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Research paper

A new chitooligosaccharide specific lectin from snake gourd (*Trichosanthes anguina*) phloem exudate. Purification, physico-chemical characterization and thermodynamics of saccharide binding

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ABSTRACT

A new lectin has been purified to homogeneity from the phloem exudate of snake gourd (*Trichosanthes anguina*) by affinity chromatography on chitin. The snake gourd phloem lectin (SGPL) specifically binds chitooligosaccharides and their inhibitory potency increased with increase in size. PAGE and SDS-PAGE studies indicate that SGPL is a heterodimer, in which the two subunits (48 and 53 kDa) are joined by disulfide bonds. Consistent with this, electrospray-ionization mass spectrum yielded the exact mass of the protein as 104,621.8 Daltons. CD studies showed that SGPL contains about 9% α-helix, 39% β-sheet, 20% β-turns and 32% unordered structures and that saccharide binding does not significantly affect its secondary and tertiary structures. Titration calorimetric studies indicate that the dimeric lectin binds two ligand molecules [$(GlcNAc)_{3-6}$] with association constants determined at 25 °C being 1.7×10^5 and 3.6×10^5 M^{-1}, for chitotriose and chitohexaose, respectively. Binding of all the chitooligosaccharides is governed by enthalpic forces, whereas the contribution from binding entropies was unfavorable. These results suggest that the SGPL-saccharide interaction is stabilized by hydrogen bonding and van der Waals' interactions. Enthalpy–entropy compensation was observed for the SGPL-chitooligosaccharide interaction, suggesting that water molecules play a key role in the binding process.

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1. Introduction

Lectins, a class of carbohydrate-binding and cell-agglutinating proteins, are ubiquitous in nature, being found in a wide variety of organisms such as plants, animals including human beings as well as microbes and fungi [1,2]. The unusual ability of lectins to specifically recognize carbohydrate structures has made them important tools in the study of glycoconjugates in solution and on cell surfaces, in mapping neuronal pathways etc [1–3]. Certain lectins stimulate mitogenesis in lymphocytes whereas some others exhibit a preferential recognition of tumor cells. Lectins are used in a number of clinical and biomedical applications such as blood typing, fractionation of cells for bone marrow transplantation, enzyme replacement therapy for treatment of Gaucher's disease etc [1,3,4].

Among plant lectins, legume seed lectins are the most thoroughly studied family of proteins due to their high abundance and have been characterized well with respect to macromolecular properties and carbohydrate binding specificity [3]. Studies on lectins from other plant families are significantly fewer, and therefore it is important to investigate them and characterize their carbohydrate binding characteristics in detail. Since many species from Cucurbitaceae are cultivated in large quantities in different countries as they form part of the diet, it is of considerable interest to purify and characterize lectins from this family. During the last 30 years or so several lectins have been isolated from the seeds and the phloem exudate of cucurbit species [5–14].

For more than a decade our laboratory has been working on the purification and characterization of lectins from Cucurbitaceae species and reported the purification and characterization of galactose-specific lectins from the seed extracts of several cucurbit

Abbreviations: SGPL, snake gourd phloem exudate lectin; PPL, pumpkin phloem exudate lectin; MCL, *Momordica charantia* lectin; SGSL, snake gourd (*Trichosanthes anguina*) seed lectin; LAA, *Luffa acutangula* agglutinin; CIA, *Coccinia indica* agglutinin; WGA, wheat germ agglutinin; UDA, *Utrica dioica* agglutinin; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$ and $(GlcNAc)_6$, chitooligosaccharides containing, respectively, 2, 3, 4, 5 and 6 GlcNAc residues; DSC, differential scanning calorimetry; Q-TOF, quadrupole time-of-flight; LC-MS, liquid chromatography-mass spectrometry; ITC, isothermal titration calorimetry; CD, circular dichroism; PBS-βME, 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 0.02% sodium azide and 10 mM β-mercaptoethanol.

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species [cf. 9–12]. More recently we started to investigate lectins from the phloem exudate of cucurbits and reported a rapid affinity method for the purification of the pumpkin (*Cucurbita maxima*) phloem exudate lectin (PPL) and characterized it with respect to macromolecular properties and thermodynamics of carbohydrate binding [13,14].

In the present study we report the purification and physicochemical characterization of a new chitooligosaccharide-specific lectin from snake gourd (*Trichosanthes anguina*) phloem exudate. Circular dichroism studies show that the secondary structure of the snake gourd phloem exudate lectin (SGPL) consists predominantly of β-sheet with relatively less α-helical content. Temperature dependent CD and differential scanning calorimetric (DSC) studies indicate that the protein undergoes thermal unfolding around 70 °C. Results of isothermal titration calorimetric (ITC) studies indicate that the dimeric SGPL binds to two ligand molecules and that the binding reaction is driven predominantly by enthalpic forces.

2. Materials and methods

2.1. Materials

Snake gourd fruits were obtained from local vendors. β-Mercaptoethanol, chitin (from crab shells), chitooligosaccharides $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$ and $(GlcNAc)_6$ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulphate, calcium chloride, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, trichloroacetic acid, sodium deoxycholate and acetic acid were obtained from local suppliers and were of the highest purity available.

