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High-density ligand attachment to brominated allyl matrices and application to mixed mode chromatography of chymosin

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Abstract

Allylated cellulose, agarose and methacrylate matrices, activated with allyl bromide or allyl glycidyl ether, were modified by aqueous bromination, preferably with N-bromosuccinimide. Amine, thiol and sulphite ligands were attached efficiently and in high densities (1–1.5 mmol/g dry) to the brominated matrices, using concentrated reaction mixtures. Organic solvents were not required. Bromohydroxypropyl matrices and amine or carboxylate derivatives could thus be used to produce matrices for all major forms of adsorption chromatography. This chemistry was used to prepare high-density mercaptoalkyl acid matrices, which were successfully applied to mixed mode purification of crude chymosin and compared with matrices used previously. © 1997 Elsevier Science B.V.

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1. Introduction

In a previous report, efficient activation of polyhydroxylate matrices with allyl bromide and allyl glycidyl ether was described [1] and advantages over conventional epoxide chemistries [2,3] proposed. Allyl groups could be modified to more reactive forms by halogen addition to the double bond. The halogenated matrix could be substituted with nucleophiles such as amine ligands. Addition of bromine in an organic solvent has been used to prepare vicinal dibromide derivatives of allyl Sepharose [4,5]. Addition of aqueous halogen solutions (especially bromine water) was used to prepare halohydrin derivatives of cellulose, dextran and agarose [6,7]. At alkaline pH, halohydrin groups are converted to epoxides by intramolecular etherification [8]. It was therefore presumed that ligand substitution at pH≥9 would proceed similarly to that described for epoxide-activated matrices [2,9]. Oxidation of allyl groups might also be used to generate epoxides directly [10–12]. Bromine water has been used before to oxidise polysaccharide hydroxyl groups to aldehydes [13]. However, this reaction is unlikely to compete significantly with bromohydrin formation because the hydroxyl oxidation is slow, whereas reaction with alkene groups is rapid.

Bromine water has been preferred over other halogens for halohydrin formation because addition occurs readily and derivatives are reactive but reasonably stable [6,7]. However some dibromide will form as well as bromohydrin [14]. The latter is preferred because it is converted to a reactive epoxide at alkaline pH. Dibromide groups, especially

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the secondary bromide, are likely to react less efficiently than bromohydrins and lead to a higher level of residual bromine after ligand attachment [14]. Furthermore, a less noxious reagent than bromine would be preferred. Bromohydrin formation using N-bromosuccinimide (NBS) and N-bromoacetamide has been described for simple alkenes [15–17] and insignificant dibromide formation was expected. NBS was preferred over other halogenation reagents (e.g. N-chlorosuccinimide) on the basis of price and expected reactivity of the bromohydrin product.

Previously, substitution of brominated matrices with sodium sulphite was much less efficient if the activation reagent was allyl bromide rather than allyl glycidyl ether, at high activation levels [1]. The latter reagent produces matrices with a four-atom spacer arm between the cellulose backbone and the allyl group. It was presumed that inefficiency with allyl bromide matrices was due to steric effects. Allyl bromide is preferred over allyl glycidyl ether to minimise manufacturing costs. Also, it is much easier to obtain very high activation levels of Perloza with allyl bromide. Therefore, improvement of substitution methods to increase efficiency and consequently lower the residual bromine content in the final product was sought.

Perloza bead cellulose is a robust, inexpensive matrix with good chromatographic properties [18–21]. Activation limitations have been overcome by the use of allyl reagents [1]. Efficient manufacture of high-ligand-density Perloza derivatives is preferred for some hydrophobic and mixed mode chromatography applications [22–24].

In this report, modification of allyl matrices with bromine water and aqueous NBS are compared for bromination of allyl Perloza. Optimisation of reaction conditions for substitution of highly activated matrices with amine, thiol and sulphite ligands is also reported. The usefulness of these methods is tested by application of high-density mercaptoalkyl acid derivatives of Perloza to mixed mode chromatography of chymosin.

2. Experimental

Thiolacetic acid, 4-vinyl pyridine, mercaptoacetic acid, cysteamine HCl and 6-bromohexanoic acid were from Janssen Chimica, Geel, Belgium; diethylaminopropylamine and sodium bisulphide were from Aldrich-Chemie (Steinheim, Germany) or Aldrich Chemical Co. (Milwaukee, WI, USA); (1*s*,2*s*)-(+)-2-amino-1-phenyl-1,3-propanediol, iminodiacetic acid and butyrolactone were from Sigma Chemical Co. (St. Louis, MO, USA); Perloza was from ICS (Prague, Czech Republic); hydrogen peroxide (30% w/v H_2O), sodium borohydride, *n*-butylamine, ethanolamine, diethylamine, tetraethylammonium hydroxide, tetrabutylammonium iodide and N-bromosuccinimide were from BDH (Dorset, UK); bromine was from Hopkin and Williams (Essex, UK); thiourea and 45% trimethylamine were from Merck (Munich, Germany); ϵ-caprolactone was from Acros (Geel, Belgium); and sodium sulphite was from May and Baker (Manchester, UK). All other chemicals were analytical grade. Purified and crude, clarified chymosin were from Genencor International (Palo Alto, CA, USA).

4-Mercaptobutyric acid was prepared from butyrolactone and thiourea [25]; and 6-mercaptohexanoic acid prepared analogously from caprolactone and thiourea, or from 6-bromohexanoic acid and sodium bisulphide [26]. 4-Mercaptoethylpyridine HCl was prepared from 4-vinyl pyridine and thiolacetic acid by an adaptation of the synthesis of 2-mercaptoethylpyridine described by Vinton [27]. An optimised preparation gave 308 g (theoretical 318 g, yield 97%), m.p. 189–190°C, lit. 189°C [28]. Full details are provided elsewhere [29].

Allyl bromide (AB) and allyl glycidyl ether (AGE) matrices were prepared by the methods reported previously [1].

