

# Aluminium induced esterase activity and isozyme pattern in barley root tip

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## ABSTRACT

Changes in the activity of esterase as well as changes in the viability of root cells and some growth parameters were analysed during cultivation of barley seedlings in the artificial substrate under Al stress conditions. Aluminium-induced elevated esterase activity correlated with Al uptake, root growth inhibition and increased Evans blue uptake in the barley root tips. Analysis of isozyme pattern of esterase revealed one anodic and one cathodic esterase isozyme induced by Al-treatment. The possible role of elevated esterase activity during Al stress is discussed.

**Keywords:** aluminium uptake; cell death; esterase activity; isoesterases

Proteins capable of hydrolyzing ester bonds are present in many isoforms in all living organisms. Mainly in animals some of these are characterized by their substrate specificity indicating their biological function (Aldridge 1993). However, the most esterases have a broad range of substrates without a known specific biological function. For the longest time esterases have been used as a marker system of somatic embryogenesis and organogenesis (Chibbar et al. 1988, Coppens and Dewitte 1990). Esterases were also coupled with a degree of resistance in barley to powdery mildew (Hwang et al. 1982), and in nitrogen fixing symbioses; gene coding for protein with esterase activity expressed early in nodule development (Pringle and Dickstein 2004). In the cell wall pectin methylesterases are responsible for demethylation of cell wall polygalacturonans. This reaction affects pH of apoplast and ionic concentration thus regulating several hydrolytic enzymes and rigidity of cell wall (Micheli 2001). Similarly to pectin methylesterases, acetyl esterases have been reported to play an important role in cell wall modifications (Bordenave et al. 1995).

Cell wall esterases by cleaving ester linkages increase negatively charged matrix of the cell wall, which is a crucial target site for Al toxicity. Horst (1995) proposed a model, where binding of Al to sensitive binding sites of apoplast and competition with other ions for these binding sites determines Al-induced inhibition of root elongation. Due to the rapid Al entry into the cell (Silva et al. 2000) similar toxic effects can occur also on the negatively charged molecules of cytoplasm.

Several Al toxicity mechanisms were described in numerous plant species (see reviews Matsumoto 2000, Rout et al. 2001) and all of the precise molecular and biochemical mechanisms of Al toxicity have not yet been fully shown.

The aim of the present study was the characterization of the impact of aluminium on esterase activity in relation to Al-induced root growth inhibition and cell death. Change in esterase isozymes during Al stress was characterized as well.

## MATERIAL AND METHODS

### Plant material and growth conditions

Seeds of barley (*Hordeum vulgare* L.) cultivar Jubilant were incubated in distilled water for 4 h at 20°C in darkness. After this short imbibition the seeds germinated between two sheets of filter paper fully moistened with distilled water under the same conditions as during the imbibition. After 20 h germinated seeds (with about 0.5 cm long roots) were transferred to the artificial substrate (PERLIT, Czech Republic) freshly moistened with 2mM CaCl<sub>2</sub> solution, pH 4.0 (control), or with 2mM CaCl<sub>2</sub> containing 2 or 4mM AlCl<sub>3</sub> solution, pH 4.0 (Al-treated) for 3 days under the same conditions as during the imbibition. Root length was measured by a ruler and excised root tips were used immediately for analysis or stored at -70°C until analyzed. Each experiment was repeated at least five times with 200 replicates.

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Supported by the Science and Technology Assistance Agency, Project No. APVT-51-001002.0.

## Aluminium uptake

Hematoxylin staining was used for the determination of Al uptake (Ownby 1993). Freshly harvested roots, washed in distilled water for 15 min were stained with 0.2% hematoxylin and 0.02%  $\text{KIO}_3$  solution for 15 min at room temperature. After washing with distilled water for 15 min 10 root tips (0.5 cm) were excised and soaked in 200  $\mu\text{l}$  of 1M HCl for 1 h. Optical density of the released stain was measured at 490 nm.

## Determination of cell death

The loss of plasma membrane integrity was evaluated by spectrophotometric assay of Evans blue staining (Baker and Mock 1994). Roots were stained in 0.25% (v/v) aqueous solution of Evans blue for 15 min at room temperature. The stained roots were washed three times with distilled water, for 10 min each. Root tips (5 mm) were excised and soaked in N,N-dimethylformamide for 1 h at RT. The optical density was measured spectrophotometrically at 600 nm.

