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Journal of Chromatography A, 814 (1998) 71–81

JOURNAL OF CHROMATOGRAPHY A

Hydrophobic charge induction chromatography: salt independent protein adsorption and facile elution with aqueous buffers

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Received 29 July 1997; received in revised form 18 May 1998; accepted 18 May 1998

Abstract

A new form of protein chromatography, hydrophobic charge induction, is described. Matrices prepared by attachment of weak acid and base ligands were uncharged at adsorption pH. At low ligand densities, protein adsorption was typically promoted with lyotropic salts. At higher ligand densities, chymosin, chymotrypsinogen and lysozyme were adsorbed independently of ionic strength. A pH change released the electrostatic potential of the matrix and weakened hydrophobic interactions, inducing elution. Matrix hydrophobicity and titration range could be matched to protein requirements by ligand choice and density. Both adsorption and elution could be carried out within the pH 5–9 range.

Keywords: Hydrophobic charge induction chromatography; Stationary phases, LC; Proteins

1. Introduction

Hydrophobic interaction chromatography (HIC) is typified by adsorption of proteins to a moderately hydrophobic surface in the presence of high concentrations of a lyotropic salt such as ammonium sulphate. HIC matrices typically have a low ligand density (e.g. phenyl-Sepharose CL4B: approximately 40 μmol/ml) to facilitate protein recovery by decreasing salt gradients [1,2]. Nevertheless desorption can require the use of ethylene glycol [3,4] or harsher procedures [5,6]. At higher ligand densities, the necessity for harsh elution conditions would increase. High ligand density (>80 μmol/ml) matrices have been used for mixed mode hydrophobic/ionic chromatography of chymosin [7]. This enabled high capacity, hydrophobic adsorption without lyotropic salts. Because chymosin was adsorbed at both high and low ionic strength, a pretreatment step of salt addition, or removal by dialysis, dilution or ultrafiltration was not required. Despite strong adsorption, exploitation of matrix charged groups allowed simple recovery of chymosin with aqueous buffers.

The matrices used for chymosin purification contained secondary amine linkages or carboxyl groups and were significantly charged over the pH 4–9 range. Adsorption to an uncharged surface was only possible at pH extremes. Nonspecific electrostatic interactions could result in lower capacity and product purity and/or matrix fouling problems. The latter possibility was demonstrated by the fouling of matrices containing protonated amine groups by crude chymosin samples [7]. Furthermore, charged

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0021-9673/98/$19.00
PII: S0021-9673(98)00436-1

groups could interfere with adsorption of target proteins other than chymosin. In its preferred form, adsorption is carried out under conditions which do not cause electrostatic repulsion between the protein and the matrix. A pH change across the isoelectric point of the protein is used to drive elution by electrostatic repulsion. This dictates the pH requirements for chromatography and may often be incompatible with the preferred range for protein stability.

If carboxyl/amine groups were replaced with weaker acids or bases such as imidazole, an uncharged matrix form could be obtained within the pH 4–10 range. Ideally, partially or fully charged forms could also be obtained within this range. This would allow protein interaction with two distinct matrix surfaces without the requirement of extreme pH or the need to cross the isoelectric point. Adsorption would not be complicated by nonspecific electrostatic interactions.

Histamine and histidine Sepharose matrices have been used for protein adsorption at low ionic strength [8]. The matrices were charged at adsorption pH, due to the presence of amine and/or carboxyl groups as well as imidazole, and proteins were eluted by addition of salt. Pyridyl matrices have been used for salt promoted 'thiophilic' adsorption, particularly of immunoglobulins [9,10]. Desorption was obtained by a decrease in ionic strength because of the low ligand densities used. Weak adsorption of nucleic acids to pyridyl matrices has also been described [11]. Other pyridyl matrices used for immunoglobulin purification had stronger adsorption properties but contained tertiary amine groups, which would be charged at adsorption pH [12]. Salt independent protein adsorption (between 0 and 2 *M* NaCl) was described for a weak cation-exchange Amberlite resin [13,14]. It was claimed that this resin was uncharged at adsorption pH (4–4.5) although base titration of the protonated resin has shown that the resin buffers from pH 3.3 upwards [S.C. Burton, unpublished data]. Adsorption to an uncharged surface would therefore require strongly acidic pH.

The use of high ligand densities should lead to high capacity adsorption at both high and low ionic strengths for many proteins. This might reduce the level of purification, compared to gradient elution from HIC matrices. Further resolution may be obtained by using pH steps for elution, exploiting surface charge differences of proteins. Equally, lower ligand densities could be used for proteins which bind very strongly to conventional HIC matrices. In contrast to HIC, efficient recovery might be obtained by a pH change, without polarity reducing agents, solvents or detergents. Furthermore, the regeneration properties may be superior to standard HIC matrices [15].

