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The *Urtica dioica* Agglutinin Is a Complex Mixture of Isolectins[1]

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ELS J. M. VAN DAMME, WILLEM F. BROEKAERT, AND WILLY J. PEUMANS*
Laboratorium voor Plantenteelt, KU Leuven, Kardinaal Mercierlaan, 92, B-3030 Leuven, Belgium

ABSTRACT

Rhizomes of stinging nettle (*Urtica dioica*) contain a complex mixture of isolectins. Ion exchange chromatography with a high resolution fast protein liquid chromatography system revealed six isoforms which exhibit identical agglutination properties and carbohydrate-binding specificity and in addition have the same molecular structure and virtually identical biochemical properties. However, since the *U. dioica* agglutinin isolectins differ definitely with respect to their amino acid composition, it is likely that at least some of them are different polypeptides coded for by different genes.

Plant lectins are a heterogeneous group of (glyco-)proteins sharing their unique ability to recognize and bind specific sugars or sugar containing molecules. At present over 100 different phytohemagglutinins have been isolated, either from seeds or vegetative tissues, and characterized in detail with respect to their molecular structure, biochemical properties, and sugar binding specificity. Although most of these lectins can be considered as single molecular species, a number of them represent more or less complex mixtures of closely related molecular forms usually designated as isolectins. One of the best known examples of isolectins is the *Phaseolus vulgaris* phytohemagglutinin (PHA)[2] which can be resolved into five isolectins by ion exchange chromatography (5, 7). In this case the origin of the five isoforms can be explained by the *in vivo* association of erythrocyte (E)-reactive and lymphocyte (L)-reactive subunits into five different tetrameric forms. Multiple forms of lectins are also well documented for allopolyploid cereal species such as hexaploid wheat (*Triticum aestivum*), tetraploid wheat (*Triticum turgidum*), and *Triticosecale*. In these cases, however, the origin of the isolectins lies in the simultaneous expression of lectin genes located on homoeologous chromosomes of each of the individual genomes in the allopolyploid cells (10,16).

In this paper we describe the complex isolectin composition of the stinging nettle (*Urtica dioica*) lectin. Evidence is presented that a total preparation of this small, single-chain lectin contains six isolectins which can be separated by ion exchange chromatography and exhibit similar but not identical biochemical properties.

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[2] Abbreviations: PHA, *Phaseolus vulgaris* hemagglutinin; UDA, *Urtica dioica* agglutinin; FPLC, fast protein liquid chromatography.

MATERIALS AND METHODS

Material. Rhizomes of stinging nettle were collected during the winter. After thorough washing with tap water, they were cut into small pieces and used immediately or stored at −20°C.

Isolation of *Urtica dioica* Agglutinin (UDA). Total UDA was isolated by affinity chromatography on chitin as described previously (12).

Crude separation of total UDA on Sulfopropyl (SP)-Sephadex. A total affinity-purified UDA-preparation, dialyzed against 30 mM Tris-acetate (pH 5.0) was loaded on a column of SP-Sephadex (type C 50 from Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the same buffer. After washing the column with buffer the lectin was eluted with a linear gradient of increasing Tris-acetate (pH 5.0) concentration (from 30 to 300 mM). Fractions were collected and further used for the isolation of the individual UDA isolectins. Experimental details are described in the legends to the figures.

Separation of UDA Isolectins by High Resolution FPLC. UDA preparations, dissolved in and dialyzed against Na-formate buffer (50 mM formic acid-NaOH, pH 4.0) were analyzed with a Pharmacia FPLC system equipped with a Mono-S (type HR 5/5) cation exchange column. Lectin samples were loaded on the Mono-S column equilibrated with formate buffer and after washing the column with 4 ml of the same buffer eluted with a linear gradient of increasing NaCl concentration. Experimental details are given in the legends to the figures.

Analysis Methods. Protein was determined according to Lowry *et al.* (8). SDS-PAGE was done on 12.5 to 25% polyacrylamide gradient gels using a discontinuous system as described by Laemmli (6). Amino acid compositions of individual UDA isolectins were determined as described previously (11). Lectin samples were hydrolyzed (in sealed glass tubes under an N_2 atmosphere) for 24 h at 110°C in 6 M HCl containing 0.02% β-mercaptoethanol. After hydrolysis, HCl was removed by evaporation, and the residue was dissolved in citrate buffer (pH 1.9). Amino acid analysis was done with a Biotronik LC 2000 amino acid analyzer. This apparatus allows a separation of all 20 amino acids on a single column of Durrum resin, using an elution programme consisting of four different buffers. Amino acids were directly quantified using an Infotronics integrator. Cysteine was determined as cysteic acid after hydrolysis in performic acid. All analyses were done in triplicate.

