Proteolytic activities in *Phaseolus vulgaris* cotyledons under copper stress

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Abstract The changes in the protease activities of bean cotyledons were investigated in response to copper stress. Assays using synthetic substrates and specific protease inhibitors followed by activity measurements and electrophoresis analysis allowed to study the classes of enzymes involved in the storage protein mobilization during the germination of bean (*Phaseolus vulgaris* L) seeds, and then identify which ones were affected in the presence of 200 μ M CuCl₂ in the imbibition medium. Copper treatment affected embryo growth and total protease activity. The results of SDS-gelatin-PAGE show that Cu excess led to a decrease in protease activity of 45 to 66 kDa. Moreover, cysteine-, aspartic- and metallo-protease activities were markedly lowered under copper stress, while serine-protease one was enhanced as well as its activity dependent abundance in comparison with control. However, the relative distribution of major cysteine protease in H₂O-germinated seeds was significantly diminished after Cu exposure. Thus, copper excess can disturb the nitrogen freeing from reserve tissues at enzymatic level; differential responses of protease classes are discussed, notably, cysteine protease in the way of storage protein mobilization and serine protease in protective mechanism one.

Keywords Bean · Copper · Cotyledon · Endoprotease · Germination

Abbreviations

ACA	Azocaseinolytic activity
AP	Aspartic protease
CP	Cysteine protease
DAN	Dicyclohexylaminenitrite
E-64	L-trans-epoxysuccinyl-leucylamide-4-guanidino-butane
MP	Metalloprotease
NEM	N-ethylmaleimide
PMSF	Phenylmethylsulfonylfluoride
SP	Serine protease
STI	Soybean trypsin inhibitor

Introduction

Regulated proteolysis is directly or indirectly involved in most cellular processes (Callis 1995; Schaller 2004). Among the functions assigned to proteolysis are: (a) the supply of amino acids needed to make new proteins and (b) the removal of abnormal proteins (Vierstra 1996; Palma et al. 2002). These molecular mechanisms are related to numerous developmental events including seed germination during which storage protein mobilization is a fundamental component (Müntz 1996). Research interests have focused on physiological, biochemical and molecular processes relevant for agriculture including the breakdown of reserve proteins during seed germination concomitant with the allocation of nitrogen resources (free amino acids) to seedling (Ryan and Walker-Simmons 1981; Schaller 2004). Thus, the role of proteases in germination is essential.

Most proteases act either on the interior of peptide chains (endopeptidases) or on termini (exopeptidases). Exopeptidases can be classified according to the substrate specificity as aminopeptidases or carboxypeptidases (Dalling 1986;

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Huffaker 1990). Endopeptidases are classified according to their catalytic mechanism, which implies specificity in the enzyme active sites. In plants, four classes of endopeptidases have been described: serine proteases (SP, EC.3.4.21), cysteine proteases (CP, EC.3.4.22), aspartic proteases (AP, EC.3.4.23) and metalloproteases (MP, EC.3.4.24) (Rawling and Barrett 1994; Powers et al. 2002). For SP and CP, the hydroxyl or sulfhydryl groups of the active-site amino acids act as the nucleophile during catalysis. Aspartic proteases contain two aspartic residues essential for catalytic activity and rely on an activated water molecule as nucleophile. Metalloproteases rely on a divalent cation for activity, most commonly zinc, or cobalt or manganese.

On the other hand, seed germination is triggered by tissue imbibitions (uptake of water) and, after an apparent lag phase, is followed by the elongation of the radicle and thereafter of the whole embryonic axis (Bewley 1997). Heavy metals affect seed germination by causing respiratory disturbances (Smiri et al. 2009, 2010) and limiting the nutriment availability (Mihoub et al. 2005; Rahoui et al. 2008, 2010; Sfaxi-Bousbih et al. 2010a, b, c). However, little is known about the behaviour of breakdown of storage proteins under Cu stress, and the effects on proteolysis during seed germination have not been studied thus far.

In our previous work, we have pointed out that Cu stress inhibits the mobilization of cotyledonary globulin and albumin (Karmous et al. 2011). The aim of the present study was to investigate the impact of 200 μM CuCl_2 on the activity of four classes of protease in germinating bean seeds.

Materials and methods

Germination and copper treatment conditions

Seeds of bean (*Phaseolus vulgaris* L. var. soisson nain hatif) were germinated in the presence of H_2O or 200 μM CuCl_2 and cotyledons were collected at different times (0, 3, 6 and 9 days) according to Karmous et al. (2011).

Protein extraction and quantification

Cotyledons were homogenized in a mortar at 4 °C with five times their fresh weight of extraction medium (50 mM Tris–HCl pH 7.5, 5 mM β -mercaptoethanol). The homogenate was centrifuged for 30 min at 20,000 $\times g$, and the supernatant was used for protease activity measurements.

Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as the standard.

Protease assay

Two hundred μL of enzyme extract and 200 μL of azocasein (5 $\text{mg}\cdot\text{mL}^{-1}$ in 100 mM sodium citrate pH 5.5) were incubated for 1 h at 37 °C. The reaction was stopped by addition of 2 mL of 10 % (v/v) trichloroacetic acid. After 10 min on ice, the samples were centrifuged at 20,000 $\times g$ for 10 min; 500 μL supernatant was then added to 1 mL of 1 M NaOH, and the absorbance was taken at 440 nm. The extinction coefficient $E_{1\%}^{\text{azocasein in 1 M NaOH}} = 37 \text{ L}\cdot\text{cm}^{-1}\cdot\text{g}^{-1}$ was used to calculate the azocasein degradation activity.

To examine the effect of Cu ions on azocaseinolytic activity (ACA) in vitro, control extract was mixed with 200 μM CuCl_2 and incubated for 30 min at 4 °C before enzymatic assay.

The optimum pH for ACA was determined by measuring the synthetic substrate hydrolysis over pH range 4 to 9.5. The following buffers (0.1 M) were used for the indicated pH ranges: Na-citrate, 4 to 5.5; Tris-maleate, 6 to 8.5; glycine, 9 and 9.5.

Protease inhibitors were prepared as the following stock solutions: E-64 (50 μM), iodoacetamide (1 mM), iodoacetate (1 mM), NEM (1 mM), STI (100 μM), and EDTA (10 mM) in water, PMSF (1 mM) in ethanol, 1-10 phenanthroline (10 mM), pepstatin (10 μM) and DAN (10 mM) in methanol. Inhibitors were first preincubated for 15 min at ambient temperature with enzyme extracts prior to azocasein addition, and activities were measured as described above. Control assays were carried out with the corresponding solvents.

Polyacrylamide gel electrophoresis

The proteases in cotyledon extracts were separated by SDS-gelatin-PAGE. Regular SDS-PAGE (Laemmli 1970) was performed, except that the 7.5 % resolving gel was impregnated with 0.2 % (w/v) gelatin. Aliquots containing equal protein amount (90 μg) were mixed with reducing or non-reducing buffer (0.1 M Tris–HCl pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue in the presence or absence of 5 % β -mercaptoethanol, respectively). After electrophoresis, the gels were washed for renaturation in Triton X-100 (2.5 % (v/v) for 1 h and incubated in Na-citrate pH 5.0 for 3 h at 37 °C. Gels were stained with Coomassie Brilliant Blue R250 containing 0.4 % amidoblack. After destaining, protease activities were detected on a dark-blue background as colourless bands.

Statistical analysis

The experiment was repeated at least twice. The presented data are from representative experiment. The mean values ($\pm\text{SE}$)

were compared for significance of differences at $p < 0.05$ using ANOVA test.

Results and discussion

Effect of copper stress on embryo growth

In a previous work, we have shown that $200 \mu\text{M}$ CuCl_2 delay germination and biomass mobilization from storage tissues of bean seeds (Karmous et al. 2011). In the present study, until the 3rd day, we report no effect of Cu treatment on the embryo growth, while the latter was significantly reduced by the day 6, and reached 70 % of decrease after 9 days, as compared to control (Fig. 1). Thus, the hypothesis of the nitrogen starvation in the embryonic axis, coupled with the Cu-imposed delay in growth (Karmous et al. 2011) is suggested from the recognition of the failure in albumin and globulin hydrolysis, which results in the decrease in the availability of amino acids after the exposure to the pollutant. In this investigation, we attempt to understand the Cu effect on the proteolytic system.

Effect of copper stress on global protease activity

The control ACA was rapidly stimulated following the imbibition, while no change in the total protease activity was observed after 3 days of germination in the presence of Cu (Fig. 2). At day 9, the inhibition by copper treatment was approximately 60 %, compared to control. This finding corroborates numerous reports that heavy metals cause a decrease in protease activity; Cd (Bishnoi et al. 1993; Shah and Dubey 1997; Kuriakose and Prasad 2008), Pb (Mohan

Fig. 1 Embryo length of bean seeds germinated in H_2O (0) or $200 \mu\text{M}$ Cu. Values are the means of five measurements with standard error. Each measurement was performed with 80 germinating seeds

