Isolation and partial characterization of an *N*-acetylgalactosamine-specific lectin from winter-aconite (*Eranthis hyemalis*) root tubers

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A lectin was isolated from root tubers of winter aconite (*Eranthis hyemalis*) by affinity chromatography on fetuin–agarose, and it was partially characterized with respect to its biochemical, physicochemical and carbohydrate-binding properties. The *Eranthis hyemalis* lectin is a dimeric protein (M_r 62000) composed of two different subunits of M_r 30000 and 32000, held together by disulphide bonds. It is especially rich in asparagine/aspartic acid, glutamine/glutamic acid and leucine, and contains 5% covalently bound carbohydrate. Hapten inhibition assays indicated that the winter-aconite lectin is specific for *N*-acetylgalactosamine. In addition, the lectin exhibits a pronounced specificity towards blood-group-O erythrocytes. The winter-aconite lectin is the first lectin to be isolated from a species belonging to the plant family Ranunculaceae. It appears to be different from all previously described plant lectins.

At present, over 100 plant lectins have been isolated and characterized with respect to their biochemical and molecular structure and carbohydrate-specificity (Goldstein & Hayes, 1978). In the past, lectins have mostly been studied in dry seeds, and especially in seeds of legumes. However, during the last few years, increasing interest has been drawn to lectins occurring in various types of vegetative tissues of a number of plant species. Well-known examples of typical non-seed lectins are the tuber, leaf and fruit lectins from Solanaceae species (Allen *et al.*, 1978; Kilpatrick, 1980), phloem lectins from Cucurbitaceae species (Sabnis & Hart, 1978; Allen, 1979), root lectins from pokeweed (*Phytolacca americana*) (Waxdal, 1974) and white bryony (*Bryonia dioica*) (Peumans *et al.*, 1984) and bark lectins from black locust (*Robina pseudoacacia*) (Horejsi *et al.*, 1978) and elder (*Sambucus nigra*) (Broekaert *et al.*, 1984).

In the present paper we describe the isolation and partial characterization of an *N*-acetylgalactosamine-specific lectin from root tubers of winter aconite (*Eranthis hyemalis*), a representative of the plant family Ranunculaceae.

Abbreviations used: EHL, *Eranthis hyemalis* lectin; PBS, phosphate-buffered saline [1.5 mM-KH₂PO₄/10 mM-Na₂HPO₄ (pH 7.4)/3 mM-KCl/140 mM-NaCl].

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Materials and methods

Materials

Winter-aconite plants were collected locally in March and the tubers were either used immediately or stored at -20°C .

All reagents used were analytical grade. Fetuin–agarose was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Extraction and purification of the lectin

Winter-aconite tubers (20 g) were cut into small pieces and homogenized with a mixer in 250 ml of ice-cold PBS containing 5 mM-thiourea. After being left on ice for 30 min, the homogenate was decanted and the settled slurry re-extracted by mixing in another 250 ml of ice-cold PBS. Then both fractions were combined, stirred for 1 h in the cold and centrifuged (10000g, 10 min). The resulting supernatant was decanted and frozen overnight at -80°C . After thawing, the material that coagulated during freezing was spun down by centrifugation (10000g, 10 min) and the resulting supernatant was taken off and filtered through filter paper (Whatman 3 MM) (to remove any particulate material). Then the clear filtrate was applied to a column (5 ml bed volume) of fetuin–agarose equilibrated with PBS. Unbound protein was eluted by washing the column with PBS and

1 M-NaCl consecutively until the A_{280} fell below 0.01. Finally the bound lectin was desorbed with distilled water (Fig. 1a). It is worth mentioning that lactose (which, as is discussed below, is an efficient inhibitor of the agglutination activity of the winter-aconite lectin) did not desorb the lectin even when applied at a concentration as high as 0.3 M. Since other lectins such as elder bark lectin

(Broekaert *et al.*, 1984) and *Bryonia dioica* agglutinin (Peumans *et al.*, 1984) could easily be desorbed from the same column with lactose, the unusual behaviour of the winter-aconite lectin is, most probably, an intrinsic property of this lectin.