In this report, the preparation and titration properties of weakly ionisable hydrophobic matrices of varying ligand density is described. The salt promoted chromatographic properties of low ligand density (<50 μmol/ml) pyridyl and imidazolyl matrices were compared with those of phenyl-Sepharose CL4B, using myoglobin, ribonuclease, chymotrypsinogen, lysozyme and ferritin. The adsorption of trypsin, chymotrypsinogen and lysozyme to matrices of higher ligand density, at high and low ionic strength was also studied. High ligand density matrices (>100 μmol/ml) were also used for purification of crude recombinant chymosin and their performance compared with mixed mode matrices.

2. Experimental

Diethylaminopropylamine, 4-(3-aminopropyl)morpholine, 3-(aminomethyl)pyridine, 4-(aminomethyl)pyridine, 1-(3-aminopropyl)imidazole, 2-(aminomethyl)benzimidazole HCl, 4- hydroxyphenylacetic acid, 2-mercaptobenzimidazole, 4-mercaptopyridine, 3,5-dichlorosalicylic acid, tyramine HCl and *p*-aminobenzamidine dihydrochloride were from Aldrich (Steinheim, Germany); 4-hydroxy-3-nitrobenzoic acid, 3-chloro-4-hydroxyphenylacetic acid, 2-(aminomethyl)pyridine and 2-mercaptopyridine from Janssen (Geel, Belgium); ethyldimethylaminopropylcarbodiimide (EDC), (−)-phenylpropanolamine, histamine, 2-mercapto-1-methylimidazole, lysozyme, ribonuclease, myoglobin, trypsin (type IX), chymotrypsinogen and ferritin from Sigma (St. Louis, MO, USA); crude trypsin from Alliance Foods (Invercargill, New Zealand) and crude *A. awamori* chymosin from Genencor (Palo Alto, CA, USA). Ethoxycarbonylethoxydihydroquinoline (EEDQ) and 4-mercaptoethylpyridine hydrochloride were prepared as described previously [7,16]. 3-,5-Dibromotyramine was pre-

pared from tyramine hydrochloride by the method of Zeynek [17]. Other reagents were analytical grade. Perloza MT (various grades) bead cellulose were from Tessek or ICS, both of Prague, Czech Republic.

Preparation of carbonyldiimidazole (CDI) activated matrices and aminocaproic acid derivatives [7], allyl activated matrices, N-bromosuccinimide modified derivatives and aminophenylpropanediol Perloza [16,18] was described previously. Epichlorohydrin activated Sepharose was prepared by a proprietary method. A trypsin affinity matrix (5 μmol/ml *p*-aminobenzamidine) was prepared by substitution of brominated allyl glycidyl ether Perloza [16].

2.1. Ligand attachment

2.1.1. Amine ligand substitution of CDI activated and aminocaproic acid matrices

A 5 *M* excess of ligand was used. Liquid amines were mixed directly with dioxan solvated, CDI activated Perloza [7]. Aminomethylbenzimidazole hydrochloride was converted to a free base by dissolution in water and pH adjustment to 11.5 with 7.5 *M* NaOH. Water was removed by rotary evaporation leaving NaCl crystals and a viscous liquid. This free base was prepared and used fresh because it became discoloured when stored. Tyramine and 3,5-dibromotyramine were prepared likewise from their hydrochlorides. The solids obtained after evaporation were dissolved in dimethyl sulfoxide (DMSO)–water (75:25, v/v) (8% solutions). Amine ligands were also reacted with aminocaproic acid Perloza (1.57 mmol/g dry) and EDC at pH 4.7 [19]. A 5 *M* excess of EDC and ligand were used plus the minimum water needed for their dissolution [7]. After reaction, the matrices were washed with 0.1 *M* HCl and water to remove excess reagents and byproducts.

2.1.2. Carboxyl ligands condensation with amine matrices

Ligands were attached to an amine matrix (1.24 mmol/g dry), prepared by reaction of a 10 *M* excess of diaminohexane with CDI activated Perloza [20]. A 5 *M* excess of ligand and coupling reagent were used. Dichlorosalicylic acid (10% solution in ethanol) was attached using EEDQ. EDC was used for all other reactions. Hydroxyphenylacetic acid and chlorohydroxyphenylacetic acid were dissolved in water and 7.5 *M* NaOH to pH 5 (12.5% solutions). Nitrohydroxybenzoic acid was prepared likewise but as a 10% solution, pH 6.4. After attachment, matrices were washed with 0.1 *M* NaOH and water. Any amine groups remaining after a repeat attachment reaction were capped with 1 *M* sodium acetate and EDC, at pH 4.7.

2.1.3. Thiol ligand attachment

Initially, ligands were attached to epichlorohydrin activated Sepharose at room temperature and to brominated allyl Perloza at 60°C. A typical reaction mixture was 10 g activated matrix, 10 ml of 1 *M* phosphate buffer, pH 7.2, 50 mg $NaBH_4$ and a 5 *M* excess of ligand. Mercaptobenzimidazole was first dissolved in ethanol or DMSO. Subsequently, reaction pH was increased to 10.5 for mercaptoethylpyridine (MEP) and 11.5–12 for mercaptomethylimidazole attachment to brominated allyl matrices. These latter reactions were carried out at room temperature for 16–24 h or at 60°C for 4–6 h [18]. Low ligand density MEP derivatives were prepared by IR lamp catalysed addition to allyl matrices [21]. After reaction, matrices were washed out with 0.1 *M* HCl and water.

