m*M* NaCl. The gel was then rinsed with equilibration buffer and with 50 m*M* Tris pH 8.5. Serine protease was eluted using an electroeluter primarily designed for elution of proteins from acrylamide gels (at 5 mA for 4 h at 4°C). This approach (seldom used though the principle was described many years ago [138]) yielded serine protease contaminated by one single other protein. The purification factor was 65 and yield was 12% [137]. (This report also describes another elegant procedure to purify the same enzyme in an inactive state but suitable for structural studies. Partially purified enzyme was incubated with a covalent inhibitor H_2N-Phe-Pro-Arg-CMK modified with biotin at its N-terminus. Labeled enzyme was recovered after adsorption on avidin agarose by electroelution from the beads. A similar procedure was applied by others to purification of apopain, a protease implicated in mammalian apoptosis [139]).

6.1.2. Inhibitors with peculiar specificities: pop corn inhibitor and Erythina trypsin inhibitor

Though aprotinin or STI inhibit a number of serine proteases, many of them are not bound by these inhibitors. This is the case, e.g., for factor XIIa. Systematic screening of plant extracts for inhibitory activity directed against factor XIIa (and other proteases of the coagulation cascade) allowed discovery of some inhibitors endowed with interesting specificities [140]: Pop corn inhibitor (PCI), from corn seeds, was found to inhibit factor XIIa. Purified PCI was used after immobilization for purification of factor XII [141]. Partially purified factor XII was dialyzed in 25 m*M* Tris–HCl pH 8, 0.1 *M* NaCl (with additives to suppress unwanted activation of factor XII to factor XIIa; benzamidine 2 m*M*, hexadimethrine bromide 50 mg/l and EDTA 0.1 m*M*). It was loaded on a column of PCI agarose equilibrated in the same buffer. Factor XII was eluted during washing of the column with equilibration buffer but later than the bulk of contaminating proteins. The purification factor 283 and yield 37% were probably underestimated due to the presence of inhibitors in the buffer. It is worth noting that immobilized factor XII is retarded on the PCI column, though it is the zymogen of factor XIIa. It is nevertheless probable that interaction takes place at the latent protease active site: other cases of proteic inhibitor–zymogen interactions are known (such complexes, e.g. BPTI trypsinogen, have been crystallized and studied by X-ray diffraction) and in fact, binding of the inhibitor is thought to induce reorganization of the zymogen conformation to a conformation close to that obtained after physiological activation [142].

The leguminosae *Erythrina latissima* and *Erythrina caffra* contain inhibitors (ETI) for trypsin and TPA. It seems that, if inhibition of trypsin by ETI follows the general mechanism of protease inhibition (inhibition of trypsin by a stoichiometric amount of inhibitor, cleavage and resynthesis of the critical peptide link in reactive loop), inhibition of TPA does not obey the standard mechanism (inhibition of TPA by ETI is obtained at an ETI/TPA ratio much larger than one, TPA does not cleave reactive site and does not resynthesize reactive site previously cleaved by trypsin) [143]. ETI binds to the active site of TPA (proteolysis or mutation of the inhibitor reactive loop abolishes binding to TPA) but fit of the inhibitor loop in the TPA active site might be not as tight as usually seen (ETI binds DFP-inhibited TPA) [144]. Also, it is probable that other sites, distant from the respective reactive sites, are necessary for binding of the two proteins [143–145].

ETI in immobilized form is very efficient for purification of TPA. In the first report which described purification of TPA on immobilized ETI [144], supernatant from melanoma cells was loaded on the column equilibrated in pH 7.4 buffer con-

taining 0.4 *M* NaCl and 0.1% Triton X 100. TPA was eluted with 1.6 *M* KSCN dissolved in equilibration buffer.

Note: chromatography on immobilized ETI was used to discriminate, in a preparation of recombinant mutated TPA, the molecules which were correctly folded: only molecules able to hydrolyse TPA chromogenic substrates or plasminogen (the TPA natural substrate) were retained on an ETI column while improperly folded enzymatically inactive, immunologically cross reactive material passed through [146].

6.2. Chromatography of collagen degrading enzymes on immobilized collagen or gelatin

Matrix metallo proteases can be purified through immobilized gelatin agarose chromatography [74,75,77,78,98,99,147–152]. Proteases from natural sources, supernatants from cultured cell lines or recombinant enzymes are usually loaded on columns equilibrated in buffer pH 7.5 containing 10 m*M* $CaCl_2$ and often 150 to 600 m*M* sodium chloride. The column is rinsed after loading with equilibrating buffer but use of washing buffers of similar composition but with increased ionic strength (e.g. 1 *M* NaCl [150]) or containing 1 *M* arginine [147] has been described. Elution of retained proteases is usually obtained with the addition to the equilibrating buffer of 5 to 10% dimethyl sulfoxide in buffer containing 0.4 to 1 *M* NaCl.