2.1. General reaction methods

Reactions at room temperature were mixed by rotation (Ballmill roller or Cole-Parmer Roto-Torque rotator) or shaking (Ika Vibra-mix). Reactions at elevated temperature were incubated in a water bath without mixing. Reactions were carried out in glass vials or jars. Allyl derivatives of Perloza 100 fine (80–100 μm beads) were used unless otherwise indicated. Activated matrices were identified by reagent type and volume used, expressed as ml/g suction-dried matrix or as a percentage. The units

used are 'g' for suction-dried and 'g dry' for oven-dried matrix weights. Titration data are expressed accordingly in 'mmol/g' or 'mmol/g dry'.

2.2. Halogenation

2.2.1. Bromine water

Bromination of unbuffered allyl Perloza with 2% bromine water was similar to the titration method described previously [1], except that a surplus (approximately 10%) was used to ensure completeness. Bicarbonate buffer (2 ml of 0.5 *M*, pH 6.5 per g matrix) was used for bromine water addition [7] in the experiment comparing this method with N-bromosuccinimide addition. After 2–5 min, reaction mixtures were transferred to a sintered funnel, washed with water and suction-dried.

2.2.2. N-Bromosuccinimide (NBS)

An initial reaction used 5 ml DMSO per g matrix [16]. Subsequently, only aqueous solvation was used. A 1.1–1.5 *M* excess of NBS over allyl groups and 1–10 ml of water or 0.1 *M* phosphoric acid/g Perloza was used. In one experiment, excess NBS was washed out with water after 60 min and the completeness of allyl group reaction determined by bromine water titration. Otherwise, reaction was for 30 min and excess NBS was consumed at the end of reaction by addition of KBr (plus phosphoric acid for mixtures not already acidified) to produce bromine and succinimide. A 1.2 *M* NBS excess, 30-min reaction, followed by dropwise addition of sufficient 1 *M* HBr to generate bromine colour, was the standard method for bromination of all allyl matrices. After bromination, matrices were again washed with water to remove reagents and suction-dried.

2.3. Ligand substitution reactions

2.3.1. Amine ligands

These were mixed with brominated matrices for 48–72 h at room temperature. Some diethylaminopropylamine samples were also reacted for 24 h at 60°C. Extra water (5 ml/g Perloza) was included in initial diethylaminopropylamine mixtures. Iminodiacetic acid was prepared as a 1.5 *M* solution by dissolution of 4 g in 7.5 *M* NaOH (approximately 7.5 ml) and water to a final pH of 11 and volume of 20 ml. Aminophenylpropanediol was prepared in the base form and used as described previously [22]. Other amines were used without modification. The molar excess of amine used was between 10 and 50 before optimisation and 5–10 afterwards.

2.3.2. Sodium sulphite

The standard mixture used was 200 mg sodium sulphite, 1 g brominated matrix and 5 ml water. Reaction was either at 60°C for 8 h, or room temperature for 24 h. Variations were the use of less water (1 ml), pH adjustment (with 1 ml of 1 *M* sodium carbonate), solvent inclusion (1 ml of dioxan or DMF), or addition of tetraethylammonium hydroxide (0.1 ml). Another variation was the inclusion of tetrabutylammonium iodide (0.1 g), either as a solid or dissolved in 2 ml of dioxan.

2.3.3. Thiol ligands

For initial reactions, mercaptoacetic acid (MAA) was adjusted to pH 7 or 10.5–11 with 2 *M* NaOH. Cysteamine and mercaptoethylpyridine hydrochlorides were dissolved in water (3 ml/g ligand) and the pH adjusted to 7.5, 9 or 10 with 2 *M* NaOH. A 5 molar excess of ligand (over original allyl groups) was used. Reaction mixtures also contained 3–5 ml of 1 *M* phosphate buffer (pH 7, 7.5 or 9) or 1 *M* carbonate (pH 10) and 2–3 mg sodium borohydride per g brominated matrix. Reactions were for 24 h at 60°C, except for samples at pH 10–11, which were reacted at room temperature. The volume of the above thiol reactions varied between 8 and 12 ml/g matrix.

Subsequently, reaction volumes were reduced to 2–3 ml/g. Reaction of mercaptoethylpyridine was optimised using a 3 *M* excess prepared at pH 10–11, mixed at 60°C for 5 h or room temperature for 24 h. Likewise, the NBS derivative of stock 8% AB Perloza (1 g) was reacted with solutions of MAA and cysteamine, adjusted to pH 10 with 10 *M* NaOH. Less water was used to dissolve MEP, MAA and cysteamine and buffer was reduced to 0.5–1 ml of 1 *M* carbonate, pH 11, per mmol of ligand. A similar procedure was used for reaction of the same stock-activated matrix (2 g) with 0.3 ml of mercaptohexanoic acid and 0.4 g of mercaptobutyric acid. Extra water was used to dissolve mercaptobutyric acid and

the initial reaction volume was 5 ml/g matrix. For a second reaction, the volume was reduced to 3–4 ml/g.

2.4. Oxidation of allyl Perloza

Stock 27% AGE Perloza (1 g) was mixed with 100 μl of H_2O_2, 5 ml water and 1 ml diethylaminopropylamine for 48 h at (i) 60°C and (ii) room temperature, washed and titrated for amine groups. A third sample of 27% AGE Perloza (1 g) was mixed with 100 μl of H_2O_2, 5 ml water and 1 ml of glacial acetic acid at room temperature for 48 h. The washed product was reacted for 24 h at room temperature with 1 ml diethylaminopropylamine and titrated as above.

