ACID PHOSPHATASES IN SEEDS AND DEVELOPING SEEDLINGS OF SQUASH (*CUCURBITA FICIFOLIA*)

IRENA LORENC-KUBIS

Institute of Biochemistry, University of Wrocław, Tamka 2
50-137 Wrocław, Poland

(Received: January 18, 1994. Accepted: March 22, 1994)

ABSTRACT

Changes in protein content and acid phosphatase activity were followed during germination (imbition through seedlings development) in extracts from cotyledons of squash (*Cucurbita ficifolia*). It has been shown that the activity of acid phosphatase was initially low and then increased to a maximum after 6 days of imbibition. Acid phosphatases were isolated from cotyledons of seeds and from 6-, 10- and 22-days old seedlings by extraction the proteins with 0.1 M acetate buffer pH 5.1, precipitation with ethanol and by affinity chromatography on con A-Sepharose. Two glycoprotein enzymes AcPase Ba and AcPase Bb which differ in their affinity to immobilized con A were obtained. Both acid phosphatases retained the enzyme activity after binding to free con A. Rocket affinity electrophoresis of AcPase Ba and AcPase Bb, isolated from cotyledons of seeds and seedlings, revealed differences in their ability to bind to con A during seeds germination and seedling development indicating changes in their sugar component. Con A was found to activate both enzymes. The enzymes cross-reacted with monospecific antibodies raised against grass seed acid phosphatase Ba indicating an antigenic relationship between squash and grass acid phosphatases.

KEY WORDS: Acid phosphatase, con A-binding, cotyledons, *Cucurbitaceae*, glycoproteins, immunological relationship.

INTRODUCTION

Seed germination involves the loss of stored reserves from the embryonic cotyledons as the root-shoot axis of the young seedling expand. During the germination the activity of various proteins changes (Bøg-Hansen et al. 1974; Harley and Beevers 1986; Moris et al. 1985, Wilimowska-Pelc et al. 1991) which may indicate a regulatory function of these proteins in the biochemical mechanism of germination. Earlier it has been shown that acid phosphatase activity changes during germination of seeds (Karczewska et al. 1987; Teno et al. 1987) and that the level of enzyme activity in grass seeds is related strictly to their germination ability (Lorenc-Kubis and Morawiecka 1984).

Acid phosphatases which occur in seeds and tissues of higher plants are glycoproteins (Kruzel and Morawiecka 1982; Lorenc-Kubis and Morawiecka 1980; Ferens and Morawiecka 1985) and bind to lectins (Lorenc-Kubis and Bøg-Hansen 1981; Lorenc-Kubis and Morawiecka 1986).

Rocket affinity electrophoresis with con A revealed that AcPases isolated from seeds and tissues of grass seedlings (Lorenc-Kubis and Morawiecka 1986) and blue lupine (Lorenc-Kubis 1989) differ in their ability to bind to free con A, what may reflect changes in their sugar component.

Very little is known about acid phosphatases in germinating seeds and developing seedlings of *Cucurbitaceae*. Thus, the temporal changes in squash (*Cucurbita ficifolia*) cotyledonary protein content and acid phosphatase activity during seed germination and seedling development have been investigated.

Besides, studies on some properties of isolated AcPases, their interaction with lectins have been undertaken.

MATERIALS AND METHODS

Plant material

The study was carried out on seeds and seedlings of squash (*Cucurbita ficifolia*). Seeds were sterilized by soaking them in 90% acetone for 5 min then 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 for 22 d with 10-12 h of daily light. No fungal contamination was observed on the germinating seeds. The seedlings were harvested after 3-, 6-, 10- and 22-d and the cotyledons of seedlings were dissected.

Extraction of acid phosphatase

Ground cotyledons of dry seeds and cotyledons of 3-, 6-, 10- and 22-d old seedlings were homogenized with 3 vols (w/v) of 0.1 M sodium acetate buffer, pH 5.1 at 40 °C and extracted for 1 h with constant mechanical stirring. After centrifugation (12 000 x g, 30 min) the pellet was discarded and the supernatant was used for purification of acid phosphatase.

