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QUARTERLY

Comparative biochemical analysis of lectin and nuclease from *Chelidonium majus* L.

Ewa Fik[1]✉, Michele Dalgalarrondo[2], Thomas Haertlé[2] and Anna Goździcka-Józefiak[1]

[1]*Institute of Molecular Biology and Biotechnology, Międzychodzka 5, 60-371 Poznań, Poland*
[2]*Groupe Protéines, Laboratoire d'Etude des Interactions des Molécules Alimentaires, Institut National de la Recherche Agronomique, BP 71627, 44316-Nantes Cdx 03, France*

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It has been recently recognized that lectins exhibit other activities besides hemagglutination. Previously we have found that purified lectin from *Chelidonium majus* showed DNase activity (Fik, Goździcka-Józefiak & Kędzia, 1995, *Herba Polon*. 41, 84–95). Comparison of lectin and DNase from the sap from leaves and roots of *Chelidonium majus* proved that both these compounds are composed of 24 kDa monomer subunits which have an identical N-terminal sequence but differ in amino-acid composition and degree of glycosylation. Possible interrelationship between lectin and DNase is discussed.

Lectins are carbohydrate binding proteins that are widely distributed among plants, animals and microbes [1]. Mature seeds are the main source of plant lectins, however, lectins are also present in small amounts in other tissues such as leaves and roots. The majority of plant lectins are secretory proteins that accumulate either in the vacuole or extracellular matrix [2]. Most plant tissues contain one lectin, but in some cases they, contain two (or more) lectins that differ in their biological properties [3–5].

Generally, lectins are glycoproteins consisting of subunits ranging in molecular mass from 25 to 35 kDa, arranged as dimers or tetramers, and existing as multiple isoforms sharing similar biochemical properties [6]. The subunits are usually identical or very similar, made up of single polypeptide chains that are encoded by different genes or by a family of closely related genes [7, 8]. In addition to their carbohydrate binding activity, some of the lectins such as those of hemagglutinin, galactosidase, polynucleotide adenosine glucosidase, or ribosome inactivating proteins show also other biological activities [9]. The lectin isolated from the sap of *Chelidonium majus* L. (further referred to as CM lectin) is

✉To whom correspondence should be addressed.

Abbreviations: CMD, DNase isolated from *Chelidonium majus* L.; CML, lectin isolated from *Chelidonium majus* L., i.d., inside dimension.

associated with the other protein which exhibits DNase activity. Both proteins have oligomeric structure, each being composed of 24 kDa monomer subunits differing in amino-acid composition charge and carbohydrate content [10]. CM lectin stimulates significantly proliferation of human lymphocytes, shows hemagglutination activity towards human erythrocytes of group B, has antimicrobial properties against multiresistant enterococci and staphylococci [11], and induces apoptosis in the CHO (Chinese hamster ovary) and epithelial cells of rat and mouse thymus cell lines [12]. The protein with DNase activity exhibit weak mitogenic and bacteriostatic effect [11, 12].

The aim of the present study was to explore the relation between lectin and DNase of *Chelidonium majus*.

MATERIALS AND METHODS

Plant material. *Chelidonium majus* plants were collected in the neighbour hood of Poznań during summer. The milky sap was isolated from leaves and roots of the plants and stored at −70°C or −20°C prior to being used.

Extraction and isolation of glycoproteins. Proteins were extracted from the yellow milky sap from leaves and roots of *Chelidonium majus*. The crude protein extract was prepared according to Diffey & Stillman [13]. Protein fraction precipitated by ammonium sulphate (final concentration 0.8 M) was dissolved at 0.02 M Tris/HCl buffer containing 30% glycerol and was loaded on a Con A-Sepharose column equilibrated with 0.02 M Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl. The column was eluted with the same buffer and a linear gradient of 0 to 0.2 M methyl-α-D-glucopyranoside (methylglucoside) in the same buffer. Fractions of about 300 μl were collected and the absorbance at 280 nm, hemagglutination and DNase activity of all fractions were determined. The fractions showing lectin and DNase activity were pooled and proteins were precipitated with acetone. Protein concentration was determined according to Lowry *et al.* [14]. Purity of the separated proteins was checked by SDS/PAGE according to Laemmli [15] and by RP-HPLC.