Gelatin columns show some selectivity: MMP3 (stromelysin) is not adsorbed [74,75,78]; proMMP9 was eluted faster than proMMP2 when a column of gelatin agarose, on which these two activities were bound, was developed with 5% DMSO in Tris–HCl pH 8.0, 1 *M* NaCl [148].

It is worth noting that matrix metallo proteases or proforms are retained on gelatin columns complexed to the natural inhibitor TIMP, if present in starting material.

Interestingly, bacterial proteases which degrade collagen can also be purified on immobilized collagen. Clostridial collagenase was retained on collagen Sepharose equilibrated in 5 m*M* Tris–HCl pH 7.4, 0.5 m*M* $CaCl_2$; 50 m*M* Tris–HCl pH 7.4, 5 m*M* $CaCl_2$ eluted a partially proteolyzed 92 kDa form while a 96 kDa form was eluted by 50 m*M* Tris–HCl pH 7.4, 5 m*M* $CaCl_2$ containing 1.5 *M* NaCl [153].

Clostridial collagenase does not bind to a collagen column in the absence of calcium or other divalent cations. However, lanthanide ions can be used in place of calcium with the advantage that enzymatic activity, and hence, on-column collagen hydrolysis is repressed: Evans described that in presence of 0.4 m*M* Sm^{3+}, collagenolytic activity, present in a commercial preparation, was retained on a collagen column equilibrated in 30 m*M* Tris–HCl buffer pH 7.0, 0.2 *M* NaCl, while other contaminating proteases passed through. The enzyme was eluted in homogenous form with 1 m*M* EGTA [154].

7. Application of hydrophobic interaction chromatography to protease purification

Hydrophobic interaction chromatography (HIC) is a powerful technique for protein purification, e.g. the technique was used in several papers we have referenced above for other reasons. Several types of supports have been used for protease purification by HIC. Supports with alkyl substituents and immobilized poly(ethylene glycol) (or an equivalent to this latter support, obtained by covering a reversed-phase chromatography support with a polyoxyethylene type detergent [155]) have been used, but phenyl Sepharose seems to be the preferred HIC support.

Procedures used for protease purification by HIC are obviously somewhat variable depending on the proteases involved, as shown in some following examples which illustrate how to promote binding to the HIC support if needed, (by addition of salts) and the use of decreasing ionic strength gradients or of detergents to promote desorption. Examples of desorption through ethylene glycol will also be given below.

Urinary kallikrein was purified on octyl Sepharose as follows [156]. Pig urine was made 2 *M* with $(NH_4)_2HPO_4$ and adjusted to pH 7.5. After clarification, the sample was loaded on an octyl Sepharose column equilibrated in 2 *M* $(NH_4)_2HPO_4$ buffer pH 7.5. After washing of the column with the same buffer, enzyme was eluted by a gradient from 2 *M* to 0.05 *M* $(NH_4)_2HPO_4$. The purification factor was

289 with a yield of 49% (protease was purified to homogeneity by two further chromatographic steps, on aprotinin agarose and Sephadex G 100).

A metallo protease, endopeptidase 24.16B was purified from rat testes by a multi-step procedure [157]. Two HIC steps were used, the first was on a TosoHaas butyl 650-S column. Partially purified enzyme was loaded on the column equilibrated in 30% saturated ammonium sulfate in 20 m*M* Tris–HCl buffer pH 7.5. After column washing, enzyme was eluted by a decreasing gradient of ammonium sulfate. Yield for this step was 70% and the purification factor 2.6. A chromatographic procedure on a TosoHaas phenyl 650-S column was performed similarly but the equilibrating mobile phase contained 20% saturated ammonium sulfate. Yield for this step was 18% and the purification factor 2.5.

ACE from human renal cortex was purified on phenyl Sepharose followed by immobilized lisinopril chromatography [158]. With regard to the HIC step, CHAPS extracted enzyme was dialyzed against 150 m*M* potassium phosphate buffer pH 8 and applied to the HIC column equilibrated in the same buffer. After column washing with this buffer, retained enzyme was eluted by a gradient of 0–10 m*M* CHAPS in equilibration buffer. (Yield and purification factor were not given).

Two recent reports claim that phenyl Sepharose applied to protease purification behaves, in fact, as an affinity chromatography packing; in other words, that phenyl sepharose is no more a non specific hydrophobic sorbent but interacts with proteases at the active site or in its immediate vicinity.

A metallo protease from *Proteus mirabilis* was purified by a single chromatographic step on phenyl Sepharose [159]. Protease containing bacterial culture supernatants were loaded after filtration on a phenyl Sepharose column equilibrated in 50 m*M* Tris–HCl buffer pH 8.0. After column washing, retained protease was eluted through 50 m*M* Tris pH 11. Eluted protease was then concentrated and freed from a contaminating pigment by anion-exchange chromatography. Although quantitative data on yield and purification factors are lacking, presented electrophoresis gels demonstrate high purity of the protease.

