IMMUNOLOGICALLY RELATED LECTINS FROM STEMS AND ROOTS OF DEVELOPING SEEDLINGS OF *CUCURBITA FICIFOLIA*: PURIFICATION AND SOME PROPERTIES OF ROOT AND STEM LECTINS

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ABSTRACT

Hemagglutinating activity has been found in acetate extracts from roots and stems of squash seedlings (*Cucurbita ficifolia*). The hemagglutinating activity changes during seeds germination and seedling development. Dot blot and Western blot techniques have shown that proteins from these vegetative tissues cross-reacted with antibodies raised against endogenous cotyledons lectin CLBa and Con A.

Lectins were isolated from stems and roots of 6-day old seedlings by precipitation with ethanol, affinity chromatography on Con A-Sepharose, gel filtration on Bio-gel P100 and separated by electrophoresis on polyacrylamide gel. Three purified lectins (RLA₁, RLA₂, RLA₃) were obtained from roots and four from stems (SLA₁, SLA₂, SLA₃, SLA₄). The purified lectins from roots and stems agglutinated all human red blood cells, but sheep erythrocytes were most sensitive to agglutination. The hemagglutination of the root lectins RLA₂ and RLA₃ was inhibited by a very low concentration of arabinose, while RLA₁ of xylose and GalNAc. Arabinose and Xylose were also found to be the most effective inhibitors of all stem lectins.

KEY WORDS: Lectins, Ara/Xyl specificity, immunological relationship, seedlings, roots, stems, *Cucurbita ficifolia*.

INTRODUCTION

Lectins are carbohydrate binding proteins that are widely distributed in plant kingdom. They are proteins which not only possess a sugar-binding site, but in addition also a binding site for hydrophobic or ionic residues (Rüdiger 1966). Plant lectins have intensively been studied and the steadily increasing number of them have been purified and characterized.

A function for lectin has not been unequivocally demonstrated, however several observations suggest that they may be involved in an intracellular sucrose-transporting system and cell wall metabolism of the plant (Pusztai 1991). They may play a role in tissue/organ development.

Plant lectins occur in different types of vegetative tissues especially of storage tissues. Well known examples of such lectins are those found in tubers (Chatelain et al. 1994), bulbs (Van Damme and Peumans 1989, 1991), roots (Waxdal 1974) and bark (Horejsi et al. 1978). Weak hemagglutinating activity was also found in extract of stems, leaves and fruits (Kilpatrick et al. 1980; Etzler 1986; Pusztai 1991).

Comparative biochemical, biological and immunological studies on lectins from different species of grass have shown that they are closely related to each other and probably represent a family of evolutionary conserved proteins (Peumans et al. 1982a, 1982b, 1982c; Peumans and Stinissen 1983; Stinissen et al. 1983).

Earlier we have shown that in seeds and cotyledons of developing seedlings from *Cucurbita ficifolia* (Lorenc-Kubis et al. 1993) and other species of Cucurbitaceae (Lorenc-Kubis, Uram-Walaszczyk 1999) are presented lectins which are immunologically related to endogenous seeds lectin (CLBa) and have common antigenic sites with Con A.

To find out whether the vegetative part of seedlings (roots and stems) contains also lectins, and if so, whether they are related to cotyledons lectin CLBa and to Con A, we made a partial survey of the occurrence and immunological relationships of lectins from roots and stems of developing seedlings of *Cucurbita ficifolia*.

In this paper we also describe the isolation, purification and partial characterization of a lectin from roots and stems of 6 day old seedlings of *Cucurbita ficifolia*.

MATERIAL AND METHODS

Plant material

The study was carried out on roots and stems of squash seedlings (*Cucurbita ficifolia*).

The seeds were from Plant Seeds Corporation in Wrocław.

Germination of seeds. Dry seeds were sterilized by soaking them in 30% H₂O₂ for 10 min and washed with distilled water according to Piche et al. (1985). The seeds were sown in a moist germination bed of filter paper and allowed to germinate at 22-

-24°C. Germination was performed in the dark for 2 days at room temperature, and then the plants were exposed to a light/dark cycle (12 hours light/12 hours dark). The seedlings were harvested after 3-, 6-, 10- and 22-days and the stems and roots of the seedlings were dissected.

