Purification and biochemical characterization of a vacuolar serine endopeptidase induced by glucose starvation in maize roots

Franck JAMES*, Renaud BROUQUISSE*‡, Claude SUIRE†, Alain PRADET* and Philippe RAYMOND*

*Institut National de la Recherche Agronomique, Station de Physiologie Végétale, BP 81, F-33883 Villenave d'Ornon Cedex, France, and †Institut de Biochimie et de Génétique Moléculaire, CNRS, UPR 9026, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France

An endopeptidase (designated RSIP, for root-starvation-induced protease) was purified to homogeneity from glucose-starved maize roots. The molecular mass of the enzyme was 59 kDa by SDS/PAGE under reducing conditions and 62 kDa by gel filtration on a Sephacryl S-200 column. The isoelectric point of RSIP was 4.55. The purified enzyme was stable, with no auto-proteolytic activity. The enzyme activity was strongly inhibited by proteinaceous trypsin inhibitors, di-isopropylfluorophosphate, 3,4-dichloroisocoumarin and PMSF, suggesting that the enzyme is a serine protease. The maximum proteolytic activity against different protein substrates occurred at pH 6.5. With the exception of succinyl-Leu-Leu-Val-Tyr-4-methylcoumarin, no hydrolysis was detected with synthetic tryptic, chymotryptic or

peptidylglutamate substrates. The determination of the cleavage sites in the oxidized B-chain of insulin showed specificity for hydrophobic residues at the P2 and P3 positions, indicating that RSIP is distinct from other previously characterized maize endopeptidases. Both subcellular fractionation and immunodetection *in situ* indicated that RSIP is localized in the vacuole of the root cells. RSIP is the first vacuolar serine endopeptidase to be identified. Glucose starvation induced RSIP: after 4 days of starvation, RSIP was estimated to constitute 80 % of total endopeptidase activity in the root tip. These results suggest that RSIP is implicated in vacuolar autophagic processes triggered by carbon limitation.

INTRODUCTION

Marked changes occur when plant cells are deprived of carbohydrates [1–5]. As in natural starvation, which occurs in higher plants during senescence [6,7], shading [4,8] or after harvest [9], maize roots incubated without an exogenous carbon source substitute protein and lipid metabolism for sugar metabolism through autophagic processes [1,2,5]. There are many reports of degradation and synthesis of specific proteins during starvation. In response to starvation, plant cells decrease the activity of enzymes involved in sugar metabolism and respiration [2,5] and in nitrate reduction and assimilation [10]. In contrast, the activity of enzymes involved in fatty acid oxidation [11] and in amino acid [10] and protein degradation [12,13] increase. Moreover, the expression of various proteins [14–19] has been shown to be sugar-dependent. Thus it seems that starved plant cells are able to induce the synthesis of new or pre-existing enzymes that are involved in the response to starvation.

The co-ordinated response of plant cells to starvation suggests a fine control of proteolysis. This could be mediated by several populations of proteases implicated in various catabolic processes: (1) specific nuclear and cytosolic proteolysis, (2) organellar proteolysis and (3) lysosomal/vacuolar proteolysis (micro- and macro-autophagy or protein importation into the vacuole) (reviewed in [20,21]). Because each cellular compartment is affected by the carbon limitation, these different proteolytic systems might be involved, although to differing extents, in the cellular response to starvation. Degradation of proteins followed by amino acid catabolism and oxidation could supply carbon to the tricarboxylic cycle [10]. In a previous study we showed that starvation-induced protein degradation in the maize root tips was correlated with a transient increase in free amino acids and a rise in endopeptidase and carboxypeptidase activities [13]. The

endopeptidase activities involved in the response to starvation seemed to be different from those present in non-starved tissues, indicating that the level and nature of endoproteolytic activities were controlled by the level of sugars during the starvation period [13].

Here we describe the purification to apparent homogeneity of a new vacuolar serine endopeptidase, responsible for 80 % of total endopeptidase activity in maize roots starved for 4 days. The biochemical properties and the subcellular localization of the enzyme are discussed in relation to the starvation process.

EXPERIMENTAL

Chemicals

Leupeptin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), pepstatin, *p*-chloromercuribenzenesulphonate (PCMBs), phosphoramidon, 1,10-phenanthroline, PMSF, 3,4-dichloroisocoumarin, chymostatin, benzamidine, iodoacetamide, di-isopropylfluorophosphate, soybean trypsin inhibitor (STI) and egg trypsin inhibitor (ETI) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Na₂EDTA was from Merck (Darmstadt, Germany). Carrier-free Na¹²⁵I (5.6 × 10⁸ Bq/μg) was purchased from Amersham (Les Ulis, France). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H + L) were from Sanofi (Marnes-la-Coquette, France).

Plant materials and incubation conditions

Maize seeds (*Zea mays* L. cv. Dea; Pioneer France Maïs, Toulouse, France) were soaked for 3 h in water and germinated for 3 days on layers of wet filter paper; primary root tips 3 mm long or primary roots 3–4 cm long were then excised and either

Abbreviations used: Bz, benzoyl; Cbz, benzyloxycarbonyl; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; ETI, egg trypsin inhibitor; FITC, fluorescein isothiocyanate; Mec, 4-methylcoumarin; NA, β -naphthylamide; NHPHNO₂, *p*-nitroanilide; PCMBs, *p*-chloromercuribenzenesulphonate; RSIP, root-starvation-induced protease; STI, soybean trypsin inhibitor; Succ, *N*-succinyl.