2.5. Titration

A Radiometer ETS822 autotitrator was used to titrate 1 g matrix samples suspended in 5 ml of 1 *M* NaCl. Underivatised and allyl-activated matrices were reacted with MAA for 16 h at 60°C and the resulting carboxylate matrices washed, suction-dried, titrated to pH 8 and oven-dried by the methods described previously [1]. Mercaptoalkyl acid and sulphite-substituted matrices were washed, titrated and dried analogously. Iminodiacetate matrices were also washed with HCl and titrated with Convol 0.1 *M* NaOH to pH 7 and 11. A titration curve of iminodiacetic acid Perloza was obtained by incremental addition of titrant. Other amine matrices were washed with 0.1 *M* HCl, converted to the free base by washing with 0.1 *M* NaOH and titrated with Convol 0.1 *M* HCl to pH 4. Pyridyl matrices were treated likewise except they were titrated to pH 3 and corrected for a blank titration value. Diethylaminopropylamine titration results were halved to obtain molar values.

2.6. Elemental analysis

Analyses of samples, oven-dried for 1.5 h at 110°C, were carried out by the Chemistry Department, University of Otago, Dunedin, New Zealand.

2.7. Chymosin chromatography and analysis

The optimal conditions for chymosin adsorption to alkyl carboxylate matrices [22] were used for mercaptobutyric acid and mercaptohexanoic acid Perloza. Load buffer was 10 m*M* citrate+0.5 *M* NaCl, pH 4.4. The intermediate buffer was 10 m*M* citrate, pH 4.4, and elution was with 20 m*M* citrate, pH 6.2. For aminophenylpropanediol Perloza, load buffer was 10 m*M* citrate+0.5 *M* NaCl, pH 5.5. The intermediate buffer was 10 m*M* citrate, pH 5.5, and elution was with 50 m*M* HCl/KCl, pH 2 [22].

Matrix samples (1.3–1.5 ml) were packed in Pierce columns (0.7 cm I.D.) and equilibrated with load buffer. Crude chymosin was adjusted to the appropriate adsorption pH with 2 *M* citric acid and 4 ml loaded by gravity at approximately 0.5 ml/min. The column was then washed with load buffer followed by low ionic strength intermediate wash, at 0.8 ml/min. A 5-ml flowthrough fraction of peak absorbance was collected for electrophoresis and activity analyses. The next 15 ml was also tested for any loss of chymosin activity. Matrices were eluted at 0.5 ml/min. Elution peaks (5 ml) were collected for protein and activity assays. Samples of peak elution absorbance (6–8 ml) were collected on separate runs (using 5 ml crude chymosin) for electrophoresis. Chromatography runs were monitored with a Pharmacia UV-1 monitor and chart recorder or a Biorad Econosystem.

Total protein of original and elution samples was assayed by the bicinchoninic acid method [30]. Chymosin activity of 0.2-ml samples was assayed by clotting time with skim milk (4 ml) at 37°C [22]. A Pharmacia Phast system and 20% homogeneous gels were used for SDS PAGE (separation file 111). Silver staining was used (development file 210) but formaldehyde content of the fixer was reduced from 0.04 to 0.033%. Original chymosin broth was diluted 1.5× with the standard Phast buffer. Elution samples were used without dilution. Molecular mass markers were from Sigma.

For chromatography of purified, lyophilised chymosin, a standard 1 mg/ml solution was prepared by dissolution in load buffer. Mercaptobutyric acid Perloza samples (1.8 ml) were packed in Pierce columns (0.8 cm I.D.). Chymosin (0.4 ml) was applied to equilibrated columns which were then

washed for 45 min with 10 m*M* citrate+0.5 *M* NaCl, pH 4.4. This was followed by a 20-min wash with 40 m*M* citrate, pH 6.2, and an 8-min wash with 0.1 *M* NaOH. A flow-rate of 1 ml/min was maintained. Full scale absorbance was 0.09. Adsorption and elution samples were collected for activity analysis.

3. Results and discussion

3.1. Bromination

Initially reaction with bromine water was used both to titrate activated matrices and to prepare reactive bromohydrin intermediates for substitution of amine, thiol and sulphite nucleophiles. Bromination was rapid and excess bromine readily washed out. However, it was thought that dibromide formation could limit the efficiency of subsequent substitution reactions. Also, bromine water could not be prepared and stored in bulk because it decolourised slowly, even in the dark.

Reaction of allyl Perloza with a 1.2–1.5 *M* excess of N-bromosuccinimide (NBS), for 0.5–1 h, appeared to result in complete reaction of allyl groups (negative alkene test with bromine water), whether water or a mixture of DMSO and water was used for solvation. Unless Br^+ was reduced, there should be no Br^- available and dibromide formation should therefore be minimised or eliminated [31]. Direct proof was not obtained but small differences in the subsequent ligand substitution levels were found (Table 1). Nevertheless, the results obtained by the two methods did not differ greatly and other factors influenced the selection between them.

Aqueous bromination with NBS was adopted as the method of choice. In addition to the proposed reaction specificity advantages, bromine dissolution was not required and noxious fumes were avoided. Buffering was not required, whereas bromine water addition is an acid-producing reaction and the importance of pH control has been reported [7]. Indeed, acids which do not interfere with bromination could promote the action of NBS [32], although the matrices produced at acidic and neutral pH were indistinguishable by titration of their sulphite derivatives [29]. No indication of bromine formation occurred in either reaction but a bright orange colour appeared upon addition of bromide ions, at acidic pH. This indicated both that NBS had not been exhausted and that bromide ion formation during the reaction, if any, was insignificant. Addition of HBr allows a visual check of completeness of reaction and wash-out of excess NBS. NBS is inexpensive and because the molar excess used is low, its contribution to overall cost is minor, even at high activation levels.

Table 1
Ligand substitution levels of NBS and bromine water-modified allyl Perloza

Ligand/matrix	Titration (mmol/g dry)	
	(NBS)	(Br_2)
Ethanolamine/30% AGE	0.83	0.79
Diethylaminopropylamine/27% AGE stock	0.67	0.62
Diethylaminopropylamine/7% AB stock	0.69	0.66
Diethylaminopropylamine/10% AB stock	0.88	0.79
Mercaptoacetate/27% AGE stock	0.94	0.99
Mercaptoacetate/7% AB stock	0.94	1.00
Mercaptoacetate/10% AB stock	0.87	1.06

The 30% AGE Perloza sample used for ethanolamine had an MAA titration of 0.177 mmol/g. MAA titration values for stock allyl Perloza matrices are given in Table 2. Elemental analysis of the ethanolamine samples gave lower results (0.6 and 0.55 mmol/g dry) but the difference was maintained. Mercaptoacetate (sodium) was reacted at pH 10.5.