Agglutination Assays. Agglutination assays were carried out in small glass tubes containing in a final volume of 0.1 ml, 80 μl of a 1% suspension of untreated or trypsin-treated erythrocytes and 20 μl of a lectin solution. Agglutination was determined visually after 1 h at room temperature.

Induction of Interferon in Fresh Human Lymphocytes. Experiments to check the induction of interferon in fresh human lymphocytes by each of the six isolectins were carried out as described previously (12).

RESULTS

Indications for Presence of Isolectins in Total UDA. Since lectin preparations obtained by affinity chromatography on chitin are usually contaminated with other chitin-binding proteins, an additional ion exchange chromatography step was included in the purification scheme developed for UDA a few years ago (12). Although in the original large-scale purification of UDA the lectin eluted in a more or less symmetrical peak from an SP-Sephadex column (and in addition yielded a single polypeptide band upon SDS-PAGE) (12), a closer examination under conditions allowing higher resolution revealed the presence of at least three distinct peaks in the elution pattern of UDA from an SP-Sephadex column (Fig. 1). Since, in addition, all three peaks exhibited agglutination activity and yielded single polypeptide bands of 8.5 kDa upon SDS-PAGE (results not shown), there was no doubt left that UDA is a mixture of isolectins. To obtain a better resolution, the same UDA preparation was analyzed using a high resolution FPLC system which eventually demonstrated the presence of six different isolectins (Fig. 2).

Isolation of UDA Isolectins. All six isolectins which can be distinguished in the elution pattern of total UDA upon chromatography on the Mono-S column were isolated by ion exchange chromatography. In a first step about 250 mg of UDA were chromatographed on an SP-Sephadex column in order to obtain a crude separation of a reasonable amount of material (Fig. 1). Fractions were collected, pooled as indicated in Figure 1, and brought to 60% (relative concentration) ammonium sulfate by adding the solid salt. After being left in the cold overnight the precipitated lectin was centrifuged (15 min, 30,000*g*), dissolved in an appropriate volume of formate buffer (so that the lectin concentration was about 5 mg/ml) and extensively dialyzed against the same buffer. After dialysis lectin fractions were centrifuged (15 min, 20,000*g*), filtered (to remove any particulate material) and applied (in 5-mg portions) on the Mono-S column for subsequent chromatography. Peak fractions were collected (manually), pooled, fourfold diluted with formate buffer, and rechromatographed on the Mono-S column until single symmetrical peaks were obtained. Control chromatograms of each isolectin are shown in Figure 2. In addition, equal amounts of all six isolectins were combined and chromatographed in order to demonstrate that their elution positions correspond to these of the six peaks which can be distinguished in the elution pattern of total UDA.

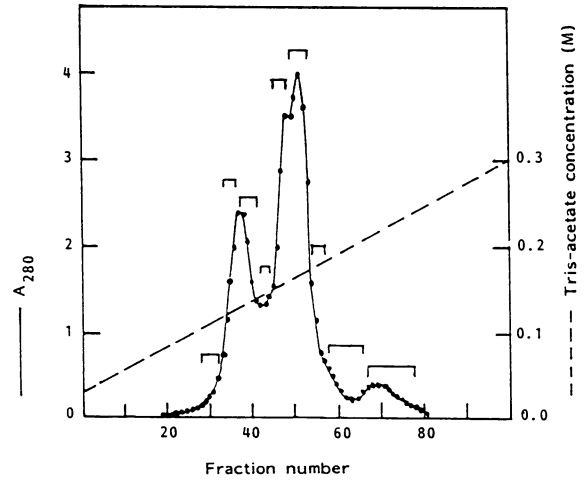

FIG. 1. Ion exchange chromatography of total UDA. About 250 mg of total UDA was applied to a column (35 cm × 2.5 cm) of SP-Sephadex and eluted with a linear gradient (600 ml) from 30 to 300 mM Tris-acetate (pH 5.0). Fractions (6 ml) were collected and pooled into 9 groups (as indicated by the brackets) for further purification of the isolectins.

