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Shielding of protein–boronate interactions during boronate chromatography of neoglycoproteins

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Abstract

A method for separating glycoproteins on a boronate column under conditions which suppress the interactions between the protein moiety and the boronic acid ligand has been developed. A model system consisting of non-glycosylated chymotrypsin and maltose-modified chymotrypsin (cht-mal) was utilised in the investigations. Chymotrypsin was chosen as the model protein because of its known interaction with boronate. By coupling maltose to chymotrypsin, a neoglycoprotein was created which has the property of binding to the affinity matrix both via the protein moiety and via the carbohydrate residues. The introduction of a so-called shielding reagent into the buffer solutions during chromatography resulted in the prevention of the protein–boronate interactions while the carbohydrate–boronate interaction was little influenced. Different types of, mainly low-molecular-mass, polyhydroxyl chemicals were screened in order to correlate the shielding efficiency to the chemical structure of the investigated compounds. Polyhydroxyl chemicals with a conformation that allows the formation of tridentate complexes with the boronate anion provided the highest shielding efficiencies.

Keywords: Boronate columns; Molecular shielding; Protein–boronate interaction; Chymotrypsin; Proteins; Glycoproteins

1. Introduction

The ability of boronate to form complexes with hydroxyl groups in carbohydrates is exploited in boronate affinity chromatography [1]. The interaction is not only specific for carbohydrates, as any compound containing hydoxyl groups in a suitable geometry will form a moderately stable complex with the boronate. As a consequence, boronate chromatography can, for example, be used for the separation of glycoproteins, nucleosides and catechol compounds [2–4]. A variety of other functional groups, such as α-hydroxycarboxylic acids, aromatic α-hydroxy acids and amides can also interact with boronates. These functional groups can be found in compounds such as lactic acid, salicylic acid, salicylamide and steroids [5,6].

There are also examples in the literature of

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interactions between boronates and non-glycosylated proteins, such as β-lactamases [7], subtilisin BPN' [8], trypsin [9], α-chymotrypsin (α-cht) [10], pepsin [11] and β-amylase [12]. It has been postulated that the complex formed between the boronate and the enzyme mimics the transition state complex. Enzyme inhibition studies have been used to investigate this hypothesis [8,9,13]. Others are however of the opinion that secondary interactions are responsible for the complex formation [12,14].

Although boronate chromatography was introduced in the 1970s, the number of successful applications in the purification of glycoproteins is still limited. One explanation of this may be the formation of protein–boronate complexes which reduce the capacity and purification efficiency of the method. Boronate chromatography would be a much more powerful tool in glycoprotein purification if it were possible to facilitate the process of interacting of boronate ligands with the carbohydrate moieties of glycoproteins by selectively preventing the interactions with protein moieties.

A technique for suppressing non-specific interactions in dye affinity chromatography has been developed [15,16]. This concept is termed molecular shielding. A polymeric substance, called the shielding reagent, is adsorbed onto the affinity matrix via multi-point attachment to the immobilised ligands. This binding is relatively weak compared to the specific interaction between the ligand and the target biomolecule. The shielding reagent therefore specifically protects the ligand from weak, non-specific interactions. The strong specific interactions are not affected and can take place even in the presence of the shielding reagent. The result is a significant improvement in the chromatographic efficiency.

The objective of the present study was to evaluate the possibility of applying the molecular shielding concept to boronate chromatography, and to explore if the concept could be extended by replacing the polymeric shielding reagent adsorbed onto the stationary phase with a low-molecular-mass shielding reagent in the mobile phase. A well-defined model system, consisting of cht and maltose-modified chymotrypsin (cht-mal), was chosen for the experiments. In this way, any discrepancy caused by heterogeneous samples was avoided.