‡ To whom correspondence should be addressed.

used immediately or incubated for starvation treatment. Incubation conditions were essentially as already described [5,13]. Before each renewal of incubation medium, the roots were rinsed thoroughly with pure (Milli Q) water. For protease localization experiments on stems or leaves, 3-day-old seedlings were grown for a further 4 days in pots containing a mixture of peat and vermiculite (50:50, v/v), in a phytotron at 24 °C and 80 % relative humidity either in the light (photon flux density 300 $\mu\text{mol/s per m}^2$, 14 h per day) or in complete darkness (etiolated seedlings). Seedlings were watered daily with 150 ml of diluted culture medium. Stems and leaves were then harvested and frozen immediately in liquid nitrogen for protein extraction.

Preparation of crude extracts

Crude extracts of root tips (3 mm long), mature roots, germinated seeds (3 days after imbibition), coleoptiles (5 days after imbibition), stems, green leaves or etiolated leaves were used for proteolytic assay and immunodetection experiments. Fresh or frozen tissues were crushed at 4 °C in a mortar in grinding medium (0.4 ml per g fresh weight) containing 20 mM Mops, pH 7.0, 10 mM 2-mercaptoethanol and 3 % (w/v) polyvinylpyrrolidone. The brei was transferred to a 1.5 ml micro-centrifuge tube and the mortar was rinsed with grinding medium, which was then pooled with the brei. The homogenate was squeezed through a single layer of Miracloth (Calbiochem, Meudon, France) and centrifuged at 10000 *g* for 15 min. The supernatant was desalted through an Econo Pac 10-DG column (Bio-Rad) equilibrated with 10 mM Mops (pH 7.0).

Purification of the root-starvation-induced protease (RSIP)

All steps were performed at 4 °C.

Step 1

Approx. 150 g of primary roots 3–5 cm long was first incubated for 4 days in sugar-depleted medium A, and then homogenized in a Waring Blendor with 300 ml of 20 mM sodium acetate, pH 4.8, containing 10 mM 2-mercaptoethanol and 3 % (w/v) polyvinylpyrrolidone. The homogenate was filtered through one layer of Miracloth and two layers of 50 μm nylon netting (Nylon Blutex; Tripette et Renaud, Lyon, France) and centrifuged at 20000 *g* for 20 min. The crude supernatant was used for the further purification step.

Step 2

The crude supernatant was applied to a 2.5 cm $\times$ 42 cm column of Sepharose Q Fast Flow (Pharmacia) equilibrated with 10 mM sodium acetate (pH 4.8)/2 mM 2-mercaptoethanol. The column was washed at 120 ml/h with 200 ml of the same equilibration buffer and then bound proteins were eluted at the same flow rate with 200 ml of the equilibration buffer containing 0.6 M NaCl. Fractions of 40 ml were collected and those containing the major peak of proteolytic activity were combined and concentrated in an Amicon stirred cell with a YM-30 membrane.

Step 3

The concentrated sample was applied to a Sephacryl S-200 HR (Pharmacia) gel-filtration column (2.5 cm $\times$ 70 cm) equilibrated with 50 mM Tris/HCl (pH 7.6)/50 mM NaCl and eluted at 1 ml/min. The active fractions corresponding to the major activity peak (25 ml each) were pooled and concentrated (Amicon stirred cell, YM 30 membrane).

Step 4

The concentrated fraction was diluted 1:10 with a solution of 20 mM methylpiperazine, pH 5.7, and applied to an FPLC Mono-P HR 20/5 chromatofocusing column (Pharmacia) equilibrated with 20 mM methylpiperazine, pH 5.7 as the starting buffer. Proteins were eluted at 0.5 ml/min with a pH gradient from 5.7 to 4 generated by the addition of an Ampholine solution (PBE 74; Pharmacia) diluted 1:10 in water and brought to pH 4.0 with iminoacetic acid. Active fractions of 1 ml were pooled and concentrated by ultrafiltration on membrane cones (Amicon, CF25 Centriflo system).

Step 5

The concentrated sample was brought to 50 mM potassium phosphate buffer (pH 7.0)/1.7 M $(\text{NH}_4)_2\text{SO}_4$ and applied to an FPLC Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 100 mM potassium phosphate (pH 7.0)/1.7 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with the same buffer and proteins were eluted, at 0.5 ml/min, with a 15 min linear gradient from 1.7 to 0 M $(\text{NH}_4)_2\text{SO}_4$. The proteolytic activity peak coincided exactly with only one peak of protein absorbance. The purity of the enzyme was assessed by native PAGE and SDS/PAGE [25].

Determination of molecular mass

The molecular mass of the purified enzyme was determined by gel filtration on the Sephacryl S-200 HR column, equilibrated with 50 mM Tris/HCl (pH 7.6)/100 mM NaCl and calibrated with the following proteins as standards: alcohol dehydrogenase (150 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12 kDa).

Protease activity assays

Protein labelling

Casein, BSA and ovalbumin were radiolabelled with ^{125}I by the chloramine-T method [22]. The initial specific radioactivity and concentration of ^{125}I -labelled protein were approx. 10^4 c.p.m./ μg and 0.5 $\mu\text{g}/\mu\text{l}$ respectively.

Assays with iodinated proteins

For routine assays on crude extracts or chromatographic isolates, 40 μl of extract or isolate, 40 μl of pH 6.5 buffer mixture (50 mM acetic acid/50 mM Mes/100 mM Tris) [23] and 10 μl of ^{125}I -labelled protein (5000 c.p.m./ μl) were incubated for 10–20 min at 37 °C. The reaction was stopped with 100 μl of 30 % (w/v) trichloroacetic acid and the samples were centrifuged for 10 min at 6000 *g*; 100 μl of supernatant was mixed with 2 ml of scintillation solution and radioactivity was measured with a liquid-scintillation analyser (Tri-Carb 2000CA; Packard, Meriden, CT, U.S.A.). The linearity of casein degrading activity was checked.

