

Activity of some enzymes in barley caryopses during imbibition in aluminium presence

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ABSTRACT

Peroxidase, superoxide dismutase, acid and alkaline phosphatase, esterase and glucosidase activities were studied during imbibition of barley caryopses in the presence of aluminium. Antioxidative enzymes (peroxidase, superoxide dismutase) showed elevated activity already 2 h after the onset of imbibition in the presence of Al. In contrast hydrolytic enzymes (phosphatases, glucosidase, esterase) were only moderately activated at low Al concentrations (1–2 mM), while strong inhibition was observed at higher Al concentrations (4–8 mM). In *in vitro* conditions 8 mM Al had no effect on the activity of acid phosphatase, moderately inhibited alkaline phosphatase and glucosidase and strongly esterase activity. During imbibition of caryopses in solution without Al an increase of the pH value of the imbibition solution from 4 to 6 has occurred, while in the presence of Al the shift in pH value was less expressive and dependent on Al concentration. At 8 mM Al concentration no change in the pH value of imbibition solution was observed. The SDS-PAGE analysis of polypeptides released to the imbibition solution in the presence of Al revealed the accumulation of two polypeptides with relative molecular mass of 35 and 18 kDa. The release of 96 and 27.5 kDa polypeptides was completely inhibited at 8 mM Al concentration. These results confirmed that Al is able to influence different physiological processes already during seed imbibition and early growth phases of barley seedlings.

Keywords: spring barley (*Hordeum vulgare* L.); aluminium stress; seed coat proteins; SDS-PAGE; peroxidase; superoxide dismutase; acid and alkaline phosphatase; non-specific esterase; β -glucosidase, pH-regulation

Aluminium has toxic effects on plant growth at pH values below 5.0; therefore it became an important factor limiting crop productivity on acid soils. The first visible symptom of Al toxicity is the inhibition of root growth, which can occur within 1–2 h after exposure to Al (Ryan et al. 1993). Proposed physiological mechanisms of Al toxicity and of root growth inhibition include alterations of the plasma membrane properties (Cakmak and Horst 1991), modifying of the cation-exchange capacity of the cell wall (Horst 1995), interfering with signal transduction (Jones and Kochian 1995), binding to polynucleotides (DNA or RNA) etc. Various strategies of Al tolerance have been suggested. Al-chelating ligands such as organic acids (malate, citrate, oxalate) (Ryan et al. 1995) or phosphates (Pellet et al. 1996) released into the rhizosphere prevent root tissues from toxic Al forms. Detoxification of Al in the cytoplasm by internal chelating ligands (e.g. organic acids, phytochelatins, metallothionins) has been reported in Al-accumulating plants (Matsumoto 2000, Ma et al. 2001). Characterisation of Al stress-induced genes reveals others mechanisms leading to Al tolerance. More than 20 genes have already been isolated in wheat (Snowden et al. 1995, Cruz-Ortega et al. 1997), tobacco (Ezaki et al. 1995, 1997) and *Arabidopsis* (Richards et al. 1998). Since some of these Al-in-

duced genes are coding for antioxidant enzymes (glutathion S-transferase, peroxidase, superoxide dismutase), a strong connection between Al stress and oxidative stress in plants has been suggested (Cakmak and Horst 1991, Richards et al. 1998). The key role of antioxidant enzymes is to reduce or scavenge reactive oxygen species such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radical. Hydrolytic enzymes play important role during germination in mobilization of endosperm reserves.

The aim of the present study was the characterization of the impact of aluminium on some antioxidative and hydrolytic enzymes released from barley caryopses. During early stages of imbibition in the presence of different concentrations of Al, changes in the composition of extruded proteins to imbibition solution were characterised as well.