To ensure the purity of the *Eranthis hyemalis* lectin (EHL), the affinity-purified lectin was further purified by hydroxyapatite and ion-ex-

Fig. 1. Affinity chromatography (a), hydroxyapatite chromatography (b) and DEAE-Bio-Gel ion-exchange chromatography (c) of EHL

(a) Crude extract from the tubers (20g fresh wt. homogenized in 500 ml of PBS containing 5 mM-thiourea) was applied to a fetuin-agarose column (5 ml bed volume). Unbound protein was eluted with PBS and NaCl (1 M) until the A_{280} fell below 0.01, and the lectin was desorbed with distilled water. (b) Affinity-purified lectin was loaded on to a hydroxyapatite column (10 ml bed volume). Unbound impurities were washed off with 0.5 M-NaCl, and the lectin was eluted with 0.3 M-potassium phosphate buffer, pH 7.2. (c) Peak fractions of EHL from the hydroxyapatite column were applied to a column of DEAE-Bio-Gel (10 ml bed volume). After the column had been washed with 10 mM-Tris/HCl buffer, pH 8.7, the lectin was eluted with a salt gradient (0–0.4 M-NaCl in the same Tris buffer).
 ●—●, A_{280} ; ○—○, agglutination titre; ·····, NaCl gradient.

change chromatography. The lectin-containing fractions desorbed from the fetuin-agarose were applied to a column (10 ml bed volume) of hydroxyapatite (granular 80–200-mesh ASTM; Merck, Darmstadt, Germany) equilibrated with distilled water. Unbound impurities were washed off with 0.5 M-NaCl, until the A_{280} fell below 0.01. Then the lectin was eluted with 0.3 M-potassium phosphate buffer, pH 7.2 (Fig. 1b), dialysed against 10 mM-Tris/HCl buffer, pH 8.7, and applied to a column (10 ml bed volume) of DEAE-Bio-Gel (Bio-Rad Laboratories, Richmond, CA, U.S.A.) equilibrated with 10 mM-Tris/HCl buffer, pH 8.7. After the column had been washed with the same buffer, the lectin was eluted with a linear gradient (50 ml) of 0–0.4 M-NaCl in 10 mM-Tris/HCl buffer, pH 8.7 (Fig. 1c). Peak fractions were collected, dialysed against PBS and used immediately or stored at -20°C .

The yield and efficiency of the purification procedure are summarized in Table 1.

Agglutination assays

Agglutination assays were carried out, in small glass tubes, in a final volume of 0.1 ml containing 80 μl of a 1% suspension of untreated or trypsin-treated erythrocytes and 20 μl of crude extracts or lectin solutions (each serially diluted with 2-fold increments). Agglutination was monitored visually after the tubes had been left for 1 h at room temperature.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Total neutral sugar was determined by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956), with D-glucose as standard. Amino sugars in a hydrolysed sample were determined in an amino acid analyser.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed with a discontinuous system (Laemmli, 1971) on 12.5–25%-(w/v)-acrylamide gradient gels.

Amino acid analysis

Lectin samples were hydrolysed (in sealed glass tubes under an N_2 atmosphere) for 24, 48 and 72 h at 110°C in 6 M-HCl containing 0.02% 2-mercaptoethanol. After hydrolysis, HCl was removed by evaporation, and the residue was dissolved in sodium citrate buffer, pH 1.9. Amino acid analysis was done with a Biotronik LC 2000 amino acid analyser. This apparatus allows a separation of all 20 amino acids on a single column of Durrum resin, by using an elution program consisting of four different buffers. Amino acids were directly quantified by using an Infotronics integrator. Tryptophan was determined after hydrolysis with 3 M-mercaptoethanesulphonic acid. Cysteine was determined as cysteic acid after hydrolysis in performic acid.

