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Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (*Galanthus nivalis*) bulbs

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A lectin was isolated from snowdrop (*Galanthus nivalis*) bulbs by affinity chromatography on mannose-agarose. It is a tetrameric protein composed of 4 identical subunits of 13 kDa which are not held together by disulphide bridges. The *G. nivalis* agglutinin exhibits exclusive specificity towards mannose. Although it readily agglutinates rabbit erythrocytes it is completely inactive with human red blood cells.

Characterization; Lectin; Mannose; Purification; (*Galanthus nivalis*)

1. INTRODUCTION

The major proportion of the plant lectins which have been purified and characterized hitherto has been isolated from seeds or vegetative tissues of species belonging to the class Magnoliatae (also called dicotyledons or dicots). Indeed until recently only those isolated from a few Gramineae species such as wheat (*Triticum aestivum*, *T. turgidum*), rye (*Secale cereale*), barley (*Hordeum vulgare*), rice (*Oryza sativa*) and couch grass (*Agropyrum repens*) [1,2] were obtained from representatives of the class Liliatae (also called monocotyledons or monocots). During the last few years, however, evidence has accumulated that phytohemagglutinins are more widespread among monocots. In addition, the isolation and characterization of monocot lectins from such species as tulip (*Tulipa gesneriana*) [3,4], meadow saffron (*Colchicum autumnale*) [5] and the orchid twayblade (*Listera ovata*) [6] have demonstrated that they are distinct from dicot lectins and exhibit several unique properties. This report deals with another monocot lectin from snowdrop (*Galanthus nivalis*) bulbs which has the unique property of recognizing exclusively mannose.

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Abbreviations: GNA, *Galanthus nivalis* agglutinin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Material*

Snowdrop (*G. nivalis*) bulbs were collected locally at the end of the growing period (around the beginning of May). Unless used immediately they were stored dry (at room temperature). Mannose-agarose (Selectin 10) was obtained from Pierce Chemical Company, Rockford, IL, USA). Phenyl Sepharose was a product of Pharmacia Fine Chemicals (Uppsala, Sweden).

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2.2. *Isolation of the* G. nivalis *agglutinin*

Whole bulbs (50 g) were homogenized with a blender in 500 ml of 1 M $(NH_4)_2SO_4$ and the homogenate centrifuged (15 min; 20000 × g). The resulting supernatant was decanted, frozen overnight at −80°C and recentrifuged (15 min; 20000 × g). During the second centrifugation the suspended material formed as a result of the freezing and thawing was completely spun down and a clear extract was obtained, which was applied to a column (10 ml bed volume) of Selectin 10 equilibrated with 1 M $(NH_4)_2SO_4$. Unbound proteins were eluted with 1 M $(NH_4)_2SO_4$ (until the A_{280} of the eluate fell below 0.01) and the lectin desorbed with 20 mM (unbuffered) 1,3-diaminopropane. Alternatively, the lectin can be desorbed with mannose, but high concentrations of sugar (at least 0.2 M) are required and even then the lectin elutes in a large volume (about 200 ml for a column of 10 ml bed volume). Since affinity-purified GNA preparations were slightly contaminated with polyphenolic compounds an additional purification step was included. Lectin fractions (desorbed with 1,3-diaminopropane) were pooled, brought to 1 M $(NH_4)_2SO_4$ by adding solid salt and applied to a column (10 cm × 1.6 cm) of phenyl Sepharose equilibrated with 1 M $(NH_4)_2SO_4$. After washing the column with 50 ml of 1 M $(NH_4)_2SO_4$ the lectin was eluted with distilled water. Under these conditions GNA eluted as a sharp peak leaving behind the (brown) polyphenolic compounds. To ensure complete purity of our GNA preparation, the lectin fraction obtained after hydrophobic interaction chromatography on phenyl Sepharose was dialyzed against 20 mM 1,3-diaminopropane-HCl (pH 9.0) and chromatographed on a Pharmacia Mono-Q anion-exchanger column using a Pharmacia fast protein liquid chromatography system (type GP 250). About 5 mg of lectin was loaded on the Mono-Q column equilibrated with 20 mM 1,3-diaminopropane, pH 9.0. After washing the column with 4 ml of buffer the lectin was eluted using a linear gradient of increasing NaCl concentration (from 0 to 0.4 M) in the same buffer. Peak fractions were collected and used for further analysis.