Assay with synthetic substrates

N-Benzoylarginine-*p*-nitroanilide (Bz-Arg-NH-PhNO₂), *N*-benzoyltyrosine-*p*-nitroanilide (Bz-Tyr-NH-PhNO₂), *N*-benzyloxycarbonyl-Leu-Leu-Glu- β -naphthylamide (Cbz-Leu-Leu-Glu-NA), *N*-benzyloxycarbonyl-Gly-Gly-Arg-7-amido-4-methylcoumarin (Cbz-Gly-Gly-Arg-Mec), *N*-succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin (Succ-Ala-Ala-Phe-Mec) and *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Succ-Leu-Leu-Val-Tyr-Mec) were assayed with purified enzyme for

determination of substrate specificity. The assay mixture consisted of 10 μ l of synthetic substrate (5 mM in DMSO) and 140 μ l of buffer mixture (see above) with 1 μ g of enzyme. After incubation for 1 h at 37 °C, the 7-amino-4-methylcoumarin or naphthylamine radicals released were measured fluorimetrically (excitation at 380 nm, emission at 460 nm; excitation at 335 nm, emission at 410 nm respectively), and the 4-nitroaniline radicals were measured spectrophotometrically at 410 nm. Measurement of endoproteolytic activity with azocasein as a substrate was performed on purified enzyme as previously described [13].

Determination of the optimum pH

The pH dependence of the proteolytic activity against iodinated casein, iodinated serum albumin, iodinated ovalbumin or azocasein as substrates was determined by using the three-component buffer mixture described above [23].

Effect of inhibitors

The effects of inhibitors on the enzymic activity were studied on purified RSIP (2 μ g in 40 μ l). Protease inhibitors were prepared as the following stock solutions: antipain (10 mM), leupeptin (20 mM), E-64 (10 mM), iodoacetamide (0.1 M), PCMBs (10 mM), phosphoramidon (1 mM), Na₃EDTA (0.5 M), STI and ETI (10 mM) were in water; PMSF (0.2 M) and benzamidin (0.1 M) were in ethanol; 3,4-dichloroisocoumarin (10 mM) and chymostatin (10 mM) were in DMSO; 1,10-phenanthroline (0.2 M) and pepstatin (5 mM) were in methanol; di-isopropyl-fluorophosphate (0.5 M) was in isopropanol. Inhibitors were first preincubated at ambient temperature for 15 min with RSIP before the addition of ¹²⁵I-labelled casein (see above), and activities were measured after 10 min reaction time. Control assays were conducted with solvent alone.

Hydrolysis of insulin by RSIP

Oxidized B chain of bovine insulin (100 μ g) was incubated at 37 °C in 10 mM potassium phosphate buffer, pH 6.5, with 1 μ g of purified RSIP. At intervals, 25 μ l aliquots of sample were mixed with 25 μ l of buffer 1 [0.1% trifluoroacetic acid in water/acetonitrile (90:10, v/v)] and frozen in liquid nitrogen until analysis. For peptide analysis, samples were quickly thawed and immediately applied to a C₁₈ reverse-phase HPLC column (Vydac, Hesperia, CA, U.S.A.) equilibrated with buffer 1. After a 10 min wash with buffer 1, peptides were separated at a flow rate of 0.7 ml/min by a 30 min linear gradient from 100% buffer 1 to 100% buffer 2 [0.1% trifluoroacetic acid in water/acetonitrile (40:60, v/v)]. Peptides were detected at 214 nm and collected. Peptides were then identified (1) by the analysis of their amino acid N-terminal sequence, with an Applied Biosystems 470A Protein Sequencer, by the method of Edman and Henschen [24], and (2) by their compositions in total amino acids. For amino acid analysis, peptides were first hydrolysed in evacuated sealed tubes with 100 μ l of 6 M HCl at 110 °C for 24 h and then neutralized with 6 M NaOH. Amino acids were analysed as described previously [10].

Electrophoresis

Native PAGE and SDS/PAGE were performed with 8.5% (w/v) and 12.5% (w/v) polyacrylamide gels respectively by the procedure of Laemmli [25]. Gels were fixed, stained with Coomassie Blue and destained by standard methods.

Immunological and blotting methods

Polyclonal antibodies against maize endopeptidase (RSIP) were produced by subcutaneous injections of purified enzyme into a New Zealand white rabbit as described in [26]. Serum was stored at -80 °C in 0.02% NaN₃. RSIP-specific antibodies were purified as described in [27] and immediately used for immunoblotting determination and immunofluorescence microscopy.

For immunoblots, proteins from native PAGE or SDS/PAGE were transferred to nitrocellulose membrane (BA 85; Schleicher and Schull, Ecquevilly, France) for 1 h at 4 mA/cm² in a Bio-blot semi-dry system (Eurogentec, Tileman, Belgium). Blots were blocked with Tris-buffered saline containing 0.2% Tween-20 and 5% (w/v) non-fat milk powder. RSIP was detected with purified anti-RSIP antibodies plus goat anti-(rabbit IgG)-alkaline phosphatase conjugate (Sigma). Signal quantification on immunoblots was done by a scanning densitometer (Ultrosan DL, LKB). For immunoprecipitation experiments, equal caseinase activities in different crude extracts (100 c.p.m./min) or in purified protease fractions (500 c.p.m./min) were incubated for 1 h at 25 °C with increasing volumes of immune or preimmune serum. Immune complexes were incubated for 1 h at 25 °C with a 2-fold (IgG-binding) excess of Protein A-Sepharose and then centrifuged for 5 min at 10000 g. The caseinase activity was then measured in each supernatant fraction.