Fig. 2 ACA in cotyledons of bean seeds germinated in H_2O (0) or $200 \mu\text{M}$ Cu. Values are the means of four measurements with standard error. Each measurement was performed in an extract obtained from several cotyledons. • Effect of Cu ions on ACA in vitro: extract of 9 days-control was mixed with $200 \mu\text{M}$ CuCl_2 and incubated for 30 min at 4°C before enzymatic assay

and Hosetti 1997) and Ni (Maheshwari and Dubey 2007). The inhibition could not be explained only by the direct effect of the metal ion on the enzymatic proteins, since ACA was reduced less than 20 % after Cu incubation in vitro with the control enzymatic extract (9 days of H_2O -germination; Fig. 2). Thus, it seems that copper stress can operate in more ways than one with regard to the mechanism of enzymatic inhibition, although metal ions can bind with important functional groups, since they have a high affinity for O-, or N- or S-ligands of the enzyme proteins (Van Assche and Clijsters 1990) and may cause proteases inactivation (Palma et al. 2002). Effectively, heavy metals can induce cellular redox imbalance leading to oxidative stress (Sharma and Dietz 2009). The generation of reactive oxygen species is often considered to reflect a rather indirect metal effect of cellular disregulation; notably, the oxidation of side chains of amino acid residues and the formation of protein-protein covalent cross-linkage that inactivate or denature the protein function (Stadtman 1993; Davies 2001).

It is important to point out that, in the experiments just described, the activity on the synthetic substrate azocasein was conducted at pH 5.5. This choice was dictated by the results of a study of the effect of pH on ACA of 9-days germinated seeds. The maximum activity occurred at pH 5.5 (Fig. 3). The acidic optimum pH agrees with the fact that the proteases of the reserve tissues are derived from the protein bodies, after steps of intracellular protein transfer through the endomembrane system to the deposition in the storage vacuoles (Müntz 1996).

Fig. 3 Effect of pH on ACA of cotyledonary extract of 9 days-germinated bean seeds. Values are the means of four measurements with standard error. Each measurement was performed in an extract obtained from several cotyledons. Maximum activity (100 %)=15.12±2.03 µg azocasein/min/g FW. The following buffers (0.1 M) were used for indicated pH ranges: Na-citrate, 4 to 5.5; Tris-maleate, 6 to 8.5; glycine, 9 and 9.5

The results of SDS-gelatin-PAGE (Fig. 4) show that Cu treatment leads to a decrease in protease activity of 45 to 66 kDa (region A). However, in the presence of β-mercaptoethanol, an enhancement of gelatinase activity was seen, but Cu had no effect on it (region B). This finding would indicate that the enzymes responsible for B activity should be (i) either unsuitably separated monomers which remained masked in high molecular weights region, (ii) or sulfhydryl enzymes (CP) exhibited by the reducing agent.

Effect of copper stress on protease classes

To assess the different types of proteases, various specific inhibitors for each class were assayed against azocasein-degrading activities: 1-10 phenantroline and EDTA for MP, PMSF and STI for SP, E-64, NEM, iodoacetamide, iodoacetate for CP and pepstatin A and DAN for AP (Table 1). On the basis of 1-10 phenantroline, PMSF, E-64 and pepstatin A inhibitions, MP, SP, CP and AP were found to account for 29, 5, 56 and 23 % of the total endoproteolytic activities in the cotyledons of control 9 days-germinated seeds. The inhibition concentrations indicated in Table 1 were used to investigate the changes in the activities of the four protease classes as a function of H₂O or Cu treatments at each point of duration (Fig. 5).

As recorded for the total ACA (Fig. 2), the activities of control protease classes increased rapidly in control seeds (Fig. 5). However, in the presence of Cu, inhibition of CP and MP activities exceeds 80 % after 9 days of germination. Similar decline was found at day 6 for AP activity. However, SP activity was stimulated over 100 % by Cu (9 days; Fig. 5).

Fig. 4 Protease activity analysis of 90 µg proteins (per lane) from cotyledons of 9 days-germinated bean seeds in H₂O or 200 µM Cu. Gels (7.5 % polyacrylamide with 0.2 % gelatin, w/v) were run under reducing (+β-mercaptoethanol) or non-reducing (-β-mercaptoethanol) conditions and stained with Coomassie Brilliant Blue R250 containing 0.4 % amidoblack. Clear bands on dark background indicate sites of protein degradation. Brackets (A and B) denote major protease activities. M, molecular weight markers (kDa). Experiment was performed in duplicate

The obtained data confirm numerous reports that endoprotease classes are sensitive to the action of abiotic and biotic stresses, especially heavy metal exposure (Djebali et al. 2007; Domash et al. 2008).