## Protein extraction and sample preparation

Root tips (1 cm) were ground to a fine powder in a cold mortar in liquid nitrogen and the resulting powder was rehomogenised in 100mM Tris/HCl buffer, pH 8.0 with homogenisator (Heidolph DIAX 900). After filtration the homogenate was centrifuged at 1500 g for 5 min, then at 12 000 g for 15 min and finally at 100 000 g for 30 min (Beckman L8-M). The resulting supernatant (soluble fraction) was used for analysis after concentrating and passing through Sephadex G-25 using 10mM Tris/HCl buffer, pH 8.0. Proteins were fractionated using anion exchange column (UNO Q, Bio-Rad) equilibrated with 10mM Tris/HCl buffer, pH 8.0. The adsorbed proteins were eluted with linear gradient of 0–1.0M NaCl in the same buffer. Proteins were quantified with *Bovine Serum Albumin* as a standard by the method of Bradford (Bradford 1976).

## Enzyme assays

Enzyme activities were determined photometrically using microplate reader (SLT-Laborinstruments, Austria). Specific enzyme activities were expressed as OD/ $\mu\text{g}$  protein (OD – optical density). Changes in enzyme activities were expressed as a percentage of control. Assay for non-specific esterase (EC 3.1.1.3) contained 100  $\mu\text{l}$  of 100mM Tris/HCl buffer, pH 7.2; 50  $\mu\text{l}$  4-nitrophenylacetate (2 mg/ml

solubilized in 20% acetone) and sampled according to Ward and Bamforth (2002). Activity was measured at 405 nm against the control reaction without sample.

## Gel electrophoresis and activity staining

The basic proteins were separated under non-denaturing conditions on 7% slab polyacrylamide gels using the cathodic system (Reisfeld et al. 1962), the acidic and neutral proteins on 7% slab polyacrylamide gels using the discontinuous buffer system (Laemmli 1970). The esterase activity on the gels was detected by using 0.04% 1-naphthyl acetate in 20% acetone and 50mM Tris/HCl pH 7.2 as substrate and with subsequent color development with 0.04% Fast blue RR salt.

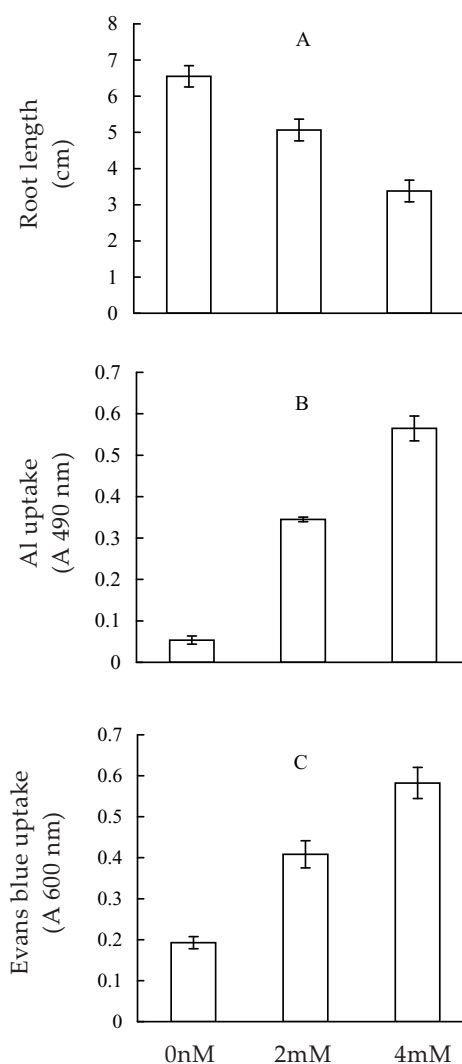


Figure 1. Root growth (A), aluminium uptake (B) and Evans blue uptake (C) of barley roots grown in the presence of 0, 2 and 4mM Al; data represent the means  $\pm$  SE ( $n = 5$ )

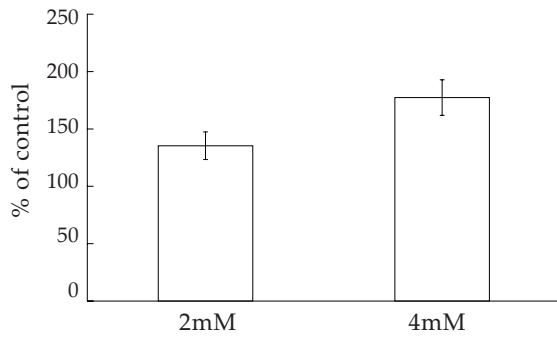


Figure 2. Esterase activity in barley root tips grown in the presence of 2 and 4mM Al; data represent the means  $\pm$  SE ( $n = 5$ )

## RESULTS

To mimic natural soil conditions in our experiments we used an artificial substrate for cultivation of young barley seedlings. Due to the high binding capacity of this substrate application of 2 and 4mM Al concentration was required to achieve 75 and 50% root growth inhibition compared to control plants (Figure 1A). Root growth inhibition was correlated with Al uptake (Figure 1B) and increased Evans blue uptake induced by 2mM and 4mM Al (Figure 1C). Elevated esterase activity was detected already at 2mM Al and raised further with increasing Al concentration representing at 4mM Al about 175% of control roots (Figure 2). After separation on anion exchange column esterase activity was detected in five fractions (Figure 3). However, the Al induced esterase activity was observed only in fraction 7, where nearly two times higher esterase activity was detected in Al treated root tips compared to controls (Figure 4). Figure 5 shows native

anodic and cathodic PAGE of fraction 7, 8 and 9. One anodic and one cathodic Al-induced esterase isozyme was detected in fraction 7.