2.3. *Assays and analysis methods*

Protein was estimated as in [7]. Agglutination assays were carried out with trypsin-treated rabbit erythrocytes as in [4]. SDS-PAGE was done on a 12.5–25% polyacrylamide gradient gel using a discontinuous system as in [8]. Amino acid and carbohydrate analysis were carried out as in [9], the neutral sugars being analyzed by GLC and the amino sugars on the amino acid analyzer. Tryptophan was determined spectrophotometrically.

3. RESULTS

3.1. *Purification of GNA*

Since preliminary experiments with crude extracts from snowdrop bulbs indicated that the agglutinating factor they contain exhibited specificity towards mannose, GNA was purified by affinity chromatography on immobilized mannose. Both the extraction and affinity chromatography were done in 1 M $(NH_4)_2SO_4$ for two reasons. Firstly, the activity of polyphenol oxidases is strongly reduced and secondly, GNA binds much stronger to the affinity column than for instance in PBS. Under the conditions described virtually all lectin activity was retained on the Selectin 10 column (which bound up to 10 mg lectin per ml bed volume). Although the affinity-purified GNA was virtually pure (as can be judged by SDS-PAGE analysis shown in fig.1), an additional

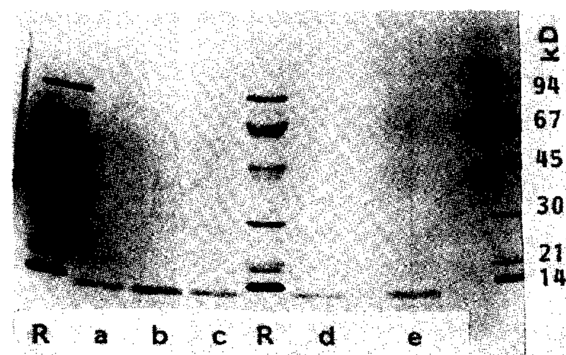

Fig.1. SDS-PAGE of purified GNA. Lanes: a, affinity-purified GNA; b, GNA after chromatography on phenyl Sepharose; c–e, GNA after ion-exchange chromatography. All samples were reduced except that in lane e. About 25 μg protein was loaded per lane. Lanes R contain M_r marker proteins: lysozyme (M_r 14300), soybean trypsin inhibitor (M_r 21000), carbonic anhydrase (M_r 30000), ovalbumin (M_r 45000), bovine serum albumin (M_r 68000) and phosphorylase *b* (M_r 93000).

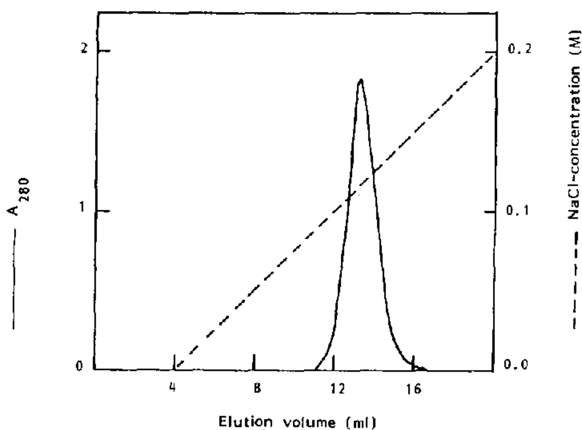

Fig.2. Ion-exchange chromatography of purified GNA. 5 mg of our final GNA preparation was chromatographed on a Mono-Q column (type HR 5/5 from Pharmacia Fine Chemicals) using a Pharmacia FPLC system. After washing the column with 4 ml of 20 mM 1,3-diaminopropane-HCl (pH 9.0), the lectin was eluted using a linear gradient (44 ml) of increasing NaCl concentration (from 0 to 0.4 M) in the same buffer. The flow rate was 2 ml/min.