Protein determination

Proteins were quantified by the method of Bradford [28]. Bovine γ -globulin was used as the protein standard.

Subcellular fractionation

All steps were performed at 4 °C. A protocol for subcellular fractionation of sunflower coleoptiles [29] was adapted for maize root tips. Maize root tips (400; approx. 1 g fresh weight) that were either non-starved or had been starved for 24 h were gently crushed in a smooth-bottomed mortar in 5 ml of homogenization buffer [170 mM Tricine/NaOH (pH 7.5)/10 mM KCl/1 mM MgCl₂/1 mM EDTA/1 mM 2-mercaptoethanol/1 mM dithiothreitol/0.6 M sucrose/9 g/l BSA]. The homogenate was filtered through three layers of Miracloth and centrifuged at 600 g for 10 min. The supernatant was layered carefully on a discontinuous sucrose gradient. All sucrose solutions contained 1 mM EDTA, pH 7.5. The gradient consisted of 5 ml of 30%, 5 ml of 40%, 10 ml of 50% and 10 ml of 60% (w/v) sucrose. The tubes were then centrifuged at 83000 g for 90 min. Fractions of 1 ml were collected along the gradient, from the bottom to the top of the tube, and analysed. RSIP was immunodetected and then quantified by scanning in each fraction, after SDS/PAGE and electrotransfer to nitrocellulose membrane (see above). Marker enzymes were assayed in each fraction, and regions of the gradient corresponding to the different cell compartments were identified: fumarase (mitochondria) and phosphoenolpyruvate carboxylase (cytosol) as described in [5], NADP:glyceraldehyde-3-phosphate dehydrogenase (plastid) as in [29] and α -mannosidase (vacuole) as in [30]. The integrity of mitochondria and plastids was determined as in [31].

Microscopy and immunofluorescence methods

Root tips were fixed 4 h at room temperature in 25 mM NaH₂PO₄/Na₂HPO₄ (pH 7.3)/100 mM sucrose/4% (w/v)

paraformaldehyde/0.5 % (v/v) glutaraldehyde (EM grade). After a 30 min wash in 100 mM phosphate buffer, pH 7.2, tips were embedded in Epon. Sections (1.5 μ m) were processed as described in [32]. After removal of the resin, tissues were incubated first with anti-RSIP antibodies, then with FITC-conjugated goat anti-rabbit IgG (H+L). The primary antibody was derived from the crude serum (55.6 mg/ml protein), purified serum (2.8 mg/ml protein) or purified IgG (0.68 mg/ml protein) isolated from the crude antiserum as described in [32]. Antibodies were diluted into 100 mM sodium phosphate buffer, pH 7.3, containing 200 mM NaCl, 0.1 % BSA and 0.1 % (v/v) Triton X-100. Control sections were incubated with preimmune serum in place of the primary antibody. Sections were counterstained with 0.01 % Evans Blue in saline phosphate buffer (pH 7.3).

RESULTS

Preliminary experiments showed that, in whole maize roots as well as in maize root tips [13], the maximum increase in endoproteolytic activity occurred after 4 days of carbohydrate starvation, whereas no increase was observed in sugar-supplied (0.2 M glucose) tissues (results not shown). Thus whole roots were chosen, instead of root tips, as starting material for protein purification because their specific endoproteolytic activity was higher and large quantities of tissue could be more easily obtained.

Endopeptidase purification

The crude supernatant obtained after the first step contained a large quantity of protein (230 mg) associated with a high total endopeptidase activity (Table 1). The application of the crude supernatant to Sepharose Q Fast Flow, at pH 4.8 (step 2), resulted in the retention of the major part of total endopeptidase activity (results not shown). Although the recovery of total endopeptidase activity was low (20.5 %) (Table 1), this step was necessary to remove polyphenols and microsomal fragments that were not retained on the column. After elution, the active fractions were pooled, concentrated and subjected to gel filtration on Sephacryl S-200 (step 3), which separated the major endopeptidase activity peak from the bulk of the higher-molecular-mass contaminating proteins (results not shown). The 45-fold purification factor was associated with a small loss of activity (Table 1). The endopeptidase pool was then chromatofocused on Mono P (step 4) and only one caseolytic activity peak was eluted at pH 4.55, providing an estimate of the isoelectric point of the enzyme. The purification factor at this step was 3 with no significant loss of activity (Table 1). Finally the active pool was applied to a Phenyl-Superose column and a relatively hydrophobic fraction

Figure 1 SDS/PAGE (A) and PAGE (B) analysis of the RSIP preparation obtained after Phenyl-Superose chromatography

The purified RSIP preparation was revealed by Coomassie Brilliant Blue coloration after SDS/PAGE [12.5 % (w/v) gel] (lane 3) or 8.5 % (w/v) PAGE (lane 7). Purified antibodies were used to immunodetect RSIP in crude extracts of maize root (lanes 2 and 6) or in the Phenyl-Superose preparation (lanes 1 and 5). Pure RSIP (2 μ g) was loaded in tracks 1, 3, 5 and 7 and 100 μ g of total protein in 2 and 6. The molecular masses of markers, separated on SDS/PAGE [12.5 % (w/v) gel] (lane 4), are shown in kDa.

was eluted at approx. 200 mM $(\text{NH}_4)_2\text{SO}_4$. This final step provided a purified enzyme showing a single band of protein after SDS/PAGE and native PAGE (Figure 1, lanes 3 and 7). Under denaturing conditions, the apparent molecular mass of the protease was calculated to be 59 ± 2 kDa (Figure 1). It was estimated to be 62 ± 2 kDa by gel filtration on Sephacryl S-200 (results not shown). These results indicate that the native enzyme is monomeric.