3.2. Initial substitution reactions of brominated allyl Perloza

The sulphite substitution efficiency of brominated AGE matrices was apparently greater than 95% [1]. However some residual bromine was found by elemental analysis, especially for highly activated samples. The sulphonate derivative of a repeat 30% activated matrix (0.315 mmol/g bromine water titration) had a titration of 0.272 mmol/g. A small amount of unreacted bromine (0.19 mmol/g dry) was found by elemental analysis but the substitution efficiency (86%) was still high.

The first AB matrices tested were very highly activated (bromine water titration) but the ligand substitution level following bromination was low. For example, an AB matrix (0.46 mmol/g) produced a sulphonate matrix of 0.22 mmol/g (48% efficiency). This suggested that either the bromine water titration was misleading or that the reactivity

of AB derivatives was poorer. Mercaptopropionic acid titration later confirmed the bromine water values. High elemental bromine levels (1.6 mmol/g dry) were also consistent with inefficient substitution.

This poor reactivity could be due to steric factors, exacerbated by the shorter spacer arm, or different proportions of bromohydrin and dibromide formation. Substitution of sodium sulphite will result in formation of a negatively charged matrix surface. At high densities, this will repel sulphite ions, limiting their access to remaining activated groups. By contrast, primary amine ligands will react in the uncharged (unprotonated) form and, if the pH is maintained above 11, the secondary amine linkages formed will also be neutral. Charge shielding effects should be most significant for AB-activated matrices because distances between groups and flexibility will be lower due to the shorter spacer arm. The effects might be reduced by increasing the ionic strength of the reaction medium, and especially by use of concentrated ligand solutions. Surfactants and organic solvents might also facilitate access of ligands to the matrix surface.

3.3. Amine substitution

Low substitution efficiencies were found for both NBS and bromine water modified allyl Perloza (1 g), reacted with diethylaminopropylamine (1 ml) and 5–6 ml of water (Table 1). Efficiency was improved (to 70% for an 8% AB Perloza sample) by omission of water but reaction at higher temperature (60°C) had little effect. Because of the volatility of most of the amine ligands used, further reactions were confined to room temperature. High substitution efficiencies were obtained with stock allyl matrices (Table 2), using *n*-butylamine, diethylamine and trimethylamine at the same ratio of amine to cellulose (1 ml:1 g) but without added water (Table 3). Substitution of brominated AGE Perloza was only slightly more efficient than for AB derivatives. Substitution levels were consistently 80–90% of MAA titration values (Table 2) and there was no indication of a limit of substitution for AB-activated matrices. Subsequent reactions using much less reagent (10 *M* excess diethylamine or trimethylamine) were equally effective.

Table 2
Stock allyl Perloza activation levels

Matrix	Titration	
	(mmol/g)	(mmol/g dry)
AGE 27%	0.153	1.17
AB 7%	0.180	1.36
AB 8%	0.202	1.51
AB 10%	0.252	1.67

Matrices were reacted with MAA and the product titrated. The values were not corrected for the control titration (0.005 mmol/g).

High substitution levels were also found with disodium iminodiacetate solutions (5 *M* excess). Charge repulsion effects due to carboxylate groups could occur, but the use of concentrated (1.5 *M*) iminodiacetic acid solutions might have counteracted this effect. The level of iminodiacetic acid substitution was determined by titration with NaOH to pH values of 7 and 11. The titration curve of an iminodiacetic acid matrix (Fig. 1) demonstrated that only one carboxyl group was titrated (up to pH 7) and that an end point of pH 10 was adequate for imine titration.

The products of these amine substitutions can be used for immobilised metal ion affinity [33] and ion-exchange chromatographies and exemplify generic amine substitution.

3.4. Improved substitution of AB activated matrices by sodium sulphite

Various reagents and conditions were used to try

Table 3
Substitution levels of various amine ligands on brominated allyl Perloza

Chemistry (%)	Ligand	Titration	
		mmol/g	mmol/g dry
AB 10%	butylamine	0.192	1.43
AB 7%	butylamine	0.146	1.17
AGE 27%	butylamine	0.136	1.07
AB 10%	trimethylamine	0.212	1.42
AB 7%	trimethylamine	0.147	1.17
AB 7%	diethylamine	0.159	1.15
AB 10%	iminodiacetate	0.187	1.15
AB 7%	iminodiacetate	0.139	1.04
AGE 27%	iminodiacetate	0.145	1.01

Stock allyl Perloza matrices were brominated by the NBS/water method. Substitution reactions were at room temperature for 48 h.