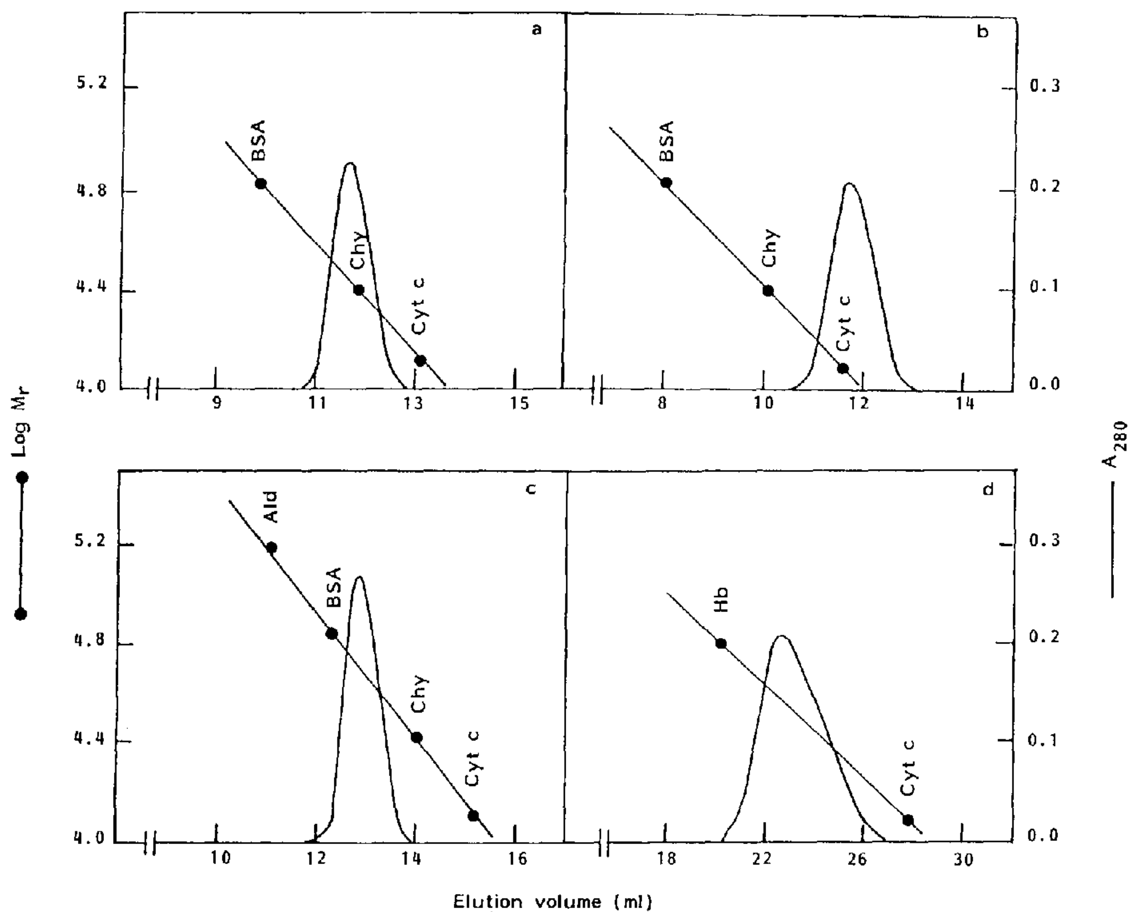

Fig.3. Gel filtration of GNA. (a) Chromatography of 1 mg denatured (with 6 M guanidine HCl) GNA on a Pharmacia Superose 12 (type 10/30) column with 6 M guanidine HCl as running buffer. (b) Chromatography of 1 mg denatured and iodoamidated [10] GNA on the same Superose 12 column with 6 M guanidine HCl as running buffer. (c) Chromatography of 1 mg native GNA on the same Superose 12 column with PBS containing 0.2 M mannose as running buffer. (d) Chromatography of 1 mg native GNA on a Sephadex G-100 column (28 cm × 1.4 cm) with PBS containing 0.2 M mannose as running buffer. Elution positions of M_r marker proteins are indicated: aldolase (Ald, M_r 160000), bovine serum albumin (BSA, M_r 68000), haemoglobin (Hb, M_r 65000), chymotrypsinogen (Chy, M_r 25000), cytochrome *c* (Cyt c, M_r 12500).

hydrophobic interaction chromatography and ion-exchange chromatography was included to ensure complete purity of the lectin. Since our final lectin preparation yielded a single polypeptide band upon SDS-PAGE (fig.1) and in addition, eluted in a single symmetrical peak from the Mono-Q column (fig.2), it can be considered as homogeneous. The overall yield of affinity-purified GNA was 2.5 mg/g bulb tissue (fresh wt). During subsequent hydrophobic interaction chromatography and ion-exchange chromatography about 20% of the total agglutination activity was lost.