The overall purification achieved was approx. 340-fold and the apparent yield of activity was only 6 % (Table 1). However, these factors are underestimates because the crude supernatant contained multiple endopeptidase activities. The five-step purification procedure typically yielded 30–50 μ g of highly purified protease from 150 g (fresh weight) of maize roots. Because this endopeptidase was induced in starved material and exclusively located in roots (see below), we called it RSIP.

Immunocharacterization

Rabbit polyclonal antibodies were raised against purified RSIP. Immunoprecipitation experiments showed that the activity of

Table 1 Purification of RSIP from glucose-starved maize roots

Endopeptidase activity was determined with ^{125}I -labelled casein as a substrate. The initial specific radioactivity of ^{125}I -labelled casein was 10^4 c.p.m./ μ g. Activities, expressed in c.p.m./min, were measured as described in the Experimental section.

Step	Protein activity (mg)	Total activity (c.p.m./min)	Specific activity (c.p.m./min per mg)	Purification factor (fold)	Recovery (%)
Crude extract	228.6	278660	1219	1	100
Sepharose Q	38.66	57125	1478	1.2	20.5
Sephacryl S-200	0.98	54340	55450	45.5	19.5
Mono P	0.32	51273	160228	131.4	18.4
Phenyl-Superose	0.042	17276	412140	338.1	6.2

Figure 2 Determination of RSIP stability by immunoblotting

Purified RSIP (5 μ g) was incubated for 3 h at 37 °C in 30 μ l of a 50 mM potassium phosphate buffer (lane 1), or for 3 h (lane 2) and 6 h (lane 3) in 30 μ l of a crude extract of mature maize roots starved for 4 days. Total extracts separated by SDS/PAGE [12.5% (w/v) gel] under reducing conditions followed by immunodetection with purified anti-RSIP antibodies. The molecular masses of prestained molecular mass markers (lane 4) are shown in kDa.

purified RSIP was 90 % and 98 % inhibited on addition of 10 and 20 μ l of crude antiserum respectively (results not shown). Further purification of antibodies by antigen-immobilized affinity [27] was used to increase their specificity for RSIP. The purified antibody was found to be specific for RSIP because it gave a single band on immunoblots against both the crude extract and the purified enzyme in SDS/PAGE (Figure 1, lanes 1 and 2) as well as in native PAGE (Figure 1, lanes 5 and 6).

Biochemical characterization

Stability of the enzyme

The stability of the purified RSIP was tested under various storage conditions. Enzyme activity at pH 7.0 was stable: after 1 h the loss of activity was only 25 % at 50 °C, whereas no change was observed at 25 or 4 °C (results not shown). After 5 days at -20 °C, the conservation of RSIP was improved in the presence of 5 mM 2-mercaptoethanol and 5 % (v/v) glycerol. Under these conditions activity was not significantly modified after multiple cycles of freezing and thawing (results not shown). The positive effect of 2-mercaptoethanol suggested the presence of an important thiol group in the protease. The effect of pH on the storage stability of RSIP was also estimated after 5 days of conservation at -20 °C (results not shown); it seems that the enzyme was stable at a large range of pH values (5–9) but was less active after conservation under more acidic conditions (pH 3). No autoproteolytic degradation was observed when purified enzyme was incubated at 37 °C (Figure 2, lane 1). However, when RSIP was incubated at 37 °C in the presence of a crude extract of maize roots starved for 4 days, degradation peptides were detected on immunoblots (Figure 2, lanes 2 and 3). These results suggest that in a crude extract RSIP was degraded by other proteases and not by autoproteolysis.

Substrate specificity and optimum pH

The activity of the protease was assayed with several 125 I-labelled proteins (Table 2). RSIP degraded the three iodinated protein substrates; the activity was the highest with BSA and casein. Azocasein was also found to be a good substrate for pure RSIP. However, RSIP exhibited little or no activity against

Table 2 Hydrolysis of different protein or synthetic substrates

Assays were performed as described in the Experimental section. Abbreviation: n.d., not detected.

Substrate	Concentration	Activity
125 I-labelled protein substrate	(c.p.m. per assay)	(c.p.m./min per μ g of protein)
Casein	50 000	410
BSA	50 000	580
Ovalbumin	50 000	160
Synthetic peptide substrate	(mM)	(pmol/min per μ g of protein)
Bz-Arg-NHPhNO ₂	0.3	n.d.
Bz-Tyr-NHPhNO ₂	0.3	n.d.
Cbz-Leu-Leu-Glu-NA	0.3	n.d.
Cbz-Gly-Gly-Arg-Mec	0.3	n.d.
Succ-Ala-Ala-Phe-Mec	0.3	n.d.
Succ-Leu-Leu-Val-Tyr-Mec	0.3	1.1
Azoprotein substrate	(mg/ml)	(units/mg of protein)
Azocasein	1.8	2.8

Figure 3 Kinetics of degradation of the insulin B chain by RSIP

The insulin B chain (100 μ g) was incubated in a 10 mM potassium phosphate buffer (pH 6.5) with 1 μ g of pure RSIP in a final volume of 120 μ l. At the times indicated, 0 min (A), 5 min (B), 15 min (C) and 4 h (D), aliquots of the reaction mixture were removed and peptides were analysed on a HPLC reverse-phase column by monitoring absorbance at 214 nm, as described in the Experimental section. Peptides from 1 to 15 were clearly separated from the insulin B chain peak (I).

various synthetic substrates; only Succ-Leu-Leu-Val-Tyr-Mec was hydrolysed but at a low rate (Table 2).

