HEMAGGLUTINATING AND ACID PHOSPHATASE (AcPASE) ACTIVITIES IN DEVELOPING SEEDLINGS OF FOUR SPECIES OF CUCURBITACEAE

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ABSTRACT

The acid phosphatase and hemagglutinating activities of four species of Cucurbitaceae were determined during seeds germination and seedlings development. In all cases traces of enzyme and hemagglutinating activities were found in dry and imbibed seeds. In developing seedlings of *Cucumis sativus* the activities increased to maximum on the 3rd day while in other species on the 6th day of germination and then fell down. Dot blot and Western blot techniques have shown that in seeds and seedlings of all investigated species present were proteins which cross-reacted with antibodies raised against lectins: CLBa and Con A.

It has been shown that proteins from seeds and seedlings of *Cucurbita maxima* var. *bambino*, *Cucurbita pepo* var. *giromontia* and *Cucumis sativus* had more pronounced antigenic similarity to lectin CLBa (from Cucurbitaceae) than Con A, while proteins from cotyledons of *Cucurbita pepo* var. *patissonina* reacted better with antibodies raised against Con A (the lectin from Papilionaceae) than with CLBa.

KEY WORDS: Lectins, acid phosphatases, immunological-relationship, seedling development, cotyledons, Cucurbitaceae.

INTRODUCTION

Lectins represent a heterogeneous class of proteins or glycoproteins sharing their ability to bind specific sugars or sugar-containing (macro) molecules (Golstein I.J et al. 1980). Plant lectins have been intensively studied during the past decades by researchers in various disciplines. As a result, the biochemical, physicochemical, biological and immunological properties of a steady increasing number of lectins have been well documented.

Isolation and characterization of new monocotyledonous lectins have revealed that several distinct classes of agglutinins occur within taxonomic groups that differ not only in their molecular structure and carbohydrate-binding properties, but also with respect to their abundance, distribution throughout the plant, and the control of their expression (Van Damme et al. 1991, 1994).

Related lectins occur in taxonomically related plant species and are supposed to represent evolutionarily closely related proteins. Studies on a number of plant species revealed amino acid sequences homologies (Konami et al. 1994; Mills et al. 1994) and immunological relationship (Hankins et al. 1979) corresponding well to taxonomic relationships between these species.

Acid phosphatases (AcPases) the orthophosphoric monoester phosphohydrolases which exhibit low substrate specificity are usually, like lectins, immunologically related proteins (Lor-

enc-Kubis I. and Morawiecka 1985; Lorenc-Kubis 1986) or glycoproteins which differ in their binding to Con A (Lorenc-Kubis and Bøg-Hansen 1981; Lorenc-Kubis 1983). The presence of sugar in the molecules of AcPases makes possible their interaction with other proteins, especially with lectins.

Plant lectins occur in seeds and also in all vegetative tissues (Peumans and Van Damme 1993; Chatelain et al. 1994). Multiple lectins have been isolated from the same tissue of many plants. In Cucurbitaceae a high level of hemagglutinating activity was found in phloem exudates of three species: pumpkin (*Cucurbita maxima*), cucumber (*Cucumis sativus*) and melon (*Cucumis melo*). No lectin was detected in the seeds of these three species, nor in the developing plant until the seedlings were at least 5 days old (Sabnis and Hart 1978).

In previous studies on lectins and AcPases from squash (*Cucurbita ficifolia*) we have found that extracts of cotyledons, roots and stems of developing seedlings showed agglutinating and acid phosphatases (AcPases) activity. The agglutinating and AcPase activities at the onset of germination were rather low and increased maximally on the 6th day of germination (Lorenc-Kubis et al. 1993; Lorenc-Kubis and Bonfante 1994; Lorenc-Kubis and Kalinowska 1994).

The cotyledons and roots of germinating seeds and developing seedlings provide a useful material to study the changes in many proteins e.g. lectins and acid phosphatases. To find out whether some other species of Cucurbitaceae contain also lectins and acid phosphatases and whether the lectins are re-

lated to squash lectin CLBa or to lectin from *Canavalia ensiformis*-ConA, study on seeds and developing seedlings of four species has been undertaken.

MATERIAL AND METHODS

Plant material

The study was carried out on seeds and seedlings of four species from Cucurbitaceae: *Cucurbita maxima* var. *bambino*, *Cucurbita pepo* var. *giromontis*, *Cucurbita pepo* var. *patissonina* and *Cucumis sativus*.

