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Short communication

Simple affinity chromatographic procedure to purify β-galactoside binding lectins

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Abstract

Affinity chromatography based on the commercial resin Sepharose CL-6B was used to isolate new C1-β-type lectins from crude preparations of snake venoms (*Bothrops jararaca*, *Bothrops jararacussu*, *Bothrops newiedi*, *Bothrops moojeni*, *Lachesis muta rhombeata*). Most of the C-type lectins could be eluted with almost 100% recovery using the competitor isopropyl-β-D-thiogalactoside (IPTG) or through Ca^{2+} sequestration with EDTA. The lectin yield varied considerably among the different snake species, but *B. newiedi* venom was a particularly rich source of lectin, retaining 2.7 mg of lectin by milliliter of resin in saturating conditions. C1-α-lectins from *Crotalus durisus terrificus* venom, from the jack fruit (jacalin) and from bread fruit seeds extract (frutalin) had no affinity, either with or without Ca^{2+} added, for Sepharose CL-6B, showing that the resin is specific for C1-β-type lectins. Sepharose CL-6B used as galactose-affinity chromatography provides a simple and fast method for isolating C-type β-galactoside binding lectins from crude sample preparations.

Keywords: C-type β-galactoside binding lectins; Affinity chromatography; Lectins

1. Introduction

Lectins are glycoproteins and/or oligomeric proteins found in a diverse assortment of organisms and have the extraordinary property of binding specifically, reversibly, and non-covalently to carbohydrates. Some lectins recognize sequences of two or more saccharides with specificity towards both inter-residue glycosidic linkages and anomeric configuration. For these reasons, lectins have proved to be useful reagents for the identification, isolation and characterization of the carbohydrate residues of glycoconjugates. They have been described in seeds, bacteria, snails, vertebrates, mushrooms and snake venoms [1]. Those from animal are classified into four major groups: (i) C-type or calcium dependent lectins, (ii) S-type or the galactose binding galectins, (iii) the I-type lectins, and (iv) the luminal proteins of the endoplasmatic reticulum that interacts transiently with glycoproteins [2].

Those of snake venom are characterized as C-type galactose depending lectins, present a highly conserved primary structure, including the carbohydrate recognition domain (CRD), and extraordinarily distinct biological activities [3]. The toxicological importance of these snake venom molecules has been demonstrated but is not yet totally understood [4,5] and therefore the introduction of an efficient, fast and scale up method to recover C-type lectin could allow for: (i) obtaining significant amount of native protein in one single step to sustain 3D structure characterization and (ii) investigating its physiological mechanisms at cellular and/or molecular level during an envenomation accident.

Downstream processing is considered a critical step in the commercial development of biotechnological products. Of the different techniques available, affinity chromatography remains one of the most powerful methods [6] and is employed as a basic tool in glycoproteomics studies [7]. In a previous study we have observed that a 32 kDa protein from *Bothrops jararacussu* venom (BJ-32) adsorbed non-specifically in an inhibitor-affinity column [8]. Thus, now we show that this interaction is dependent on galactose moieties exposed in the matrix (Sepharose

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CL-6B). In addition, we have investigated its interaction and demonstrated that the gel filtration resin Sepharose CL-6B may function as an affinity matrix to purify C1-type β-galactoside dependent lectins. This was accomplished by purifying several snake venom lectins from specimens belonging to the *Bothrops* (*Bothrops jararaca*, *B. jararacussu*, *Bothrops newiedi* and *Bothrops moojeni*) and *Lachesis* (*Lachesis muta muta*) genera.

2. Experimental

2.1. Material, reagents and chemicals

Sepharose™-CL 6B was from Amershan Biotechnology. Isopropyl-β-D-thiogalactoside (IPTG), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol (BME) and SDS–PAGE protein marker kit were from Sigma-Aldrich (St Louis, Missouri, USA). Polyvinylidene difluoride (PVDF) and protein assay were purchased from Bio-Rad Labs (CA, USA). All other reagents and chemicals were from Merck (Darmstadt, Germany). *B. jararacussu*, *B. moojeni*, *B. newiedi*, *B. jararaca*, *C. durissus* and *L. muta* venoms were extracted manually from several specimens kept at the Vital Brazil Institute and stored frozen at −20 °C until use. The lectins jacalin and frutalin were purified from *Artocarpus integrifolia* and *Artocarpus incise* seeds as described previously [9,10].

2.2. Chromatographic assay

The venoms (200 mg) were dissolved in water (3.0 mL), centrifuged (10.000 × *g*, 20 min, 4 °C) and the buffer exchanged with 20 mM Tris–HCl buffer containing 150 mM NaCl and 50 mM $CaCl_2$ (Tris–buffer), pH 7.5, using centricon P10 filters. After a new centrifugation step, as above, the clear supernatant was filtered and subjected to Sepharose CL 6B-column (3 mL), previously equilibrated with Tris–buffer. After extensive washing, the adsorbed proteins were eluted with 20 mM Tris–HCl, pH 7.5, containing either 10 mM EDTA (buffer A), 100 mM IPTG (buffer B) or 1 mM HCl, pH 3.0, containing 150 mM NaCl (buffer C). The eluent was collected on ice and the Tris–buffer changed to phosphate buffer 50 mM, pH 7.2, using P10 centrifugal filters. The protein content of the fractions was monitored by the absorbance change at 280 nm and estimated according to Lowry's method [11], using bovine serum albumin as a standard.