Results

Occurrence, agglutination properties and carbohydrate-specificity of the lectin in winter-aconite tubers

Crude extracts from winter-aconite tubers were found to exhibit a reasonable agglutination activity. Preliminary experiments indicated that the lectin is blood-group-O-specific, since titres obtained with erythrocytes of that blood group are about 10 times those obtained with group-A and group-B erythrocytes (Table 2). The carbohydrate-specificity of the agglutinin in crude extracts was determined by hapten inhibition tests with a series of simple sugars (*N*-acetylgalactosamine, galactose, glucose, *N*-acetylglucosamine, galactosamine, glucosamine, lactose, melibiose, fucose, arabinose, ribose, fructose, trehalose, sorbose,

Table 1. *Purification of EHL*

The procedure started from 20 g of winter-aconite tubers, homogenized in 2×250 ml of PBS. Further details are given in the Materials and methods section. One unit of agglutination activity corresponds to the amount of lectin required for agglutination of 1 ml of trypsin-treated human blood-group-O erythrocytes.

Purification step	Volume (ml)	Total protein (mg)	Total agglutination activity (units)	Specific agglutination activity (units/mg of protein)	Recovery (%)
Crude extract	500	924	200 000	216	100
Freezing (-80°C)	500	749	200 000	267	100
Affinity chromatography on fetuin-agarose	50	27.8	180 000	6471	90
Hydroxyapatite chromatography	25	22.5	160 000	7111	80
Ion-exchange chromatography on DEAE-Bio-Gel	36	19.1	140 000	7330	70

Table 2. *Blood-group-specificity of crude tuber extract and purified EHL*

The crude extract was prepared by homogenizing tuber tissue in 5 vol. (w/v) of PBS. Specific activity is expressed as the minimal concentration (in $\mu\text{g/ml}$) required for agglutination.

Blood group	Titre of crude extract with trypsin-treated human erythrocytes	Specific activity of purified EHL	
		With untreated human erythrocytes	With trypsin-treated human erythrocytes
A	200	100	1
B	200	100	1
O	2000	10	0.1

Table 3. *Carbohydrate-binding specificity of EHL in crude extract and purified preparations*

Agglutination activity was assayed with trypsin-treated human blood-group-O erythrocytes. The agglutination titre of the crude extract was 10 (which corresponds to a lectin content of about $1 \mu\text{g/ml}$).

Carbohydrate	Minimal concentration required for 50% inhibition of agglutination (mM)	
	With crude tuber extract	With purified EHL ($1 \mu\text{g/ml}$)
N-Acetylgalactosamine	0.25	0.25
Lactose	1.0	1.0
Galactose	2.0	2.0
Melibiose	3.0	3.0
Fucose	6.0	6.0
Ribose	9.5	9.5
Arabinose	11.5	11.5
Raffinose	12.5	12.5
Galactosamine	16.0	16.0
Fetuin	100*	100*

* Results expressed in $\mu\text{g/ml}$.

xylose, sucrose, maltose, sorbitol and mannose). As shown in Table 3, N-acetylgalactosamine was the best inhibitor, being respectively 4, 8 and 12 times as potent as lactose, galactose and melibiose. Fucose, ribose, arabinose, raffinose and galactosamine also inhibited the agglutination, but only at higher concentrations. The agglutination of tuber extract was also efficiently inhibited by fetuin (0.1 mg/ml).

Purification of EHL

EHL could be purified by affinity chromatography on fetuin-agarose. The lectin was quantitatively bound (Fig. 1a) and could easily be desorbed with distilled water. Further purification by hydroxyapatite and ion-exchange chromatography yielded a homogeneous preparation. As shown in Table 1, the yield is about 1 mg/g of tuber tissue (on a fresh-weight basis). Taking into consideration an overall yield of 70% (Table 1), EHL represents about 3% of the total soluble protein in the crude extract. Since in extracts from other parts of the plants no agglutination activity could be detected, EHL has to be considered as a tuber-specific protein.