2. Materials and methods

2.1. Materials

Anthrone, D-arabinose, D-mannitol, 1-*O*-methyl-α-D-glucopyranoside, 1-*O*-methyl-α-D-mannopyranoside, D-sorbitol, tris(hydroxymethyl)aminomethane (Tris), *m*-aminophenyl boronic acid agarose (APBA, product No. A-8312, 40–80 μmol APBA per ml packed gel) and α-chymotrypsin (E.C.3.4.21.1, C.4129) were purchased from Sigma (St. Louis, MO, USA). *S*-(+)-Erythrulose hydrate, *N*-tris(hydroxymethyl)methylacrylamide, (1*R*,3*R*,4*R*,5*R*)-quinic acid, pentaerythritol, D-ribose, 1,3,5-tris(2-hydroxyethyl)cyanuric acid, D-threitol and xylitol were obtained from Aldrich (Milwaukee, WI, USA). Glycerol, D-/L-lactic acid and polyvinyl alcohol (M_r ~115 000) were obtained from BDH (Poole, UK). *N*-Tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid, 1,1,1-tris(hydroxymethyl)ethane and triethanolamine were obtained from Fluka (Buchs, Switzerland). Merck (Darmstadt, Germany) supplied D-maltose, D-lactose, D-glucose, D-galactose, D-fructose and sucrose. Neopentyl glycol and trimethylolpropane were generous gifts of Perstorp (Perstorp, Sweden). Bio-Rad protein dye reagent concentrate (catalogue No. 500-0006) was bought from Bio-Rad (Hercules, CA, USA) and was utilised according to the instructions given by the supplier. Sodium phosphate, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulphonic acid) (EPPS), sodium cyano-borohydride, sodium chloride, hydrochloric acid and acetic acid were of analytical grade. All chemicals were used without further purification. Dialysis membrane (Spectra/Pro1 Membrane M_r cut-off: 6000–8000) was purchased from Spectrum Labs, Inc. (Ft. Lauderdale, GA, USA).

2.2. Protein assay

The absorbance at 280 nm was measured and the concentration of cht was calculated as: $[\text{Cht}]_{\text{mg/ml}} = 0.49A_{280\ \text{nm/ml}}$ [17]. This method was used when there was no contribution to the absorption at 280 nm from other components in the sample. The Bio-Rad protein assay was utilised when there was interference. This assay technique is based on the

Bradford method [18]. A 5.0 ml volume of diluted dye reagent (1 part concentrated dye reagent was mixed with 4 parts distilled, de-ionised water) was added to 100 μl of the standard and sample solutions. Cht was chosen as the standard. After incubation at room temperature for at least 5 min, the absorbance was measured at 595 nm.

2.3. Carbohydrate assay

The carbohydrate content of the neoglycoproteins was analysed using the anthrone–sulphuric acid method [19]. The sample (1 ml, 10–50 μg/ml) was mixed with 2 ml anthrone–sulphuric acid reagent (0.2 g anthrone dissolved in 100 ml concentrated sulphuric acid) and incubated for 10 min in boiling water. The absorbance was measured at 620 nm after the temperature of the sample had reached room temperature. Glucose was used as the standard [20].

2.4. Modification of α-chymotrypsin with maltose

Maltose was coupled to cht using the reductive amination method [21] with a slight modification [20]. Chymotrypsin (5 mg) was dissolved in 0.1 *M* sodium phosphate buffer (1 ml, pH 7.2). Sodium cyanoborohydride (20 mg) and maltose (20 mg) were added to the solution. The mixture was incubated at room temperature for 3 days and dialysed several times against 10^{-5} *M* HCl at 4°C for 24 h. The final sample consisted of non-glycosylated cht and cht-mal. The overall molar ratio of maltose to cht in the sample was 12.1.

2.5. Chromatographic experiments

2.5.1. The chromatographic system

All chromatographic experiments were carried out on a Delta Prep 3000 system purchased from Waters (Milford, MA, USA). The system is composed of a 600E system controller, a 484 tunable absorbance detector and a pump, model 600. The fraction collector, model 201, was bought from Gilson (Middleton, WI, USA). All chromatographic columns were supplied by Bio-Rad.

2.5.2. Chromatography of native chymotrypsin under non-shielding conditions

APBA agarose was packed into a column (3.9× 0.7 cm I.D.) and equilibrated with 0.05 *M* EPPS–NaOH (pH 8.5). Cht (5 mg) was dissolved in the same buffer (1 ml) and applied to the column. The column was washed thoroughly with the same buffer until no protein absorption was detected in the effluent. Acetic acid (0.05 *M*, pH 4.5) was applied to elute the bound protein. The flow rate was 0.2 ml/min during the whole chromatographic process.