The purified protease was maximally active at pH 6.5–7.0

Figure 4 Localization of the RSIP cleavage sites on the insulin B chain

Peptides (numbered 1 to 15) obtained from insulin B chain digestion and separation on a reverse-phase HPLC column (Figure 3) were identified by analysis of their N-terminal sequence and by their amino acid compositions. Arrows indicate the different cleavage sites deduced from the sequences of peptides cleaved by RSIP. A comparison of RSIP cleavage sites with those of other, well-characterized, endopeptidases [34,37] is also shown.

against casein, BSA and ovalbumin (results not shown). Azocasein was maximally degraded at pH 6.1, which might be due to some modifying effect of its sulphanilamide chromophore [33].

Specificity of the cleavage site

The specificity of cleavage site was studied by following the action of the purified protease on the oxidized insulin B chain. Chromatographic analysis of peptide fragments released during digestion showed that insulin was hydrolysed rapidly (Figures 3A, 3B and 3C). With a substrate-to-enzyme mass ratio of 100:1, approx. 80 % of the insulin was hydrolysed within 15 min and 100 % within 30 min. After 5 min of hydrolysis, four major peptides were found (numbered 5, 12, 13 and 14) (Figure 3B). From 5 to 15 min, secondary hydrolysis products were generated besides the former major peptides (numbered from 1 to 15) (Figure 3C). After several hours, less specific cleavage sites occurred, providing a large number of small peptides (Figure 3D). The abundance of generated peptides was an indication of the large domain of activity of RSIP. However, the identification of the peptides released during short-term hydrolysis clearly demonstrated a preference for cleavage sites (Figure 4) with hydrophobic amino acids located at the P2 and, to a lesser extent, the P3 positions : Phe¹-Val², Leu¹¹-Val¹², [Glu¹³]-Ala¹⁴, Ala¹⁴-Leu¹⁵, Leu¹⁷-Val¹⁸ and Phe²⁴-Phe²⁵.

Effect of inhibitors

The inhibitor study was intended to determine the mechanistic class of RSIP. The effect of various compounds on the activity of the purified protease is shown in Table 3. All the serine protease inhibitors tested were strong inhibitors of caseolytic activity (90–100 % inhibition), except benzamidine, which was practically ineffective. The strong inhibition of the purified enzyme by PMSF, which was not reversed by L-cysteine or 2-

Table 3 Effect of various inhibitors on protease activity

Enzyme (2 µg) was preincubated with different inhibitors for 15 min before the addition of ¹²⁵I-labelled casein. Activity is expressed as a percentage of the control (100% of activity represented 9000 c.p.m. for 10 min reaction time). Control assays were performed with solvent alone. Abbreviations: DFP, di-isopropylfluorophosphate; 3,4DCI, 3,4-dichloroisocoumarin.

	Inhibitor concentration (mM)	Activity (% of control)
Control		100
PMSF	5	3
DFP	5	12
3,4DCI	0.1	11
STI	0.01	0
ETI	0.01	8
Benzamidine	5	95
E-64	0.5	108
N-Ethylmaleimide	5	91
Iodoacetamide	1	82
PCMBS	0.5	28
Chymostatin	0.1	2
Leupeptin	0.2	90
EDTA	10	93
1,10-Phenanthroline	10	88
Phosphoramidon	0.01	92
Pepstatin	0.05	101

mercaptoethanol in the assay medium (results not shown), confirmed the assignment of RSIP to the serine protease class. Proteinaceous inhibitors, STI and ETI, and the microbial inhibitor chymostatin were also efficient inhibitors of endopeptidase activity. In contrast, cysteine protease inhibitors such as E-64, N-ethylmaleimide, iodoacetamide or leupeptin had no

Figure 5 Determination of the RSIP localization of by subcellular fractionation

The different compartments were separated by centrifugation on a discontinuous sucrose gradient, and marker enzyme activities were measured along the gradient. The densities of the different sucrose layers are indicated at the top. Maize root tips were non-starved (open symbols) or starved for 24 h (filled symbols). Fractions of the gradient were also immunoblotted for RSIP immunolocalization (open bars, non-starved; filled bars, starved for 24 h). The absolute value of 110% in fractions from starved root tips is 5-fold that from non-starved root tips.

marked effect on RSIP activity. However, PCMBs caused 72 % inhibition, suggesting the presence of at least one essential thiol group in the protease. Metalloprotease inhibitors (1,10-phenanthroline, EDTA and phosphoramidon) and aspartic protease inhibitor (pepstatin) did not inhibit RSIP activity.

Subcellular localization of RSIP

The subcellular localization of the enzyme in the root tips, either starved for 24 h or non-starved, was studied by discontinuous density gradient centrifugation. The locations of RSIP and the different marker enzymes along the gradient are shown in Figure 5. The recovery of the various enzyme activities on the gradient, compared with crude extract [5], was close to 100 %. Immunodetection of RSIP showed that it was located at the top of the gradient, in the 20 and 30 % sucrose layers. With non-starved material RSIP appeared as a double peak and the immunosignal was one-quarter to one-fifth as strong as that from starved root tips. Comparison with marker enzyme patterns clearly showed that RSIP was not associated with plastidial or mitochondrial compartments. Indeed, three populations of plastid were located along the sucrose gradient: in the pellet, at the 50–60 % interface and at the top of the gradient (Figure 5). Similar organellar integrity (results not shown) was observed in starved and non-starved conditions: 85 % for the pellet and 60 % at the 50–60 % interface, whereas NADP:glyceraldehyde-3-phosphate dehydrogenase activity in the top of the gradient could be attributed to broken plastids (5 % integrity). Similarly, in non-starved maize root tips (Figure 5), three different peaks of fumarase activity were detected. The integrity of the mitochondria was 91 % at the 40–50 % interface, declined to 30 % at the 30–40 % interface, and was only 5 % at the top of the gradient. After 24 h of starvation the 30–40 % peak became undetectable. As with