Reverse phase HPLC (RP-HPLC). The HPLC equipment consisted of an auto-sampling injector model 231 (Gilson, France), with Millennium solvent delivery model 996 (Waters Associates, Milford, U.S.A.), a peak separator model 2150 and a fraction collector (ISCO, Lincoln, Nebraska, U.S.A.). The active fractions (CML and CMD) separated by affinity chromatography were digested by trypsin and pepsin and fractionated by RP-HPLC on a Nucleosil C-18 column (4 mm i.d. × 25 cm) equilibrated with solvent A (0.11% trifluoroacetic acid, pH 2.5). The elution was carried out for 40 min using a gradient from solvent A to solvent B (80% acetonitrile, 20% H_2O, 0.09% trifluoroacetic acid). Both the column and the solvents were maintained at 40°C. The flow rate was 0.6 ml/min and the absorbance was recorded at 214 nm. The fractions collected were dried in a Speed Vac Concentrator (Speed Vac Plus S.C. 110A (SAVANT)).

Hemagglutination assay. Assays for hemagglutination activity were performed as described by Kabat & Mayer [16]. The aliquots contained in a final volume of 80 μl: 50 μl of 1 M NaCl and 0.5 mM $CaCl_2$ in 0.05 M Tris/HCl buffer, pH 7.5, 25 μl of the suspension of washed 3 times human erythrocytes (group A, AB, B, 0), and 5 μg of the lectin solution. After gentle mixing, the aliquots were incubated for 30 min at 37°C or for 24 h at 4°C. Agglutination was monitored macroscopically.

DNase activity. DNase activity was assessed colorimetrically as described by Burton [17].

Amino-acid analysis. After acid hydrolysis under vacuum in 6 M HCl for 24 h at 110°C in Pico Tag Stations (Waters), amino acids were

derivatized with PITC (phenylizothiocarbamyl aminoacid) according to Bidlingmeyer *et al.* [18] and quantified by RP-HPLC on a Pico Tag C-18 column (3.9 mm i.d. × 15 cm, Waters).

Dried samples were dissolved in 95% 2 mM Na_2HPO_4, pH 7.4, containing 5% acetonitrile. The column was equilibrated with solvent C (0.14 M CH_3COONa in 6% acetonitrile containing 0.5 ml TAE (Tris/acetate/EDTA buffer), pH 6.4, and eluted with a gradient from solvent C to solvent D (40% H_2O, 60% acetonitrile). Both the column and the solvents were maintained at 38°C. The flow rate was 1 ml/min and the absorbance was recorded at 254 nm.

Deglycosylation of glycoproteins. Samples of glycoproteins were incubated successively with 1 M and 2 M HCl for 1, 1.5, 3 and 4 h at 50°C. The deglycosylated proteins were digested by trypsin and pepsin and the peptides were separated by RP-HPLC.

Amino-acid sequence determination. The N-terminal sequence of amino acids was determined by Edman degradation with the Applied Biosystems 477A protein sequencer. Internal sequence were determined in proteins purified by RP-HPLC. The amino acid analysis was performed on Applied Biosystems 120A derivatizer, in which free amino acids were converted to phenyl thiohydantoin amino acids (PTH-amino acids), followed by separation on a PTH C-18 185 μm reverse phase column (2.1 μm i.d. × 220 cm, Applied Biosystems) and detection at 254 nm.

Staining of polyacrylamide gel. For detection of glycoproteins in polyacrylamide gel the carbohydrate-specific periodic acid Schiff (PAS) staining method was used as described by Carlson [19]. The whole incubation was performed by slow agitation at room temperature.

Isoelectric focusing (IEF). Isoelectric points of the lectin and DNase was measured using the LKB-2117 MULTIPHOR II (Bromma, Sweden). About 5–10 μg of protein was applied on the gel. The solution of 0.33% aspartic acid and 0.37% L-glutamic acid was added to the anode, and that containing 0.4% arginine and lysine to the cathode. Focusing was carried out in 5% ultrathin layer of polyacrylamide gel Servalyt Precote (Serva), containing 2% ampholine, pH 3–10, at 2°C for 4 h. The gel was then fixed in 20% trichloroacetic acid (TCA), stained with 0.1% Serva blue W in 5% TCA, and destained in 1% TCA.

Polyacrylamide gel electrophoresis. SDS/polyacrylamide gel electrophoresis and urea/polyacrylamide gel electrophoresis of glycoproteins was carried out according to Laemmli [15], using 10% polyacrylamide as the separating gel and 5% polyacrylamide as the stacking gel. The proteins were reduced by heating at 100°C in the presence of 2-mercaptoethanol for 5 min. Then, gels were calibrated using the following standard proteins: phosphorylase *b* (M_r 97 000), bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000) and α-lactalbumin (M_r 14 200). Gels were fixed and stained with Coomassie blue R250.2. Alkaline urea/PAGE electrophoresis and electrophoresis under acidic conditions at pH 4.3 were after Bollag & Edelstein [20].