2.5.3. Evaluation of potential shielding reagents

A column (12.6×1.0 cm I.D.) packed with APBA agarose was equilibrated with 0.05 *M* sodium phosphate, 0.5 *M* NaCl, pH 7.0. Cht (30 mg) was dissolved in 10 ml of the same buffer and loaded onto the column. The column was washed thoroughly with the same buffer until no protein absorption was detected in the effluent. Elution was carried out by applying a linear concentration gradient of the investigated reagent dissolved in 0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, pH 8.0. The total gradient elution volume was 20 times the bed volume. The column was finally rinsed with acetic acid (0.05 *M*, pH 4.5). The flow rate was 0.8 ml/min during loading and washing and 1.0 ml/min during elution.

The shielding efficiency of each reagent investigated was determined from its chromatogram as follows. The *total amount of bound cht*, T (mg), was defined as the sum of the cht in the elution peak, E (mg), and in the acetic acid peak, C (mg). The *elution percentage,* E/T (%), was defined as the fraction of the bound cht eluted from the column by the reagent. The *optimum concentration* (M) of the reagent was defined as the concentration corresponding to the highest point of the elution peak (Fig. 1). The shielding efficiency was evaluated by combining the *optimum concentration* and the *elution percentage*. A reagent with a high shielding efficiency thus provides a high *elutionpercentage* at a low *optimum concentration*.

2.5.4. Chromatography of a mixture of cht and cht-mal under shielding conditions and rechromatography of selected fractions

An APBA agarose column (10×0.7 cm I.D.) was

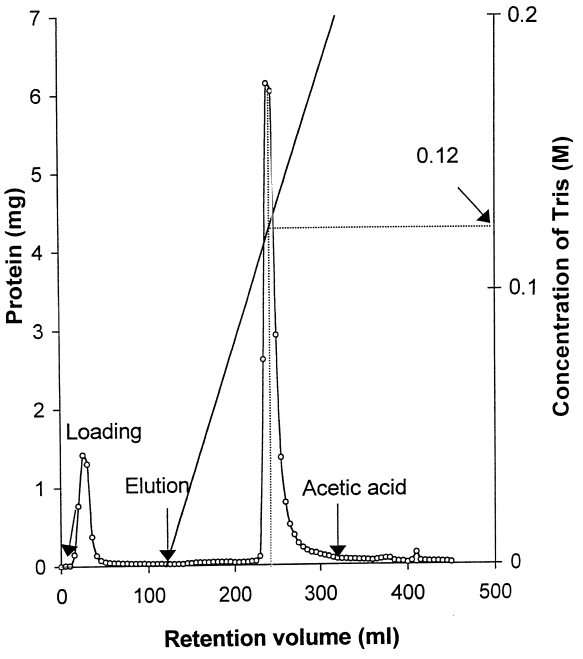

Fig. 1. Results of chromatography of native chymotrypsin on a boronate column; evaluation of the shielding efficiency of Tris. Sample: 30 mg of native cht dissolved in 10 ml loading buffer. Support: APBA agarose. Column size: 12.6×1.0 cm I.D. Loading buffer: 0.05 *M* sodium phosphate, 0.5 *M* NaCl, pH 7.0. Elution start buffer: 0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, pH 8.0. Elution end buffer: 0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, 0.2 *M* Tris (shielding reagent), pH 8.0. The linear increase in Tris concentration started after a retention volume of 125 ml and ended after a retention volume of 325 ml. Flow rate: 0.8 ml/min during loading and 1.0 ml/min during elution.

equilibrated with buffer containing a shielding reagent, for example Tris (0.12 *M* Tris–HCl, 0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, pH 8.0). The sample (4 mg) consisting of both non-glycosylated cht and cht-mal was dissolved in the same buffer and applied to the column. The column was washed with the same buffer until there was no detectable protein in the effluent. The bound protein was then eluted using acetic acid (0.05 *M*, pH 4.5). The flow rate was 0.2 ml/min during binding and washing and 0.4 ml/min during elution.

The breakthrough and acetic acid elution fractions obtained from chromatography as described above were dialysed thoroughly against 0.12 *M* Tris–HCl, 0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, pH 8.0. The breakthrough fraction was then applied to the same column and rechromatographed according to the same protocol. The same procedure was carried out with the acetic acid elution fraction.