Figure 6 Intracellular immunolocalization of RSIP in a maize root tip

Longitudinal section of a maize root tip starved for 24 h at the boundary between the cortex and the medulla, approx. 700 μm above the apical meristem. (A) Test section incubated with purified anti-RSIP antibodies. The tonoplast is generally underlined by peripheral fluorescent precipitates, and the sap of the largest vacuoles (V) is strongly labelled. Unlabelled structures are counterstained with Evans Blue; their red fluorescence is considerably weaker than the FITC labelling and is poorly visible or invisible in black and white. (B) Control section corresponding to (A) incubated with preimmune serum instead of the purified antibodies. Vacuoles are not labelled and the counterstained nuclei (N) are clearly visible. Magnification $\times 590$.

plastids, the fumarase activity peak at the top of the gradient corresponded to broken mitochondria.

Both phosphoenolpyruvate carboxylase (cytosol marker) and α -mannosidase (vacuole marker) activities were located at the top of the gradient, with either starved or non-starved material (Figure 5). Under these extraction conditions, vacuoles were broken and their contents remained in the supernatant fraction. The RSIP pattern resembles the α -mannosidase pattern more than the phosphoenolpyruvate carboxylase one; particularly, both RSIP and α -mannosidase exhibit a double peak with non-starved material, and the same increase in activity occurred in starved root tips. However, these similarities were not convincing enough to allocate RSIP to the vacuole rather than to the cytosol. Thus the detection of RSIP *in situ* by immunofluorescence was necessary to determine whether the enzyme was exclusively localized in the vacuole or in the cytosol.

RSIP was weakly detected in unstarved excised root tips. However, after 24 h of starvation, RSIP was clearly expressed from the excision plane (at 3 mm from the tip) to the area of cell lengthening located between 0.5 and 1 mm above the apical meristem (Figure 6). In this area the tonoplast was underlined by peripheral precipitates, and the vacuole sap (not the cytosol) was strongly labelled in differentiating vascular tissues and in epidermis. Young vacuoles from meristematic cells at the root apex were not labelled. Cells close to the excision plane were degenerating and large zones were strongly labelled. After 48 h of starvation, RSIP was expressed in the meristematic cells of the

Figure 7 Distribution of RSIP in various organs of maize whole plants

(A) Crude extracts (100 μ g of proteins) prepared from coleoptile (1), green leaf (2), etiolated leaf (3), stem (4), germinating seed (5) and roots 3 cm long (6) from maize plants were analysed by SDS/PAGE followed by immunoblotting with the purified anti-RSIP antibodies. (B) Equal amounts of caseolytic activity obtained from different maize tissues (100 c.p.m./min) were mixed with anti-RSIP antiserum.

apex, the vacuoles of which had tonoplast underlined by sharp fluorescent labelling (results not shown).

RSIP abundance in different maize tissues

To determine whether the same endopeptidase polypeptide was expressed in different maize cell types, proteins in crude extracts of roots, stems, germinated seeds (minus coleoptile and primary root), coleoptiles, green leaves and etiolated leaves were separated by SDS/PAGE, transferred to nitrocellulose membrane and probed with purified antibody. When equal amounts of protein were loaded on a gel, only root tissues exhibited an immunosignal (Figure 7A). In addition, no inhibition of caseolytic activity was observed when the crude extracts of the different tissues were subjected to immunoprecipitation tests with anti-RSIP antibodies (Figure 7B). These results suggest that RSIP was specifically localized in roots and absent or undetectable in aerial tissues or in germinating seeds.

RSIP root distribution under starved and non-starved conditions

The exclusive occurrence of RSIP in roots raises the question of its distribution as a function of the differentiation state of the cells before and after starvation. Preliminary results had shown that when mature roots 3–4 cm long were subjected to glucose starvation, increased endoproteolytic activity was observed in both the mature and the meristematic regions of the root (results not shown). Thus crude extracts of starved and non-starved root tips and more mature subapical segments were subjected to SDS/PAGE and RSIP immunodetection experiments. When equal amounts of endopeptidase activity were separated, RSIP could be detected in both parts of the root (Figure 8A, lanes 1 and 3). Signal intensity was stronger in the mature section than in the root tip. After 4 days of starvation, the intensity was higher

Figure 8 Distribution of RSIP in non-starved maize roots and in roots starved for 4 days

Equal amounts of caseolytic activity (100 c.p.m./min) were analysed either by SDS/PAGE followed by immunoblotting with purified anti-RSIP antibodies (A) or by immunoprecipitation tests with crude antiserum (B). Root tips: non-starved (1, $\circ$) and starved (2, $\bullet$); mature roots: non-starved (3, $\square$) and starved (4, $\blacksquare$).

in both parts of the root, showing an enrichment of RSIP content (Figure 8A, lanes 2 and 4). When equal amounts of protein were separated, RSIP signal was barely detectable in non-starved root tips, whereas it was clearly visible in root tips starved for 4 days (results not shown). The same overexpression was observed, although to a smaller extent, in mature segments (results not shown). Immunoprecipitation tests of equal caseolytic activity (100 c.p.m./min) (Figure 8B) showed that almost 80% inhibition was obtained in both root tips and mature sections after 4 days of starvation, whereas only 10–30% of inhibition was obtained in non-starved tissues (Figure 8B). When mature roots and root tips were incubated for 4 days in the presence of 0.2 M glucose, no significant changes in the immunosignal intensity or in the percentage inhibition of caseolytic activity were observed (results not shown). These results show that RSIP (1) is a constitutive endopeptidase, (2) occurs mainly in differentiated cells and at a basal level in the root apex, (3) is overexpressed in root tissues subjected to starvation treatment, and (4) is the major endopeptidase (as measured by its caseolytic activity) present in glucose-starved root tissues.