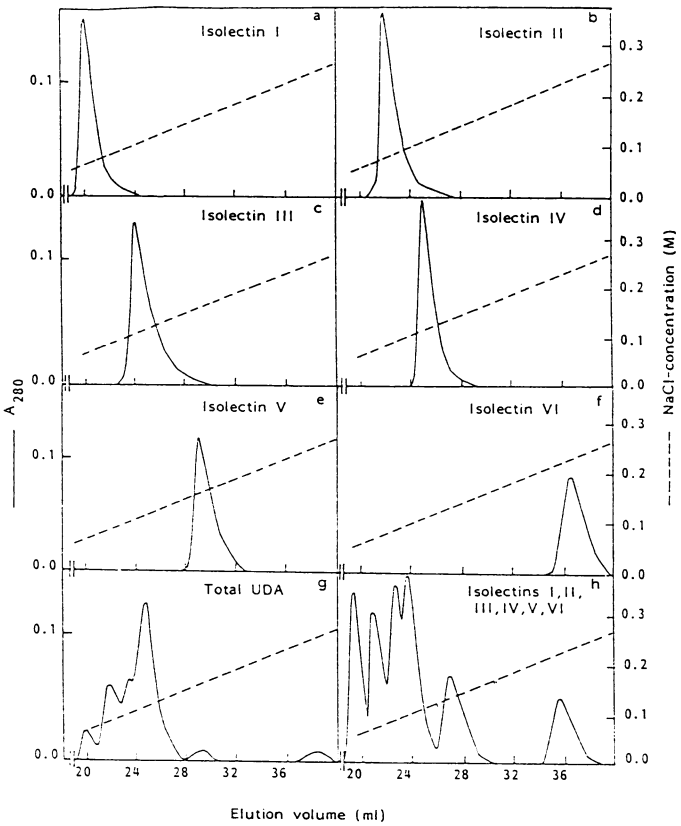

FIG. 2. Ion exchange chromatography of purified UDA isolectins, total UDA and a mixture of equal amounts of all six isolectins on a Mono-S column. Lectin samples were loaded on the column and after washing the column with 4 ml of Na-formate buffer (50 mM, pH 4.0) eluted with a linear gradient (44 ml) from 0 to 0.4 M NaCl in the same buffer.

Molecular Structure of Different UDA Isolectins. Individual UDA isolectins were analyzed by SDS-PAGE and gel filtration. All six isoforms migrated on the SDS polyacrylamide gel with an apparent M_r of 8.5 kDa (Fig. 3) and eluted from a Sephadex G-100 column with an apparent M_r of 3 kDa (results not shown). It is evident therefore that all six isolectins in their native form occur as monomers of M_r 8.5 kDa. With respect to their amino acid composition also the UDA isolectins strongly resemble each other. Indeed as shown in Table I they differ from each other only by one or a few amino acids. Although some of these differences may be due to experimental errors (especially for the most abundant amino acids such as cysteine, glycine and serine) the absence of leucine in isolectin II and the observed differences between the ratios of less abundant amino acids (such as, *e.g.*, alanine/valine and lysine/histidine) leave no doubt that UDA isolectins differ with respect to their amino acid composition. Additional evidence for our results is provided by the work of Chapot *et al.* (2). These authors found at least two different NH_2-terminal sequences by Edman degradation of (enzymatically deblocked) UDA. In addition, upon tryptic digestion of reduced and iodoamidated total UDA they obtained numerous peptides, the total length of which surpassed that of a single UDA polypeptide chain. It should also be mentioned that none of the UDA isolectins was found to contain covalently bound carbohydrate (at least when assayed for total carboyhdrate by the phenol sulphuric acid method described by Dubois *et al.* (3). At this point the question has to be addressed whether the observed differences between the UDA isolectins could be due to differential proteolysis (during extraction and purification) or some