2.5.5. Chromatography of a mixture of cht and cht-mal under non-shielding conditions and rechromatography of selected fractions

An APBA agarose column (10×0.7 cm I.D.) was equilibrated with the loading buffer A (0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, pH 8.0) containing no shielding reagent. A sample (4 mg) consisting of non-glycosylated cht and cht-mal was dissolved in the same buffer and applied to the column. The column was washed with 7 bed volumes of the same buffer. The bound protein was then eluted with buffer B (0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, 0.12 *M* Tris, pH 8.0), followed by buffer C (0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, 0.5 *M* Tris, pH 8.0) and finally acetic acid (0.05 *M*, pH 4.5). The flow rate was 0.2 ml/min during binding and washing and 0.4 ml/min during elution.

The fraction eluted by buffer B containing 0.12 *M* Tris was collected and dialysed against the loading buffer A and rechromatographed. The sugar content of this fraction was also analysed.

3. Results and discussion

3.1. Chromatography of native chymotrypsin under non-shielding conditions

It has been known since the 1970s that cht can interact with boronates [9,22,23]. The interaction can be assumed to be a combination of direct interactions between the enzyme and the boronate anion, and contributions from additional functionalities i.e. hydrophobic structures. Phenyl boronate is one example of a compound which can give rise to hydrophobic interactions [14].

The binding of cht to APBA agarose was studied in the present work. Cht was applied to the column under the conditions suggested by the manufacturer of the matrix (see Materials and methods). As shown in Fig. 2, the major part of the cht was found in the acetic acid elution peak, indicating relatively strong binding between the boronate ligand and cht. In other experiments to investigate the effect of differ-

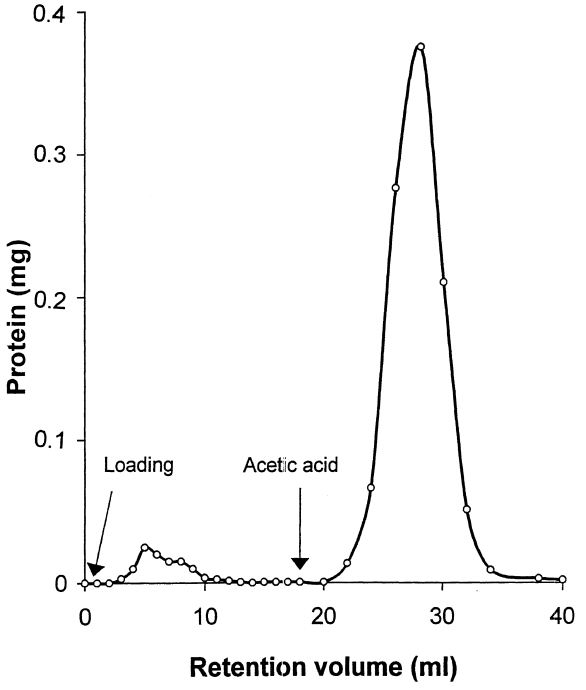

Fig. 2. Results of chromatography of native chymotrypsin on a boronate column under non-shielding conditions. Sample: 5 mg of native cht dissolved in 1 ml loading buffer. Support: APBA agarose. Column size: 3.9×0.7 cm I.D. Loading buffer: 0.05 *M* EPPS–NaOH, pH 8.5. Elution buffer: 0.05 *M* acetic acid, pH 4.5. Flow rate: 0.2 ml/min during the whole process.

ent buffer systems on the binding of cht to the boronate column, it was observed that cht exhibited strong interactions with the boronate ligand, regardless of whether the buffer was 0.05 *M* EPPS–NaOH (pH 8.5) or 0.1 *M* sodium phosphate (pH 7.0) containing 0.5 *M* NaCl (data not shown).

3.2. Evaluation of potential shielding reagents

The fact that native cht interacts with the boronate ligand indicates that the separation efficiency of boronate chromatography of glycoproteins would be impaired by involving a competitive substance to the protein in the chromatographic system. A strategy was designed to suppress the unwanted protein–boronate interactions, while at the same time allowing the carbohydrate–boronate interactions to take place. This was done by extending the molecular shielding concept by continuously adding a so-called

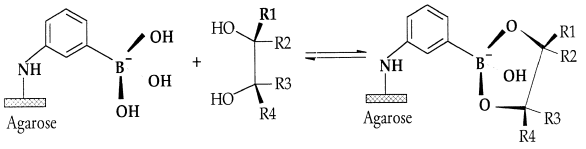

Fig. 3. Interaction between a boronate anion with a tetrahedral conformation and polyhydroxyl compounds.

shielding reagent to the mobile phase during the whole chromatographic process. The ability of one polymeric and 27 low-molecular-mass polyhydroxyl compounds to reduce the cht-boronate interactions was evaluated. The outcome of one of these experiments (using Tris) is presented in Fig. 1. The elution percentage (E/T,%) was 99.5% in this case. The optimum concentration of Tris corresponding to the highest point of the elution peak was 0.12 M.