Extraction of proteins and ethanol precipitation

Stems and roots of developing seedlings were homogenized with 3 vols (w/v) of 0.1 M sodium acetate buffer, pH 5.1. The extraction was performed, at 4°C for 1 h with constant mechanical stirring. After centrifugation (12 000 g for 30 min) the pellet was discarded and the supernatant was used for protein estimation and hemagglutinating activity.

The proteins from crude extract of roots from 6 day old seedlings were precipitated with ethanol to a final concentration of 60% as described by Lorenc-Kubis (1994). The precipitated proteins were centrifuged at 12 000 g × 30 min 4°C and the pellet was dissolved in 0.1 M acetate buffer pH 5.6.

Analytical methods

Protein concentration was determined according to Lowry et al. (1951) bovine serum albumin (BSA) was used as standard.

Total carbohydrate estimation was performed by phenol-sulfuric acid method (Dubois et al. 1956)

Acid phosphatase activity was measured as described by Ferens and Morawiecka (1985) using 5 mM sodium p-nitrophenyl phosphate as a substrate, pH 5.1 at 37°C. The amount of p-nitrophenol was measured spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the amount of acid phosphatase releasing 1 µmol of p-nitrophenyl phosphate after 1 min under the assay condition.

Hemagglutination assay was performed at room temperature in microtiter U-shaped plates using serially two-fold dilutions of the test materials. Human erythrocytes A, O, B were purchased from Blood Bank of Wrocław. The sheep erythrocytes were purchased from the Agriculture Academy of Wrocław. The erythrocytes were washed three times in PBS (phosphate buffered saline).

A 25 µl of extracts or lectin solution was serially diluted with 25 µl of PBS buffer and mixed with 25 µl of 3% (v/v) erythrocytes in PBS.

A hemagglutination unit was defined as the minimum amount of lectin giving a positive reaction after 60 min at room temperature.

Affinity chromatography was performed at room temperature. The ethanol precipitated material was suspended in 0.1 M sodium acetate buffer, pH 5.6 and applied to a Con A-Sepharose column (10 cm long, 3 cm i.d, Pharmacia Uppsala, Sweden) equilibrated with 0.1 M acetate buffer, pH 5.6 containing 1 M NaCl and Mg²⁺, Mn²⁺, Ca²⁺ at 1 mM concentration. The proteins not adsorbed on the column were removed by washing with the same buffer. The bound proteins were displaced from the column with 15 mM and 300 mM α-methyl-D-mannopyranoside (Sigma, USA). Active fractions were pooled and dialysed against water and concentrated by ultrafiltration.

Gel filtration chromatography. Chromatography of samples on Biogel P-100 (2.5 × 98 cm) was performed at 4°C. The column was equilibrated and eluted with 0.05 M acetate buffer, pH 5.6. Fractions of 2 ml were collected at a flow rate of 2 ml/hr.

Carbohydrate inhibition assay. For the carbohydrate inhibition assay, 25 µl of carbohydrate solution (400 mM) was serially diluted with 25 µl of PBS buffer and mixed with 25 µl of lectin solution before addition of 50 µl of 3% sheep erythro-

cytes. The mixture was incubated for 60 min at room temperature. The inhibitory activity was defined as the minimum concentration of inhibitor (mM) required for complete inhibition of erythrocyte agglutination after 1 h.

Polyacrylamide gel electrophoresis. Non-denaturing PAGE was performed on 12% gel slabs in TRIS-glycine buffer, pH 8.3 according to Davis (1964). Samples containing 80-100 µg protein of crude extracts from cotyledons, stems and roots or 10-20 µg of pure lectin were subjected to electrophoresis. The gel was stained for detection of protein with 0.4% Coomassie Brilliant Blue R-250 in 50% TCA.

Preparative electrophoresis was performed according to Davis (1964) on 12% gel slabs. The active fractions after gel filtration on Biogel P-100 were collected and concentrated under N₂ using an Amicon PM-10 membrane and loaded onto polyacrylamide gel. After electrophoresis the gel was cut into two parts. One part was stained for proteins, the other was cut onto slices from which the proteins were eluted with 0.1 M acetate buffer pH 5.6 containing Mg²⁺, Ca²⁺ and Mn²⁺ at 1 mM concentration.