3.2. *Molecular structure and biochemical properties*

Both reduced (with 2% β-mercaptoethanol) and unreduced GNA migrated as a single polypeptide band of M_r 13000 upon SDS-PAGE (fig.1). Since, in addition, a single symmetrical peak of M_r 12000 was obtained upon gel filtration in 6 M guanidine hydrochloride of both unreduced and iodoamidated GNA (fig.3), the lectin probably contains a single polypeptide. Gel filtration of native GNA in the presence of 0.2 M mannose on both a Superose 12 and a Sephadex G-100 column indicated an M_r around 50000 (fig.3) (in the absence of mannose GNA strongly bound to both gel filtration gels), a value which was confirmed by sucrose density gradient centrifugation (not shown). It is evident therefore that GNA is a tetrameric protein built up of 4 identical subunits of M_r 13000. Amino acid analysis indicated high contents of asparagine/aspartic acid, glycine, serine and leucine; no methionine could be detected. The lectin does not contain any amino sugar and only low levels of neutral sugars, it is likely that these are contaminants and that the protein is not glycosylated (table 1). Purified GNA is stable within the pH range between 3 and 12, and withstands heating at 70°C for 10 min. Even after heating in a boiling water bath for 10 min, 20% of the initial agglutination activity was retained. The agglutination activity is fully preserved after reduction with 1 M β-mercaptoethanol, which indicates that GNA subunits are not held together by disulphide bridges.

3.3. *Carbohydrate-binding specificity and agglutination properties*

GNA readily agglutinates rabbit erythrocytes,

Table 1

Amino acid composition of GNA

Amino acid	mol%
Asx	15.4
Thr	7.7
Ser	10.5
Glx	7.2
Pro	4.1
Gly	12.0
Ala	3.5
Cys	1.7
Val	4.8
Met	0.0
Ile	5.4
Leu	8.9
Tyr	5.0
Phe	2.1
His	1.0
Lys	4.2
Trp	3.0
Arg	3.5
Total	100.0
Sugar	
Xyl, Fuc, Ara	<0.1
Man	1.2
Gal	1.4
Glc	2.2

Table 2

Carbohydrate-binding specificity of GNA

Sugar, polysaccharide or glycoprotein	Concentration required for 50% inhibition of the agglutination of trypsin-treated rabbit erythrocytes in the presence of 10 μg/ml GNA
Mannose	9 mM
Glycogen	1 mg/ml
Amylose	1 mg/ml
Dextran	0.5 mg/ml
Thyroglobulin	1 mg/ml

Other sugars which were tested (L-fucose, D-glucose, maltose, D-fructose, sucrose, trehalose, cellobiose, melibiose, galactose, raffinose, lactose, rhamnose, arabinose, ribose, xylose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, glucosamine, galactosamine) were not inhibitory at concentrations below 200 mM; also the glycoproteins fetuin, asialofetuin and ovomucoid had no inhibitory effect at concentrations below 5 mg/ml

minimal lectin concentrations required being 1.8 and 0.8 μg/ml with untreated and trypsin-treated cells, respectively. Human red blood cells, however, irrespective of their type are not agglutinated even at GNA concentrations as high as 5 mg/ml. The carbohydrate-binding specificity of GNA was determined in hapten inhibition assays. From all mono- and oligosaccharides tested only D-mannose was inhibitory. Also polymers of glucose such as amylose, glycogen and dextran had some inhibitory effect. Finally, from the glycoproteins tested only thyroglobulin was slightly inhibitory but only at a relatively high concentration (table 2).

4. DISCUSSION

Snowdrop bulbs contain reasonable amounts of a lectin which can readily be isolated by affinity chromatography on immobilized mannose. This lectin, which is the first to be isolated from a species of the family Amaryllidaceae, differs with respect to its molecular structure from all known monocot (and dicot) lectins such as those from grasses, tulip and meadow saffron [1–5]. In addition, GNA exhibits also a very particular carbohydrate-binding specificity which is distinct from that of the mannose/glucose binding lectins from legumes such as jackbean (*Canavalia ensiformis*) and pea (*Pisum sativum*) in that GNA recognizes exclusively mannose. A similar exclusive specificity towards mannose has been observed for a lectin from leaves of the orchid twayblade (*L. ovata*). However, the *L. ovata* agglutinin definitely differs from GNA with respect to its affinity for glycoproteins since its agglutination activity is strongly inhibited by thyroglobulin and asialofetuin which as shown in table 2 have very little effect on that of GNA. Moreover, GNA does not bind to fetuin-agarose whereas this affinity matrix is well suited for the isolation of the *L. ovata* agglutinin.

GNA represents apparently a new type of plant lectin with a unique carbohydrate-binding specificity. Since this lectin can easily be isolated in reasonable amounts from readily available material it can be of great potential use as a biochemical tool.

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