As has been reported earlier [14], an essential requirement for the interactions of boronate with polyhydroxyl compounds is that the boronate must have a tetrahedral conformation (Fig. 3). Alkaline conditions can easily promote the conformational change of the boronate anion from planar trigonal to tetrahedral (Fig. 4). Therefore, all the screened chemicals were presented in an alkaline buffer (0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, pH 8.0) to favour the maximal interaction between the boronate ligand and the shielding reagent candidates.

The shielding efficiencies of all the compounds investigated are listed in Table 1. They are classified as *Group I, II* or *III* according to their shielding efficiency. This classification can be considered a guide in the choice of a suitable shielding reagent.

Group I comprises chemicals with a high shielding efficiency. Over 95% of the bound cht was eluted using no more than 0.25 *M* of these shielding reagents. Substances belonging to this group contain either the structure fragment $(HOCH_2)_3C-$ or

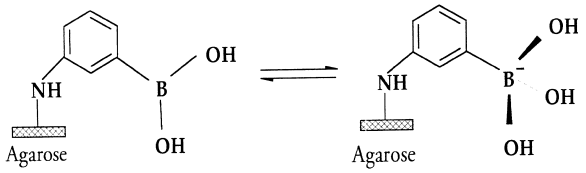

Fig. 4. Transition of planar boronic acid to a tetrahedral boronate anion under alkaline conditions.

Table 1
Shielding efficiency of polyhydroxyl chemicals

	Polyhydroxyl chemicals	Optimum concentration of polyhydroxyl chemicals (M)	Elution percentage of the bound cht (%)
Group I			
(1)	Pentaerythritol	0.08	>99
(2)	Tris(hydroxymethyl)aminomethane (Tris)	0.12	>99
(3)	Triethanolamine	0.12	>99
(4)	*N*-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid	0.13	>99
(5)	1,1,1-Tris(hydroxymethyl)ethane	0.13	>99
(6)	D-Ribose	0.15	99
(7)	(1*R*, 3*R*, 4*R*, 5*R*)-Quinic acid	0.16	97
(8)	*N*-Tris(hydroxymethyl)methyl-acrylamide	0.17	99
(9)	Trimethylolpropane	0.25	>99
Group II			
(10)	D-Mannitol	0.31	>99
(11)	D-Sorbitol	0.32	>99
(12)	D-Fructose	0.33	>99
(13)	Xylitol	0.33	97
(14)	D-Threitol	0.34	97
(15)	Polyvinyl alcohol (M_r ~115 000)	0.34 (monomer)	90
(16)	D-Lactose	0.35	90
(17)	D-Arabinose	0.37	99
(18)	D-Galactose	0.43	>99
(19)	*S*(+)-Erythrulose hydrate	0.45	96
(20)	D-Maltose	0.61	99
(21)	D-Glucose	0.65	90
(22)	Sucrose	0.80	95
Group III			
(23)	1-*O*-Methyl-α-D-mannopyranoside	0.78	83
(24)	1,3,5-Tris(2-hydroxyethyl)cyanuric acid	0.62	70
(25)	D-/L-Lactic acid	1.0	70
(26)	Neopentyl glycol	1.0	55
(27)	1-*O*-Methyl-α-D-glucopyranoside	>1.0	35
(28)	Glycerol	1.0	0

$(HOCH_2CH_2)_3N$. These structure fragments are able to form tridentate complexes involving all three hydroxyl groups of the boronate anion, as shown in Fig. 5a. *Group I* consists of the following chemicals: *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, 1,1,1-tris(hydroxymethyl)ethane, trimethylolpropane, tris(hydroxy-methyl)aminomethane, pentaerythritol, triethanolamine and *N*-tris(hydroxymethyl)methylacryl-amide. D-Ribose and (1*R*, 3*R*, 4*R*, 5*R*)-quinic acid also belong to *Group I* since the overall conformation of their hydroxyl groups allows the formation of tridentate complexes (Fig. 5b) and as a result of this a high shielding efficiency is obtained.

