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Increases in α-mannosidase activity in the arbuscular mycorrhizal symbiosis of *Allium schoenoprasum*

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Abstract Mycorrhizal and nonmycorrhizal roots of *Allium schoenoprasum* were tested for activities of α-mannosidase, β-glucosidase and arabinosidase. Mannosidase activity was higher by a factor of two in mycorrhizal than in nonmycorrhizal root extracts. The apparent molecular weight of the enzyme was 152 kDa and its K_M was 1.25 mM in colonized roots and 1.85 mM in uncolonized roots. α-Mannosidase activity was further characterized by an acid pH optimum and Zn^{2+} dependency. No significant differences could be found between mycorrhizal and nonmycorrhizal roots for β-glucosidase and arabinosidase activities.

Key words Arbuscular mycorrhiza · α-Mannosidase · Glycosidases · *Allium schoenoprasum*

Introduction

The biochemical mechanisms by which an arbuscular mycorrhizal (AM) symbiosis is established are still unknown. A useful approach towards a more detailed understanding is to screen colonized and uncolonized root systems for enzyme activities. Glycosidic enzymes apparently play an important role in the presymbiotic and symbiotic phases of AM. Fungal hydrolytic enzymes are required for the penetration of host tissues, and the involvement of cellulase, hemicellulases and pectinases has been suggested in several studies (Garcia-Romera et al. 1990, 1991; Garcia-Garrido et al. 1992). By the activity of glycosidic enzymes, oligosaccharides are released from polysaccharides and act as signal molecules for defense reactions of plants against pathogens (McNeil et al. 1984; Ryan and Farmer 1991). Thus, glycosidic enzymes may play a key role in processes of recognition and compatibility. In AM a matrix is formed between host cell membrane and fungal cell wall which consists of polysaccharides and proteins similar to precursors of the primary plant cell wall (Bonfante-Fasolo et al. 1990). The matrix has been suggested to be a carbon source for the fungus (Harley and Smith 1983; Peretto et al. 1995). If so, glycosidic enzymes should also be important for the production and utilization of this material. Other enzymes like chitinases may be part of a system that allows the host to control the proliferation of the fungus by means of carefully regulated defense reactions. Isoforms of chitinase specific for the symbiosis (Dumas-Gaudot et al. 1992) can contribute to this regulation. However, the precise involvement of glycosidic enzymes for AM establishment and function has not been well addressed, and further key enzymes of the host-fungus interaction may exist. Therefore, in the present study the effect of AM colonization upon activities of several glycosidases in roots of *Allium schoenoprasum* was assessed.

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Materials and methods

Seeds of chives (*Allium schoenoprasum* L.) were grown in an autoclaved mixture of sand/perlite/garden soil (8:2:1) under standard greenhouse conditions. For AM inoculation, 2–3 well-infected plants of *A. schoenoprasum* were transferred to pots with seeds or 50-day-old target plants. Experiments were conducted in two parallel sets from December to March, with sampling 60 and 90 days after seed inoculation and 30 days after inoculation of precultivated plants.

At harvest, roots were cut off 0.5 cm beneath the hypocotyl and pooled from 2–3 pots. Samples for the quantification of AM colonization were taken and root extracts were prepared. Colonized root length was determined according to McGonigle et al. (1990) after trypan blue staining (Koske and Gemma 1989). Root extracts were obtained from a given fresh weight which was pulverized with pestle and mortar in liquid nitrogen. Extraction buffer [50 mM trisodium citrate pH 5.0, 1 mM dithioerythrol, 3% (w/v) polyvinylpolypyrrolidon] was added to a ratio of 1:5 (w/v) and the resulting slurry was filtered through two layers of gauze and one layer of Miracloth (Calbiochem, San Diego, Calif.). The filtrate was centrifuged for 10 min at 10 000 *g*. The supernatant was

separated, the pellet was stirred up again, recentrifuged and the supernatants were pooled. The combined supernatants were subjected to a third centrifugation. The resulting supernatant was used as soluble protein extract either immediately or after storage in aliquots at –20 °C.