## DISCUSSION

Barley contains several forms of esterases with broad range of functions, hydrolyzing ester bonds in different types of metabolites. Multiple forms of esterases and their differential expression during embryogenesis and organogenesis suggested their important role in several physiological processes (Coppens and Dewitte 1990). In this paper we reported that Al treatment increases the activity of two esterase isozymes. The major portion of adsorbed Al is localized in the apoplast, where it binds to the negatively charged groups of cell wall pectins. Esterases increase the number of free carboxyl group of pectic matrix e.g. by demethylation or deacetylation of cell wall materials; therefore the number of possible binding sites for Al increases. One of the reported mechanism of Al tolerance is phosphate precipitation of cell wall bound Al (Marienfeld and Stelzer 1993). Al resistant barley cultivar contained two times higher amount of Al-phosphate in the root surface region than Al sensitive cultivar (Millard et al. 1993). Therefore it is possible that elevated esterase activity contributes to formation of more binding sites for Al in the cell wall, which delay Al uptake into deeper root tissues. In contrast to these observations, transgenic potato plants over-expressing pectin methylesterase were more Al-sensitive than the wild type, suggesting that higher amount of cell wall bound Al to pectic matrix enhanced sensitivity of roots to Al (Schmohl et al. 2000). However, these

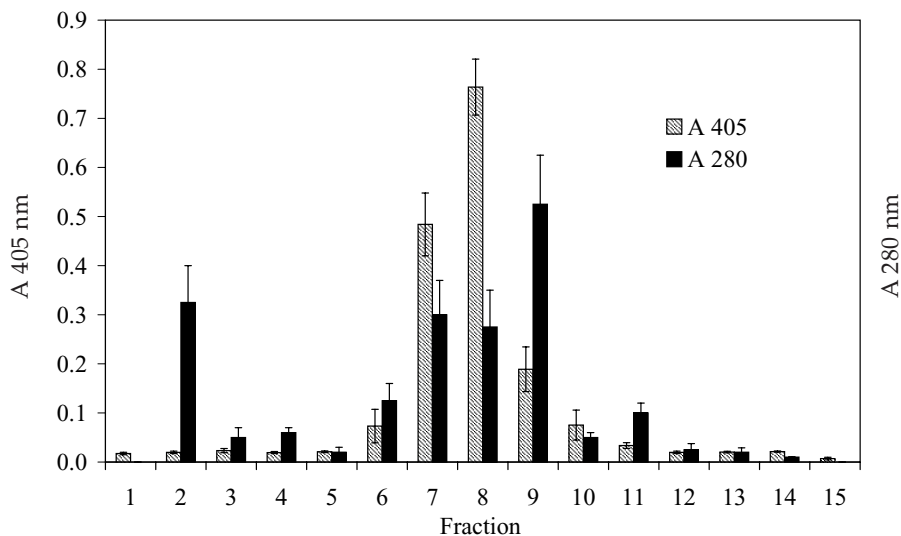


Figure 3. Separation of proteins isolated from barley root tips on anion exchange column (A 280) and esterase activity in individual fractions (A 405); data represent the means  $\pm$  SE ( $n = 5$ )

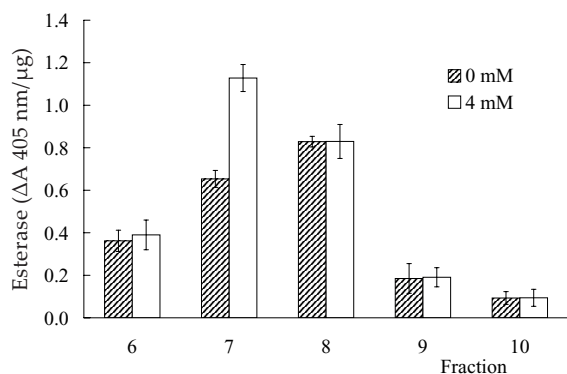


Figure 4. Esterase activity in barley root tips grown in the presence of 0 or 4mM Al in the fractions 6, 7, 8, 9 and 10 after separation on anion exchange column; data represent the means  $\pm$  SE ( $n = 5$ )

plants were cultivated in hydroponics where the outer layer of the root surface is disturbed. Delise et al. (2001) reported that accelerated cell turnover on the root surface might help to protect deeper cell layers during Al-stress. Theoretically, these cells with higher esterase activity may contain more Al binding sites; therefore more Al can be