Seeds were sterilized by soaking in acetone as described by Lorenc-Kubis (1994) and then were sown in a moist germination bed of filter paper which was watered daily with tap water, 22–24°C. Until day 2 all seeds were incubated in the dark, then the plants were exposed to a light/dark cycle (12 hours light/12 hours dark). During the light phase the plants were illuminated by four light sources, 100 W each. The seedlings were harvested after 3-, 6-, 10- and 14-days and the cotyledons, stems and roots of seedlings were dissected.

Extraction of proteins

Cotyledons of dry seeds were ground and homogenized with 6 vols and cotyledons of developing seedlings 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, 30 min) the pellet was discarded and the supernatant was used for analysis of acid phosphatase and hemagglutinating activities.

Analytical methods

Protein was estimated by the method of Lowry et al. (1951) and determined spectrophotometrically at 280 nm.

Acid phosphatase activity was measured by following the release of p-nitrophenol from 5 mM sodium p-nitrophenyl phosphate in 0.1 M sodium acetate buffer, pH 5.1 at 37°C, as described by Ferens and Morawiecka (1985). The amount of p-nitrophenol was measured spectrophotometrically at 410 nm. One unit of enzyme activity corresponds to the hydrolysis of 1 µmol of p-nitrophenyl phosphate per 1 minute under the assay conditions.

Hemagglutination assay was performed at room temperature in microtiter U-sharped plates using serial two-fold dilutions of the test materials. Defibrinated human erythrocytes A, O, B were purchased from Blood Bank of Wrocław. The sheep erythrocytes were purchased from the Agricultural Academy of Wrocław. The erythrocytes were washed 3 times in PBS (phosphate buffered saline).

Extracts of 25 µl were 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.

Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Davis (1964) at pH 8.3 in 12% gel. Samples containing 80–100 µg proteins of crude extracts of cotyledons were subjected to electrophoresis. The gel was stained for proteins detection with 0.4% Coomassie Brilliant Blue R-250 in 50% TCA.

For detection of acid phosphatase activity the gels were soaked in 0.2 M acetate buffer, pH 5.1 for 20 min and then incubated at 37°C in 0.1 M acetate buffer, pH 5.1 containing 0.1% sodium α-naphthyl phosphate and 0.04% Fast blue B (until the transparent zones were visible). Then the gels were soaked in 7% acetic acid.

Polyacrylamide gel electrophoresis (SDS-PAGE) was achieved on 15% polyacrylamide gel slabs according to Laemmli (1970). Samples containing about 200 µg of proteins were denaturated in the presence of 1% SDS and 2% β-mercaptoethanol by heating for 3 min. in 100°C. Proteins in the gel were stained with 0.1% Coomassie Brilliant Blue or with silver nitrate according to Merrill et al. (1981).

Phosphorylase b from rabbit muscles (94 kDa), bovine serum albumine (67 kDa), hen ovalbumine (43 kDa), human erythrocytes carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine lactalbumine (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 0.5% 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, diluted 1: 2000 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.

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.

RESULTS AND DISCUSSION

Acid phosphatase and hemagglutinating activity in seeds and developmental seedlings of 4 species of Cucurbitaceae

Proteins from cotyledons of dry and imbibed seeds and 3-, 6-, 10- and 14-day old seedlings were extracted with 0.1 M acetate buffer, pH 5.1. Acid phosphatase and hemagglutinating activities were monitored during seeds germination and seedlings development. Changes in protein content and acid phosphatase activity in cotyledons of seeds and developmental seedlings are shown in Fig. 1A, B, C, D.

It has been shown that AcPase activity in all examined species, like in cotyledons of squash *Cucurbita ficifolia* (Lorenc-Kubis 1994), was relatively low at the onset of germination, increased on the 6th day (except *Cucumis sativus*) and then fell down. In cotyledons of cucumber (*Cucumis sativus*) the highest AcPase activity observed in 3 day old seedlings can be caused by a faster development of seedlings when compared to the other species.

The study of germinating seeds and developmental seedlings showed also marked variation in haemagglutinating activity in the extracts of cucumber (*Cucumis sativus*) and patisson (*Cucurbita pepo* var. *patissonina*). The protein content and the agglutination of sheep erythrocytes by the extracts from dry seeds and cotyledons of developmental seedlings of *Cucumis sativus* and *Cucurbita pepo* var. *patissonina* are presented in Fig. 2A, B.