Abbreviations used: AcPase – acid phosphatase; p-NPP-p – nitrophenyl phosphate; PAGE – polyacrylamide gel electrophoresis; Con A – concanavalin A. LcA – Lens culinaris agglutinin; WGA – wheat germ agglutinin; SBA – soy bean agglutinin.

Ethanol-precipitation

The proteins were precipitated with ethanol to a final concentration of 60%. The process was carried out at -20°C . Cooled ethanol was gradually added to the supernatant with constant stirring. After being kept at this temperature for 24 h, the mixture was centrifuged at $6000 \times g$ for 20 min. The precipitate was suspended in 0.1 M sodium acetate buffer, pH 5.6 and dialyzed against this buffer. After 24 h solid material was removed by centrifugation at $12\,000 \times g$ for 20 min and an equal volume of 0.1 M acetate buffer, pH 5.6 containing 2 M NaCl and Mg^{2+} , Mn^{2+} , Ca^{2+} (chloride forms) at 2 mM concentration was added.

Affinity chromatography

Affinity chromatography was carried out at room temperature as described earlier (Lorenc-Kubis 1989). The material was 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 Mg^{2+} , Mn^{2+} , Ca^{2+} at 1 mM concentration. The proteins not adsorbed on the column were removed by washing with the same buffer. The active 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, or in the stream of cold air.

Ion-exchange chromatography

The active fractions eluted from con A-Sepharose column with 15 mM-(AcPase Ba) and 300 mM α -methyl-D-mannopyranoside (AcPase Bb) were dialysed against 0.05 M acetate buffer, pH 4.6 and then subjected to chromatography on SP-Sephadex C-50 (15 cm long, 3 cm i.d.; Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The proteins were eluted with increasing NaCl concentration (0.05 to 1.0 M). The enzymes were eluted from the column with 0.1 M NaCl and 0.5 M NaCl in 0.05 M sodium acetate buffer, pH 4.6.

Analysis of acid phosphatases

Acid phosphatase activity was measured as described in Ferens et al. (1985) using 5 mM p-NPP as substrate in total volume of 2 ml. The amount of p-NP was measured spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the amount of acid phosphatase releasing 1 μmol of p-NP at pH 5.1 at 37°C after 1 min.

Lectin-acid phosphatase interaction experiments were performed with 1–5 $\mu\text{g}/\text{ml}$ of enzyme and 10–50 $\mu\text{g}/\text{ml}$ of Con A (Pharmacia, Uppsala, Sweden) as in Lorenc-Kubis (1989).

Double affinity diffusion with free lectins: Con A, LcA, SBA and WGA was performed in 10 mg/ml agarose gel (pH 8.6). The lectins (10 μl of 10 mg/ml) were placed in the outside wells, and the acid phosphatase (10 μl of 1 mg/ml) was placed in the centre well. The diffusion took place during 48 h.

Rocket affinity electrophoresis was performed according to Bøgg-Hansen et al. (1977).

Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Davis (1964) at pH 8.3 in 12% gel. Samples containing 20–100 μg proteins of crude extracts of cotyledons were subjected to electrophoresis. Gels were stained for proteins with 0.4% Coomassie brilliant blue R-250 in 50% TCA. For detection of acid phosphatase activity the gel was 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% so-

dium α -naphthyl phosphate and 0.04% Fast blue B (until the transparent zones were visible). Then the gels were soaked in 7% acetic acid.

The Immunodiffusion test was performed according to Ouchterlony (1967).

Protein precipitates were stained on washed and dried immunoplates (Axelsen et al. 1973) with Coomassie brilliant blue R-250. Acid phosphatase activity was revealed on the plates by the diazocoupling method. The washed and dried plates were incubated at 37°C in 0.1 M acetate buffer, pH 5.1 containing 1 mg/ml sodium α -naphthyl phosphate as substrate and 0.4 mg/ml Fast Blue B salt. The acid phosphatase activity in the immunodiffusion test, was revealed on the plates before staining for protein.