2.3. Sequence analysis

NH_2-terminal amino acid sequence of protein samples was determined by automated Edman degradation using a gas-phase protein microsequencer (Model PSQ-1; Shimadzu, Kyoto, Japan) and the conditions described before [9].

2.4. Agglutination assay

The agglutination assay was performed in U-bottom microtiter plates (Falcon, Becton Dickinson, NJ) using a constant amount (50 μg) of the *B. jararacussu* purified lectin (BJ-32), mixed with 10^8 phosphate buffer-saline (PBS) washed parasites *Leishmania* sp. and 1% human blood red cells (BRC, type A) in a final volume of 100 μL. The results were read after 4 h (BRC, 37 °C) and after 8 h (parasites, 28 °C) of incubation.

3. Results and discussion

The "*Sepharose*™" gel is a polymeric matrix obtained from 2,3-dibromopropanol cross-linked (CL) agarose chains, each one containing approximately a molecular mass of 120 kDa. This resin is devoid of ionic character and when mounted in a column is suitable for gel filtration chromatography. The present contribution shows that in addition to its gel-filtration property the CL-6B resin under certain conditions is useful as a C1-β-galactoside type lectin binding-matrix. The lectin yield per mg of crude venom varied considerable among the different snake venom species samples, as indicated in Table 1. *B. newiedi* venom was a particularly rich source of lectin, furnishing 2.7 mg of purified lectin by millileter of resin in saturating conditions (data not shown).

The venom and the seed proteins could be eluted from Sepharose CL-6B columns with either 1 mM Tris–HCl, pH 7.5, containing 10 mM EDTA (buffer A) or 100 mM IPTG (buffer B). In both cases the elution occurred efficiently suggesting that either the ion Ca^{2+} sequestration or the anomeric recognition, respectively, are important events involved in the lectin adsorption (Fig. 1). However, monosaccharides as galactose, glucose and manose were inefficient as or had no effect (data not shown) contrasting with other affinity galactose resins [12,13]. Alternatively, it was shown that 1 mM HCl, pH 3.0, containing 0.5 M NaCl (buffer C) elutes the lectins as well, probably through reversible protein denaturation. In all cases the lectins retained activity, as demonstrated by agglutination test either with BRC or *Leishmania* sp., after restoration of the optimum pH or elimination of the EDTA by dialysis (Table 2).

Crotalus durisus terrificus convulxin, the C1-jacalin and C1-frutalin lectins were not adsorbed. Since these two last proteins are known to be α-anomeric binding lectins [13], Sepharose CL-6B resin was characterized as specific for C1-β-type lectins. This feature is supported by the fact that the IPTG was able to elute specifically the proteins from the column. In addition, our

Table 1
Yield of C1-galactose bind lectins from various snake venoms and seeds

Snake venom	Total of protein (mg) recovered/3 ml resin	Amount of each lectin (%) present in the venoms
Bothrops newiedi	4.96	2.48
Bothrops jararacussu	3.38	1.69
Lachesis muta rhombeata	2.50	1.25
Bothrops moojeni	1.04	0.52
Bothrops jararaca	0.55	0.27
Crotalus durisus terrificus	ND	–
Artocarpus integrifolia (Jacalin)	ND	–
Artocarpus incise (frutalin)	ND	–

Yields are based on material specifically eluted from Sepharose-CL6B affinity columns, and are expressed as milligrams of lectin protein recovered/mg wet weight of venom or lectin applied (200 mg of each sample). ND = none detected.

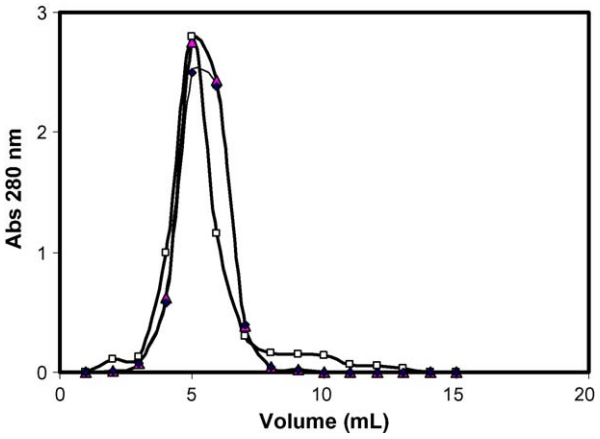

Fig. 1. Affinity chromatography purification of the lectin from *B. jararacussu* venom (200 mg) by a Sepharose-CL 6B column (3 cm × 1 cm, I.D.). The adsorbed proteins were eluted with 20 mM Tris–HCl, pH 7.5, containing 10 mM EDTA (▲), or containing 100 mM IPTG (□), or 1 mM HCl, pH 3.0, containing 150 mM NaCl (■).