2.2. Matrix titration methods

2.2.1. Small ion titration of unreacted spacer arm carboxyl or amine groups

Carboxylate matrix derivatives (1 ml settled bed) were washed with 0.1 *M* NaOH to convert weak base groups to their unprotonated form and residual acid groups to the Na^+ form. Unbound Na^+ was removed by washing with up to 50 bed volumes of water, or until baseline was reached. Na^+ retained on the matrix by carboxyl groups was displaced with 0.1 *M* acetic acid. The sodium acetate eluted was detected with a Bio-Rad Econosystem conductivity meter. Control matrices (4-aminomethylpyridine CDI Perloza and 95% substituted phenylpropanolamine aminocaproic acid CDI Perloza) containing a known proportion of carboxyl groups (0 and 0.011 mmol/g respectively) were tested likewise. Amine matrix derivatives were titrated similarly except they were converted to the hydrochloride form with 0.1 *M* HCl and excess chloride removed with water. Chlo-

ride ions retained by amine groups were displaced by 0.1 *M* Tris (free base).

2.2.2. Acid base titration

A Radiometer ETS822 autotitrator was used to titrate 1 g matrix samples suspended in 5 ml of 10 m*M* or 0.5 *M* NaCl. Imidazolyl and pyridyl matrices were washed with base and water and titrated with Convol 0.1 *M* HCl to pH 3.3. Some pyridyl matrices were titrated further to pH 2.5. Morpholine matrices were titrated to pH 4. Weak acid matrices were adjusted to pH 12 and titrated down to pH 4. Values were corrected for control titrations of unmodified Perloza. Titration curves were obtained by incremental addition of titrant.

2.3. Model protein chromatography

Matrices (5 ml) for ammonium sulphate promoted chromatography were packed in 15×0.8 cm columns with a peristaltic pump at 1 ml/min. The ferritin solution was diluted twofold with 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)+1.2 *M* ammonium sulphate, pH 7.5 (buffer A). Lyophilised proteins were prepared as 1% solutions in buffer A. A Pharmacia FPLC system was used for salt gradient chromatography. Matrices were equilibrated in 90% buffer A, a 100-μl sample injected and washed for 2 min with 90% A. Chromatograms were developed by a 40-min gradient to 100% buffer B (20 m*M* HEPES, pH 7.5) and 12 min at 100% B. If required, 25 m*M* acetate (pH 5), 100 m*M* acetate (pH 4), and 0.1 *M* NaOH were delivered by a peristaltic pump. Regeneration was with a 2-min gradient to 90% A and 8 min at 90% A. The flow-rate was 1 ml/min.

For chromatography in the absence of ammonium sulphate, matrices (1.5 ml) were packed in 5×0.7 cm columns and the effluent was monitored by a Bio-Rad Econosystem. Buffer B+0.5 *M* NaCl was used for protein dissolution, column equilibration and washing and buffer B for an intermediate wash. Elution was with 50 m*M* acetate, pH 5 or 5.5. Calcium chloride (10 m*M*) was included in wash buffers for trypsin chromatography. Ferritin elutions were with 100 m*M* formate, pH 3. Tris (30 m*M*, pH 8.2) replaced HEPES for the experiment comparing trypsin purification on mercaptomethylimidazole and aminobenzamidine Perloza. Protein recovery was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). An 0.1 *M* NaOH wash was used to monitor completeness of protein removal.

2.4. Chymosin chromatography

Matrices (1.3–1.5 ml) for chymosin adsorption were packed in 5×0.7 cm columns. They were equilibrated and washed at 0.8 ml/min with 10 m*M* citrate±0.5 *M* NaCl, pH 5.5 or 6. Crude chymosin was adjusted to pH 5.5/6 with 2 *M* citrate buffer and 3 ml loaded by gravity at approximately 0.5 ml/min. Bound chymosin was eluted with 50 m*M* HCl/KCl, 200 m*M* acetic acid+HCl or 100 m*M* malonic acid, all pH 2. The initial 5 ml flowthrough fraction was collected for activity analysis. Elution peaks were collected for electrophoretic, protein [22] and activity assays. Electrophoresis was carried out as described previously [18]. Chymosin activity was monitored at 37°C using 4 ml skim milk. Samples which failed to clot within 30 min were classed as inactive [7]. A standard curve for clotting time was prepared using varying amounts (40–100 μl) of crude chymosin. Matrix regeneration was with 0.1 *M* HCl and NaOH. Chromatography was monitored with a Pharmacia UV-1 monitor or a Bio-Rad Econosystem.

3. Results and discussion

3.1. Ligand selection and attachment

3.1.1. Titratable group options

Matrices containing weak acids with pK_a values of 7–10 and bases with pK_a values of 4–7 were chosen. Phenol and its halogenated or nitrated derivatives, morpholine, imidazole, pyridine and benzimidazole were considered the most suitable of many weakly ionisable groups [23,24]. The thiol group was not considered because of its reactivity. The other ligand requirement was a functional group suitable for reaction with an activated matrix. Ligands are grouped below on this latter basis.