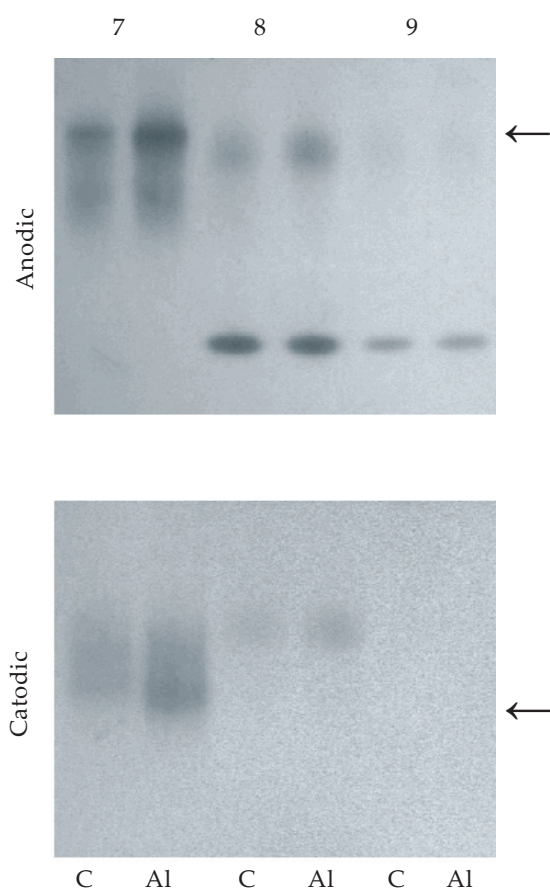


Figure 5. Anodic and cathodic PAGE of esterase isozymes in the fractions 7, 8 and 9 in the presence of Al and in control (C) seedlings; the Al-induced isoesterases are indicated by arrow

immobilized and remaining root tissues essential for root growth can be protected.

The plant cell wall is more than a simple skeleton around cells. It is a metabolically active dynamic part of the cell, which requires active changes of its components and its modification especially during cell expansion. Hydrolytic enzymes such as endoglycanases, glucosidases, and esterases participate in altering cell wall (Cosgrove 2001). Pectin methylesterases by removing methyl groups from pectins generate protons. Consequently, cell wall expansion mediated by acid growth is induced and several hydrolytic enzymes are activated. Free carboxyl group on pectins inhibited hydrolysis of p-nitrophenyl acetate catalyzed by pectin methyl-esterases (Nari et al. 1991) therefore binding of Al to the carboxyl group may enhance esterase activity in the cell wall. It was suggested that not only the degree of esterification, but also changes in ester bonds between polysaccharides play crucial role during expansion of the grass cell wall (Kim and Carpita 1992). The cleavage of ester bonds between polysaccharides can physically weaken the wall structure during wall extension. This mechanism is used for cell wall degradation by some fungi, by secretion of enzymes with esterase activity cleaving cross-links between plant cell wall polymers (García-Conesa et al. 2001). Al induced increase in cell wall rigidity can be weakened by the action of cell wall esterases.

The role of esterases in many cases is associated with cell wall metabolism but little is known about their intracellular function. One of their most interesting functions in the cytoplasm is the metabolism of many pesticides and pollutants entering the cell (Cummins et al. 2001). Al can block negatively charged sites of molecules in cytoplasm and in this manner prevent the creation of ester bonds. These Al blocked negative charges of molecule can activate cytoplasmic esterase similarly to the cell wall esterases (Nari et al. 1991). Mukherjee et al. (2004) reported that esterase variation of *Lemna minor* is a potential biomarker of heavy metal pollution. Precise localization, isolation and characterization of Al-induced esterase isozymes are required for the understanding of their role during Al-stress – that is the subject of our ongoing experiments.

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Received on July 26, 2004

## ABSTRAKT

### Aktivita esterázy indukovaná hliníkem a izozymy esterázy v kořenových špičkách ječmene

Změny v aktivitě esterázy a změny v růstové schopnosti buněk kořene, stejně jako změny růstových parametrů byly analyzovány po dobu kultivace klíčících rostlinek ječmene na umělém substrátu v podmínkách Al stresu. Zvýšená aktivita esterázy indukovaná Al korelovala s příjmem Al, inhibicí růstu kořene a zvýšením příjmu barviva Evansové

modři do kořenových špiček. Analýza izozymů esterázy poukázala na jeden anodický a jeden katodický esterázový izozym, které byly indukovány po působení Al. Prezentované výsledky poukazují na možnou funkci zvýšené esterázové aktivity po dobu Al stresu.

**Klíčová slova:** příjem hliníku; buněčná smrt; aktivita esterázy; izoesterázy

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