Root extracts containing 60 μg protein in 100 μl were subjected to a screening procedure for glycosidic activity using agar plates. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. The presence of glycosidases was indicated by the release of p-nitrophenol (PNP) from glycoconjugates. Substrates were from Sigma, Deisenhofen: PNP-α-D-mannopyranoside for α-mannosidase (α-D-mannoside-mannohydrolase; E.C. 3.2.1.24), PNP-β-D-glucopyranoside for β-glucosidase (β-D-glucoside glucohydrolase; E. C. 3.2.1.21), PNP-α-L-arabinofuranoside for arabinosidase (α-L-arabinofuranoside-arabinofuranohydrolase; E.C. 3.2.1.55). Agar plates contained 1% (w/v) agar, 50 mM trisodium citrate buffer pH 5.0 and 1 mM substrate. Samples were applied to 7-mm-diameter wells cut into the agar. Commercially available enzymes (Sigma, Deisenhofen) were used as positive controls. Incubation was for 20 h at 37 °C and the released PNP was made visible by addition of 0.2 M Na_2CO_3.

To quantify enzyme activities, root extracts were tested in photometric assays. 0.1 ml of extract was added to 1.5 ml preheated test solution consisting of 50 mM trisodium citrate pH 5.0, and 5 mM substrate (modified after Paus and Christensen 1972; Tagawa and Kaji 1988). For the β-glucosidase assay, sodium acetate was used instead of citrate (modified after Deshpande and Ericsson 1988). Samples of 0.5 ml were taken after 0, 30 and 60 min incubation at 37 °C and transferred to 2.5-ml aliquots of 0.2 M Na_2CO_3. Absorbance at 400 nm was measured in a Uvikon 860 photometer (Kontron, Munich). Enzyme activity in U = 1 μmol/min was determined with a PNP standard. The K_M value of α-mannosidase was determined with extracts from roots colonized by *Glomus mosseae* 90 days after inoculation. Proteins were concentrated by precipitation with 90% $(NH_4)_2SO_4$ and desalted by dialysis. Substrate concentration was varied between 0.1 and 10 mM. Dependence of the enzyme reaction upon divalent cations was tested by addition of 1 mM EDTA and/or 5 mM $ZnSO_4$.

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed in slab gels in a Mini Protean II apparatus (Bio-Rad, Munich) at 4 °C, according to Davis (1964), with a pre-electrophoresis to prevent inactivation of enzymes by ammonium persulfate (Gabriel 1971). α-Mannosidase activity was visualized by a modified method of Gabriel and Wang (1969). Afterwards the gels were silver-stained (Blum et al. 1987). The apparent molecular weight of α-mannosidase was determined by the method of Hedrick and Smith (1968) in nondenaturing gels using the marker proteins urease, β-amylase, bovine serum albumin and ovalbumin (Sigma).

Spores were isolated by wet sieving, decanting (Gerdemann and Nicholson 1963) and centrifugation on sucrose density gradients (25% sucrose, 5 min at 1000 *g*). They were surface sterilized for 20 min with 2% (w/v) chloramine T, 0.02% (w/v) streptomycin sulfate and 0.05% (v/v) Tween 40. Aliquots of 500 spores were incubated for 21 days under greenhouse conditions near the roots of 30- to 50-day-old plants of *A. schoenoprasum* in the soil mixture described above. After this period of time, spores were reisolated and again surface sterilized. 10 000 spores of *Glomus intraradices* or 1000 spores of *G. caledonium* were crushed on ice with a micropestle. After the addition of 100 μl 50 mM trisodium citrate buffer pH 5, the extract was centrifuged for 1 min at 10 000 *g*. The supernatant was used as crude extract. α-Mannosidase activity was measured as follows: 50 μl crude extract and 50 μl test solution (10 mM substrate, 50 mM trisodium citrate pH 5.0) were mixed and incubated at 37 °C for 1 h. Then 0.2 ml Na_2CO_3 was added and the absorbance was measured as described above.

Results

Plants in several physiological and developmental stages were used to test the involvement of glycosidases in the AM symbiosis. None of the uninoculated control plants showed AM infection in any of the experiments. In the plants inoculated with *G. intraradices* a continuous increase in infected root length was detectable throughout 90 days (Fig. 1).