3.1.2. Amine ligands

Ligand attachment to CDI activated and/or carboxylate matrices was through uncharged urethane and/or amide linkages. Because CDI groups should react completely by ligand attachment or hydrolysis [25], the desired ligand moiety should be the only titratable group on these matrices. However, carbodiimide catalysed ligand attachment to carboxylate matrices resulted in a mixture of ionisable groups if reaction was incomplete. This could not be quantified by base titration but unreacted carboxyl groups could be detected by the release of sodium ions and associated conductivity peak. When matrices containing carboxylate groups were washed with NaOH followed by water, the return to baseline conductivity was slow and a sharp peak obtained with acetic acid elution [26]. A peak height $\leq$20% of a control matrix (0.011 mmol/g carboxyl groups) was considered acceptable. A rapid return to baseline conductivity and no peak with acetic acid were found for 4-aminomethylpyridine directly attached to CDI Perloza.

3.1.3. Carboxylic acid ligands

Carbodiimide chemistry was used for ligand attachment to diaminohexane CDI Perloza. Unreacted matrix amine groups were monitored by small ion titration, similar to that described for carboxylate groups, except that a Tris solution was used to detect chloride counterions [26]. Complete reaction was harder to obtain than for amine ligand reactions with carboxylate matrices, possibly due to poorer solubility of carboxylic acid ligands at reaction pH (4.7).

3.1.4. Thiol ligands

Ligands were attached to epoxide activated Sepharose or brominated allyl matrices through uncharged thioether bonds. A side reaction, attributed to substitution by the pyridyl rather than the thiol group, occurred when MEP or 4-mercaptopyridine was reacted at pH 7.5. This was not found when MEP was attached at pH 10–11, presumably due to the greater reaction efficiency of the thiolate ion [16]. Therefore this pH range was preferred for thiol ligand reactions. Side reactions were not found with mercaptomethylimidazole or mercaptobenzimidazole.

3.1.5. Preferred methods

Thiol ligands were preferred because activation and ligand attachment chemistry was simple, aqueous and inexpensive. However, these ligands were often expensive and the available range limited. The facile synthesis of MEP [16] suggested that preparation of a greater range of thiol ligands might be preferred for economic as well as reactivity advantages. The second choice was attachment of amine ligands to CDI matrices. Condensation chemistry was required for the nitro and halo phenol ligands tested here. Alternatively phenol matrices such as tyramine CDI Perloza could be modified with an aqueous halogen or tetranitomethane. [27].

3.2. Titration curve analysis of matrices

No evidence of base titratable groups was found for pyridyl matrices washed with HCl and water. This indicated that hydrochloride was displaced and the free base reformed during the water wash. Therefore, weak base matrices were titrated with HCl, despite the fact that unambiguous endpoints could not always be determined. Initially, weak acid matrices were washed with HCl and water and titrated with NaOH. However, titration kinetics were very slow above pH 8. Therefore samples were adjusted to pH 12, with 1 *M* NaOH, and titrated with HCl.

The location of the matrix linkage on the pyridine ring was significant. The pK_a values were highest when the linkage was *para* and lowest when *ortho* to the ring nitrogen. The type of attachment bond also influenced the pK_a. Derivatives of 2-mercaptopyridine and 2-mercaptobenzimidazole had lower pK_a values than their 2-aminomethyl counterparts. In general, attachment to the solid-phase resulted in lower pK_a values for bases and higher values for acids compared to typical values for simple molecules [16,17]. Titration data are summarised in Tables 1 and 2 . The measured pK_a typically varied by one pH unit between low ionic strength (10 m*M* NaCl) and high ionic strength (0.5 *M* NaCl) conditions. The pK_a values were regularly spaced across the preferred pH 4–10 range. The titration ranges of pyridyl and imidazolyl matrices were considered especially useful because uncharged forms were

Table 1
Titration data for weak base matrices

Ligand	Start pH	pK_a	pH 90% titrated
Diethylaminopropylamine	10.5	9.3	8.4
Aminopropylmorpholine	8.9	7.1	6.2
Aminopropylmorpholine (10 m*M*)	8.05	5.8	4.8
Aminopropylimidazole	8.0	6.25	5.2
Histamine	8.4	6.85	5.6
Aminomethylbenzimidazole	6.75	4.8	4.0
4-Aminomethylpyridine	7.2	4.7	3.7
3-Aminomethylpyridine	6.7	4.2	3.3
2-Aminomethylpyridine	6.1	4	3.0
Mercaptomethylimidazole	6.9	5.3	3.8
4-Mercaptopyridine	7.2	4.8	3.7
2-Mercaptopyridine	5.4	2.8[a]	1.9[a]
4-Mercaptoethylpyridine	7.0	4.8	3.7
2-Mercaptobenzimidazole	6.2	4.1	3.0

All matrix samples (1 g) were suspended in 5 ml of 0.5 *M* NaCl except that marked 10 m*M* for which 10 m*M* NaCl was used. Titration below pH 4 was corrected by subtraction of values obtained for an unmodified Perloza control.
[a] Accuracy of values is likely to be lower due to the relatively large correction for dilution required at very acidic pH.

obtained between pH 7 and 8.5 yet most were significantly ionised at pH $\leq$5.