Fig. 1. Changes in protein content and acid phosphatase activity in cotyledons of germinating seeds and seedlings of four species of Cucurbitaceae at different developmental stages. Data are the mean of four replications.

The haemagglutinating activity, similarly to the activity of acid phosphatase was low at the onset of germination, and increased rapidly to the maximum on the 3rd day (*Cucumis sativus*) or the 6th day (*C. pepo* var. *patissonina*) of germination. In 14-days old seedlings only a trace activity was found. Examination of various parts of the plants revealed a rapid reduction in lectin level with plant maturation (Nir et al. 1966).

We have shown earlier that lectin of cotyledons is involved in crossed-linking of some proteins and is localized in the protein bodies (Lorenc-Kubis and Bonfante 1994).

The low acid phosphatase and haemagglutinating activities in seeds of all investigated species suggest that AcPase and lectin may exist in a complexes with other molecules from which they are released during seeds germination and seedling development (Lorenc-Kubis et al. 1994). This could explain the increase of enzyme and agglutination activities observed on 3rd or 6th day of germination. (Lorenc-Kubis and Bonfante 1994).

We have found that roots and stems of cucumber and patisson seedlings contained lectins at concentrations four or more orders of magnitude lower than those in the cotyledons. The lower lectin activities in stems, roots and leaves than in co-

tyledons was also observed in developing seedlings from *Erythrina corallodendron* (Nir R. et al. 1996) and in seedlings of rye (Ferens-Sieczkowska et al. 1989).

Electrophoretical analysis

Crude extracts of dry seeds and cotyledons of developmental seedlings from all investigated species were electrophoresed on polyacrylamide gel (PAGE), pH 8.3 and stained for acid phosphatase activity. The results are presented in Fig. 3.

In extracts from dry seeds of all investigated species acid phosphatase was detectable only as a trace single band with a low electrophoretic mobility. In cotyledons of 3-days old seedlings only one band with the enzyme activity was detectable, while in extracts of 6-days old seedlings two major bands appeared and then diminished during development of seedlings. The only exception was *C. pepo* var. *giromontis* which showed two distinct bands with enzyme activity in cotyledons of 6 and 14-days old seedlings. The increase of the number of acid phosphatase bands in extracts of developing seedlings may indicate the heterogeneity of the enzyme probably caused by the release of glycoprotein AcPases from the complexes with lectin.

Fig. 4. Western blot analysis of cotyledon proteins from seeds and developing seedlings of Cucurbitaceae: A – *Cucurbita maxima* var. *bambino*; B – *C. pepo* var. *giromontis*; C – *C. pepo* var. *patissonina*; D – *Cucumis sativus*.

Samples (200 µg of protein) of crude extracts 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 antibody was detected with goat anti-rabbit IgG-peroxidase complex and was visualized with 4-chloro-1-naphthol.

Lane: a – dry seeds; b – imbibed seeds; c – 3-day; d – 6-day; e – 10-day; f – 14-day old seedlings.

Antigenical similarities between some proteins from cotyledons of Cucurbitaceae (Cucurbita maxima var. bambino, C. pepo var. giromontis, C. pepo var. patissonina, Cucumis sativus) and lectins: Con A or CLBa

The cross-reactivity between antibodies raised against con A (the lectin from a Papilionaceae plant) or endogenous lectin CLBa and proteins from extracts of seeds and cotyledons of developing seedlings has been examined by the Western blotting technique. The proteins from crude extracts of seeds, and cotyledons of seedling resolved by SDS-PAGE were transferred on to NC membrane and treated with antibodies as described in "Materials and Methods". The results are presented in Fig. 4A, B, C, D.

Western blot analyses were carried out to determine if proteins from Cucurbitaceae seeds and seedlings are immunologically related to CLBa and Con A lectin. It has been shown that in cotyledons of dry and imbibed seeds and 3 days old seedlings of *Cucurbita maxima* and *Cucumis sativus*, the proteins with a low molecular weight (20 and 27 kDa) gave a sharp cross reaction with antibodies raised against lectin CLBa. In the case of proteins of dry and imbibed seeds from *C. pepo* var. *giromontis* and *C. pepo* var. *patissonina* only a weak positive immunological reactivity was observed. In developing seedlings of all investigated species proteins mainly with a molecular weight 40-67 kDa, cross reacted with antibodies raised against CLBa.