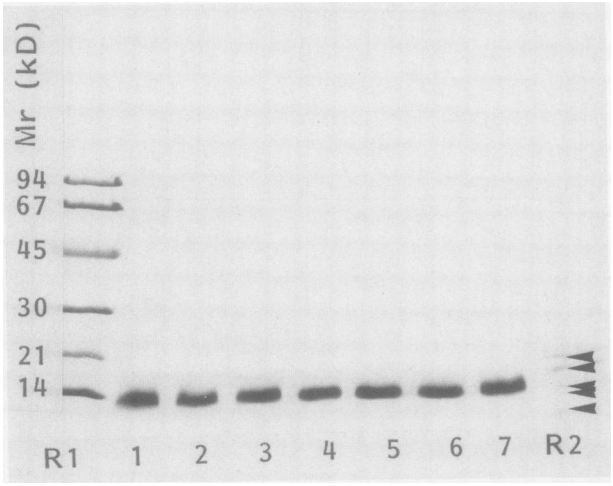

FIG. 3. SDS-PAGE of UDA isolectins. About 25 μg of isolectins I, II, III, IV, V, VI, and total UDA were loaded in lanes 1, 2, 3, 4, 5, 6, and 7, respectively. M_r reference proteins are shown in lane R; lane R_1: lysozyme (M_r 14,300), soyabean trypsin inhibitor (M_r 21,000), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 45,000), BSA (M_r 68,000), and phosphorylase *b* (M_r 93,000). Lane R_2 myoglobin (intact, M_r 17,201), myoglobin I + II (M_r 14,632), myoglobin I (M_r 8,235), myoglobin II (M_r 6,383), myoglobin III (M_r 2,560). The position of the M_r reference proteins in lane R_2 is indicated by *arrowheads*.

Table I. *Amino Acid Composition of UDA Isolectins*

Amino Acid	Isolectin					
	I	II	III	IV	V	VI
	residues/mol					
Cys	16	16	16	16	16	16
AsX	7	8	7	7	7	6
Thr	2	2	1	2	1	1
Ser	9	9	10	10	8	9
GlX	7	6	8	7	6	6
Pro	5	5	4	4	4	4
Gly	18	18	18	17	18	18
Ala	3	2	3	3	3	2
Val	2	3	2	2	2	2
Met	0	0	0	0	0	0
Ile	1	1	1	1	1	1
Leu	1	0	1	1	1	1
Tyr	3	3	3	3	3	3
Lys	1	1	2	2	1	3
His	2	2	2	2	2	2
Arg	6	7	6	8	8	9
Phe	0	0	0	0	0	0
Trp	ND[a]	ND	ND	ND	ND	ND
Total[b]	83	83	84	85	81	83

[a] Not determined. [b] exclusive tryptophan.

type of post-translational modification. The former possibility is rather unlikely since (a) native UDA is extremely resistant to proteases (*e.g.* trypsin and chymotrypsin) and (b) all UDA isolectins migrate with the same apparent M_r upon SDS-PAGE (Fig. 3). Differential post-translational modifications cannot be excluded. However, since all six UDA isolectins have the same M_r and none of them is glycosylated possible differences in both proteolytic cleavage and glycosylation (which are the most common post-translational modifications of plant proteins) are unlikely.

Carbohydrate-Binding Specificity, Agglutination Properties, and Induction of Human γ-Interferon in Lymphocytes by Different Isolectins. Since total UDA is known to exhibit carbohydrate-binding specificity exclusively toward GlcNAc and GlcNAc-oligomers (14), the carbohydrate-binding specificity of the individual UDA isolectins was investigated by determining the minimal concentration of GlcNAc-oligomers of defined chain length required for 50% inhibition of the agglutination activity of each isolectin. As shown in Table II, no differences could be observed between the isolectins. The same holds true for the specific agglutination activity and blood group specificity, since as is indicated in Table III the same concentrations are required of each isolectin to obtain agglutination with either human or rabbit erythrocytes. Finally it is worthwile to mention that all UDA isolectins induce the synthesis of γ-interferon in fresh human lymphocytes (Table IV). Indeed, although because of the complexity of the whole procedure and the variability inherent to the bioassay, the figures shown in Table IV do not allow an exact quantitative comparison between the different isolectins, there is no doubt that all six isoforms strongly enhance the antiviral activity of the lymphocytes.