3.3. Model protein chromatography

3.3.1. Elution with a salt gradient

The salt promoted chromatographic properties of imidazolyl and pyridyl matrices were compared with phenyl-Sepharose CL4B, using myoglobin, ribonuclease, chymotrypsinogen, lysozyme and ferritin. Similar lysozyme and chymotrypsinogen retention times were found for MEP (35 μmol/ml) and phenyl-Sepharoses (Table 3). Myoglobin and ribonuclease retention times were very short, especially for MEP Sepharose. Similar results were obtained on MEP, 4-aminomethyl pyridine and mercaptomethylimidazole Perloza matrices (24, 30 and 48 μmol/ml respectively), although lysozyme was eluted after chymotrypsinogen. Myoglobin was retained longer on thiol ligand matrices. Retention times were increased on matrices of higher ligand density. Lysozyme failed to elute in the gradient or at low ionic strength from a more highly substituted MEP Perloza (50 μmol/ml). Ferritin was not eluted at pH 7.5 from any of the matrices. Strongly adsorbed proteins were recovered from pyridyl matrices by a step down to pH 5.5 (lysozyme) or pH 3.5 (ferritin). Ferritin was not eluted from phenyl-Sepharose at pH 3.5. It was only partially eluted with 0.1 *M* HCl and NaOH.

Table 2
Titration data for weak acid matrices

Ligand	Start pH	pK_a	pH 90% titrated
Aminocaproic acid	3	5.2	6.5
Aminocaproic acid (10 m*M*)	3.3	6.1	7.5
Nitrohydroxybenzoic acid	4	6.4	7.5
Nitrotyrosine/ethanolamine	5	7.2	9.0
Dichlorosalicylic acid	5	7.2	9.3
Dibromotyramine	5	7.7	9.3
Chlorohydroxyphenylacetic acid	6.5	9.8	10.8
Hydroxyphenylacetic acid	7.5	10.7	11.2
Tyramine	7.5	10.7	11.3

Matrix samples were suspended in NaCl solutions as described for Table 1. The start pH was determined after washing with 0.1 *M* HCl and water. Titration from pH 12 down to 4 was corrected for an unmodified Perloza control.

Table 3
Retention times of model proteins on phenyl-Sepharose and weak base matrices

Matrix	Ribonuclease	Myoglobin	Lysozyme	Chymotrypsinogen
Phenyl-Sepharose	10	12	26	32
MEP Sepharose 35	6	7	27	34
AMP Perloza 30	6	7	20	N.D.
AMP Perloza 65	14	13	43	42
MEP Perloza 25	7	12	24	17
MEP Perloza 50	21	26	∞	47
MIM Perloza 45	N.D.	17	36	32

The column bed was 5 ml. Buffer A was 20 m*M* HEPES+1.2 *M* ammonium sulphate, pH 7.5; Buffer B was 20 m*M* HEPES, pH 7.5. After an initial 2 min at 90% A, a 40-min gradient to 100% B was applied, followed by 12 min at 100% B. Flow-rate was 1 ml/min and units used for retention are minutes, 6 min represents zero retention time. AMP=4-aminomethylpyridine, MEP=mercaptoethylpyridine and MIM=mercaptomethylimidazole. Numerical values of matrices represent the ligand density (μmol/ml).

Although some differences were found, these results were consistent with hydrophobic adsorption. Differences in salt promoted protein adsorption properties of 2-mercaptopyridine and phenyl-Sepharoses have been reported [9] and attributed to specific thiophilic interactions. The thioether linkage was considered important for thiophilic adsorption. The order of retention was not greatly affected by the linkage for the proteins tested here, although adsorption to thioether matrices was stronger at comparable ligand densities.

3.3.2. Elution by a pH change

Although proteins of weak to moderate hydrophobicity were eluted from phenyl-Sepharose in the salt gradient, ferritin was not desorbed. In contrast, any protein not desorbed from ionisable matrices by the salt gradient was easily recovered by a small pH change. For hydrophobic proteins or foulants which are difficult to recover from conventional HIC matrices, ionisable matrices should provide similar adsorption properties yet allow simple, rapid elution and matrix regeneration.

At higher ligand densities, recovery of many moderately hydrophobic proteins with a salt gradient from phenyl or pyridyl matrices would become difficult, due to an increase in multipoint interactions [28]. At high ligand density, adsorption independent of ionic strength has been reported for mixed mode chromatography [7]. In contrast to HIC, salt is not required to promote adsorption, and hence consumable use is reduced and subsequent removal downstream is not required. These attributes minimise extra processing steps and costs and are particularly significant at large scale.

