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Journal of Chromatography A, 925 (2001) 115–121

JOURNAL OF CHROMATOGRAPHY A

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Separation of mistletoe lectins based on the degree of glycosylation using boronate affinity chromatography

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Received 28 March 2001; accepted 14 May 2001

Abstract

A mixture of two mistletoe lectins (MLs) has been separated according to the degree of glycosylation using boronate affinity chromatography. The mistletoe lectins, mistletoe lectin I (MLI) and mistletoe lectin III (MLIII) with degrees of glycosylation of 6.1 and 3.8%, respectively, were used in the investigation. MLI exhibited a higher retention time than MLIII due to its higher degree of glycosylation. Separation was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The developed method may lead to new applications for the boronate affinity technique, as well as provide an alternative separation method for MLs.

Keywords: Affinity adsorbents; Lectins; Boronate; Glycoproteins; Proteins

1. Introduction

Boronate affinity chromatography has long been used to purify glycoproteins [1]. Separation is accomplished due to the unique capacity of the boronate ligand to form complexes with substances containing hydroxyl groups [2–5]. Nevertheless, most applications are based on group-specific properties of the boronate ligand by which glycoproteins can be separated from nonglycosylated substances [6,7]. Few studies have addressed the separation of individual glycoforms which share the same polypeptide backbone but differ with respect to the sugar moiety (i.e. identical polypeptides with oligosaccharides differing either in sequence or in content) [8]. The subtle differences between the glycoforms may lead to difficulty in achieving separation with good resolution. However it is of great importance to obtain a pure glycoform in order to understand the relationship between a glycoprotein and its biological activity [8,9].

Mistletoe lectins (MLs) are a group of glycoproteins extracted from the mistletoe plant [10–12]. They are of interest because of their potent immunomodulating and cytotoxic effects, which can be applied in pharmacology, immunology and cancer therapy [13–16]. In spite of their extensive range of applications, reports on the development of methods

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00967-0

for separating MLs and their glycoforms are scarce. The methods used today are mainly derived from those developed in the 1980s which employed sugar-mediated affinity matrices, such as partially hydrolysed Sepharose 4B, galactosyl Sepharose 6B or lactosyl Sepharose 6B [17–21]. Using these matrices, three MLs with different sugar specificity have been isolated from mistletoe extracts [17–21]. Mistletoe lectins I (MLI) and III (MLIII) exhibit specificity to D-galactose and *N*-acetylgalactosamine, respectively, while mistletoe lectin II (MLII) has an affinity to both D-galactose and *N*-acetylgalactosamine [17,18]. The glycosylation pattern (carbohydrate content and structure) of these three MLs also exhibits slight differences, which may be of biological significance [21,22]. A variety of factors can influence the properties of isolated MLs and their glycan pattern [15,22,23]. Since mistletoe is a semi-parasitic plant, the characteristics of the host tree, such as species, age, sex or location, are important for the content and composition of isolectin groups in the plant [22]. Therefore, the isolation and identification of different glycoforms of MLs may play a potentially important role in probing their biological and therapeutic functions.

The considerable interest in both extending the separation capacity of boronate chromatography and developing new efficient purification methods for MLs led to our study. In this paper, we report on a method of using boronate affinity chromatography to separate a mixture of MLI and MLIII based on their different degrees of glycosylation.

2. Material and methods

2.1. Materials

m-Aminophenyl boronic acid agarose (APBA agarose, product number A-8312, 40–80 μmol APBA per ml packed gel), *N*-(2-hydroxyethyl)piperazine-*N′*-(3-propanesulphonic acid) (EPPS) and sodium chloride (NaCl) were bought from Sigma (St Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was obtained from BDH (Poole, UK). Merck (Darmstadt, Germany) supplied D-galactose. Bio-Rad protein dye reagent concentrate (catalogue No. 500-0006) was bought from Bio-Rad and was used according to the instructions supplied by the producer (Hercules, CA, USA). Sepharose 4B was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). MLI and MLIII (suspended and preserved in 3 *M* ammonium sulphate) were purified via lactosyl affinity chromatography followed by MonoS ion-exchange chromatography at the Institute of Phytochemistry, University of Witten/Herdecke, Germany [21,22]. The total sugar contents of MLI and MLIII were 6.1 and 3.8%, respectively [22]. All chemicals were used without further purification. Dialysis membranes (Spectra/Prol Membrane M_r cut-off: 6000–8000) were purchased from Spectrum Labs. (Ft. Lauderdale, GA, USA).