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) in 15% polyacrylamide gel slabs. The gels were stained for protein with Coomassie Brilliant Blue R-250 or the proteins were transferred onto NC membrane. Phosphorylase b from rabbit muscles (94 kDa), bovine serum albumin (67 kDa), hen ovalbumine (43 kDa), human erythrocytes carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine lactalbumin (14.4 kDa) were used for molecular mass calculation.

Electroblotting. The proteins resolved by SDS-Page were transferred onto NC membranes using 25 mM TRIS-192 mM glycine/20% (v/v) methanol buffer, pH 8.3 according to Towbin et al. (1979). 0.03% SDS was added to the transfer buffer. After the transfer (2 hr, 200 mA) the NC membrane was blocked overnight with 0.4% gelatin in TBS (Tris buffered saline) and then washed with TBS. The immunoreaction was carried out by 2 hr incubation with rabbit antibodies anti-Con A or anti-CLBa (the lectin from squash cotyledons) diluted 1: 1500 in TBS containing 0.5% gelatin followed by 2 hr treatment with the peroxidase-labelled goat antibodies anti-rabbit IgG (Boehringer Mannheim), diluted 1: 10 000 in TBS with 0.05% Tween 20. The washing between incubation with the 1st and the 2nd antibodies and before the final staining were done with TBS in a standard manner. The peroxidase reaction was carried out using as a substrate 0.05% DAB (diaminobenzidine) or 0.05% 4-chloro-1-Naphthol in TBS, containing 0.01% H₂O₂.

Dot blot. 3 µl of samples (30 µg) from crude extracts of roots and stems or 2 µl (2-5 µg) of purified lectins were directly applied on NC membrane, blocked with 0.25% gelatin in TBS, washed with TBS and then incubated with rabbit antibodies raised against Con A and CLBa as described above in electroblotting.

Antibodies were purified from rabbit antiserum raised against purified lectin CLBa from squash cotyledons (*Cucurbita ficifolia*) according to Harboe and Ingild (1973). Antibodies against Con A (*Canavalia ensiformis* lectin) developed in rabbit were obtained from Sigma Immuno Chemicals.

Lectin-reactivity on blots. Glycoprotein detection on the blots was performed according to the method of Olden and Yamada (1977) under conditions described by Clegg (1982). The blots were first wetted with PBS and quenched for 1 hr at room temperature with 3% BSA. Then they were probed with Con A (10 µg/ml) in PBS followed by HPR solution treatment (20 µg/ml PBS) or with other lectins: AAA (*Aleuria aurantia*) and GNA

(*Galanthus nivalis*) conjugated with digoxigenin. The enzyme conjugated probes were detected with 0.05% DAB solution in 50 mM Tris/HCl, pH 7.6 containing 0.01% H_2O_2 .

RESULTS AND DISCUSSION

Proteins from seeds and vegetative tissues of *Cucurbita ficifolia* seedlings were extracted with 0.1 M acetate buffer pH 5.1 and the agglutinating activity was monitored during seeds germination and seedling development. In stems of germinating seedlings like in cotyledons (Lorenc-Kubis et al. 1993, and roots (Lorenc-Kubis et al. 1994) the hemagglutinating activity was relatively low on the onset of germination, but increased on the 6 th day and then fell down. The protein content and the agglutination activity of sheep erythrocytes by the extracts of germs and stems of developing seedlings are present in Fig. 1.

Fig. 1. Changes in protein content and hemagglutinating activity in stems from *Cucurbita ficifolia* seedlings at different developmental stages. Data are the mean of four independent experiments.

Changes in hemagglutination activity were also observed during rye germination (Ferens-Sieczkowska et al. 1989), in root-stocks and leaves of german iris during the vegetative period (Ferens-Sieczkowska and Morawiecka 1993) and in the extracts prepared from *Ulvia Lactuca* collected in different months of the year (Sampaio et al. 1996). Examination of various parts of plant (*Erythrina corallodendron*) produced from the germinating seeds, revealed a rapid reduction in lectin level with the plant maturation up to one month (Nir et al. 1996).