Trypsin and chymotrypsinogen adsorption properties at high and low ionic strength were studied on Perloza matrices of varying ligand (mercaptomethylimidazole) density (Fig. 1). At 48 μmol/ml, a sharp flowthrough peak was found for chymotrypsinogen, indicative of nonadsorption. Trypsin was not adsorbed either but the broad flowthrough peak indicated that it was significantly retarded compared to chymotrypsinogen. Both proteins were increasingly retarded at higher ligand densities. At 89 μmol/ml trypsin was adsorbed and eluted as a sharp peak after pH adjustment to 5.5. Purification of crude trypsin on this matrix appeared qualitatively comparable to that obtained with an aminobenzamidine Perloza affinity matrix (Figs. 2 and 3). Chymotrypsinogen was also adsorbed at higher ligand densities (115–140 μmol/ml). Salt independent adsorption of lysozyme to 4-aminomethylpyridine Perloza (180 μmol/ml) was also found. It was eluted by a pH change to 5. This allowed purification of lysozyme because contaminant ovalbumin was not adsorbed.

Nonspecific ionic interactions, which could prevent adsorption of a target protein or lead to contaminant binding, were eliminated from the adsorption process, unlike mixed mode. Similar hydrophobic adsorption, independent of ionic strength, would be anticipated for many proteins. The simplicity of operation, low salt requirement and high product concentration in elution fractions should be especially beneficial for large scale use. Because adsorption is to an uncharged surface and elution is

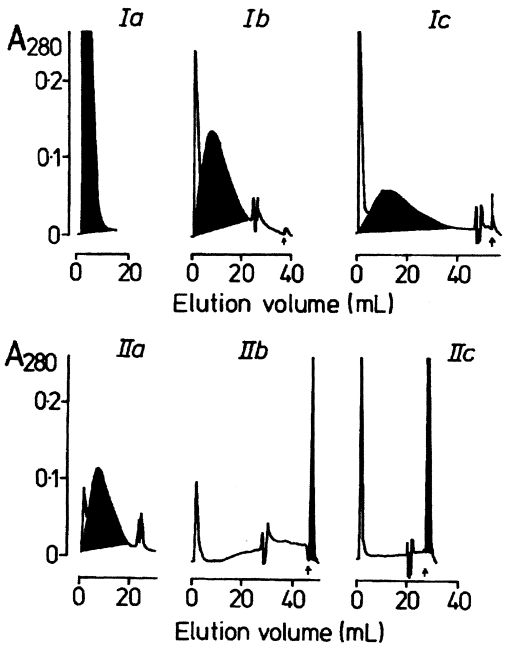

Fig. 1. Adsorption of (I) chymotrypsinogen and (II) trypsin on mercaptomethylimidazole Perloza of varying ligand density. Matrices (1.5 ml) were packed into 5×7 cm columns and equilibrated with 20 m*M* HEPES+10 m*M* $CaCl_2$+0.5 *M* NaCl, pH 8.2. Ligand densities were 48, 77 and 89 μmol/ml for a, b, and c, respectively. Trypsin and chmyotrypsinogen were prepared as 10 mg/ml solutions in equilibration buffer, and 0.1 ml loaded onto the column. The column was washed with equilibration buffer followed by 20 m*M* HEPES+10 m*M* $CaCl_2$. The length of these wash steps was varied to compensate for elution rate differences. Only the high ionic strength wash was used for chromatogram Ia. The buffer change for other traces is characterised by small absorbance increases and decreases. An arrow marks a buffer change to 50 m*M* formic acid. The shaded areas denote eluted chymotrypsinogen (I) and trypsin (II).

induced by titration of some or all ionisable groups to the charged form, this process is called hydrophobic charge induction.

3.4. Chymosin adsorption to weak base matrices

Mixed mode adsorption of chymosin to high ligand density hexylamine Sepharose at both high and low ionic strength, has been reported previously [7]. However, chymosin was not adsorbed at pH 5.5, high ionic strength to diethylaminopropylamine–aminocaproic acid Perloza (1.57 mmol/g dry), a hexyl matrix with surface amine groups. Weak adsorption was attributed to the surface location of the charged group compared to the buried secondary amine linkage of the hexylamine matrix. Nor was chymosin adsorbed to aminopropylimidazole–aminocaproic acid Perloza under these conditions. Although it had a lower proportion of ionised groups than the diethylaminopropylamine matrix at pH 5.5, it was still greater than 50% (Table 1).

Chymosin was adsorbed at both high and low ionic strengths to a matrix with a lower pK_a, 4-aminomethylpyridine–aminocaproic acid Perloza, at the same pH. This suggested that hydrophobic interactions necessary for chymosin adsorption were disrupted above a certain threshold of surface charged groups. The pyridyl ligand was also expected to be more hydrophobic than aminopropylimidazole. Indeed a pyridyl matrix without the hexyl spacer arm, 4-aminomethylpyridine CDI Perloza (1.54 mmol/g dry), also bound chymosin at high ionic strength. Other high ligand density pyridyl and benzimidazolyl matrices also adsorbed chymosin likewise, without a spacer arm.

The elution principle was the same as for hexylamine Sepharose, charge repulsion at a pH below the isoelectric point. A higher molarity buffer (100 m*M* malonic acid) was used rather than dilute HCl [7], to counteract the buffering effect of pyridyl groups. However, elution conditions which lead to a slower change in pH can provide greater resolution of elution peaks [26].