MATERIAL AND METHODS

Caryopses of barley (*Hordeum vulgare* L.) cultivar Alfor were used in experiments. Two hundred of the caryopses were imbibed in 10 ml of a solution containing 8.2 mM CaCl₂ solution, pH 4.0 (control), or in 7.2, 6.2, 4.2 or 0.2 mM CaCl₂ containing 1, 2, 4, and 8 mM AlCl₃ solution, pH 4.0 (Al-treated) at

25°C in darkness for 2 h. After pH determination of the individual imbibition solutions, part of the solution was used for enzyme analysis. In the second part, the solution proteins were precipitated with ice-cold acetone and used for SDS-PAGE analysis.

Enzyme activities were determined photometrically using microplate reader (SLT-Laborinstruments, Austria). Each experiment was repeated at least three times. Total protein content in the extracts was determined with Bradford method using Bovine serum albumin as standard (Bradford 1976). Specific enzyme activities were expressed as OD/ μg protein (OD – optical density). Changes in enzyme activities were expressed as a percentage of control.

Determination of the activity of superoxide dismutase (SOD, EC 1.15.1.1) was based on the method of Beyer and Fridovich (1987). The reaction mixture was composed of 15 μM methionine, 70 μM 3-(4,5-dimethylthiazol-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 20 μM riboflavin, potassium phosphate buffer (0.05M, pH 7) and appropriate volume of protein extract. The reaction was initiated by illumination. The blue formazane produced by MTT photoreduction was measured at 560 nm.

The activity of peroxidase (POD, EC 1.11.1.7) was determined by monitoring the formation of guaiacol dehydrogenation product by following the increase of absorbance at 405 nm, by the method of Chance and Maehly (1995). The reaction mixture contained 0.04% guaiacol, sodium acetate buffer (0.04M, pH 5.2) and the suitable volume of protein extract. The reaction was initiated by adding 0.04% hydrogen peroxide.

The reaction mixture for acid phosphatase (EC 3.1.3.2) contained 100 μl of Na-acetate buffer (0.1M, pH 5.2), 50 μl of 4-nitrophenylphosphate (2 mg/ml) and an appropriate volume of sample. The reaction was stopped after 30 minutes of incubation at 37°C by adding 50 μl of 0.4M Na-phosphate buffer and activity was measured at 405 nm against the control reaction without sample.

For alkaline phosphatase (EC 3.1.3.1) the glycine/NaOH (0.025M, pH 10) buffer was used and 1mM MgCl_2 was added. Substrate was the same as for acid phosphatase and reaction was measured at 405 nm (Thaker et al. 1996).

The assay for non-specific esterase (EC 3.1.1.3) contained 100 μl of Tris/HCl buffer (0.1M, pH 7.2), 50 μl 4-nitrophenylacetate (2 mg/ml solubilized in 20% acetone) and sample according to Ward and Bamforth (2002).

Na-acetate buffer (0.1M, pH 5.2) was used for β -glucosidase (EC 3.2.1.21) activity analysis with 0.5 mg/0.1 ml of p-nitrophenyl-D-glucopyranoside as substrate. The reaction was stopped by

adding 0.5 volume of 10% Na_2CO_3 per reaction. The released nitrophenol was measured at 405 nm (Piślewska et al. 2002).

Precipitated proteins were dissolved in SDS-sample buffer and separated under denaturing conditions on 12% polyacrylamide slab gels (BIO RAD, USA) using the discontinuous buffer system (Laemmli 1970) and silver stained. Protein concentration was determined according to Lowry et al. (1951). The apparent molecular masses of polypeptides were calculated based on the mobilities of protein standards obtained from BIO RAD with gel documentation system (UVP 5000, England).

RESULTS

The pH value of control (without Al) imbibition solution increased during 2 hours of imbibition from 4.0 to 6.0. In Al-treated caryopses pH increment of imbibition solution was proportionally reduced with increased Al concentrations (2mM Al – pH 5.6, 4mM Al – pH 5.0) up to 8mM Al where the original value 4.0 remained unchanged during imbibition (Figure 1).