2.2. Protein assay

The absorption was measured at 280 nm and the protein concentration of MLs was calculated as $[P]_{mg/ml}=1.41A_{280\ nm}$ [21,22]. 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 interfering substances were present. The analysis is based on the Bradford method [24]. The absorption was determined at 595 nm and bovine serum albumin was chosen as the standard.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

SDS–PAGE analysis was performed on a Mini-Protean II Electrophoresis Cell from Bio-Rad. All samples were concentrated using Microsep centrifugal concentrators (M_r cut-off: 3000, Pall Filtron, MA, USA) before running the electrophoresis. Pre-cast Tris–glycine gels with a total acrylamide concentration of 10% (Labora, Sollentuna, Sweden) were used for electrophoresis, and Coomassie Blue R-250 (Sigma) was used for staining. The low-molecular-mass calibration kit was bought from Amersham Pharmacia Biotech. Electrophoresis was run at a constant 125 V until the dye marker reached the end of the gel.

2.4. Chromatographic experiments

2.4.1. The chromatographic system

The chromatographic columns were purchased from Bio-Rad. The chromatographic system consisted of an Alitea pump C-4v (Stockholm, Sweden), a Gilson fraction collector (Middleton, WI, USA) and a Waters 484 tunable absorbance detector (Milford, MA, USA).

2.4.2. Chromatography of a mixture of MLI and MLIII on a boronate column using Tris as the eluting agent

The APBA agarose, packed in a 1.0-cm diameter column to a bed height of 20 cm, was equilibrated with the loading buffer (0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5). A sample consisting of MLI (3.0 mg) and MLIII (3.2 mg), which had been separately dialysed against the loading buffer, was applied. The column was washed with the loading buffer until the protein absorption at 280 nm in the effluent reached the base line. The linear gradient elution mode was applied. The elution start buffer was composed of 0.02 *M* EPPS–NaOH and 0.5 *M* NaCl, pH 8.5, while the elution end buffer consisted of 0.02 *M* EPPS–NaOH, 0.5 *M* NaCl and 0.5 *M* Tris–HCl, pH 8.5. The total elution volume was 480 ml. Finally the column was rinsed with 0.05 *M* acetic acid (HAc), pH 4.5. A flow-rate of 30 cm/h was used during the entire process.

Based on the results obtained in the gradient elution process, an experiment was performed using the stepwise elution mode. The conditions of the column, flow-rate and loading buffer applied in the stepwise elution process were the same as those used in the gradient elution process. Two elution solutions were prepared, buffer B (0.02 *M* EPPS–NaOH+0.5 *M* NaCl+0.07 *M* Tris–HCl, pH 8.5) and buffer C (0.02 *M* EPPS–NaOH+0.5 *M* NaCl+1.0 *M* Tris–HCl, pH 8.5). MLI (0.8 mg) and MLIII (1.9 mg), separately dialysed against the loading buffer, were mixed and applied to the column. After being thoroughly washed with the loading buffer, the column was eluted with buffer B and C, one after the other. Two elution peaks were collected and analysed with SDS–PAGE.

2.4.3. Chromatography of a mixture of MLI and MLIII on a boronate column using galactose as the eluting agent

The APBA agarose, packed in a 0.7-cm diameter column to a bed height of 10 cm, was equilibrated with the loading buffer (0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5). MLI (0.7 mg) and MLIII (0.5 mg), separately dialysed against the loading buffer, were mixed and applied to the column. The column was washed with the loading buffer until the absorption at 595 nm of the protein–dye complex in the effluent had reached the base line. Elution was performed using a linear gradient of galactose (0–0.3 *M*) present in 0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5. The total elution volume was 120 ml. Finally HAc (0.05 *M*, pH 4.5) was used to rinse the column. The flow-rate used during chromatography was 60 cm/h.