RESULTS

Isolation from *Chelidonium majus* L. sap of glycoproteins: lectin and DNase was achieved in two steps: ammonium sulphate fractionation of crude extract and affinity chromatography on Con A-Sepharose column as described by Fik *et al.* [10]. Purity of the two proteins in separated fractions was confirmed by SDS/PAGE (Fig. 1). Both activities were eluted in a single peak, but within its peak lectin and DNase were distinguished in separate fractions. Each yielded a single protein band of 24 kDa, whereas the molecular mass of native lectin and DNase eluted from Sephadex G-100 column was 164 and 84 kDa, respectively [10]. It is suggested that both pro-

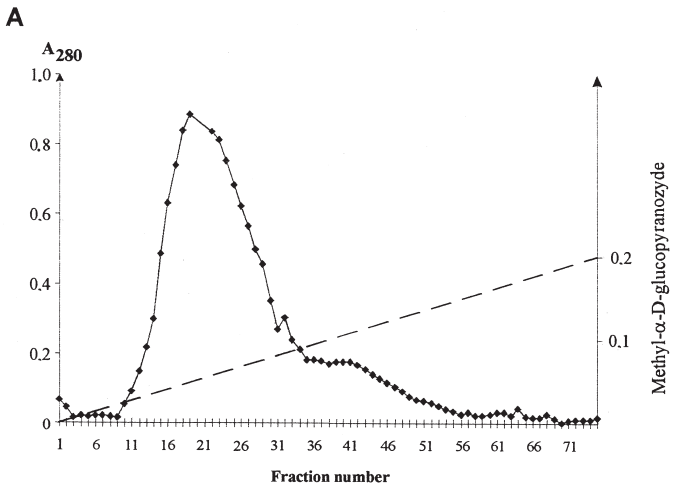

Figure 1. A. Fractionation of protein extract from *Chelidonium majus* juice by affinity chromatography on Concanavalin A-Sepharose column.

Fractions 11–20: lectin; Fractions 21–27: DNase activity.

teins studied can be oligomeric forms composed of identical subunits of 24 kDa. Both proteins were glycosylated as confirmed by positive reaction with Schiff's reagent.

Analytical isoelectric focusing of lectin indicated the existence of different isoforms with pIs of, approximately, 7.4, 8.2 and 8.5. On the other hand, CMD migrated in the opposite direction and contained poorly focusing isoforms of pH about 5.8 (Fig. 2).

Urea/PAGE carried out at alkaline pH detects lectin and a very weak DNase band (Fig. 3A). In contrast, the same electrophoresis carried out at acidic pH with reversed electrode polarity (+ on the top), showed only the band of DNase (Fig. 3B). This is clearly indicative of opposite charges of the studied proteins at pH close to 7. HPLC elution profiles of lectin and DNase are similar, showing the same elution times and displaying three peaks (one major and two smaller) (Fig. 4). This might suggest that the native lectin and DNase are formed as different combinations of different subunits which have a similar molecular mass. The proteins present in the major peak (peak 3, Fig. 4) were used for amino-acid analysis.

This analysis (Table 1) showed that the subunits of the two proteins (lectin and DNase) differ in amino-acid composition sharing, however both, a high content of AsX, Ser, Thr, Val and Leu. Lectin is also rich in Gly and Phe, and DNase in Lys (Table 1). In spite of differences in amino-acid composition of the

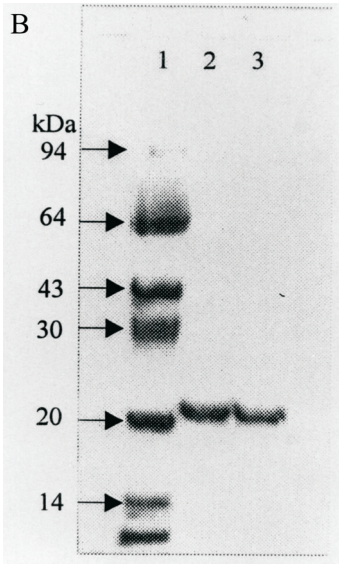

Figure 1B. SDS/PAGE of lectin and DNase after purification by affinity chromatography.