Antibodies were purified from rabbit antiserum raised against purified acid phosphatase Ba from seeds of meadow-grass (*Poa pratensis* L.) according to Harboe and Ingild (1973).

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

RESULTS AND DISCUSSION

Acid phosphatase activity during seeds germination and development of seedlings

Protein from cotyledons of dry and imbibed seeds and cotyledons of 3-, 6-, 10- and 22-d old seedlings were extracted with 0.1 M acetate buffer, pH 5.1 and the acid phosphatase activity was monitored during germination and seedlings development. The curves showing protein content, and acid phosphatase activity in cotyledons as a function of germination days are shown in Fig 1. It has been shown that the AcPase activity

Fig.1. Changes in protein content (▲-▲-) and acid phosphatase activity (●-●-) of cotyledons during seeds germination and seedlings development of squash (*Cucurbita ficifolia*).

Proteins of cotyledons were extracted with 0.1 M acetate buffer pH 5.1. Acid phosphatase activity was measured in crude extracts in conditions described in *Material and methods*. The data are means of five replications.

was relatively low at the onset of germination and then increased about 9-fold to a peak on day 6 and fell down on day 10. These results allowed to establish the time of maximum of acid phosphatase activity during germination and hence to optimize the extraction procedure. Changes in acid phosphatase activity have been demonstrated during plant growth and development (Baker and Takeo 1973, Bøg-Hansen et al. 1974, Ching et al. 1984; Tamura et al. 1982). Mioduszevska et al. (1984) described an increase of acid phosphatase activity during potato bulb maturation and completion of repose state.

Electrophoretical analysis

The proteins from crude extracts of seed cotyledons and developing seedlings were electrophoresed on polyacrylamide gel (PAGE), pH 8.6 and stained for proteins and acid phosphatase activity. The data are shown in Fig. 2. In extracts from cotyledons of dry seeds acid phosphatase activity was not detectable in these conditions or only a trace band with a low mobility was shown. This may suggest that in dry seeds the enzyme is bound to the other proteins and is hardly soluble in acetate buffer. In extracts of cotyledons from 3- and 6-d old seedlings after imbibition one major and two minor bands of enzyme activity appeared. In 22-d old seedlings one more band with a low mobility appeared.

Fig.2. Polyacrylamide gel electrophoresis of cotyledon proteins and acid phosphatase during growth of squash seedlings.

The proteins (20-100 μ g) 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 (1); the other for acid phosphatase activity (2) (incubation at 37 °C in 0.2 M acetate buffer pH 5.1 containing 0.1 % sodium α -naphthyl phosphate and 0.04 % Fast Blue B). a) -dry seeds; b) -6-d; c) -10-d; and d) -22-d old seedlings.

Fig.3. Affinity chromatography on con A-Sepharose of acid phosphatase from cotyledons of: dry seeds (a), 6-d (b), 10-d (c), 22-d old seedlings from squash (d).

The dialysed ethanol precipitated proteins (60-100 mg) were applied to con A-Sepharose column (10 cm long, 3.0 i.d.) equilibrated with 0.1 M acetate buffer pH 5.6 containing 1 M NaCl and Mg^{2+} , Mn^{2+} , Ca^{2+} at 1 mM. The adsorbed proteins were eluted from the column with 15 mM - and 300 mM - α -methyl D-mannopyranoside in acetate buffer pH 5.6. Fractions of 3ml were collected. The bars indicate the pooled fractions (o-o). Optical density at 280 nm, (●---●); acid phosphatase activity, (o---o); AcPase Ba (I), AcPase Bb (II).