The members of *Group II* exhibit moderate shielding efficiency. At least 90% of the bound cht is eluted using between 0.30 and 0.80 *M* of these shielding reagents. This group contains polyols and mono- and disaccharides. The possibility of relatively free rotation about the C–C bonds results in a number of steric conformations of the hydroxyl groups which permit complex formation with the boronate. *S*(+)-Erythrulose hydrate, D-/L-threitol, xylitol, D-mannitol and D-sorbitol belong to this group. Polyvinyl alcohol (M_r ~115 000) also allows a moderate interaction with the boronate anion. This can be explained by the fact that this polymer is rather flexible, and that some conformations possess

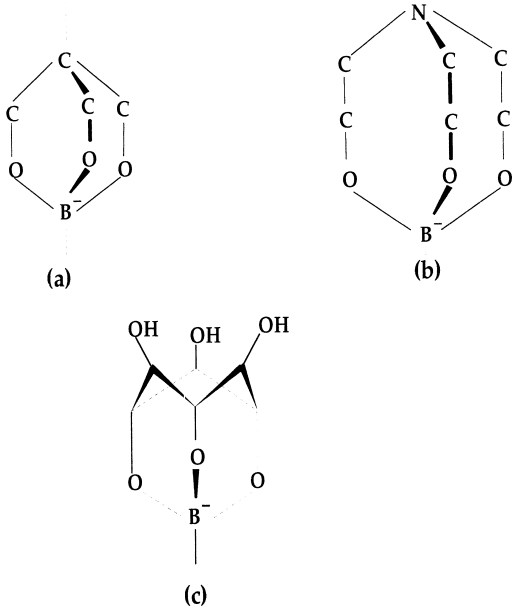

Fig. 5. Tridentate interaction of boronate with compounds containing (a) $(HOCH_2)_3C–$, (b) $(HOCH_2CH_2)_3N$ and (c) D-ribose or (1*R*, 3*R*, 4*R*, 5*R*)-quinic acid.

free hydroxyl groups suitable for binding to the boronate anion. In the case of carbohydrates, the ring conformation has more sterically fixed hydroxyl groups. However, the rotation possible is still sufficient to make the interaction with the boronate anion possible. D-Fructose and D-arabinose exhibit higher shielding efficiencies than D-maltose, D-lactose, D-glucose, D-galactose and D-sucrose. The relatively high proportion of the furanose form of fructose at equilibrium might be the explanation of this since furanoses tend to bind borate more tightly than pyranoses [24,25]. The efficiency of D-arabinose as a shielding reagent is probably due to the suitable geometry of its hydroxyl groups which facilitates complex formation.

The remaining chemicals, which have low shielding efficiency, belong to *Group III*. No more than 83% of the bound cht could be eluted using concentrations of 0.8 *M* or more. At a concentration of 1.0 *M*, the percentage of bound cht eluted from the column by glycerol, neopentyl glycol and lactic acid was 0%, 55% and 70% respectively. Although glycerol contains three hydroxyl groups, the rigid configuration of this molecule, due to the short carbon chain, makes interaction with the boronate anion involving all the hydroxyl groups impossible. Neopentyl glycol cannot form tridentate complexes with boronate since it only contains two hydroxyl groups. Lactic acid interacts with the boronate anion via charge transfer, as shown in Fig. 6 [26]. No tridentate complexes can be formed between lactic acid and boronate. Methyl-α-D-mannopyranoside and methyl-α-D-glucopyranoside do not contain C_1–OH and their shielding efficiency is reduced compared with that of D-glucose and D-galactose. This suggests that C_1–OH is important for complex formation with the boronate anion. Methyl-α-D-glucopyranoside exhibited an even lower shielding efficiency than methyl-α-D-mannopyranoside, indicating that the configuration of hydroxyl groups at C_2, C_3 and C_6 is more suitable for the interaction than that at C_2, C_4 and C_6. A single carbon/nitrogen atom is a more suitable core for the formation of tridentate complexes than 1,3,5-tris(2-hydroxyethyl)cyanuric acid.