Lane 1, protein standards: phosphorylase *b*, bovine serum albumin, ovalbumnin, trypsin inhibitor and α-lactalbumin; lane 2; lectin (fractions 10–20); lane 3; DNase (fractions 21–27).

two subunits, their N-terminal amino-acid sequence, as determined by Edman degradation of the first eighteen amino acids, is identical. It is as follows:

Ala-Asp-Thr-Ile-Val-Ala-Val-Glu-Leu-Asp-Thr-Tyr-Pro-Asn-Thr-Ser-Ile-Gly

This sequence shows high homology with that of lectin from *Dioclea grandiflora* L. from the family *Fabaceae*:

Chelidonium majus L. lectin:
ADTIVAVELDIYPNTSIG

Dioclea grandiflora L. lectin:
ADTIVAVELDSYPNTDIG [12].

DISCUSSION

Milky sap of *Chelidonium majus* is a very complex mixture of several biologically active substances such as alkaloids, carboxylic acids, amines and glycoproteins [10, 21, 22]. Two glycoproteins, one functioning as a good agglutination agent, a lectin, and another active as a nuclease termed DNase, were isolated and partially characterised [12]. Using thin-layer chromatoraphy for analysis composition of sugar after deglycosylation of each glycoprotein the sugars were separated and characterized. Sepharose, glucose, galactose and xylose were identified in lectin, and saccharose, glucose, arabinose and mannose – in DNase [10].

Probably, both glycoproteins are oligomers composed of 24 kDa monomer subunits differing in amino acid, charge and carbohydrate content. However, as indicated by electrophoresis and isoelectric focusing, it might be that some subunits can be common to both these glycoproteins. Despite clearly detectable differences in charge between lectin and DNase, their hydrophobicity is similar as indicated by RP-HPLC.

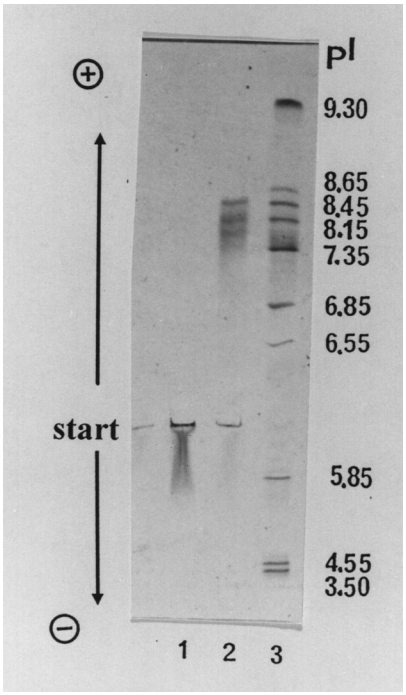

Figure 2. Isoelectric focusing patterns (pH 3–10 ampholine) of DNase (lane 1) and lectin (lane 2); gel calibration mixture (lane 3).

Analysis of the N-terminal amino-acid sequence and amino-acid composition demonstrated that the dominant components of

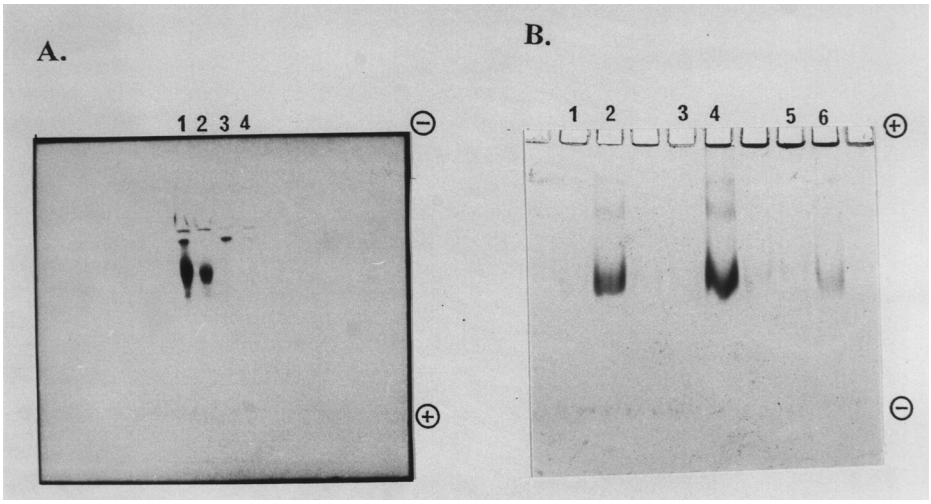

Figure 3. PAGE of lectin and DNase from *Ch. majus* at different pH.

A. Alkaline urea/PAGE at pH 8.8 of the unheated lectin, lanes 1 and 2, and DNase, lanes 3 and 4. B. PAGE in acidic conditions (pH 3.5), with reversed electrode polarity (+ on the top). Lanes 1, 3, 5, lectin, lanes 2, 4 and 6, DNase.

lectin and DNase could be products of distinct but related genes. Most of the known legume lectins are, in fact, encoded by a multigene family that arose by gene duplication and divergence from an ancestral gene [5, 23]. This family includes also the α-amylase inhibitor, which plays an important role in plant defence against animals, and some storage proteins in legumes maize, potato and cereals [24].