Purification of acid phosphatase

Acid phosphatases from cotyledons of seeds and developing seedlings were precipitated from 0.1 M acetate (pH 5.1) extracts with ethanol and then separated by affinity chromatography on con A – Sepharose. The elution profiles obtained after chromatography of proteins from coleoptiles of dry seeds and of developing seedlings (6-, 10-, and 22-d old) are shown in Fig 3. About 60% of total protein applied on the column was washed out with the starting buffer, pH 5.6. This fraction contained only a trace activity of acid phosphatase. The con A binding fraction which contained about 90% of the total activity of the enzyme was eluted from the column with 15 mM α -methyl-D-mannopyranoside (AcPase Ba), and with 300 mM solution of this sugar (AcPase Bb).

In cotyledons of dry seeds and 22-d old seedlings only a low amount of con A binding (AcPase Ba and AcPase Bb) could be detected.

It was shown that during the development of seedlings changes in the activity of AcPase Ba and Bb occurred. In cotyledons of dry seeds the activity of AcPase Bb was higher than the AcPase Ba while in cotyledons of 10-d old seedlings the activity of AcPase Ba was more than 2 times higher than that of AcPase Bb. In 6-, and 22 days old seedlings both enzymes have shown similar activity. The highest activity of acid phosphatase Ba and Bb was found in cotyledons of 6-d old seedlings. The differences in activity between the two forms of AcPase Ba and Bb may reflect some changes occurring in the molecular forms of these enzymes during seedlings development. These enzymes were further purified by ion-exchange chromatography on SP-Sephadex. The AcPases Ba and Bb were eluted from the column with 0.1 M and 0.5 M NaCl in 0.05 M sodium acetate buffer, pH 4.6.

Interaction of AcPase Ba and Bb with lectins

Both cotyledonary enzymes from all stages of development interact with free con A and form an affinity precipitate indicating that the carbohydrate part of enzymes contains exposed mannose or glucose units. Moreover the enzymes were activated by binding to con A. The increase of activity ranges between 50 to 80 per cent. The activation was reversible with addition of specific sugar inhibitors of lectin binding.

It has been shown earlier that some acid phosphatases change their properties after interaction with lectins (Lorenc-Kubis and Bøg-Hansen 1981; Lorenc-Kubis et al 1981; Ferens and Morawiecka 1985a; Morawiecka and Arabska 1982). The effect of lectins on the enzyme activity suggests a protective action of lectins perhaps by stabilizing the conformation of the enzyme (Lorenc-Kubis et al. 1981).

Rocket-affinity electrophoresis with free con A showed differences in the morphology and staining intensity between the affinity precipitates of AcPase Ba and Bb isolated from cotyledons of seedlings from different stages of development (Fig. 4). The affinity precipitate of AcPase Ba from cotyledons of dry seeds was very weak, whereas the enzyme isolated from 6-, 10- and 22-d old seedlings formed a distinct precipitate with free con A. A distinct affinity precipitate was formed also in the case of AcPase Bb isolated from cotyledons of 6-, and 10-d old seedlings. In the case of AcPase Bb from 22-d seedlings only a small precipitate was found. The differences in morphology and staining intensity of rocket-affinity precipitates of AcPase Ba and Bb may reflect changes in the sugar component of the enzymes during the germination of seeds and development of seedlings.

Fig. 4. Rocket affinity electrophoresis of 10 μ g of acid phosphatase Ba (A) and Bb (B) of cotyledons from: seeds (a) and seedlings 6-d (b), 10-d (c), and 22-d old (d) into 10 mg/ml agarose gel containing con A 50 μ g/cm². After electrophoresis the plates were washed and dried and acid phosphatase Ba and Bb were revealed by incubating the plates in 0.1 M acetate buffer pH 5.1, 37 °C containing 0.1 % sodium α -naphthyl phosphate and 0.04% Fast Blue B (until the transparent affinity precipitates were visible). Then the plates were washed with water and dried.