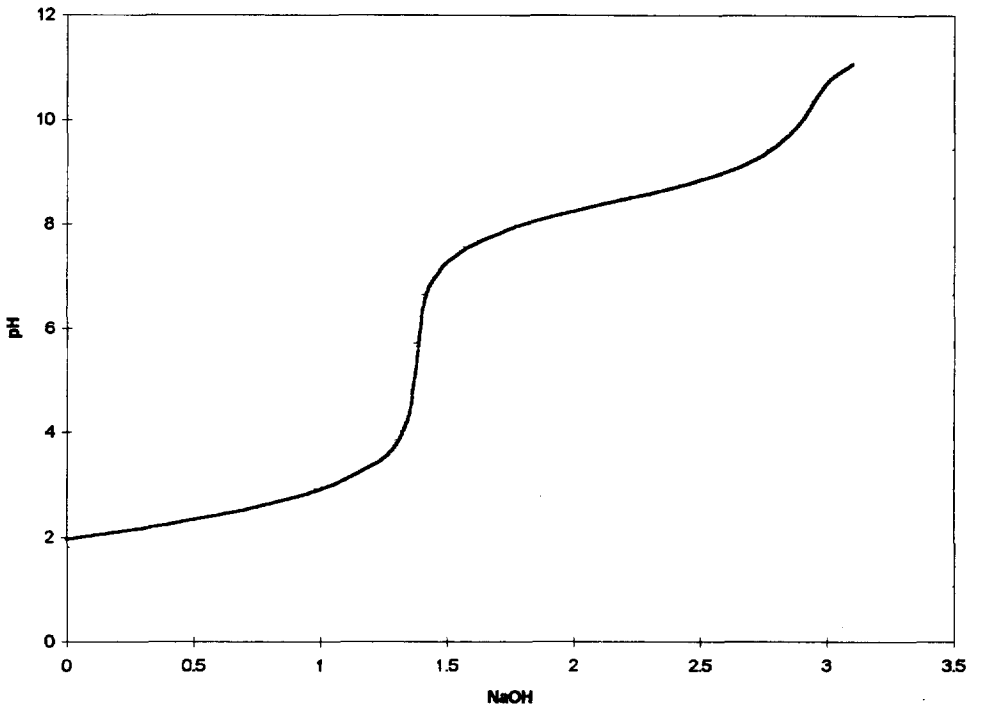

Fig. 1. Titration curve of iminodiacetic acid (AGE, NBS) Perloza. The protonated form of iminodiacetic acid Perloza (1 g) was mixed with 5 ml of 1 *M* NaCl. The suspension was titrated incrementally with 0.1 *M* NaOH.

to overcome charge shielding effects, by analogy to the methods of Paul and Ranby [34], without success (Table 4). The use of organic solvents did not improve substitution efficiency. Inclusion of dioxan had no effect on the room temperature reaction and DMF resulted in a lower level. The room temperature reaction was apparently enhanced by inclusion of a quaternary ammonium salt, tetrabutylammonium iodide, in the reaction mixture. However this difference may have been due to variation of reaction volume. Addition of dioxan-solvated tetrabutylammonium iodide (0.5 ml of a saturated solution) or NaCl to the 60°C reaction resulted in lower substitution levels. The use of tetraethylammonium hydroxide also lowered the substitution level. The most significant variables were concentration and temperature. The highest substitution level, 80% of MAA titration value (Table 4), was obtained by reaction at 60°C using 1 ml of 20% sodium sulphite per g matrix. This level might still be increased because the reaction volume could be lowered further and sulphite content raised. Propyl sulphonate (SP) de-

Table 4
Sodium sulphite substitution levels on brominated AB Perloza

Method	Titration	
	mmol/g	mmol/g dry
Room temperature		
Standard mixture	0.163	1.18
+ dioxan	0.164	n.d.
+ DMF	0.149	1.08
+ TBI	0.177	1.29
+ tetraethylammonium hydroxide	0.149	n.d.
Concentrated mixture	0.175	n.d.
60°C		
Standard mixture	0.180	1.27
+ carbonate buffer	0.184	1.39
Concentrated mixture	0.202	1.49
+ TBI, dioxan	0.178	1.34
+ NaCl	0.185	1.25

All reactions were with stock 10% AB Perloza. Bromination was with aqueous NBS. Reaction conditions are detailed in Section 2. Solvation was aqueous unless otherwise indicated. DMF, dimethylformamide; TBI, tetrabutylammonium iodide.

rivatives can be used for cation-exchange chromatography.

3.5. Thiol substitution

Initial substitutions of brominated allyl Perloza with the sodium salt of MAA (Table 1) at pH 10.5 were much less efficient than MAA addition (Table 2). The reaction mixtures were relatively dilute and carboxylate group charge shielding may have contributed to the lower efficiency. Inefficient reaction was also found at lower pH (Table 5). Substitution with cysteamine at pH 10, was also higher than pH 7. Charge shielding by amine hydrochloride groups may affect the result at the lower pH. The amine group could compete with the thiol for bromohydrin groups at pH 10–11 but this competition is not expected to be significant due to the far greater reactivity of thiols [35].

Reaction volumes were minimised to try to repeat the efficiency gains found with sulphite substitution. The efficiency of MAA and cysteamine substitution increased to 90–100%. Similar efficiency was found using mercaptohexanoic acid but a larger reaction volume was used for mercaptobutyric acid and its substitution efficiency was lower. However, mercaptoacid substitution at high activation levels (Table 5) could approach but not match the efficiency of MAA addition (Table 2). At high activation levels, the swollen volume of MAA (or sulphite)-substituted Perloza is lower and more stable to changes in ionic strength than the MAA addition form. This suggests that the lower efficiency is in fact desirable and that the sites which are not substituted have an important role in Perloza stability, which is compromised by their reaction with an ionised ligand [29].

Substitution efficiency of a brominated AB matrix (MPA titration, 0.175 mmol/g) with mercaptoethylpyridine at pH 7.5 (60°C) was also inefficient (57%). Approximately 20% of the titration occurred between pH 10.5 and 8, above the range expected for the pyridyl matrix [23]. This indicated that a substantial side reaction between activated groups and pyridine had occurred, producing (quaternary) pyridinium groups rather than thioether-linked pyridine [29]. Inefficient substitution (49%) was also found with 30% AGE Perloza (MPA titration, 0.198 mmol/g).