In the initial screening procedure for enzyme activity on agar plates, α-mannosidase, arabinosidase and some samples of β-glucosidase showed positive reactions visible as a yellow halo around the wells. Therefore, photometric tests with these enzymes were performed in order to quantify enzyme activity. Activities of β-glucosidase in the root extracts were close to the level of detection (0.7–1.6 mU/mg protein). Thus, the precision of these measurements was too low to allow the detection of differences between mycorrhizal and nonmycorrhizal plants. A higher level of arabinosidase activity was found (4.9–6.2 mU/mg protein), but no significant differences between mycorrhizal and nonmycorrhizal plants could be detected (data not shown).

α-Mannosidase activity in mycorrhizal roots was twofold higher than in nonmycorrhizal roots (Fig. 2). The level of activity found in inoculated and in uninoculated plants increased during the experiment; however, the difference between mycorrhizal and nonmycorrhizal plants was the greatest 90 days after inoculation of the seedlings with *G. intraradices* or *G. mosseae*. A similar symbiosis-related activity was detected at this stage in precultivated plants that had been inoculated with *G. intraradices* at the age of 50 days. This reflects the developmental stage of the plants rather than the period of time passed since inoculation.

α-Mannosidase activity was not detected in spore extracts of either *G. intraradices* or *G. caledonium*. Equally negative results were obtained from spores germinated in the rhizosphere of *A. schoenoprasum*. Hyphae

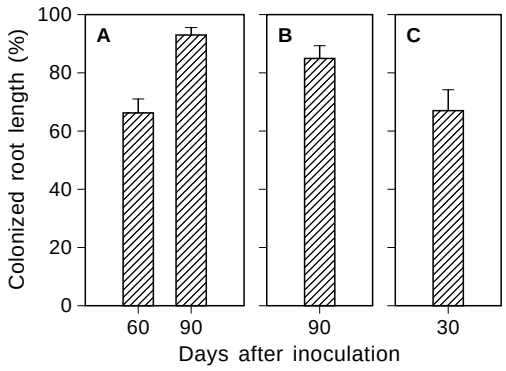

Fig. 1A–C Development of mycorrhizal infection. **A** Seeds of *Allium schoenoprasum* inoculated with *Glomus intraradices*. **B** Seeds of *A. schoenoprasum* inoculated with *G. mosseae*. **C** Precultivated plants of *A. schoenoprasum* colonized by *G. intraradices*. *Error bars* show one standard deviation

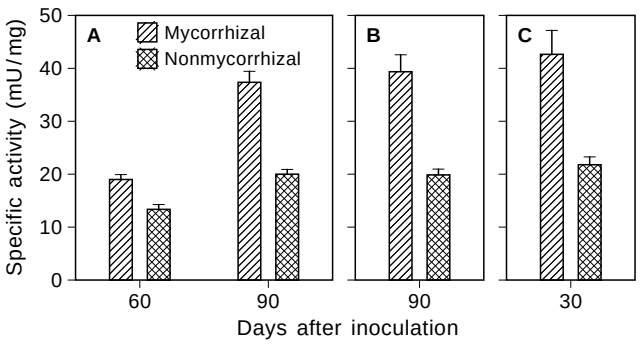

Fig. 2A–C Specific activity (mU/mg protein) of α-mannosidase in crude extracts of mycorrhizal and nonmycorrhizal roots of *A. schoenoprasum*. **A** Roots 60 and 90 days after planting and inoculation with *G. intraradices*. **B** Roots 90 days after planting and inoculation with *G. mosseae*. **C** Roots 80 days after planting and 30 days after inoculation with *G. intraradices*. Data are means of two replications. *Error bars* represent one standard deviation

attached to the spores and mycorrhizal roots demonstrated successful spore germination. Thus, direct assignment of α-mannosidase activity as a fungal enzyme was not possible.

In order to achieve further insight into the possible role of α-mannosidase for the AM symbiosis, the enzyme activity was subjected to further biochemical characterization. Direct detection of α-mannosidase in the gel after nondenaturing PAGE allowed molecular weight determination (Hedrick and Smith 1968). Following electrophoresis and enzyme assay from extracts, α-mannosidase appeared as a single yellow band with an apparent molecular weight of 152 kDa for both mycorrhizal and nonmycorrhizal roots. The higher specific activity of the enzyme in colonized roots was visible as a higher band intensity. The localized activity of α-mannosidase corresponded to a protein band visualized by silver staining (results not shown).