Table II. *Inhibitory Effect of GlcNAc and GlcNAc-Oligomers on Agglutination Activity of Total UDA and Individual UDA Isolectins*

Lectin (10 μg/ml)	Minimal Concentration Required for 50% Inhibition of Agglutination Activity in Assays with Trypsin-Treated Rabbit Erythrocytes			
	GlcNAc	$(GlcNAc)_2$	$(GlcNAc)_3$	$(GlcNAc)_4$
	mM			
Total UDA	300	1.2	0.042	0.014
Isolectin I	300	1.2	0.042	0.014
Isolectin II	300	1.2	0.042	0.014
Isolectin III	300	1.2	0.042	0.014
Isolectin IV	300	1.2	0.042	0.014
Isolectin V	300	1.2	0.042	0.014
Isolectin VI	300	1.2	0.042	0.014

DISCUSSION

As has been reported previously stinging nettle rhizomes contain considerable amounts of a lectin with some unusual properties. The so-called UDA is not only the smallest plant lectin isolated so far but is also the only lectin known at present which in its native form consists of a single polypeptide chain of less than 100 amino acid residues. In addition, UDA shows amino acid homology to wheat germ agglutinin, which is a rather unexpected finding in view of the distant taxonomic relationship between wheat and stinging nettle. The results described in this report reveal another unexpected peculiarity of UDA, namely its complex isolectin composition. There is no doubt, indeed, that the nettle lectin contains at least six molecular forms which exhibit identical agglutination properties and carbohydrate-binding specificity and in addition have the same molecular structure and virtually identical biochemical properties. However, since UDA isolectins differ definitely with respect to their amino acid composition, they probably represent different polypeptides coded for by two or more different genes. Naturally, it cannot be excluded that some of the isolectins are products derived from a single polypeptide by differential post-translational modification(s).

In fact, the idea that UDA is a complex mixture of isolectins coded for by a family of closely related genes is not so unusual since lectin multigene families have been found in a number of plant species, such as *e.g. Phaseolus vulgaris, Dolichos biflorus, Sophora japonica*, and *Griffonia simplicifolia* (4, 9, 13, 15). What is unique for UDA is the fact that it is a monomer which implies that associations of two or more different subunits in different combinations (as is the case for *e.g.* PHA) are not possible. On

Table III. *Blood Group Specificity of Total UDA and Individual Isolectins*

Lectin	Minimal Concentration Required for Agglutination of:							
	Rabbit erythrocytes		HE_A[a]		HE_B		HE_O	
	UT[b]	TT[c]	UT	TT	UT	TT	UT	TT
				μg/ml				
Total UDA	15	2.5	—[d]	25	—	25	—	25
Isolectin I	15	2.5	—	25	—	25	—	25
Isolectin II	15	2.5	—	25	—	25	—	25
Isolectin III	15	2.5	—	25	—	25	—	25
Isolectin IV	15	2.5	—	25	—	25	—	25
Isolectin V	15	2.5	—	25	—	25	—	25
Isolectin VI	15	2.5	—	25	—	25	—	25

[a] $HE_{A,B,O}$: human erythrocytes of blood group A, B, and O, respectively. [b] UT, untreated. [c] TT, trypsin-treated. [d] —, no agglutination at a lectin concentration of 2 mg/ml.

Table IV. *Indution of Human γ-Interferon in Fresh Human Lymphocytes by Total UDA and UDA-Isolectins*

Inducer	Antiviral Activity
	log_{10} *IU/ml*
Total UDA (100 μg/ml)	2.39
Isolectin (100 μg/ml):	
I	2.56
II	2.45
III	2.35
IV	2.19
V	2.86
VI	2.17
None	1.30
SEA[a] (1/1000)	3.47

[a] *Staphylococcus* enterotoxin A (this is a commonly used inducer of human γ-interferon in lymphocytes). The preparation used corresponds to a 1000-fold diluted supernatant of a *Staphylococcus aureus* culture.

the analogy of the genetic origin of isolectins in allopolyploid cereals, in which each of the individual genomes directs the synthesis of its own polypeptide (10) it could be tempting to speculate for a similar explanation for UDA. However, since populations of stinging nettles are mixtures of diploid and autotetraploid individuals (1) they could contain two isolectins at the most if they were coded for by genes located on homologous chromosomes of the different genomes present in a single cell, which is in disagreement with our results.

A final remark to be made concerns the interpretation of the results of previously reported amino acid sequence data of UDA. Chapot *et al.* (2), who determined the amino acid sequence of the NH_2-terminal of (enzymatically) deblocked UDA and a number of tryptic peptide fragments, could not explain their data on the basis of a single lectin polypeptide and postulated that UDA was either composed of two different polypeptide chains of the same size or contained at least two different isolectins. Since our results leave no doubt that UDA is a mixture of isolectins, the latter possibility is more likely.

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