3.3. Chromatography of a mixture of cht and chtmal under shielding conditions

To be a useful shielding reagent for the boronate chromatography of glycoproteins, the following requirements must be fulfilled. (a) The boronate–shielding reagent interactions must be weaker than the boronate–carbohydrate interactions. (b) The boronate–shielding reagent interactions must be stronger than the boronate–protein interactions. When this is the case, the carbohydrate–boronate

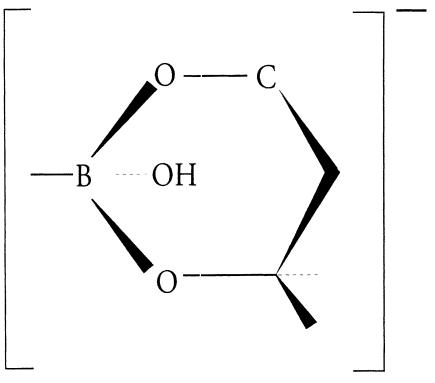

Fig. 6. Interaction of the boronate anion with α-hydroxycarboxy groups.

interactions can take place while the protein–boronate interactions are suppressed by the shielding reagent.

A sample consisting of both non-glycosylated cht and cht-mal was chromatographed on a boronate column under Tris-shielding conditions, as shown in Fig. 7a. Cht was not bound to the column and appeared in the breakthrough fraction while cht-mal was bound and was eluted from the matrix using acetic acid. The separation was further confirmed by rechromatography of both the breakthrough and the acetic acid elution fractions (Fig. 7b and c). The retention volumes of these two peaks were exactly the same in rechromatography as in the initial chromatography experiment.

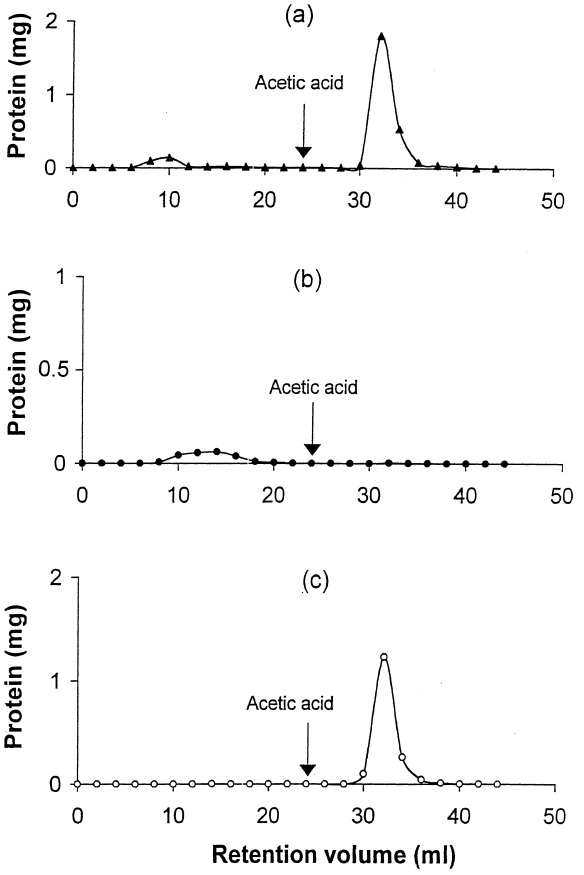

Fig. 7. Results of (a) chromatography of a mixture of cht and cht-mal, rechromatography of (b) the breakthrough fraction and (c) the acetic acid elution fraction from the initial run (a), on a boronate column under Tris shielding conditions. Sample: a mixture containing cht and cht-mal (3.5 mg) dissolved in 1 ml loading buffer. Support: APBA agarose. Column size: 10×0.7 cm I.D. Loading buffer: 0.02 *M* EPPS–NaOH, 0.12 *M* Tris–HCl, 0.5 *M* NaCl, pH 8.0. Elution buffer: 0.05 *M* acetic acid, pH 4.5. Flow rate: 0.2 ml/min during binding and 0.4 ml/min during elution. The samples used for re-chromatography: breakthrough fraction after dialysing against the loading buffer (0.35 mg), and acetic acid elution fraction after dialysing against the loading buffer (2.0 mg). The conditions were the same as those in the initial chromatography experiment.