Eluted chymosin was colourless. The purification was superior to that obtained from a mixed mode aminophenylpropanediol Perloza matrix (Table 4, Figs. 2 and 4). A load pH of 6 was preferred to minimise nonspecific ionic adsorption effects. Optimal purification was obtained with 2-aminomethylpyridine CDI Perloza. Its low titration range was well suited to the pH requirements of chymosin. After regeneration of pyridyl matrices with 0.1 *M* NaOH, no coloured foulants remained. This also contrasted with severe fouling of mixed mode amine matrices [7,16].

These results suggest that a ligand can be selected rationally on the basis of titration data to match the preferred pH conditions for the target protein. For chymosin this translated to a ligand with a neutral form between pH 5 and 6, to fit the major optimum

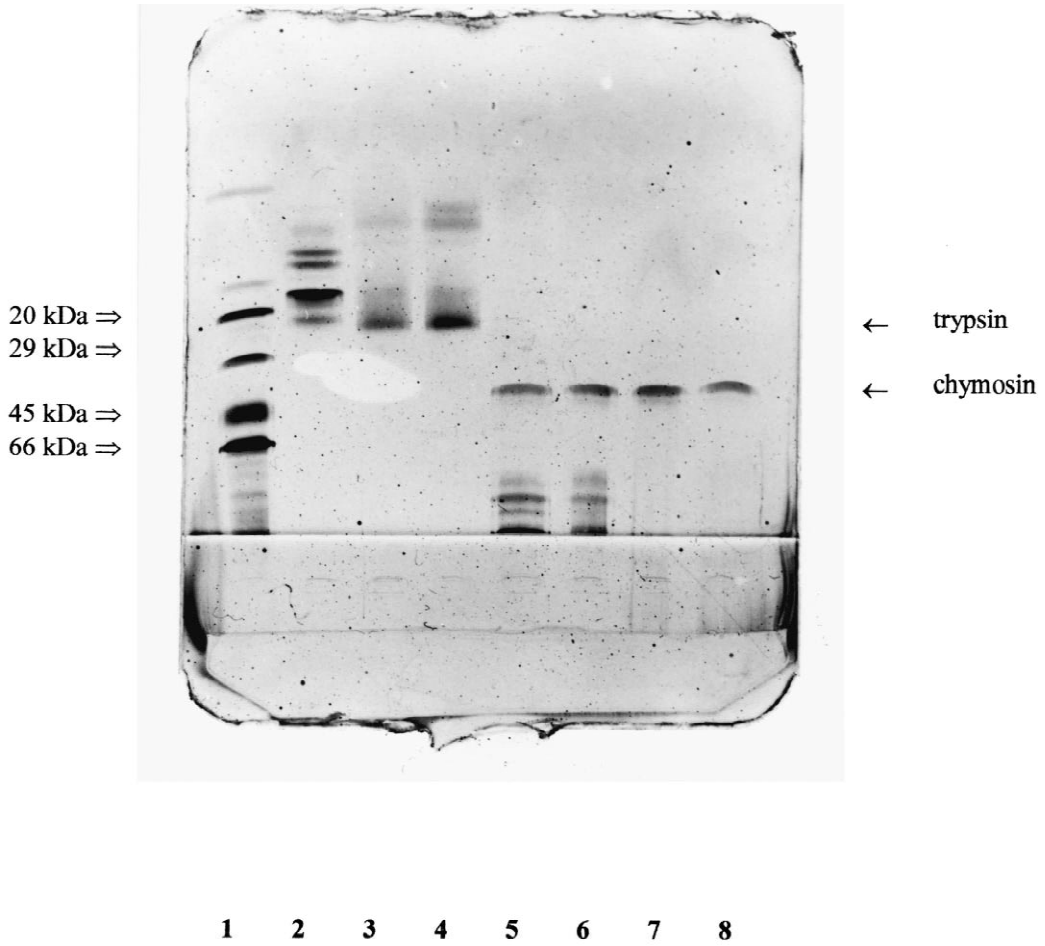

Fig. 2. 20% SDS-PAGE of chymosin and trypsin samples; Lanes: 1, M_r markers: trypsin inhibitor (20 000), carbonic anhydrase (29 000), ovalbumin (45 000) and BSA (66 000); 2, crude trypsin; 3, trypsin elution from mercaptomethylimidazole Perloza; 4, trypsin elution from aminobenzamidine Perloza; 5, crude chymosin; 6, chymosin elution from aminophenylpropanediol Perloza; 7, chymosin elution from mercaptomethylimidazole Perloza; 8, chymosin elution from 2-aminomethylpyridine-CDI Perloza.

for chymosin stability [29]. The other requirement was a partly or wholly ionised form at a pH below the isoelectric point to provide charge repulsion. The elution pH of 2 was chosen to match the lower pH zone of chymosin stability.