Concentration-dependent changes in the pH value of imbibition solutions were accompanied with the changes of some antioxidative and hydrolytic enzymes activities released from the caryopses to imbibition solution (Figure 2). During 2 h of imbibition Al-dose dependent increase in the activity of the peroxidase and superoxide dismutase was observed. At 1 and 2mM Al concentrations only slight activation of both antioxidative enzymes was exhibited, whereas application of 4 and 8mM Al caused a significant increase in enzymes activities, which represented approx. 250% that of the activity of control. In contrast, activities of hydrolytic

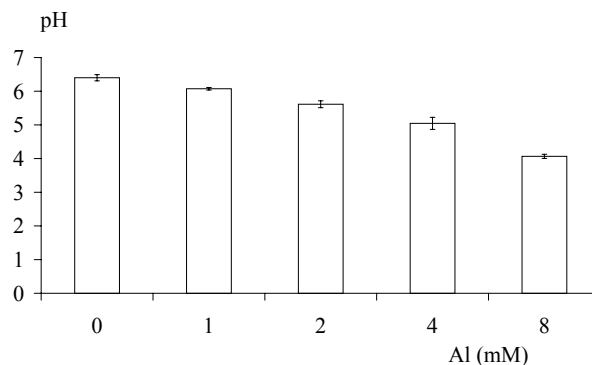


Figure 1. The effect of different concentrations of Al on pH value of imbibition solution (pH of the imbibition solution at the beginning of imbibition set to 4.0); means values \pm SD ($n = 5$)