2.4.4. Determination of the interactions between MLs and the agarose matrix

The Sepharose 4B (agarose), packed in a 0.7-cm diameter column to a bed height of 10 cm, was equilibrated with the loading buffer (0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5). MLI (1.8 mg) was loaded on to the column after being dialysed against the loading buffer. The column was washed with the loading buffer until the protein absorption at 280 nm in the effluent reached the base line. Elution was carried out using 0.05 *M* HAc, pH 4.5. The flow-rate during the entire process was 37.5 cm/h. The same procedure was applied to MLIII (3.0 mg).

3. Results and discussion

3.1. Chromatography of a mixture of MLI and MLIII on a boronate column using Tris as the eluting agent

Boronate affinity chromatography has been widely used to purify glycoproteins due to the ability of the boronate ligand to interact with compounds containing hydroxyl groups oriented in a suitable configuration [1,3]. The technique is often used to isolate glycoproteins from non-glycosylated substances [6,7]. However, the separation of different glycoproteins or glycoforms of a glycoprotein may

prove essential for our understanding of the structure–function relationships of glycoproteins. The investigation of the separation efficiency of boronate chromatography is thus of potential importance with regard to this.

The formation of boronate–hydroxyl complexes is dependent on two crucial factors: the co-ordination geometry of the boronate ligand and the configuration of hydroxyl groups [1–5]. The boronate ligand must be in the tetrahedral anion form (Fig. 1a) in order to interact with hydroxyl groups (Fig. 1b). Alkaline conditions (pH≥8.0) can facilitate the conformational change of boronate anions from planar trigonal to tetrahedral configuration, Fig. 1a [1,3]. It is usually considered that the hydroxyl groups have to be *cis* and coplanar in order to interact with the boronate ligand [1–5]. We have previously reported the effect of the configuration of the hydroxyl groups on the formation of boronate–hydroxyl complexes [25]. It was shown that some polyhydroxyl chemicals, such as Tris and triethanolamine, exhibited stronger binding to the boronate ligand than most sugars with ring structures, like galactose and maltose [25]. This conclusion was drawn as a result of the formation of a tridentate complex between the hydroxyl groups of Tris or Tris-like compounds and the boronate ligand [25,26]. This indicated that Tris could be used as a competitive eluting agent.

The chromatographic conditions for boronate affinity separation were thus chosen to favour the formation of boronate–hydroxyl complexes. The pH value of the mobile solutions is often chosen to be above 8.0, provided that the biomolecules are stable under alkaline conditions. In addition, the ionic strength may also be of importance. The negative charge on the boronate anion in its active tetrahedral form can give rise to ionic interactions, while the phenylene component of the boronate ligand can cause hydrophobic interactions. The addition of a certain amount of salt (0.1–0.5 *M*) to the operating solutions may help to suppress the non-specific ionic interactions without causing strong non-specific hydrophobic interactions [9].

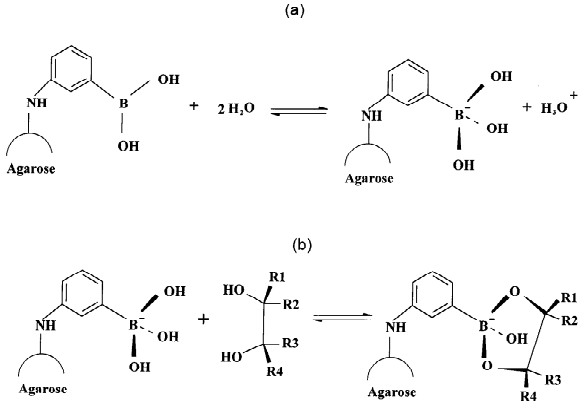

Fig. 1. Schematic presentation of: (a) the transition of planar boronic acid to a tetrahedral boronate anion under alkaline conditions, and (b) the interaction between a boronate anion with a tetrahedral conformation and polyhydroxyl compounds.

The gradient elution mode usually provides good results when separating heterogeneous samples [27]. Components with various binding affinities to the column can be eluted under favourable conditions when the elution power of an eluting agent increases continuously [27]. It is thus a useful method for determining the elution conditions of an unknown sample in an initial separation.