Improved efficiencies were obtained by reaction at pH 9, 60°C (0.168 mmol/g) and pH 10, room temperature (0.164 mmol/g). The use of a lower (3 *M*) excess of mercaptoethylpyridine, at pH 10, did not greatly effect the outcome (0.163 mmol/g). All the titrations started below pH 8, suggesting that the side reaction was no longer significant. Mercaptoethylpyridine substitution levels at pH 10.5 for stock AB and AGE matrices (Table 5) ranged between 90 and 100% of the original allyl densities (Table 2), again indicating efficient reaction. Any effect of reaction volume on substitution efficiency

Table 5
Substitution levels for thiol ligands on brominated allyl Perloza

Matrix	Ligand	Titration (mmol/g)	Substitution (mmol/g dry)
AGE, pH 7.0	mercaptoacetate	0.119	n.d.
AGE, pH 8.5	mercaptoacetate	0.137	n.d.
AB 8%*	mercaptoacetate	0.183	1.47
AB 8%*	mercaptohexanoic acid	0.178	1.14
AB 8%	mercaptobutyric acid	0.144	1.08
AGE, pH 7	cysteamine	0.120	n.d.
AGE, pH 10	cysteamine	0.141	n.d.
AB 8%	cysteamine	0.153	n.d.
AB 8%*	cysteamine	0.207	n.d.
AB 10%	mercaptoethylpyridine	0.209	1.49
AB 7%	mercaptoethylpyridine	0.162	1.20
AGE	mercaptoethylpyridine	0.166	1.11

The reaction pH was 10.5–11 unless otherwise stated. Minimal reaction volumes were used for matrices marked *. Stock Perloza matrices, brominated with aqueous NBS were used. Substitution reactions at pH 10 were at room temperature, except for those with mercaptoethylpyridine. These latter samples and all those at lower pH were reacted at 60°C.

was apparently minor. The pyridine group is unprotonated at alkaline pH and hence charge shielding is avoided. The improved substitution efficiency and insignificant side reaction at high pH compared to neutral was ascribed to far greater reactivity of the mercaptide ion compared to the mercaptan or pyridine groups.

Mercaptoethylpyridine products can be used for hydrophobic charge induction chromatography [23,24]. MAA and cysteamine products could be used for ion-exchange chromatography or for attachment of ligands containing amine or carboxyl groups to a spacer arm by condensation reactions. The preparation of an aminopropyl cellulose matrix for solid-phase peptide synthesis [36] included a 3-h (reflux) anhydrous reduction with diborane. Cysteamine (allyl/NBS) Perloza could be used instead and is simpler, safer and cheaper to prepare. Another amino matrix was prepared by ammonia treatment of epichlorohydrin-activated Sepharose [37] but this leads to crosslinking side reactions. Allyl cysteamine matrices contain a minimum 7-atom spacer arm and could also be used for the protein immobilisation and immunoaffinity chromatography [38]. For applications which require low-ligand-density matrices, efficient substitution should be simple.

3.6. Reactions with other matrices

Aqueous NBS bromination and substitution of other allyl matrices appeared to be similarly effective (Table 6). Mercaptoethylpyridine substitution of synthetic matrices (Fractogel and Sepabeads) resulted in titration values of 90–100% of the allyl values. Attachment of iminodiacetic acid was also efficient, except for Fractogel (60%). The Fractogel activation level was particularly high and, even at this lower efficiency, the ligand density obtained was still very high. These results indicated that, although the chemistry was especially useful for modification of Perloza bead cellulose, it could also be successfully applied to other supports. This might be done to achieve high ligand densities, increase efficiency, minimise side reactions and allow use of an activated support which has excellent storage properties in aqueous media [1].

3.7. Oxidation of matrix allyl groups

Attempts to oxidise matrix allyl groups to epoxides, using alkaline or acidic H_2O_2 solutions, followed by amine substitution, were apparently unsuccessful, despite using an excess (about 5 *M*) of peroxide. The amine titrations were 0.003–0.004 mmol/g, compared to the MAA titration of 0.177 mmol/g. These were consistent with a low level of ionised groups on the original matrix (a control titration of Perloza was 0.005 mmol/g) and indicated that no significant reaction had occurred. Although efficient epoxidation of allyl cellulose (in anhydrous solvents) has been reported [12], the absence of reaction in aqueous media suggested this would be an uneconomic method for activation of the double bond and it was not pursued further.

3.8. Accuracy of ligand density values

Elemental analyses of selected AGE sulphonate samples, 0.91 and 0.98 mmol sulphur/g dry, were lower than their respective titration values (1.08 and 1.16 mmol/g dry). Retitration with fresh Convol NaOH gave unchanged results. The contribution of charged groups on unmodified Perloza was not high

Table 6
Ligand substitution data for other allyl matrices

Matrix	Titration (mmol/g)		
	Allyl groups	Iminodiacetic acid	Mercaptoethylpyridine
Sepharose CL 6B	0.124	0.119	0.110
Sepabeads	0.125	0.125	0.123
Fractogel	0.400	0.239	0.357

Activation (7% AB) and bromination were by the methods optimised for Perloza. Allyl groups were titrated by MAA addition. Titration values were corrected for blank samples of Sepharose (0.003 mmol/g), Fractogel (0.006 mmol/g) and Sepabeads (0.06 mmol/g).

enough to explain this level of discrepancy. Other elemental analyses, especially of amine and sulphite substitution derivatives, were also (10–20%) lower than expected and variations of up to 0.1 mmol/g dry were found between duplicate analyses. Similar discrepancies between elemental analysis values and titration data were also reported for aminopropyl Perloza matrices [39]. Potential sources of variation are discussed elsewhere [29]. Nevertheless, the elemental analyses did confirm that very high ligand densities were obtained using allyl chemistry.

3.9. Mixed-mode chromatography of chymosin

Previously [22], mixed-mode matrices for adsorption of chymosin were prepared by anhydrous carbonyldiimidazole (CDI) or epichlorohydrin (ECH) chemistry. Perloza CDI derivatives had high capacities (50–80 mg/ml) but the chymosin capacity of a Perloza ECH aminophenylpropanediol matrix (0.043 mmol/g) was only 6 mg/ml at high ionic strength [22]. In contrast, a capacity of 40 mg/ml was found for aminophenylpropanediol Perloza (0.157 mmol/g) prepared by AGE substitution chemistry. The allyl chemistry allowed much higher ligand densities on Perloza, hence greatly increasing capacity. However the fouling of the highly substituted AGE matrix was more severe. Furthermore, the chymosin purity appeared worse than that obtained with the mixed-mode carboxylate Perloza matrices described below (Fig. 2).