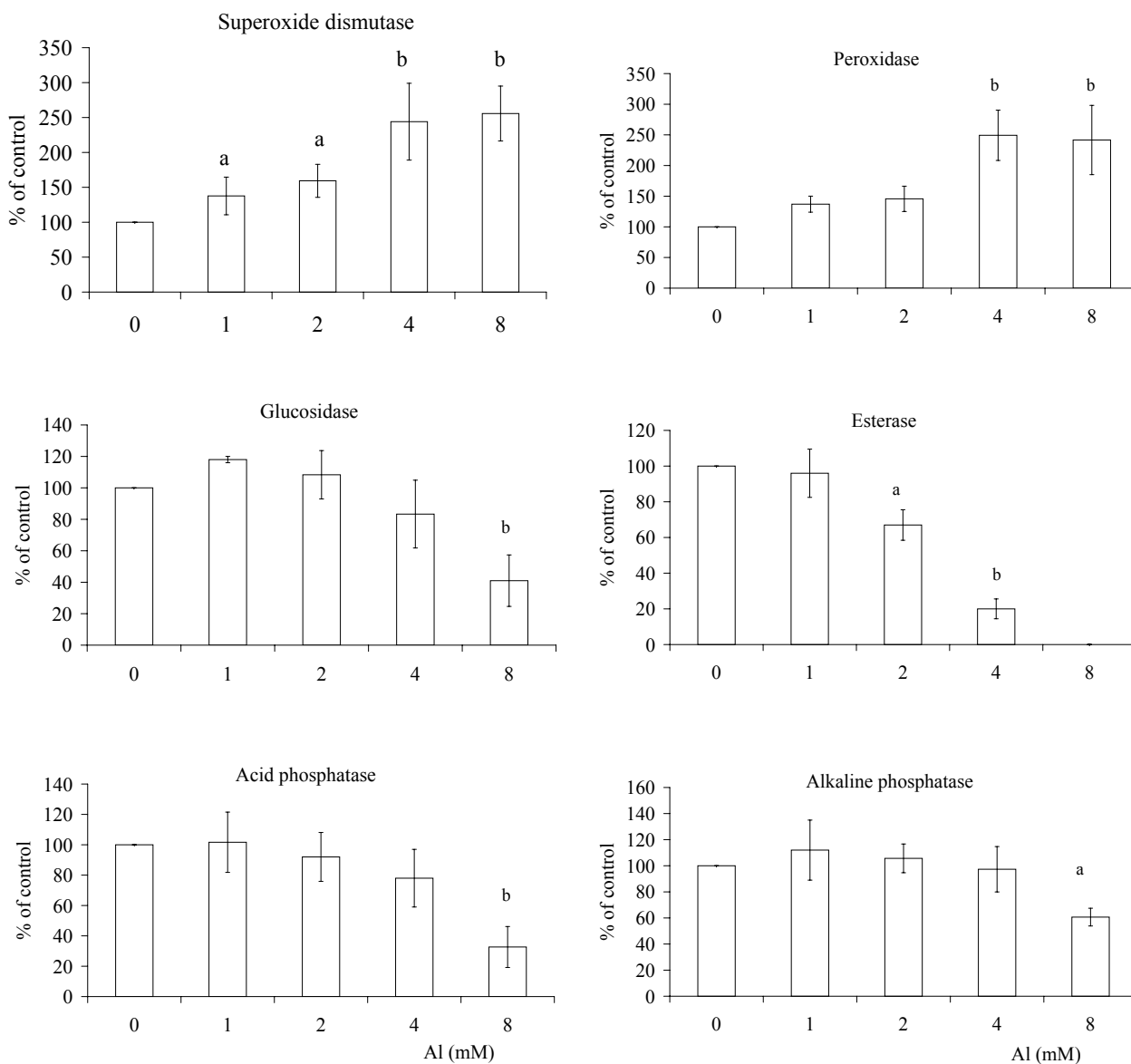


Figure 2. The effect of different concentrations of Al on the activities of superoxide dismutase, peroxidase, glucosidase, esterase, acid phosphatase, and alkaline phosphatase in the imbibition solution; means values \pm SD ($n = 5$)

enzymes (glucosidase, esterase, acid phosphatase and alkaline phosphatase) moderately increased at low Al concentrations (1–2mM) and at higher Al concentrations (4–8mM) a strong inhibition occurred. In *in vitro* conditions Al at 8mM concentration had no effect on the activity of acid phosphatase, moderate decrease was observed in the activity of alkaline phosphatase and glucosidase and a strong inhibition was found in the activity of esterase (Figure 3).

The SDS-PAGE analysis of polypeptides released into the imbibition solution revealed Al-dependent accumulation of two polypeptides with relative molecular mass of 35 and 18 kDa (Figure 4). In contrast, the release of 96 and 27.5 kDa polypep-

tides present in control (without Al) imbibition solution with increasing Al concentration (1–4mM) gradually decreased and at 8mM Al almost disappeared.

These results indicate a significant influence of Al treatment on enzyme activities and on the accumulation of some seed proteins already after a short time of imbibition of barley caryopses in the presence of Al.

DISCUSSION

Toxic effects of aluminium on plants as well as mechanisms of Al toxicity on root growth inhibition

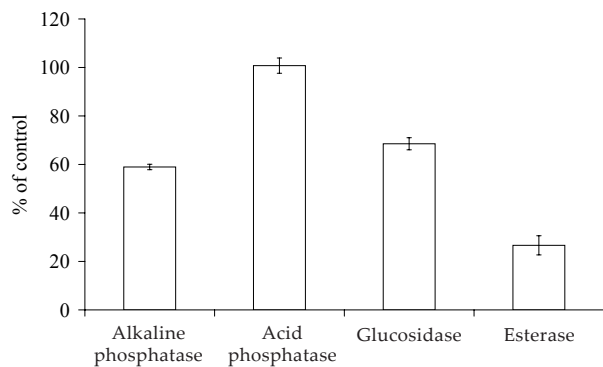


Figure 3. The effect of 8mM Al *in vitro* on glucosidase, esterase, acid phosphatase, and alkaline phosphatase activity in the imbibition solution; means values \pm SD ($n = 5$)

were studied on several plant species. But only little is known about the impact of Al on emerging roots during germination and studies about the effect of Al on caryopses during imbibition are lacking. Therefore we performed a set of analysis focused on the effect of Al on pH regulation, activities of some antioxidative and hydrolytic enzymes and changes in polypeptides accumulation during early phases of imbibition of barley caryopses.