The experiments with antibodies raised against Con A revealed that proteins from *C. maxima* and *C. pepo* var. *patissonina* had a high degree of positive immunological reactivity in fraction 20kDa.

It has been shown that proteins from *C. maxima*, *C. pepo* var. *giromontis* and *Cucumis sativus* gave sharper cross reaction with antibodies raised against CLBa (lectin from *Cucurbita ficifolia*) than with Con A, while proteins from *C. pepo* var. *patissonina* reacted much stronger with antibodies anti Con A (the lectin from Papilionaceae). Analysis of proteins from stems and roots of four Cucurbitaceae species revealed also some proteins which gave weak cross reaction with antibodies raised against lectins Con A and CLBa.

The cross reaction of some proteins from different species of Cucurbitaceae with antibodies raised against Con A and CLBa may indicate considerable antigenical similarity between lectins from different species of Cucurbitaceae and lectin from Papilionaceae plant (Con A). Immunological evidence for structural similarity among lectins has been provided for Leguminosaceae (Hankins et al. 1970), Solanaceae (Kilpatrick et al. 1980) and Gramineae (Stinissen et al. 1983; Peumans and Stinissen 1983). Cereal lectins from wheat, rye and barley are not only biochemically and physicochemically identical but also immunologically indistinguishable from each other (Peumans et al. 1982 a). Also lectin from false brome grass (*Brachypodium sylvaticum*) and rice (*Oryza sativa*) are structurally and immunologically related to wheat germ agglutinin (WGA) (Peumans et al. 1982b; Peumans and Stinissen 1992; Tsuda 1979). A study of the mannose-binding lectins from several representatives of the plant families Amaryllidaceae and Alliaceae has revealed that all mannose-binding lectins are serologically related (Van Damme et al. 1994).

The result presented in this paper demonstrated that different species of Cucurbitaceae contain proteins that are immunologically related not only to CLBa-the lectin isolated from the same family but also to Con A, a lectin from Papilionaceae. The observation may provide evidence that during the evolution some proteins of the species belonging to different

genera, as well as different families, have been highly conserved. Such a conservation may be characteristic for proteins that fulfil an important biological function.

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AKTYWNOŚĆ HEMAGLUTYNACYJNA I FOSFATAZOWA W ROZWIJAJĄCYCH SIĘ SIEWKACH CZTERECH GATUNKÓW Z RODZINY CUCURBITACEAE

STRESZCZENIE

Oznaczano aktywność kwaśnej fosfatazy oraz hemaglutynacyjną w trakcie kiełkowania nasion i rozwoju siewek czterech gatunków rodziny Cucurbitaceae.

W suchych i napęczniałych nasionach wszystkich badanych gatunków wykazano obecność śladowej aktywności zarówno kwaśnej fosfatazy jak i lektynowej. Wyraźny wzrost obu aktywności obserwowano w liścieniach 3-dniowych siewek ogórka i 6-dniowych siewek pozostałych badanych gatunków. Po tym okresie następował spadek obu aktywności.

Analiza surowych wyciągów z liścieni badanych gatunków przy pomocy technik "Dot blot" i "Western blot" wykazała obecność białek reagujących z przeciwciałami skierowanymi przeciwko lektynom CLB_a i Con A. Wykazano, że białka nasion jak i rozwijających się siewek *Cucurbita maxima* var. *bambino*, *Cucurbita pepo* var. *giromontia* jak i *Cucumis sativus* wykazywały większe antygenowe podobieństwo do lektyny CLB_a (wydzielonej z Cucurbitaceae) niż do Con A. W przypadku białek nasion jak i liścieni *Cucurbita pepo* var. *patissonina* stwierdzono znacznie większą ilość frakcji reagujących z przeciwciałami skierowanymi przeciwko Con A (lektynie z Papilionaceae) niż endogennej lektynie – CLB_a.

Duże podobieństwo antygenowe białek różnych gatunków rodziny dyniowatych do lektyny CLB_a a w szczególności do Con A wskazuje na konserwatywność tych białek.

SŁOWA KLUCZOWE: Lektyny, kwaśne fosfatazy, immunologiczne pokrewieństwo, liścienie, Dyniowate (Cucurbitaceae).