To find out whether the proteins from crude extracts of roots and stems of *Cucurbita ficifolia* interacted with antibodies raised against CLBa (the lectin from cotyledons of *C. ficifolia*) or con A (the lectin from Papilionaceae) dot blotting of the proteins sample on the NC membranes was performed. The results are present in Fig. 2. These preliminary experiments showed that proteins from examined extracts of roots and stems cross-reacted with antibodies raised against Con A and CLBa indicating antigenic similarity between the proteins. These experiments encouraged us to perform more detailed studies on the proteins by the Western blotting technique.

The proteins of crude extracts of roots and stems were resolved by SDS-PAGE and transferred on to NC membrane and treated with antibodies as described in Materials and methods. In the seedlings from different species of Cucurbitaceae (Lorenc-Kubis and Uram Walaszczyk 1999) some proteins that

Fig. 2. Immunoreactivity of proteins from crude extracts of stems and roots of developing seedlings from *C. ficifolia* with antibodies raised against lectin - CLBa [1] or Con A [2]. Dot Blot techniques. 3 μ l (30 μ g protein) of extracts of stems [A] or roots [B] was applied on NC membrane.

First antibodies: IgG anti-CLBa: [1] or IgG anti Con A. [2]. Second antibodies: peroxidase labelled goat anti-rabbit IgG at a dilution 1: 5000.

A. Proteins of crude extract of: a - dry seeds, b - extract from stems of 3 - day old seedlings, c - 6 day old, d - 22 day old seedlings.

B. Proteins of crude extract of: a - dry seeds, b - extract from roots of 3 day old seedlings, c - 6 day old and d - 22 day old seedlings.

cross reacted with antibodies anti-lectin CLBa and Con A have been found. It has been shown that roots proteins from 6 and 22 days- old seedlings of apparent molecular weight 30 kDa gave a sharp cross reaction with antibodies raised against conA and CLBa (Fig. 3). Additionally roots and stems proteins with mol. weight of 27 kDa gave a sharp immunoreactivity with antibodies raised against Con A (Fig. 4). This proteins were also found in cotyledons of 6 day old seedlings.

In cotyledons, stems and roots of *C. ficifolia* seedlings, like in cereal of Gramineae (Peumans et al. 1983), lectins with low molecular weight has been detected (Fig. 4). An extensive immunoreactivity of proteins of 14 kDa with antibodies raised against -CLBa has been demonstrated what may indicate a high antigenic relationship between lectins from Cucurbitaceae. A low degree of positive immunological reactivity was observed in the case of proteins with molecular weights of 43 and 55 kDa. Studies on lectins from a number of *Leguminosae* (Hankins et al. 1979; Ramos et al. 1996), *Solanaceae* (Kilpatric et al. 1980) and *Gramineae* (Peumans et al. 1982a, b) species revealed an immunological relationship which correspond well to taxonomic relationship between these species.

The interaction of *C. ficifolia* stems and root lectins with antibodies raised against Con A may indicate that their epitopic determinans are similar to Con A structure- the lectin from *Leguminosae*. The variation of the number of immunological related proteins in seedlings and the intensity of reaction with antibodies may suggest that during the development of seedlings some changes in the lectins occur what may indicate that they play an important biological function.

Purification of lectins from stems and roots

In our preliminary studies it was found that hemagglutinating activity could be extracted from plant tissues with 0.1 M acetate buffer pH 5.1 and recovered completely by ethanol precipitation (Lorenc-Kubis et al. 1994). Therefore, the acetate extracts from

Fig. 3. Reactivity of proteins from roots of *C. ficifolia* seedlings with antibodies raised against lectin: CLBa [1] and Con A [2]. Western blotting analysis.