Milimolar Al concentrations (1–8mM) were applied during all of our experiments; although it is known that already micromolar Al concentration can lead to a demonstration of the first visual symptoms of Al toxicity (root growth inhibition) in simple Ca^{2+} solution (Ryan et al. 1993). But it is known that early processes of plant development (germination and subsequent roots emerging) are less sensitive to Al exposure than the stage of established seedlings (Delima and Copeland 1990). Also the analyses of Al-induced inhibition of emerging barley root growth cultivated between two layers of filter paper showed that 2mM Al concentration was required to induce significant root growth inhibition on the second day after the start of germination (Tamás et al. 2003). Therefore a milimolar concentration was used in these experiments.

Numerous works reported the presence of several antioxidative and hydrolytic enzymes in dry cereal grains, which activities raised considerably after the start of seed imbibition (Fincher 1989, Jones and Jacobsen 1991). The enzymes of dry grains are characterized by high thermal and conformational stability (Kamal and Behere 2002).

One of the best-characterized antioxidant proteins is the peroxiredoxin with peroxidase activity, which plays an important role in protecting seed from oxidative damages and maintaining seed dor-

mancy (Stacy et al. 1996). Oxidative stress inhibits growth and development by arresting cell division (Reichheld et al. 1999); therefore protection from oxidative stress is crucial for seeds germination. Peroxidase and superoxide dismutase are well-characterized antioxidative enzymes induced in roots by Al in several plant species. Superoxide dismutases convert superoxide to H_2O_2 and O_2 and therefore offer the protection against superoxide-induced oxidative stress injury. Peroxidases participate in lignin biosynthesis, cell wall cross-linkage, IAA degradation, disease resistance, and convert H_2O_2 to water. Aluminium was shown to enhance SOD activity in root tips of soybean (Cakmak and Horst 1991), roots of *Arabidopsis* (Richards et al. 1998), and roots of sorghum (Peixoto et al. 1999). Lee et al. (2001) suggested that enhanced activity of SOD may function in signalling of oxidative stress, which leads to the induction of antioxidant enzymes associated with an H_2O_2 scavenging system, particularly an ascorbate-glutathione cycle. Increased activity of POD was described in soybean roots treated with Al (Cakmak and Horst 1991). Ezaki et al. (2000) suggested that the enhanced activity of anionic POD could act in conferring Al resistance by detoxifying reactive oxygen species and restricting lipid peroxidation in membrane regions. In contrast, Jan et al. (2001) proposed the increased POD activity to be part of a damage response to Al, since they showed that Al induced POD activities in Al-sensitive rice cultivar whereas in Al-tolerant cultivar they were unaffected by Al treatment.

On the other hand, production of reactive oxygen species is inevitable for cell death in the aleurone layer of cereal grains during germination (Bethke et al. 2002). Therefore mRNA encoding catalase, ascorbate peroxidase and superoxide dismutase are reduced during the early stage of germination of barley seeds (Fath et al. 2002). Probably Al-induced

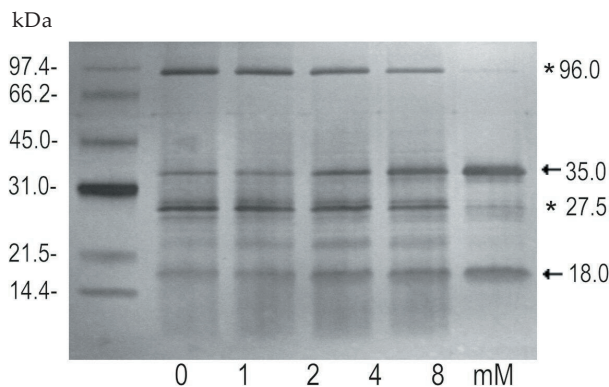


Figure 4. The effect of different concentrations of Al on the composition of proteins released to imbibition solution (arrowheads indicate induced, and asterisks reduced polypeptides)

peroxidase and superoxide dismutase plays a role in maintaining the correct level of reactive oxygen species during germination in stress conditions.