Considering the factors mentioned above, an experiment was designed to use boronate chromatography to separate a mixture of two active ingredients from a mistletoe extract, MLI and MLIII. MLI and MLIII are glycoproteins sharing a similar protein structure and subunit organisation but differing in the degree of glycosylation and glycan structure [22,28]. The mobile solutions used during chromatography were prepared by maintaining the pH at 8.5 and adding a certain amount of NaCl (0.5 *M*). Elution was performed using a linear gradient of Tris (0–0.5 *M*). As shown in Fig. 2a, two elution peaks, peak 1 (P1) and peak 2 (P2), were observed. P1 consisted of the fractions eluted by Tris between the concentrations of 0.02 and 0.07 *M*, while P2 was obtained between 0.07 and 0.24 *M*. P1 and P2 were analysed with SDS–PAGE. The results are shown in Fig. 2b. The migration position of P1 (Lane 2) was identical to that of MLIII (Lane 6) while the migration position of P2 (Lane 3) was identical to that of MLI (Lane 5). This confirmed that MLI and MLIII had been separated from each other. MLI was recovered at a higher retention volume (P2) due to its higher degree of glycosylation.

As shown in Fig. 2a, elution peak 2 (MLI) was relatively broad. This was probably due to the

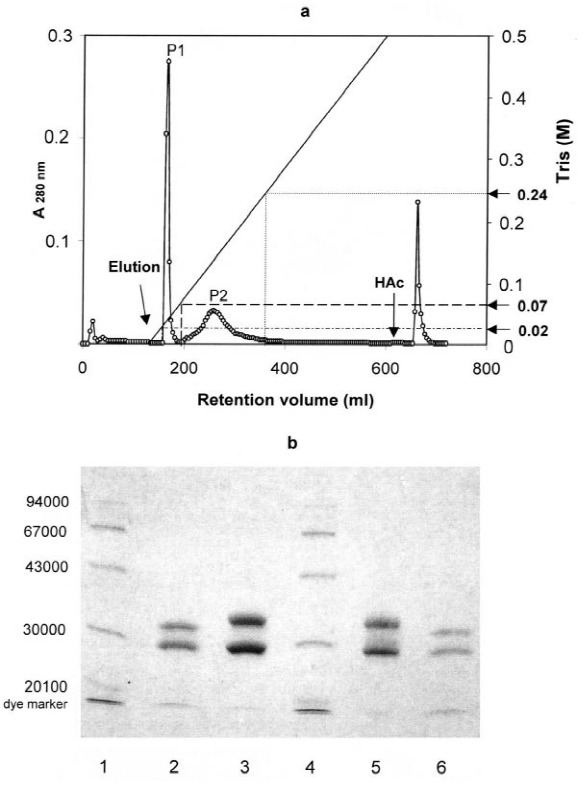

Fig. 2. The results of: (a) boronate chromatography of a mixture of mistletoe lectin I (MLI) and mistletoe lectin III (MLIII) using a linear gradient of Tris for elution, and (b) SDS–PAGE analysis of the peaks from (a). Column: 20×1.0 cm I.D. Matrix: *m*-aminophenyl boronic acid agarose (15 ml). Buffers: loading, 0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5; elution start buffer: 0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5; elution end buffer: 0.02 *M* EPPS–NaOH+0.5 *M* NaCl+0.5 *M* Tris–HCl, pH 8.5. The linear increase in Tris concentration (—) started after a retention volume of 130 ml and ended after a retention volume of 610 ml. The column was rinsed with 0.05 *M* acetic acid (HAc), pH 4.5. Flow-rate: 30 cm/h. Sample: a mixture of MLI (3.0 mg) and MLIII (3.2 mg). MLI and MLIII had been separately dialysed against the loading buffer before mixing. The protein absorption was measured at 280 nm (○). SDS–PAGE was run on the Mini-Protean II Electrophoresis Cell using a pre-cast Tris–glycine gel with a total acrylamide concentration of 10%. The gel was stained with Coomassie Blue R-250. Lanes: 1 and 4=low-molecular-mass calibration kit; 2=peak 1 (P1); 3=peak 2 (P2); 5=MLI; 6=MLIII.

diffusion of sample molecules along the column, slow transfer of sample molecules into or out of the gel matrix, or different binding affinities to the boronate ligand from the micro-heterogeneous sample molecules. However, based on the initial separation results obtained by gradient elution, the stepwise elution mode was applied in order to recover the eluted substances in a more concentrated peak. The shape of P2 was improved, as shown in Fig. 3a. Results from SDS–PAGE (Fig. 3b) confirmed that