Molecular structure of EHL

Purified EHL was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis both in the presence and in the absence of 2% (v/v) 2-mercaptoethanol. As shown in Fig. 2, non-reduced EHL migrated as a single band of M_r 62000, whereas the reduced protein yielded two bands of M_r 30000 and 32000 (indicating that the lectin molecules are built up of two different types of subunits, held together by disulphide bonds).

The M_r of native EHL was estimated by gel filtration and sucrose-density-gradient centrifugation. As shown in Fig. 3, both centrifugation and gel filtration indicated an M_r of about 60000. It therefore appears that EHL is a dimeric molecule composed of two different subunits held together by disulphide bonds.

The amino acid composition of EHL is given in Table 4. The lectin is rich in asparagine/aspartic acid ($13.53 \text{ mol/100 mol}$), glutamine/glutamic acid ($10.57 \text{ mol/100 mol}$) and leucine (9.41 mol/100 mol), but contains little methionine and cysteine. It contains also 5% covalently bound carbohydrate. No amino sugars could be detected by the amino acid analyser.

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified EHL in the presence (a) and in the absence (c) of 2% 2-mercaptoethanol

For experimental details see the text. M_r reference proteins (b) were lysozyme (M_r 14300), soya-bean trypsin inhibitor (M_r 21000), carbonic anhydrase (M_r 30000), ovalbumin (M_r 45000), bovine serum albumin (M_r 67000) and phosphorylase b (M_r 94000). Gels were stained with Coomassie Brilliant Blue.

Agglutination properties of EHL

Hapten inhibition assays indicated that crude extract and purified EHL exhibit the same carbohydrate-binding specificity (Table 3) and preferential agglutination of blood-group-O erythrocytes. Indeed, the specific agglutination activity of EHL is 10-fold lower with group-A or group-B erythrocytes (Table 2). Moreover, trypsin-treated erythrocytes (of all three blood groups) were 100 times more sensitive to EHL than were untreated cells (Table 2). Besides human erythrocytes, EHL also agglutinates rabbit erythrocytes. Minimal lectin concentrations required for agglutination of untreated and trypsin-treated rabbit erythrocytes were 0.2 $\mu\text{g}/\text{ml}$ and 3 $\mu\text{g}/\text{ml}$ respectively.

Stability of EHL under different conditions

The stability of EHL was investigated under different conditions of temperature, pH and salt

Fig. 3. Sephadex G-100 gel filtration (a) and sucrose-density-gradient centrifugation (b) of EHL

(a) To avoid interactions between EHL and the Sephadex matrix (which result in a retarded elution of the lectin), gel filtration was done in 15 mM-ethylenediamine, pH 10.5, under which condition EHL has no sugar-binding activity. Column dimensions were 1.4 cm $\times$ 28 cm. M_r marker proteins were *Ricinus communis* agglutinin (RCA, M_r 120000), haemoglobin (Hb, M_r 65000) and cytochrome c (Cyt c, M_r 12400). (b) EHL was centrifuged in a linear 12–38% (w/v) sucrose gradient (in PBS) at 2°C for 20 h at 50000 rev./min in a Beckman SW 50.1 rotor. Gradients were fractionated into 0.2 ml portions with an ISCO density-gradient fractionator and assayed for agglutination activity (—) and A_{420} (---). *Ricinus communis* agglutinin (RCA, M_r 120000) and haemoglobin (Hb, M_r 65000) were used as M_r markers.

concentration. EHL was stable over the pH range 2–12 (Fig. 4b), and it was not inactivated by NaCl at concentrations up to 5 M. However, EHL is rather sensitive to heat. Indeed, above 30°C its agglutination activity is progressively diminished (Fig. 4a). Total loss of activity was observed upon heating at 70°C.