3.5. Other proteins

The acidic pH optima of chymosin dictated use of weak base ligands with low pK_a values. The preferred pH ranges of other proteins will vary greatly but lysozyme, trypsin and chymotrypsinogen eluted at pH 5–5.5 from pyridyl and imidazolyl matrices. Therefore, the range of available ligands allows operation near neutral pH. The method can be adapted to suit individual protein isoelectric point and stability requirements by ligand selection from within this range. Matrix hydrophobicity can be manipulated by choice of ligand type or density and attachment chemistry. Although the strong adsorption obtained with high ligand density may reduce specificity, there is potential for further resolution when the pH is altered, due to isoelectric point differences.

A protein stability range of ≥2 pH units is preferred to allow adequate ligand titration. Lyso-

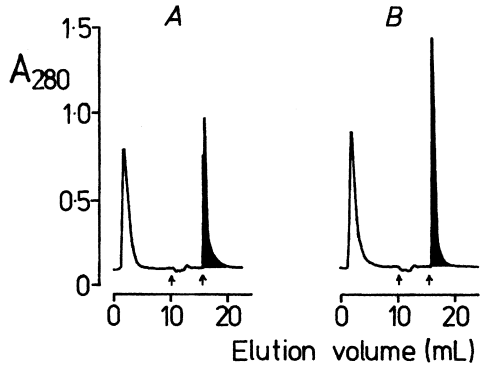

Fig. 3. Chromatography of crude trypsin; Matrices (1.5 ml) were packed into 5×0.7 cm columns and equilibrated with 30 m*M* Tris+10 m*M* $CaCl_2$ +0.5 *M* NaCl, pH 8.2. Crude trypsin (100 mg) was dissolved in 10 ml equilibration buffer, clarified by centrifugation and 1 ml loaded onto the column. The column was washed initially with equilibration buffer. Arrows mark buffer changes to 30 m*M* Tris+10 m*M* $CaCl_2$, pH 8.2 and 50 m*M* formic acid, respectively. Flow-rate was 0.5 ml/min. The ligand involved in chromatogram A is mercaptomethimidazole (89 μmol/ml) and B is aminobenzamidine (5 μmol/ml). The shaded areas denote the eluted trypsin.

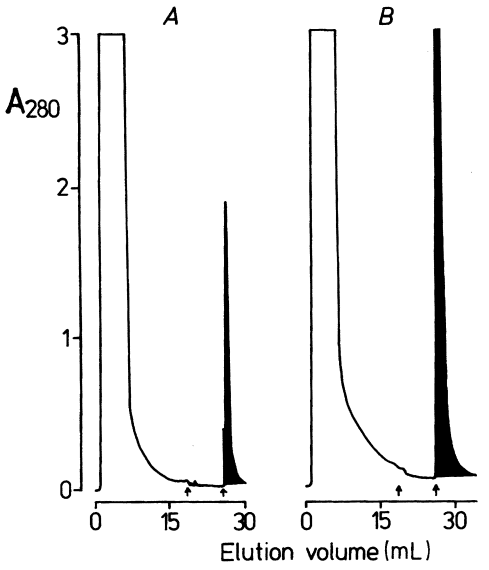

Fig. 4. Elution of chymosin from (A) mercaptomethylimidazole and (B) aminophenylpropanediol Perloza; Matrices (1.5 ml) were packed into 5×0.7 cm columns and equilibrated with 10 m*M* citrate+0.5 *M* NaCl, pH 6. Crude chymosin (3 ml) was adjusted to pH 6 and loaded and the column washed with equilibration buffer (15 ml). Arrows mark buffer changes to 10 m*M* citrate, pH 6 and 100 m*M* malonic acid, pH 2 respectively. Flow-rate was 0.5 ml/min. The ligand densities were 140 μmol/ml for A (mercaptomethylimidazole Perloza) and 0.157 mmol/g wet weight, ≈120 μmol/ml for B (aminophenylpropanediol Perloza). The shaded areas denote eluted chymosin.

zyme was adsorbed to the neutral form of pyridyl matrices (4-aminomethylpyridine or MEP Perloza) at pH 7.5 and eluted rapidly at pH 5.5. This indicated that the method could be operated over a relatively narrow pH window and demonstrated that very acidic conditions were not necessary for elution from pyridyl matrices. Because the matrix was uncharged at adsorption, the method could be carried out without crossing the isoelectric point. Greater flexibility of the operating pH range is thus possible with hydrophobic charge induction compared to mixed mode.

Table 4
Chymosin purification performance

Sample	Total protein (mg)	Activity (%)	Purification factor
Original	42	100	1
APP	5.4	73	5.7
MIM	4.2	85	8.5
2-AMP	2.3	74	13.5

Crude chymosin (3 ml, 14 mg total protein/ml) was applied to each matrix. The total protein was determined by the bicinchoninic acid method [22]. A standard curve for clotting activity was obtained using original chymosin and the activities of the eluted samples (3.5 ml) determined from this. APP=aminophenylpropanediol, 2-AMP=2-aminomethylpyridine and MIM=mercaptomethylimidazole.

Acknowledgements

The authors wish to thank Genencor International, Palo Alto, CA, USA for support and funding for this project and especially Landon Steele and Todd Becker.

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