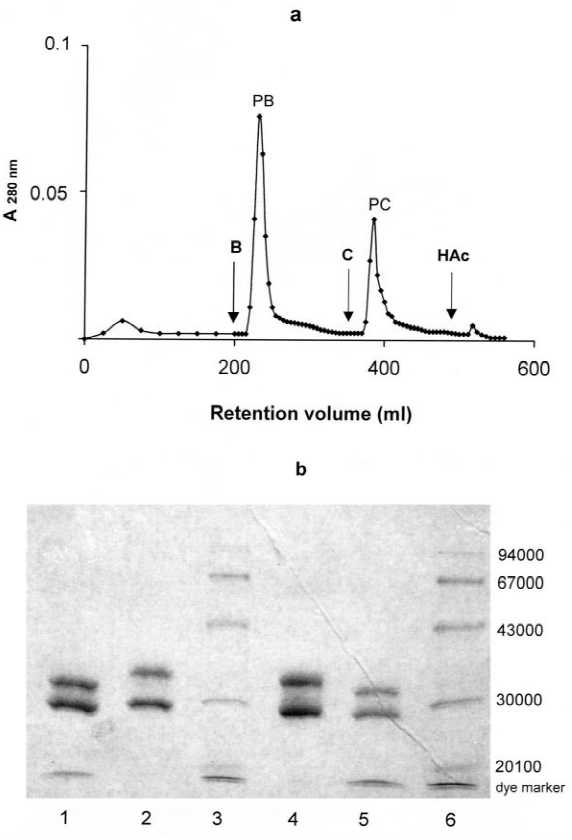

Fig. 3. The results of: (a) boronate chromatography of a mixture of mistletoe lectin I (MLI) and mistletoe lectin III (MLIII) using stepwise elution with Tris buffer, and (b) SDS–PAGE analysis of the peaks from (a). Column: 20×1.0 cm I.D. Matrix: *m*-aminophenyl boronic acid agarose (15 ml). Buffers: loading, 0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5; elution buffer B: 0.02 *M* EPPS–NaOH+0.5 *M* NaCl+0.07 *M* Tris–HCl, pH 8.5; elution buffer C: 0.02 *M* EPPS–NaOH+0.5 *M* NaCl+1.0 *M* Tris–HCl, pH 8.5. The column was rinsed with 0.05 *M* acetic acid (HAc), pH 4.5. Flow-rate: 30 cm/h. Sample: a mixture of MLI (0.8 mg) and MLIII (1.9 mg). MLI and MLIII had been separately dialysed against the loading buffer before mixing. The protein absorption was measured at 280 nm (♦). SDS–PAGE was run on the Mini-Protean II Electrophoresis Cell using a pre-cast Tris–glycine gel with a total acrylamide concentration of 10%. The gel was stained by Coomassie Blue R-250. Lanes: 3 and 6=low-molecular-mass calibration kit; 1=elution B (PB); 2=elution C (PC); 4=MLI; 5=MLIII.

the separation resolution was good. Peak B (PB) (Lane 1) presented the same migration position as MLIII (Lane 5), while peak C (PC) (Lane 2) showed the same position as MLI (Lane 4).

3.2. Chromatography of a mixture of MLI and MLIII on a boronate column using galactose as the eluting agent

It is known that MLI and MLIII exhibit different affinities to galactose [17–21], an experiment was carried out to determine if galactose could also be a suitable eluting agent for the separation of a mixture of MLI and MLIII using boronate chromatography. Nevertheless, no separation between MLI and MLIII was observed (Fig. 4). It is thus not possible to explore the binding selectivity of different MLs on a boronate column using galactose as the eluting agent.