Immunological relationship of squash acid phosphatases with grass AcPases

The acid phosphatase Ba and Bb from cotyledons of 6-d old seedlings was analysed by double immunodiffusion using antibodies raised against meadow-grass (*Poa pratensis* L.) AcPase Ba. Figure 5 shows the results of this test. Both enzymes formed an immunoprecipitate which showed acid phosphatase activity. The enzyme activity was revealed before staining for protein, which demonstrates that the same precipitation band represents both protein and enzyme activity. After interaction

Fig.5. Ouchterlony immunodiffusion of acid phosphatase Ba(A) and Bb(B) from squash cotyledons against anti-seeds acid phosphatase Ba of meadow-grass (*Poa pratensis*) antibodies.

The precipitates were first stained for acid phosphatase activity and then for protein with Coomassie brilliant blue R-250 on the washed and dried plates.

Antibodies were applied in the central well (a). The peripheral wells were filled with AcPase of: dry seeds, (1) and seedlings: 5-d (2), 10-d (3) and, 22-d old.(4).

with antibodies the AcPase remain fully active, what leads to the conclusion that the substrate-binding region is placed beyond the area of antigenic determinants. The cross-reaction of AcPases Ba and Bb with antibodies raised against acid phosphatase Ba from meadow-grass suggests that the enzymes from cotyledons of squash (*Cucurbita ficifolia*) and grass seeds have common antigenic sites. Earlier studies on immunological relationship of grass acid phosphatases (Lorenc-Kubis 1986; Lorenc-Kubis and Morawiecka 1985) and AcPases isolated from blue lupine (*Lupinus angustifolius* L.) (Lorenc-Kubis 1989) have shown that the enzymes are immunologically related proteins.

The immunological relationship between acid phosphatases from mono- and dicotyledons and the changes in enzyme activity in the presence of lectins may indicate that both of the proteins with a high degree of conservation may play an important metabolic function in plants.

ACKNOWLEDGEMENTS

The author thanks Professor Dr. hab. Bronisława Morawiecka for her helpful discussion and for reviewing the manuscript; Renata Herba and Małgorzata Dobaczewska for excellent help in the preparation of the enzyme. I am grateful to Dr. Zofia Olichwier for developing my interest in germination of squash (*Cucurbita ficifolia*) seeds.

This study was supported by K.B.N. grant, No 4047/PB/Bch/93.

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KWAŚNE FOSFATAZY NASION I ROZWIJAJĄCYCH SIĘ SIEWEK DYNI FIGOLISTNEJ (*CUCURBITA FICIFOLIA*)

STRESZCZENIE

Przeprowadzone badania nad kwaśnymi fosfatazami (AcPazami) liścieni dyni figolistnej wykazały zmiany aktywności enzymu w trakcie kiełkowania nasion i rozwoju siewk. Niską aktywność kwaśnej fosfatazy stwierdzono w suchych nasionach jak i liścieniach w początkowym okresie kiełkowania. Wyraźny wzrost aktywności enzymu obserwowano w liścieniach 6 dniowych siewek. Z liścielni nasion oraz siewk 6-, 10-, 22-dniowych izolowano kwaśne fosfatazy poprzez ekstrakcję białek 0.1 M buforem octanowym o pH, 5.1, wytrącanie alkoholem oraz chromatografię powinowactwa na con A-Sepharose. We wszystkich przypadkach uzyskano dwie grupy glikoproteinowych enzymów: AcPazę Ba i AcPazę Bb różniące się powinowactwem do immobilizowanej lektyny. W kompleksie z wolną con A enzymy te zachowywały aktywność. W rakietowej elektroforezie powinowactwa AcPazy Ba i Bb izolowane z liścieni nasion oraz rozwijających się siewek wykazywały różnice w powinowactwie do wolnej con A świadczące o zmianach zachodzących w części węglowodanowej tych enzymów. Wydzielone enzymy wykazywały podobieństwo antygenowe z kwaśnymi fosfatazami roślin jednoliściennych – traw.

SŁOWA KLUCZOWE: Kwaśna fosfataza, powinowactwo do con A, liścienie, *Cucurbitaceae*, glikoproteiny, immunologiczne pokrewieństwo.