Discussion

Winter-aconite tubers contain considerable amounts of an *N*-acetylgalactosamine-specific lectin, which can readily be isolated by affinity chromatography on fetuin-agarose. Purified EHL has been characterized and appears to be a glycoprotein composed of two non-identical subunits of M_r 30000 and 32000 held together by disulphide bonds. Since EHL is the first lectin to be isolated from a species of the family Ranunculaceae, no

Fig. 4. Heat-stability (a) and pH-stability (b) of the agglutination activity of EHL

(a) Portions of an EHL solution (0.1 mg/ml in PBS) were heated in a water bath for 3 min at the temperatures indicated, quenched cold in ice and assayed for agglutination activity. Results are expressed as percentages of the control (which was kept at 20°C) value. (b) Portions of an EHL solution (0.1 mg/ml in PBS) were adjusted to different pH values (by adding 0.1 M-HCl or 0.1 M-NaOH). After being left for 1 h at room temperature, samples were adjusted to 0.2 M-Tris/HCl buffer, pH 7.8, and assayed for agglutination activity. Results are expressed as percentages of the control (samples kept in PBS) value.

Table 4. Amino acid composition of EHL

Amino acid	Amino acid composition	
	(mol/100 mol)	(residues/molecule*)
Asp	13.53	71
Thr	6.31	33
Ser	7.34	9
Glu	10.57	55
Pro	4.64	24
Gly	7.22	48
Ala	6.44	34
Val	5.54	29
Met	1.03	5
Ile	6.96	37
Leu	9.41	49
Tyr	3.35	18
Phe	3.22	17
His	1.42	7
Lys	1.03	5
Arg	7.09	37
Trp	2.45	13
Cys	2.45	13

* Expressed as the number of residues per molecule, assuming an M_r of 59 000 (exclusive of carbohydrates).

comparison can be made with lectins from related species. Moreover, the winter-aconite lectin differs from previously described phytohemagglutinins. Indeed, EHL resembles only one plant lectin with respect to its molecular structure and sugar-binding specificity, namely that from bryony (*Bryonia dioica*) root stocks (which is also an *N*-acetylgalactosamine-specific lectin composed of two non-identical subunits of M_r 30 000 and 32 000

held together by disulphide bridges) (Peumans *et al.*, 1984). However, unlike the bryony lectin, which is not blood-group-specific, EHL preferentially agglutinates group-O erythrocytes. In addition, the specific agglutination activity of the winter-aconite lectin is 20 times higher than that of the bryony lectin. Besides differences in agglutination properties, EHL and bryony lectin differ also with respect to their abundance and distribution over the plant. Whereas the bryony lectin occurs in all vegetative tissues and represents only 0.4% of the total protein in the root stocks (Peumans *et al.*, 1984), EHL occurs exclusively in the root tubers, where it represents up to 3% of the total soluble protein.

Although EHL agglutinates preferentially blood-group-O erythrocytes, it cannot be classified with other anti-O plant lectins. Indeed, whereas the agglutinins from seeds of *Lotus tetragonobulus* (Asparagus pea) (Pereira & Kabat, 1974) and *Ulex europeus* (gorse) (Pereira *et al.*, 1979) exhibit specificity towards fucose and the *Cytisus sessifolius* (Matsumoto & Osawa, 1974) and *Laburnum alpinum* (Matsumoto & Osawa, 1971) seed lectins are best inhibited by *NN'*-diacetylchitobiose, EHL is an *N*-acetylgalactosamine-specific lectin.

The presence of high concentrations of lectin in a typical vegetative storage tissue such as winter-aconite root tubers leaves intriguing questions about its physiological role. At present, only speculations can be made. It might be possible, however, that the function of (abundant) lectin in these vegetative storage tissues is similar to that of the (abundant) agglutinins in storage organs of seeds (e.g. cotyledons of legume seeds).

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