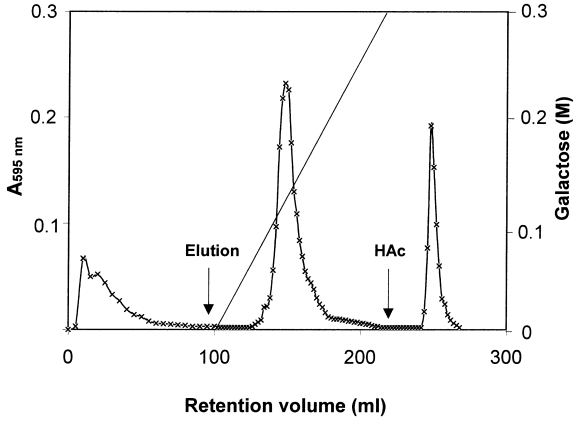

Fig. 4. Chromatography of a mixture of mistletoe I (MLI) and mistletoe III (MLIII) on a boronate column using a linear gradient of galactose for elution. Column: 10×0.7 cm I.D. Matrix: *m*-aminophenyl boronic acid agarose (4 ml). Buffers: loading, 0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5; elution start buffer: 0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5; elution end buffer: 0.02 *M* EPPS–NaOH+0.5 *M* NaCl+0.3 *M* galactose, pH 8.5. The linear increase in galactose concentration (—) started after a retention volume of 100 ml and ended after a retention volume of 220 ml. The column was rinsed with 0.05 *M* acetic acid (HAc), pH 4.5. Flow-rate: 60 cm/h. Sample: a mixture of MLI (0.7 mg) and MLIII (0.5 mg). MLI and MLIII had been separately dialysed against the loading buffer before mixing. The protein absorption was measured using a Bio-Rad reagent at 595 nm (×).

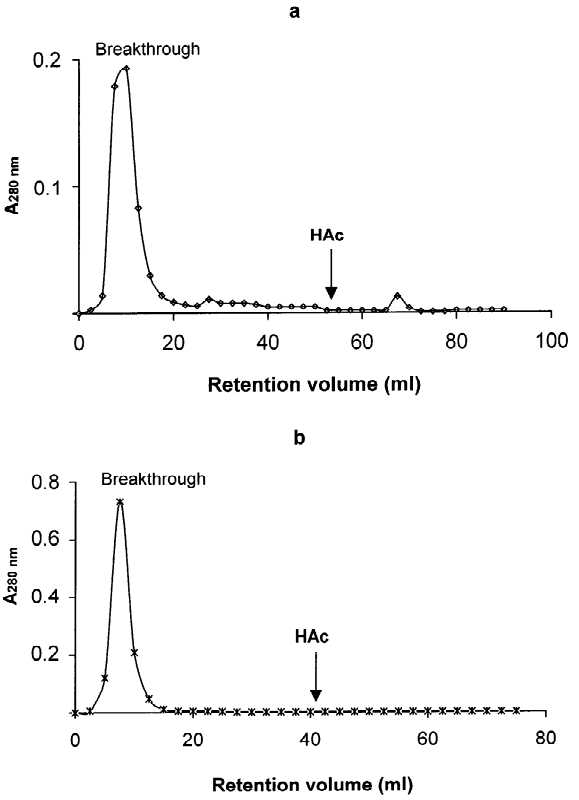

Fig. 5. Determination of the interactions of: (a) MLIII (*) and (b) MLI (◇) with the agarose matrix. Column: 10×0.7 cm I.D. Matrix: Sepharose 4B (4 ml). Loading buffer: 0.02 *M* EPPS–NaOH+0.5 *M* NaCl, pH 8.5. Elution buffer: 0.05 *M* acetic acid (HAc), pH 4.5. Sample: MLI, 1.8 mg; MLIII, 3.0 mg, both had been dialysed against the loading buffer. Flow-rate: 37.5 cm/h. The protein absorption was measured at 280 nm.

3.3. Determination of the interactions between MLs and the agarose matrix

As the boronate ligand was immobilised on the agarose, which is composed of repeating units of D-galactose and 3,6-anhydro-L-galactose, it was essential to investigate if there was any agarose–ML interaction during boronate chromatography. Fig. 5 shows the results of running MLI (a) and MLIII (b) on a Sepharose 4B column under the same loading conditions as those used in boronate chromatography. Ninety-seven percent of MLI and nearly 100% of MLIII were present in the breakthrough

fraction, suggesting that the agarose matrix underwent little or no interaction with MLs under the operating conditions used.

4. Conclusions

Boronate affinity chromatography can be used to separate different glycoforms of mistletoe lectins via a suitable choice of the chromatographic conditions. The retention volume is correlated with the degree of glycosylation.

Acknowledgements

The project was carried out within the Swedish Competence Center for Bioseparation. The digital photos of SDS–PAGE results were taken by David Karlsson, Department of Chemical Engineering II, Lund University.

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