Alternatively, a second manner to understand the impact of Cu excess on the proteolysis system during germination is to examine the changes in each protease activity dependent abundance. In fact, the behaviour of each class is discussed with respect to the pattern of its relative distribution from global proteolytic capacity. A high level of AP in ungerminated seeds (50 % of total activity) suggests a possible participation of this class in the initial cleavages of reserve proteins. Several studies have shown the involvement of AP in the maturation process of storage proteins in concert with vacuolar processing enzyme (Runeberg-Roos et al. 1994; Voigt et al. 1997). Obtained data led to the hypothesis that the AP from seeds of *P. vulgaris* are strongly active in the beginning of germination, to initiate the breakdown of storage proteins, before the massive *de novo* biosynthesis of the CP (Shutov and Vaintraub 1987; Voigt et al. 1997; Mutlu and Gal 1999). Indeed, germination appears to rise the level of CP (Bielawski et al. 1994; Nguyen et al. 1995; Sutoh et al. 1999). Here, the relative distribution of CP increased from 2 % of global proteolytic activity in ungerminated seeds to 56 % after 9 days of H₂O-germination. Thiol-dependent

Table 1 Effects of protease inhibitors on the endoproteolytic activity of bean seeds germinated in H₂O during 9 days. Values are the means of four measurements ($\pm$ SE). Each measurement was performed in an extract obtained from several cotyledons

Inhibitor	Concentration	ACA			Protease class
		$\mu\text{g azocasein/min/g FW}$	% of control	% of Inhibition	
Control	—	15.12 $\pm$ 2.03	100 %	—	
1-10 phenantroline	1 mM	10.77 $\pm$ 1.13	71 %	29 %	MP
EDTA	1 mM	14.21 $\pm$ 0.47	94 %	6 %	
PMSF	100 μM	14.32 $\pm$ 0.35	95 %	5 %	SP
STI	10 μM	14.81 $\pm$ 0.34	98 %	2 %	
E-64	5 μM	6.65 $\pm$ 1.66	44 %	56 %	CP
NEM	100 μM	13.94 $\pm$ 0.57	92 %	8 %	
Iodoacetamide	100 μM	12.32 $\pm$ 1.43	81 %	19 %	
Iodoacetate	100 μM	9.78 $\pm$ 1.00	65 %	35 %	
Pepstatin A	1 μM	11.60 $\pm$ 0.41	77 %	23 %	AP
DAN	1 mM	12.85 $\pm$ 3.20	85 %	15 %	

proteases (Nielsen and Liener 1984) as well as MP (Belozersky et al. 1990; Elpidina et al. 1991) represent the major hydrolytic activities in bean cotyledons and are major factors controlling the degradation of storage proteins in germinating seeds (Shutov and Vaintraub 1987). However, upon Cu treatment, SH-proteases activity dependent

abundance decreased dramatically as compared to the control pattern; 21 vs 56 % at 9th day.

On the contrary, the SP pattern was modified with an opposite way of CP one. The physiological significance of the increase in SP activity under copper exposure (Fig. 5) could be related to the activation of protective mechanisms

Fig. 5 CP, AP, MP and SP activities in cotyledons of bean seeds germinated in H₂O (0) or 200 μM Cu. Five μM E-64, 1 μM pepstatin A, 1 mM 1-10 phenantroline and 100 μM PMSF were, respectively, used to measure the CP, AP, MP and SP type activities. Values are the means of four measurements with standard error. Each measurement was performed in an extract obtained from several cotyledons

under stress conditions, promoting the survival of plants (Pál et al. 2006; Ahsan et al. 2007; Boojar and Goodarzi 2007; Djebali et al. 2007; Domash et al. 2008). This is possible for multifunctional SP which has been found to be involved in several physiological processes, such as senescence, programmed cell death, infection of plant cells, pathogenesis in virus-infected plants, and germination (Palma et al. 2002). Moreover, plant cells exhibit increased proteolysis during exposure to oxidative stress-inducing agents; proteins are modified, selectively recognized and degraded by proteolytic systems (Vierstra 1996). Particularly, proteolysis represents an important component together with protein oxidation in oxidative stress situations induced by heavy metals (Palma et al. 2002; Djebali et al. 2007). However, although global endoproteolytic activities can change in response to heavy metal stress (Romero-Puertas et al. 2002; Pena et al. 2006; Djebali et al. 2007), they are probably not representative of either the modification of proteolytic machinery, or the whole protein turnover. Indeed, the present work shows that AP, CP, MP and SP activities were differently modified. Among the SP potentially involved in the response to Cu-imposed protein oxidation, we can focus on trypsin and chymotrypsin-like activities, since proteasome, trypsin-, peptidyl glutamyl peptide hydrolase- and chymotrypsin-like activities can change upon heavy metals treatment (Pena et al. 2006; 2007). Further investigations are required for better understanding the significance of Cu-enhanced SP activity in bean cotyledons. In our laboratory, we have already shown the evidence of oxidative processes generation in germinating legume seeds during heavy metals exposure (Smiri et al. 2010; 2011).

In this study, we have examined the effect of copper stress on protease activity during bean seed germination. Inhibitions are seen in AP and MP activities, notably in CP one, and in proteases with molecular weights of 45 to 66 kDa. The up-regulation of SP activities argues in favor of the potential involvement of some of these enzymes in the elimination of damaged proteins.

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