Serine proteases from culture supernatants of *Thermus* spp. and *Bacillus* (strain Ak.1) and from a recombinant *Escherichia coli* (expressing *Bacillus* enzyme) were purified on columns of phenyl Sepharose equilibrated in 10 m*M* TES buffer pH 7.0, 5 m*M* $CaCl_2$. They were eluted after column washing with 50% ethylene glycol in equilibration buffer (*Thermus* enzyme) or with a gradient of ethylene glycol (20 to 50%) in equilibration buffer (*Bacillus* enzyme). High purity levels of, respectively, 90, 82 and 96% were obtained with purification factors of 5.4, 2.8 and 6.6 (yields were not given) [160].

An acid protease from *Bacillus* (strain Wai21a) was purified on phenyl Sepharose equilibrated in 20 m*M* sodium citrate pH 3.0. The protease was eluted with a 0 to 50% gradient of ethylene glycol in equilibration buffer yielding a 28% pure enzyme with a purification factor of 2.9. A metallo enzyme from *Bacillus caldolyticus* was purified on phenyl Sepharose equilibrated in 20 m*M* Bis Tris pH 6.5, 5 m*M* $CaCl_2$. Enzyme was eluted with 1 *M* toluene sulfonic acid pH 6.5 (claimed to be a substrate analog but used at high concentration). The purification factor was as high as 60 but the purity level of eluted enzyme was not given. Another metallo enzyme from *Bacillus* (strain EA1) was purified similarly. The purification factor was 5 and enzyme was 100% pure [160].

These impressive results demonstrate that HIC is indeed a powerful tool for protein (protease) purification. It is worth noting that all these enzymes were bound in mobile phases with low salt content. It may be wondered however, if these results "indicate that phenyl Sepharose adsorbs proteases through specific interaction with the active site cleft rather than by more generalized hydrophobic mechanisms" [160]. It is interesting that a series of alkyl agaroses with variable chain length have a definite lower capacity for the purified recombinant *Bacillus* enzyme than phenyl Sepharose. This might suggest "a degree of specificity for the phenyl ring of phenyl Sepharose". However, capacities of supports for proteins depend inter alia on the concentration of substituted ligands (ligand concentrations were not given). Proenzymes were shown to behave differently on phenyl agarose than enzymes but a similar observation has been made for a variety of other supports, e.g., dyes [74]. The fact that activated proteases were retained by the HIC support while the proenzymes were not might be related to modification of protein conformation, to

elimination of the activation peptide and not only to un-masking of the latent active site supposed to be the site for interaction.

With regard to the above discussion, a third recent report should be mentioned. It describes purification of a serine protease from *Thermomyces lanuginosus* [161]. Protease purification was obtained by single step chromatography on phenyl Sepharose. Clarified and concentrated supernatant was loaded on a column equilibrated in 25 m*M* sodium phosphate buffer, 1 m*M* EDTA pH 8.5, 0.25 *M* NaCl. The column was washed with 0.1 *M* phosphate buffer, then bound enzyme was eluted in homogenous form by wash buffer containing 30% ethylene glycol. Yield was 60% and the purification factor 61.

The above referenced articles demonstrate that HIC is a powerful tool for protease purification. That in some cases, HIC supports might work as affinity supports, interacting at the enzyme active site, or close to it, seems to remain an open and interesting question.

8. General comments and conclusion

Several approaches can be taken for protease purification. In some cases, a very selective inhibitor is available and may be readily immobilized. In such a situation, the choice of the purification procedure seems to be relatively straightforward. It has to be stressed however, that attention to detail may be very important as demonstrated by the marked influence of the spacer in the example of ACE purification.

Other strategies are possible however. The efficiency of immobilized Bacitracin and other cyclic peptide antibiotics (which seem to be group specific ligands for proteases) has been demonstrated in the numerous referenced reports. However, as we have noted, at least in one instance [18], a protease which was bound by Gramicidin was not retained by Bacitracin. Hence, perhaps it might be worthwhile, when purification of one protease is attempted, to check more than one immobilized cyclic antibiotic. Again, mode of immobilization is not a trivial point and may influence significantly the results [27].

The striking efficiency of HIC for protease purification has been noted.

Besides, there is place in protease purification for purely empirical procedures. Several proteases have been purified by chromatography on immobilized Reactive Red 120 or Dyematrex Green A. Screening procedures to choose both immobilized dye(s) and eluents should be considered when protease purification is planned. We would like to suggest that screening might be extended to other immobilized ligands. Reactive dyes are convenient since they can be very easily immobilized on chromatographic supports. Other relatively complex organic molecules which, as the dyes, might be able to interact with proteins because of a polycyclic backbone and polar substituents, might be considered as ligands to be empirically screened for protease purification, as also suggested elsewhere [55]. Among these organic molecules which might be screened as ligands for protease purification, antibiotics (and also non peptide antibiotics) seem to be interesting since several of them inhibit proteases and they are available at low cost.

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