Mixed-mode carboxyl matrices were obtained from reaction of (NBS) AB Perloza with mercaptobutyric acid and mercaptohexanoic acid. Chromatograms obtained with mercaptobutyric acid Perloza matrices demonstrated the requirement of high ligand density for chymosin adsorption in the presence of 0.5 *M* NaCl (Fig. 3). At low ligand density (Fig. 3I), 65 μmol/ml, chymosin passed rapidly through the column. At a higher ligand density (Fig. 3II), 83 μmol/ml, the retention time was increased and the flowthrough peak much broader, but chymosin was still not adsorbed. At a much higher density (Fig. 3III), 150 μmol/ml, no chymosin activity was found in the flowthrough, analogous to earlier results with aminocaproic acid and aminovaleric acid derivatives of CDI Perloza [22]. High-density mercaptobutyric acid and mercaptohexanoic acid matrices have been washed with in excess of 50 column volumes of load buffer without detecting any loss of activity in the effluent.

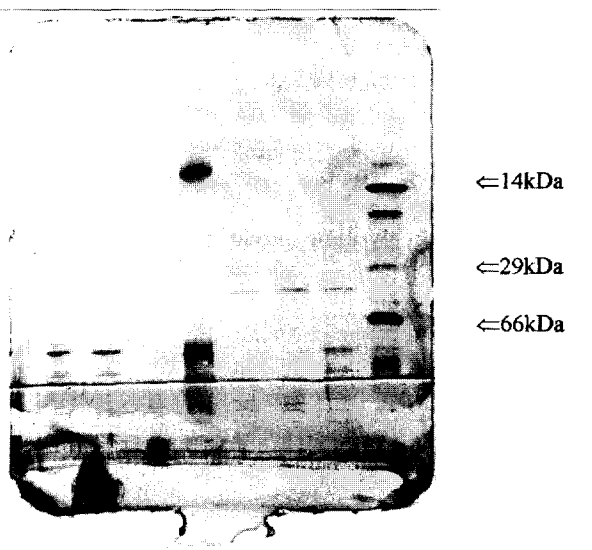

Fig. 2. 20% SDS-PAGE electrophoresis of chymosin samples. Lane 1, original chymosin; Lane 2, flowthrough fraction from mercaptobutyric acid Perloza; Lane 3, elution fraction from mercaptobutyric acid Perloza; Lane 4, flowthrough fraction from mercaptohexanoic acid Perloza; Lane 5, elution fraction from mercaptohexanoic acid Perloza; Lane 6, elution fraction from 2-aminomethylpyridine Perloza; Lane 7, elution fraction from aminophenylpropanediol Perloza; Lane 8, M_r markers (α-lactalbumin (14 000), carbonic anhydrase (29 000) and BSA (66 000)).

Chymosin was eluted from the high-density carboxylate matrices in a sharp peak, by a pH change, to induce electrostatic repulsion [22]. The resulting chymosin purity was very good. It was apparently homogeneous by SDS-PAGE (Fig. 2) and its specific activity similar to or better than that of chymosin recovered from aminocaproic acid Perloza (Table 7). This single-step recovery of high-purity chymosin demonstrated that mercaptan ligands and allyl/NBS substitution chemistry were viable alternatives to the high-ligand-density CDI-based matrices [22]. The aqueous chemistry and strong thioether linkage would allow more economic manufacture and better tolerance of regeneration and cleaning treatments. The linkage was presumed to be more hydrophobic,

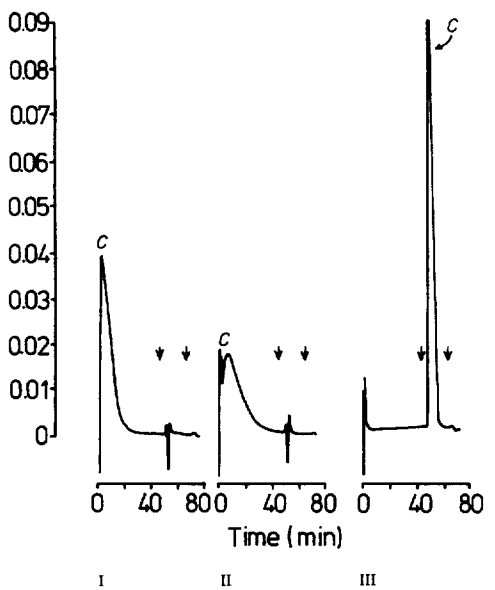

Fig. 3. Chymosin chromatography on MBA Perloza. MBA Perloza samples (1.8 ml) were packed in 0.8 cm I.D. columns and equilibrated with load buffer (10 m*M* citrate+0.5 *M* NaCl, pH 4.4). Matrices used had ligand densities of 65 (I), 83 (II) and 150 μmol/ml (III). Purified chymosin, 0.4 ml of a 1 mg/ml solution in load buffer was applied to each. Columns were washed at 1 ml/min with load buffer (45 min), 40 m*M* citrate, pH 6.2 (20 min) and 0.1 *M* NaOH (8 min). The pH change to 6.2 and the start of the NaOH wash are marked with arrows. Peaks of chymosin activity are marked C. The ordinate shows the absorbance at 280 nm.

because mercaptobutyric acid derivatives adsorbed chymosin at high ionic strength, whereas aminobutyric acid CDI Perloza did not [22]. This is consistent with the effect of the thioether linkage in hydrophobic affinity chromatography reported for thioalkyl agaroses [40].

Perloza substituted with mercaptobutyric acid, was preferred for chymosin adsorption because it has high capacity, rapid elution at a favourable pH and good regeneration properties (low fouling). This matrix was also easy to prepare, although mercaptobutyric acid is not yet commercially available.