2.2. Preparation of affinity matrix

The chitin column used for the affinity chromatographic purification of SGPL was prepared as described earlier [13,15]. Briefly, practical grade chitin from crab shells was suspended in 0.25 M NH_4OH and incubated for 60 min. The supernatant was decanted and the material was washed successively with 0.2 M NaCl, double distilled water and 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 0.02% sodium azide and 10 mM β-mercaptoethanol (PBS-βME). The material was finally suspended in PBS-βME, packed in a glass column (16 × 4 cm) and equilibrated again with the same buffer.

2.3. Extraction and purification of SGPL

Snake gourd fruits were bled by 2–3 mm deep longitudinal cuts and the exudate obtained was collected into ice-cold PBS-βME and centrifuged at 9000 rpm for 30 min in an Eppendorf 5810 R refrigerated centrifuge. The supernatant containing soluble protein was subjected to ammonium sulfate precipitation to give 80% saturation, kept at 4 °C overnight and was centrifuged at 9000 rpm for 30 min and the supernatant was discarded. The precipitate obtained was dissolved in minimum volume of water and dialyzed extensively against PBS-βME at 4 °C. The dialyzed sample was centrifuged again and the precipitate was discarded. The supernatant was subjected to affinity chromatography on a column of chitin (16 × 4 cm) that was pre-equilibrated with PBS-βME. The breakthrough obtained was reloaded to ensure complete binding of the protein. The column was then washed with PBS-βME to remove unbound proteins, monitoring absorbance of the eluant at 280 nm. When the absorbance of the column effluent fell below 0.01, the bound protein was eluted with 0.1 M acetic acid at room temperature. Fractions of ca. 5 ml were collected monitoring absorbance at 280 nm. Fractions showing high concentration of protein were pooled and the eluting acid was removed by extensive dialysis against PBS-βME. Homogeneity of the affinity purified protein was assessed by polyacrylamide gel electrophoresis under native conditions as well as upon denaturation in the presence of β-mercaptoethanol according to Laemmli [16]. Protein concentration was estimated by a modified Lowry method [17]. Assay for covalently bound neutral sugar on purified SGPL was done according to the Phenol-sulfuric acid method of Dubois et al. [18], with D-mannose as the standard.

2.4. Hemagglutination and hemagglutination-inhibition assays

Hemagglutination assays were carried out in 96 well ELISA microtitre plates. To each well containing 100 μl of serially diluted lectin or crude extract, 100 μl of a 4% rabbit erythrocyte suspension was added and mixed. The plate was then incubated at 4 °C for 1 h and then the agglutination titre was visually scored. Hemagglutination-inhibition assays were performed in the following manner using 1 mM stock solutions of the chitooligosaccharides, except for chitobiose for which a 52 mM stock was used. In the first well of the microtiter plate, 50 μl of saccharide solution was placed and serially 2-fold diluted. Then 50 μl of purified SGPL (30 μg/ml) or an appropriate amount of crude extract was added to each well. After incubating the mixture at 4 °C for 15 min, 100 μl of a 4% erythrocyte suspension was added, the plate was incubated for one hour at 4 °C and the titer was scored visually.

2.5. Effect of pH and thermal inactivation of SGPL

The dependence of agglutination activity of SGPL on the pH of the medium was investigated by dialyzing the lectin against a buffer of desired pH and then assaying for hemagglutination activity as described previously for PPL [13]. Buffers used for different pH are: 20 mM KCl–HCl (pH 2), 20 mM citrate-phosphate (pH 3–6), 20 mM phosphate (pH 7.4), 20 mM Tris–HCl (pH 8–9) and 20 mM glycine–NaOH (pH 10). All buffers contained 0.15 M NaCl and 10 mM β-mercaptoethanol.

To investigate the effect of temperature on the activity of SGPL, protein samples were incubated at different temperatures for 15 min and cooled to room temperature. The samples were then centrifuged and the clear supernatants obtained were assayed for agglutination activity as described above.

2.6. Mass spectrometry

The mass spectrum of affinity-purified SGPL was recorded using a model 6520 Q-TOF LC-MS high resolution mass spectrometer from Agilent Technologies (Santa Clara, CA, USA).

2.7. Circular dichroism spectroscopy

CD spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: http://www.jascoint.co.jp), equipped with a Peltier thermostat supplied by the manufacturer essentially as described earlier [13]. Concentration of SGPL was 0.7 μM for measurements in the far UV region (250–190 nm) and 13 μM for measurements in the near UV region (300–250 nm). Samples were placed in a 2-mm pathlength rectangular quartz cell and spectra were recorded at a scan speed of 20 nm/min with a response time of 4 s and a slit width of 2 nm. In order to investigate the effect of carbohydrate binding on the secondary and tertiary structure of SGPL, spectra were recorded in the absence as well as in the presence of 1 mM chitotetraose. To investigate the structural changes that take place in the protein

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