In *Sophora japonica* bark there are five lectins which are formed by different combinations of two types of subunits. One subunit of the bark lectin and one subunit of the seed lectins, which are closely related proteins, are products of distinct genes [25]. Also lectins can appear as different molecular forms or isoforms produced by different genes. The occurrence of multiple isoforms of lectins can be explained by differences in the degree of their glycosylation and other modifications of subunits derived from a common precursor [26]. The differences observed between lectin and DNase from *Chelidonium majus* could have also resulted from differences in post-translational processing of a common precursor.

This could be due either to differences in glycosylation or, as reported by Mandal *et al.* [27] and Young *et al.* [28] in the case of agglutinins from soybean and lentil peanut, to C-terminal proteolytic processing of subunits. Glycosylation plays an important role in biological function of native and recombinant glycoproteins. Oligosaccharides can modify intrinsic properties of proteins to which they are attached by altering their stability, protease resistance, quarternary structure as well as can modulate interaction of a glucoconjugate with other molecules [29]. Therefore is possible that the difference in carbohydrate specificity between lectin and DNase decided about aggregation of each monomer of both proteins and also suggest that their association and dissociation may be accompanied

Table 1. Amino-acid composition of lectin and Dnase (%) from *Chelidonium majus*

	Asx	Glx	Ser	Gly	His	Arg	Thr	Ala	Pro
Lectin	11.61	4.92	13.04	9.51	2.52	2.58	7.82	6.82	5.48
DNase	13.87	4.52	11.27	7.17	1.60	2.87	8.89	6.37	5.36
	MetS	Tyr	Val	ACys	Ile	Leu	Phe	Lys	Cys
Lectin	0.62	0.94	6.62	1.22	6.31	9.06	5.64	4.04	2.26
DNase	0.63	0.85	9.47	1.06	6.17	8.44	1.66	6.39	3.86

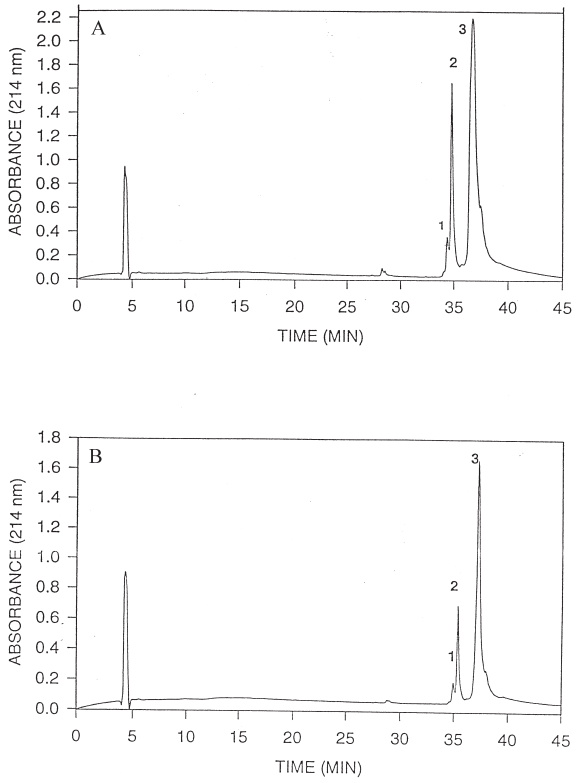

Figure 4. The elution profile from the RP-HPLC C_{18} column of the lectin and DNase from *Ch. majus* purified by affinity chromatography.

A, lectin; B, DNase.

by a change in conformation which is capable of modifying the catalytic sites of both proteins [30].

The proteins that display two types of activities: hemagglutination activity and enzymatic activity has been found previously in mung bean (α-galactosidase) and *Vicia faba* [7]. The mung bean protein can be reversibly converted by pH changes from tetrameric form, which possesses both enzymatic and hemagglutination activities to a monomeric form which possesses enzymatic activity only.

The course of evolution of plant lectins and of proteins associated with them has not been so far elucidated. Similarly, little is also known about their biological functions. It has been suggested that they play a role in plant defence against different pathogens and animals as well as in carbohydrate interactions [10, 12]. Most of them might function as storage proteins.

The sequence of genes encoding the kDa subunit proteins would provide an answer on their interesting interrelationship.

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