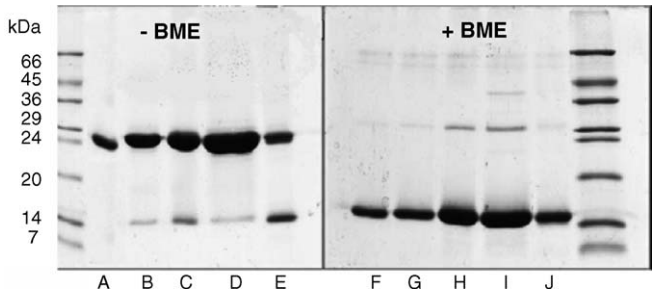

Fig. 2. SDS–PAGE (12%) analysis of lectins purified (25 μg) by affinity on Sepharose CL-6B columns, in presence (+) and absence (−) of β-mercapthoethanol (BME). (A, F) *B. jararacussu*, (B, G) *B. jararaca*, (C, H) *L. m. muta*, (D, I) *B. newiedi*, (E, J) *B. moojeni.* BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and lactalbumin (14.4 kDa) were used as standards for characterization of molecular mass. SDS–PAGE was performed using Laemmi buffers [15] and the gel was stained with Coomassie blue G-250.

studies suggest that the convulxin possesses α-anomeric affinity and show the existence of distinct specificity within C1-snake venom lectins.

Purified lectins on Sepharose-6B affinity chromatography were examined by electrophoresis in SDS–polyacrylamide gels. In the presence of SDS and without the reductor agent β-mercaptoethanol all of the proteins migrated predominantly as a duplet of protein bands with 16 and 32 kDa. Upon reduction with BME the 16 kDa protein was the predominant band (Fig. 2). This result indicates that purified *Botrops sp* and *L. m. muta* lectins appear to be formed from repeats of 16 kDa units dependent in part on disulfide bonds. Several C-type lectins found so far in snake venom are not monomers but disulfide-linked homodimers, however recently, the X-ray crystal structure of the *C. atrox* lectin revealed a decameric structure composed of two five-fold symmetric pentamers [14].

Databank comparison of the N-terminal amino acid sequences determined for the *B. jararacussu* and *B. jararaca* Sepharose CL-6B 32 kDa binding proteins (BP) confirmed that the retained proteins were Ca^{2+}-dependent lectins (data not shown). Sequences identical to *B. jararacussu* 32 kDa-BP were found for the *B. newiedi*, *B. moojeni* and *Lachesis muta rhombeata* 32 kDa CL-6B BP (Fig. 3). These observations were confirmed by the studies carried out without and with the addition of Ca^{2+} in the equilibrating buffer. A weak binding of the lectin to the resin is observed when the chromatography is performed without Ca^{2+} in the equilibrating buffer. Conversely, when the chromatography was carried out in presence of Ca^{2+}, a strong binding of the proteins to the resin was seen, being the optimum condition above 40 mM Ca^{2+} (data not shown). Overall, these studies showed the requirement of Ca^{2+} to form coordination bonds with the sugar and confirmed that all of the snake venom purified proteins in this study are authentically Ca^{2+} dependent lectins.

Snake venom	Sequence
B.jararacussu	NNCPQDWLPM NGLCYKIFDE
	....N.
B. newiedi	NNCPQDWLPM NGLCYKIFDE
B. moojeni	NNCPQDWLPM NGLCYKIFDE
L. muta rhombeata	NNCPQDWLPM NGLCYKIFDE
B. jararaca	.DCPSDWSPY EGHCYKHFIK
	....RV.TE

Fig. 3. NH_2-terminal amino acid sequences of the lectins isolated by affinity through the Sepharose CL-6B column. The isolated letters represent heterogeneous amino acids found.

Table 2
Agglutination activity of the purified *Bothrops neuwiedi* lectin

Cell type	Ca^{2+} (+)	Ca^{2+} (−)
Human BRC-A	++++	+
L.(L.) chagasi	+++	ND
L.(L.) amazonensis	+++	ND
L.(V.) guyanensis	+++	ND
L.(V.) braziliensis	+++	ND

The results were read visually after 4 h (BRC, 37 °C) and after 8 h (parasites, 28 °C) of incubation with 50 mM $CaCl_2$ (+) or without $CaCl_2$ (−) as described in Section 2. BRC, blood red cells. ND = none detected.

The Sepharose CL-6B presents excellent properties of gel filtration and as we have shown here, a new property to bind stereospecificity β-D-galactoside binding lectin-proteins. This new feature makes the method of Sepharose CL-6B-affinity chromatography an interesting alternative for proteomics analysis and three-dimensional crystallographic studies since it is fast, reliable, and consequently amendable to easy scale-up. Other advantages of the technique are: high specificity for a certain carbohydrate structure compared to other affinity chromatography gels.

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