Hydrolytic enzymes play an important role during germination in the mobilisation of endosperm reserves. They are rapidly activated during imbibition; however, some of them exist in the active form already in the aleurone layer and scutellum of dry mature grains (Fincher 1989). Glucosidase, phosphatase and protease activity was found mainly in the protein bodies and spherosomes of ungerminated seeds (Adams and Novellie 1975), however phosphatase and esterase activity was reported also from the testa of ungerminated wheat grains (Price and Ey 1970). In our previous work we reported the significant increase in acid phosphatase in barley roots during Al treatment (Huttová et al. 2002). In contrast Al at 8mM concentration markedly inhibited the release of acid phosphatase from imbibiting barley grains. Our results demonstrated that inhibition of acid phosphatase did not occur in *in vitro* conditions. Bailey et al. (1976) reported that during barley grain imbibition new acid phosphatase isoenzymes occurred, which are probably inhibited by Al. The role of secreted phosphatases is the hydrolysis of phosphate-esters from inorganic and organic phosphates, which can be disturbed at the presence of Al. Our results indicated that Al-inhibition of hydrolytic enzymes might cause phosphate deficiency during the first period of the germination. Similarly to phosphatases Al-induced inhibition of glucosidase and esterase may induce several defects in seed reserve mobilization. In addition secreted forms of hydrolytic enzymes are generally involved in plant defence mechanism in plant microbe interaction (Boller 1987). Inhibition of hydrolytic enzymes and activation of antioxidative enzymes suggest that Al-induced stress had already started in the early stage of the grain imbibition which was supported by the incapability of grains to change the pH of imbibition solution at higher Al concentration.

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Received on August 1, 2003

ABSTRAKT

Aktivita některých enzymů z obílek ječmene v prvních fázích klíčení za přítomnosti hliníku

Aktivita peroxidázy, superoxid dismutázy, kyselá a alkalická fosfatázy, esterázy a glukosidázy byla sledována v prvních fázích klíčení obílek ječmene za přítomnosti hliníku. Aktivita antioxidantních enzymů (peroxidáza, superoxid dismutáza) za přítomnosti Al se zvyšovala již v průběhu prvních dvou hodin klíčení. Aktivita hydrolytických enzymů (fosfatázy, esterázy a glukosidázy) byla nižšími koncentracemi Al (1–2mM) mírně zvyšována, vyššími (4–8mM) silně inhibována. V podmínkách *in vitro* neměl 8mM Al žádný vliv na aktivitu kyselých fosfatáz, mírně inhiboval alkalickou fosfatázu a glukosidázu a silně esterázu. V průběhu klíčení za nepřítomnosti Al se hodnota pH v roztoku, ve kterém probíhalo klíčení, zvýšila z původní hodnoty pH 4 na pH 6, za přítomnosti Al posun pH hodnoty závisel na koncentraci Al v roztoku. V případě nejvyšší použité koncentrace (8mM) nebyla pozorována žádná změna hodnoty pH klíčícího roztoku. SDS-PAGE analýza bílkovin uvolněných do klíčícího roztoku potvrdila hliníkem indukovanou zvýšenou akumulaci dvou polypeptidů s relativní molekulovou hmotností 18 a 35 kDa. Vysoká koncentrace Al

(8mM) kompletně blokovala akumulaci 27.5 a 96 kDa polypeptidů v klíčním roztoku. Prezentované výsledky potvrzují významný vliv hliníku na rostliny už ve velmi raných stádiích klíčení a růstu.

Klíčová slova: jarní ječmen (*Hordeum vulgare* L.); hliníkový stres; bílkoviny v semenných obalech; SDS-PAGE; peroxidáza; superoxid dismutáza; kyselá a alkalická fosfatáza; nescifická esteráza; β -glukosidáza, regulace pH

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