4. Conclusions

Synthesis of high-ligand-density matrices based on Perloza bead cellulose previously required the use of anhydrous CDI chemistry. Initially, allyl chemistry did not match CDI because substitution efficiency was poor. Optimised methods have been developed which have overcome this deficiency and provided a viable alternative means to obtain high ligand density. Application of such high-ligand-density matrices to chymosin purification has been demonstrated. Significantly, this chemistry is aqueous based and ligand attachment is by strong ether, thioether and/or amine bonds. The allyl matrices can be stored for long periods in aqueous media without significant change in activation level. The use of aqueous NBS to prepare the brominated intermediate is a simple, inexpensive technique which avoids the hazards of bromine storage and use. Furthermore, it has been demonstrated that this chemistry can be used for production of a wide range of matrices, of low- or high ligand density, analogous to the use of epoxide-activated matrices. The allyl chemistry is more efficient and precise than epoxide chemistry, because it is less affected by hydrolysis and crosslinking side reactions. It therefore appears well-suited to the industrial preparation of chromatographic matrices as well as laboratory applications.

Table 7
Chymosin purification performance of mixed-mode carboxylate matrices

Sample	Total protein (mg)	Activity (%)	Purification
Original (pH 4.4)	33	100	1
Aminocaproic acid Perloza	2.4	70–80	10.3
Mercaptobutyric acid Perloza	2.0	70–80	12.4
Mercaptohexanoic acid Perloza	2.3	70–80	10.8

Crude chymosin (4 ml, 8.25 mg total protein/ml) was applied to each matrix. The total protein was determined by the bicinchoninic acid method [30]. The activities of the eluted samples (5 ml) were indistinguishable from each other. Because the milk clotting assay used was crude, a range of activity has been quoted and the median value used to calculate the purification level (increase in specific activity) compared to the original crude chymosin.

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References

[1] S.C. Burton, D.R.K. Harding, J. Chromatogr. A 775 (1997) 29.
[2] R. Axen, H. Drevin, J. Carlsson, Acta Chem. Scand. B 29 (1975) 471.
[3] L. Sundberg, J. Porath, J. Chromatogr. 90 (1974) 87.
[4] J.O. Porath, J.G. Rosengren, US 3,960,720 (1976).
[5] R. Axen, J.O. Porath, P.J.E. Carlsson, US 4,048,416 (1977).
[6] C. Sawato, T. Yagi, JP 04 76,001 (1992).
[7] G.E.S. Lindgren, WO application 94/04192 (1994).
[8] J.B. McKelvey, B.G. Webre, E. Klein, Textile Res. J. 29 (1959) 918.
[9] B.A. Klyaschitskii, P.V. Kuznetsov, Russ. Chem. Rev. 53 (1984) 1010.
[10] G.B. Payne, J. Am. Chem. Soc. 81 (1959) 4901.
[11] M. Imuta, H. Ziffer, J. Org. Chem. 44 (1979) 1351.
[12] M.S. Lin, C.S. Huang, J. Polym. Sci., Part A: Polym. Chem. 30 (1992) 2303.
[13] O. Larm, E. Scholander, Carbohydr. Res. 58 (1977) 249.
[14] J. McMurray, Organic Chemistry, 2nd ed., Brooks/Cole Publishing Company, Pacific Grove California, 1988.
[15] C.O. Guss, R. Rosenthal, J. Am. Chem. Soc. 77 (1955) 2549.
[16] D.R. Dalton, V.P. Dutta, D.C. Jones, J. Am. Chem. Soc. 90 (1968) 5498.
[17] E. Schmidt, W.V. Knilling, A. Ascherl, Chem. Abstr. 20 (1926) 2979.
[18] J. Peska, J. Stamberg, J. Hradil, M. Ilavsky, J. Chromatogr. 125 (1976) 455.
[19] J. Stamberg, J. Peska, H. Dautzenburg, B. Philipp, in: T.C.J. Gribnau, R.J.F. Nivard (Eds.), Affinity Chromatography and Related Techniques, Elsevier Scientific, Amsterdam, 1982, p. 131.
[20] J. Stamberg, Sep. Purif. Methods 17 (1988) 155.
[21] P. Gemeiner, M.J. Benes, J. Stamberg, Chem. Papers 43 (1989) 805.
[22] S.C. Burton, D.R.K. Harding, Biotechnol. Bioeng., in press.
[23] S. Burton, D.R.K. Harding, presented at the Recovery of Biological Products VII, San Diego, September 25–30 1994, poster A3.
[24] L.M. Steele, S.C. Burton, D.R.K. Harding, B. Bulthuis, N.T. Becker, presented at the Recovery of Biological Products VII, San Diego, September 25–30 1994, poster C11.
[25] N. Kharasch, R.B. Langford, J. Org. Chem. 28 (1963) 1901.
[26] G. Ivanovics, L. Vargha, Chem. Abstr. 41 (1947) 3757.
[27] W.H. Vinton, US 2,607,776 (1952).
[28] L. Bauer, L.A. Gardella Jr., J. Org. Chem. 26 (1961) 82.
[29] S.C. Burton, Ph.D. Thesis, Massey University, Palmerston North, New Zealand, 1996.
[30] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem 150 (1985) 76.
[31] D.R. Dalton, V.P. Dutta, J. Chem. Soc. (B), (1971) 85.
[32] V.L. Heasley, K.E. Wade, T.G. Aucoin, D.E. Gipe, D.F. Shellhamer, G.E. Heasley, J. Org. Chem. 48 (1983) 1377.
[33] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[34] S. Paul, B. Ranby, Macromolecules 9 (1976) 337.
[35] M. Friedman, J.F. Cavins, J.S. Wall, J. Am. Chem. Soc. 87 (1965) 3672.
[36] D.R. Englebretsen, D.R.K. Harding, Int. J. Peptide Protein Res. 40 (1992) 487.
[37] I. Matsumoto, N. Seno, A.M. Golovtchenko-Matsumoto, T. Osawa, J. Biochem. 87 (1980) 535.
[38] D. Stubbings, M.O. Bubb, J.D. Conradie, Anal. Biochem. 210 (1993) 159.
[39] D.R. Englebretsen, Ph.D. Thesis, Massey University, Palmerston North, New Zealand, 1993.
[40] A. Demiroglou, H.P. Jennissen, J. Chromatogr. 521 (1990) 1.