Samples (180-200 µg of protein) of crude extract from stems or roots were resolved by SDS-PAGE in 15% polyacrylamide gel pH 8.6, electroblotted onto nitrocellulose. The blots were probed with anti CLBa rabbit IgG (1 : 1000)-[1] or anti-Con A (1 : 1500)-[2]. Bound antibodies were detected with goat anti-rabbit IgG-peroxidase complex and was visualized with 4-chloro-1- naphthol as described in Material and methods

Lane: a – imbibed seeds, b – 3 day, c – 6 day, e – 22 day old seedlings.

stems or roots of 6 day old seedlings were treated with ethanol at 60% saturation. The precipitated proteins were resolved in acetate buffer pH 5.6 and subjected to affinity chromatography using con A-Sepharose column. About 35-40% of total protein from stems and roots applied on the column was washed out with the starting buffer, pH 5.6. This fraction contained about 95% of the total hemagglutinating activity and only a trace activity of acid phosphatase. The acid phosphatase activity was bound to the column and could be eluted with 15 mM and 300 mM solution of α -methyl-D-mannopyranoside as described earlier (Lorenc-Kubis 1989).

The non binding proteins were collected and applied once again on the con A-sepharose column. The proteins with hemagglutinating activity were washed out with the acetate buffer pH 5.6 and the proteins with a trace activity of AcPase were bound to the column. This step of purification allowed to separate completely the hemagglutinating activity from the AcPase activity which were bound to con A Sepharose.

The unadsorbed fraction with hemagglutinating activity was dialysed and concentrated by ultrafiltration and subjected to gel filtration using Bio-gel P-100 column (Fig. 5). Active fractions

were pooled and after concentration by ultrafiltration were separated on 12% polyacrylamide gel. In electrophoresis three lectin fractions from roots RLA₁, RLA₂, RLA₃ and four from stems: SLA₁, SLA₂, SLA₃, SLA₄ (Fig. 6). were obtained. The protein fractions were eluted from the gel and analyzed in respect to their sugar specificity, hemagglutinating activity and interaction with some lectins.

Many plants contain a complex mixture of lectins (Van Damme et al. 1988). Several molecular forms of lectin occur in the same plant depending on the examined tissue and the devel-

Fig. 4 Reactivity of proteins from 6 day old seedlings of *C. ficifolia* with antibodies raised against lectin: CLBa [1] and Con A [2].

Samples of crude extract (180 mg protein) of: a – cotyledons, b – stems, c – roots of 6 days old seedlings were resolved by SDS-PAGE in 15% polyacrylamide gel pH 8.6, electroblotted onto nitrocellulose and probed with first and second antibodies as described in Material and method.

Fig. 5. Elution profile of *C. ficifolia* proteins from roots [A] and stems [B] of 6-day old seedlings during gel filtration on Biogel P-100. 0.8 ml of sample from roots (9.5 mg protein) or stems (20 mg) was applied on the column. Elution rate, 2 ml/h, fraction volume – 2 ml.

Fig 6. Polyacrylamide gel electrophoresis of root [A] and stem [B] lectins.

The proteins after gel filtration on Biogel P-100 were run in 12% gel at pH 8.3 according to Davis (1964). The gel was divided into two parts. One part was stained with Coomassie brilliant blue R-250. From the other the lectins were eluted as described in material and methods.

opment stage. These forms differ in their physicochemical properties and perhaps in their function (Morawiecka et al. 1989). Several molecular forms of lectin have been found in Alliaceae and Amaryllidaceae (Van Damme et al. 1994).

The root and stem lectins are glycoproteins interacting with Con A (the lectin from *Canavalia ensiformis*), AAA (*Aleuria aurantia*) and GNA (*Galanthus nivalis*) what indicates that they possess mannose/glucose, fucose and galactose in their carbohydrate moiety.

Purified root and stem lectins, as well as crude extracts from this tissues agglutinate red blood cells from human type A, B, and O and animal origin. Among various erythrocytes tested, sheep red blood cells were most sensitive to agglutination. In the case of lectins from stems, the lowest hemagglutination was observed with dog erythrocytes (data not shown).