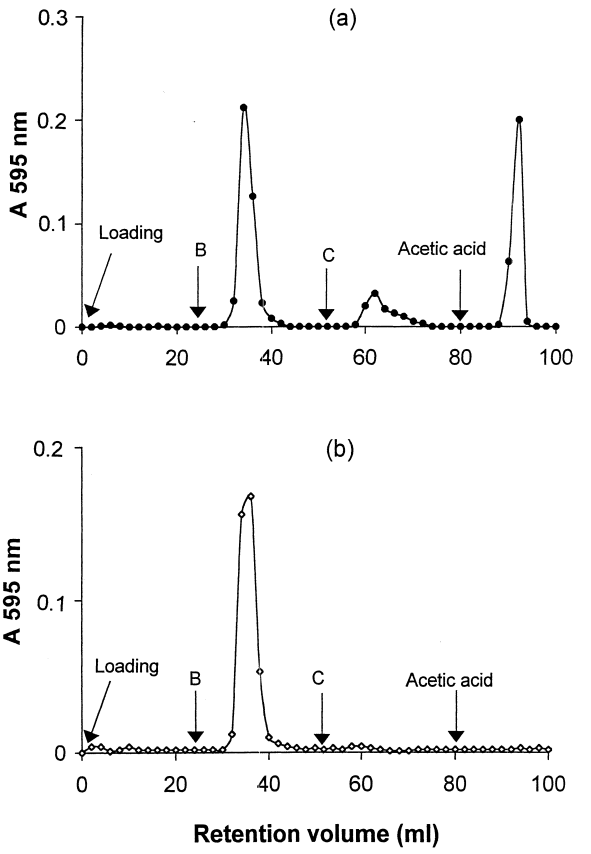

Fig. 8. Results of (a) chromatography of a mixture of cht and cht-mal and (b) rechromatography of the fraction eluted by buffer B containing 0.12 *M* Tris from the initial run (a), on a boronate column under non-shielding conditions. Sample: a mixture containing cht and cht-mal (4 mg) dissolved in 1 ml loading buffer. Support: APBA agarose. Column size: 10×0.7 cm I.D. Loading buffer: 0.02 *M* EPPS–NaOH, 0.5 *M* NaCl, pH 8.0. Elution buffer: B, 0.02 *M* EPPS, 0.5 *M* NaCl, 0.12 *M* Tris, pH 8.0; C, 0.02 *M* EPPS, 0.5 *M* NaCl, 0.5 *M* Tris, pH 8.0; and 0.05 *M* acetic acid, pH 4.5. Flow rate: 0.2 ml/min during binding and 0.4 ml/min during elution. Sample used for rehromatography: the fraction eluted by buffer B (containing 0.12 *M* Tris) collected and dialysed against the loading buffer. The conditions were the same as those in the initial chromatography experiment.

A control experiment was performed by running a neoglycoprotein sample containing cht and cht-mal on a boronate column under non-shielding conditions. Fig. 8a shows that both cht and cht-mal were bound to the boronate column after loading, indicating that cht interacts with the boronate ligand when there is no shielding by Tris. An elution buffer containing 0.12 *M* Tris was used to intentionally elute the bound non-glycosylated cht. A fraction peak was then collected and loaded for rechromatography (Fig. 8b). The retention volume was the same as that in the initial chromatography experiment (Fig. 8a), illustrating the efficient separation resolution. No sugar content was detectable in this fraction (data not shown).

To the best of our knowledge, it is recommended in the literature that Tris and other compounds containing polyhydroxyls should be avoided during boronate chromatography, since such substances can reduce the binding capacity of the boronate matrix by direct competition [14,27]. The present study shows that the well-controlled addition of such compounds containing polyhydroxyl groups can increase the separation efficiency considerably. It should also be pointed out that the presence of *cis*-diols alone in a compound is not always sufficient to bring about interactions with boronates. Suitable steric geometry of the hydroxyl groups is also required.

4. Conclusions

It is possible to suppress unwanted protein–boronate interactions during boronate chromatography by introducing a shielding reagent into the chromatographic system. Low-molecular-mass polyhydroxyl compounds exhibiting suitable conformations for the interaction with all three hydroxyl groups of the boronate anion were found to be superior shielding reagents. The results obtained are promising for the continued improvement of the separation resolution of boronate chromatography.

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