The pH optimum for α-mannosidase activity, measured photometrically was pH 5.0 for mycorrhizal roots and pH 4.7 for nonmycorrhizal roots. The K_M value of α-mannosidase for its PNP-conjugated substrate was determined to be 1.25 mM in mycorrhizal and 1.85 mM in nonmycorrhizal roots. Within 4 h, addition of 1 mM EDTA decreased α-mannosidase activity under the described conditions to 69% of the control without chelator (100%), whereas 5 mM Zn^{2+}stimulated it to 139% of the control. The inhibition caused by EDTA was reversed by addition of 5 mM Zn^{2+} to 130% of the control activity.

Discussion

The activity of α-mannosidase was found to be significantly increased in mycorrhizal roots. The effect could be equally well induced by either *G. mosseae* or *G. intraradices*. This enzyme was not detected in spores of *G. mosseae and G. intraradices*. Therefore, assignment as a plant enzyme seems likely but has yet to be proven. It remains possible that the increase in activity is due to a fungal enzyme exclusively expressed inside the host plant.

The K_M of α-mannosidase from roots of *A. schoenoprasum* varied between 1.25 mM in mycorrhizal and 1.8 mM in nonmycorrhizal roots. This variation could be due to the induction of one or several different isozymes in the two systems, as shown for chitinase in the AM symbiosis (Dumas-Gaudot et al. 1992). If so, the isozymes must have identical apparent molecular weights. Another possible mechanism could be variation in regulating substances influencing the K_M value. α-Mannosidases have been described from a wide range of organisms (Amano and Kobata 1986), and considerable variation in biochemical properties is found not only between enzymes of different species but also between isozymes localized in different tissues, organs or organelles.

Glycoproteins are glycosylated cotranslationally in the rough endoplasmic reticulum. These are processed during transport through the Golgi apparatus, mannose residues being removed by α-mannosidases I and II, and the oligosaccharide chain is modified (Kornfeld and Kornfeld 1985; Kaushal and Elbein 1989). The surface of the plant plasma membrane increases by a factor of 6–12 with the formation of arbuscules in cells (Alexander et al. 1989). This must result in enforced production of membrane proteins by the host, most of them glycoproteins (Jones and Robinson 1989), and thus an increase in processing α-mannosidase activity in mycorrhizal plants. However, processing α-mannosidases from the Golgi apparatus were shown to be strictly specific for oligosaccharide residues and, therefore, did not cleave PNP glycoconjugates (Sturm et al. 1987). Furthermore these enzymes are not stimulated by zinc ions (Kaushal and Elbein 1989; Szumilo et al. 1986). Thus, it is probable that the observed activity in mycorrhizal roots is not due to processing enzymes of the Golgi apparatus, but to less specific aryl-mannosidases from other organelles.

Van der Wilden and Chrispeels (1983) detected three isozymes of α-mannosidase in cotyledons of *Phaseolus vulgaris*. Isozymes I and II were assigned to vacuole/protein bodies, isozyme III was localized in the cell wall. All three could be detected additionally in the endoplasmic reticulum. They were also stimulated by Zn^{2+} and they cleaved PNP glycoconjugates. Kinnback et al. (1987) described three corresponding isozymes of different molecular weight from roots and nodules of *Glycine max.* Isozyme II with an apparent molecular weight of 156 kDa accounted exclusively for the activity in the peribacteroid space and can be expected to play a role in host control of the legume/*Rhizobium* symbiosis.

It is possible that in the AM symbiosis α-mannosidase exerts a control function on the mycosymbiont by cleaving oligosaccharide residues of fungal surface pro-

teins, thus rendering polypeptides more accessible to proteolytic degradation or releasing elicitor molecules. The presence of α-mannose residues at the arbuscular interface has already been demonstrated by Jabaji-Hare et al. (1990). They suggested that these residues originate from the fungal partner. In order to clarify the function of α-mannosidase in the AM symbiosis, further studies for localizing enzyme activities and characterizing isozymes are necessary.

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