Sugar specificity is one of the most important properties of lectins. This property is due to a specific interaction with particular sugars that inhibits their interaction of the lectin with the corresponding glycoconjugate, for example, during hemagglutination. The purified three lectins from roots (RLA₁, RLA₂, and RLA₃) and the four from stems (SLA₁, SLA₂, SLA₃, SLA₄) of

Cucurbita ficifolia were examined for sugar specificity (Table 1). Among the monosaccharides tested as inhibitors of agglutination of root lectins, GalNAc and Xylose were most active with lectin RLA₁, while L-Ara, was the most potent inhibitor of lectin RLA₂. The activity of these lectins was also inhibited by D-Gal, D-Ara and L-Fuc. In the case of lectin RLA₃ the hemagglutination was inhibited by D-Ara. Ribose, xylose and galactose were less effective inhibitors.

The results of the effect of monosaccharides on the hemagglutinating activity of the stems lectins shown in Table 1 indicate that arabinose and xylose were found to be the most active inhibitors of all lectins. STLA₁ was effectively inhibited by D-arabinose while three other lectins by L-arabinose. The all stem lectins were inhibited also by ribose and mannose, but the inhibitory effect of these sugars was observed at higher sugar concentration. The inhibitory effect of glucose, galactose, GLcNAc and GalNAc was not observed. The occurrence of lectins differing in carbohydrate and erythrocyte specificities within the same plant has also been reported for Leguminosae (Kortt 1988).

The presence of several immunologically related molecular forms of lectins in the same plant tissues differing in sugar specificity may indicate the different functions of these proteins depending on the developmental stage of the plant.

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TABLE 1. Carbohydrate binding specificity of root (RLA) and stem (SLA) lectins from *C. ficifolia* 6 day old seedlings. The data are means of four independent experiments.

Saccharide	Concentration causing a 50% inhibition of hemagglutination activity (mM)						
	RLA ₁	RLA ₂	RLA ₃	SLA ₁	SLA ₂	SLA ₃	SLA ₄
Glucose	200	100	50	200	200	200	200
Mannose	200	100	200	50	25	50	50
Galactose	200	12.5	25	200	200	200	200
GlcNAc	200	200	200	200	200	200	200
GalNAc	0.098	200	100	200	200	200	200
L-Arabinose	200	0.098	200	25	1.6	1.6	1.6
D-Arabinose	200	6.25	0.195	0.19	50	25	25
L-Fucose	200	25	200	25	200	200	200
D-Rybose	200	200	12.5	25	50	50	50
Xylose	0.098	200	25	1.6	6.25	3.12	3.12

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IMMUNOLOGICZNE POKREWIEŃSTWO
LEKTYN ŁODYG I KORZENI SIEWEK DYNI FIGOLISTNEJ (*CUCURBITA FICIFOLIA*):
OCZYSZCZANIE I CZĘŚCIOWA CHARAKTERYSTYKA LEKTYN KORZENI I ŁODYG

STRESZCZENIE

W surowych wyciągach z łodyg i korzeni siewek dyni figolistnej (*Cucurbita ficifolia*) wykazano obecność białek o aktywności hemaglutynacyjnej. Aktywność ta ulegała zmianom w trakcie rozwoju i wzrostu siewek. Analiza surowych wyciągów z łodyg jak i korzeni techniką „Dot blot” oraz „Western blot” wykazała obecność białek reagujących z przeciwciałami skierowanymi przeciwko endogennej lektynie liścieni – CLB_a oraz Con A.

Z łodyg i korzeni 6. dniowych siewek dyni figolistnej izolowano lektyny poprzez wytrącanie białek alkoholem, chromatografię powinowactwa na Con-A Sepharose, sączenie molekularne na Bio-gel P-100 oraz rozdział w elektroforezie w żelu poliakrylamidowym. Wydzielone trzy lektyny z korzeni (RLA₁, RLA₂, RLA₃) oraz cztery z łodyg (SLA₁, SLA₂, SLA₃, SLA₄) aglutynowały owcze erytrocyty znacznie lepiej w porównaniu z krwinkami ludzkimi. Arabinoza i ksyloza okazały się inhibitorami aglutynacji wywołanej przez lektyny zarówno korzeni jak i łodyg.

SŁOWA KLUCZOWE: Lektyny, Ara/Xyl specyficzność, immunologiczne pokrewieństwo, siewki, korzenie, łodgi, dyniowate (Cucurbitaceae).