DISCUSSION

In a previous report [13] we described the occurrence of increasing proteolytic activities in crude extracts of excised maize root tips subjected to glucose starvation. Here we describe the purification and the characterization of the major endopeptidase, RSIP, implicated in this starvation response. RSIP has been purified in four chromatographic steps (Table 1) and used for the production of polyclonal antibodies. It was shown to be a 60 kDa monomeric endopeptidase with a pI of 4.55. RSIP was strongly sensitive to serine protease inhibitors and insensitive to cysteine protease, aspartic or metalloprotease inhibitors (Table 3), and thus can be classified as a serine protease [34]. However, the stabilizing effect

of 2-mercaptoethanol on RSIP and the partial inhibition of the protease by PCMBs (Table 3) suggest the possible involvement of a cysteine residue close to the active site, or the presence of an essential thiol group in the protein structure [34].

Mature maize roots have already been reported to contain at least two endopeptidase activities, called proteases I and II [33]. Protease II did not degrade azocasein, was inhibited by serine and thiol protease inhibitors, and thus clearly differs from RSIP. Protease I, recently renamed MRP (maize root protease) [35], was also found to be a serine protease. However, it degraded azocasein optimally at pH 9–10, had a molecular mass of 54 kDa and an isoelectric point between 5 and 6 [33], which all distinguish MRP from RSIP. Moreover the study of the primary cleavage site of the oxidized B chain of insulin showed that the major cleavage site for MRP requires an alanine residue in the P1 position [35], whereas the hydrolytic attack catalysed by RSIP is independent of the nature of amino acids P1 and P1' (Figure 4). Thus, as previously suggested in [13], RSIP is a newly identified maize root protease.

The analysis of the cleavage sites (Figure 3 and 4) showed that the presence of hydrophobic amino acids at the P2 and, to a smaller extent, P3 positions seems essential for cleavage by RSIP. However, RSIP had little activity against chromogenic substrates such as Succ-Leu-Leu-Val-Tyr-Mec and was inactive against Cbz-Leu-Leu-Glu-NA and Succ-Ala-Ala-Phe-Mec, which also possess hydrophobic amino acids at the P2 and P3 positions (Table 2). Thus it is likely that other parameters, such as the structural conformation or the length of the polypeptide chain, play a role in the recognition mechanism of the cleavage site. Such substrate specificity clearly distinguishes RSIP from other serine endopeptidases, such as chymotrypsin (Figure 4), elastase or trypsin, which are active against chromogenic substrates of low molecular mass [36] and are selective for amino acids at the P1 position [37], and links it with the second major group of serine endopeptidases, for which amino acids at positions away from the scissile bond are equally important for cleavage-site specificity [37]. RSIP shares several cleavage sites with lysosomal cysteine or aspartic endopeptidases such as papain, cathepsin L or cathepsin D (Figure 4), which require hydrophobic amino acids at the P2 (and possibly P3) position [38].

With casein, BSA or ovalbumin as substrates, the activity of RSIP was maximal between pH 6 and 7. Although some plant serine proteases with acidic pH optima have been reported [39], an acidic pH optimum is a characteristic more commonly associated with cysteine proteases [38,39]. The high activity of RSIP at pH 5.5 (80% of its optimum) is consistent with its subcellular localization in the vacuole, where the pH is estimated to be approx. 5.5 [40]. Indeed, the vacuolar location of RSIP has been established by both cell fractionation and immunocytochemical studies with anti-RSIP antibodies (Figures 5 and 6). Such localization was unexpected because, so far, all the endopeptidases characterized and localized in the vacuolar/lysosomal compartment were of either a cysteine or an aspartic type, but not of a serine type, protease [38,41]. RSIP is thus the first vacuolar serine endopeptidase to be identified.

In plants, the expression of vacuolar proteases has been related to developmental processes such as seed germination, which involves the mobilization of storage protein [42] or the maturation of vacuolar protein [43]. During senescence in leaves, it has been shown that proteins are degraded and proteolytic activities increase [6,7,41,44,45], but this increase has never been proved to be related to vacuolar proteolysis [7,46]. In the carbon-starved plant cell, proteins are intensively degraded through autophagic processes [2,5,9,10], and it was suggested that vacuolar proteolysis might supply amino acids for protein synthesis or energy

production [13]. The present study shows that a vacuolar endopeptidase, specifically present in the roots of maize, is strongly induced in response to sugar starvation (Figures 7 and 8). As already proposed by Vierstra [21], this confirms that plant cells, like mammalian [47] and yeast [48] cells, are able to respond to drastic environmental changes or stress, i.e. carbon starvation, through enhanced vacuolar protein breakdown.

The role of RSIP in carbon-starved maize roots is probably to take part in the general catabolism that characterizes starved cells. Indeed, we previously showed that, in maize root tips, starvation can be divided into three phases: (1) acclimation, (2) survival and (3) cell disorganization [10]. Starvation for 4 days corresponds to the end of the survival phase during which the cell structures and the protein are non-selectively subjected to degradation. During this phase, root cells are probably the site of autophagic uptake of cytoplasm into the vacuoles, as observed in nutritionally stressed yeast [49] or animal cells [47]. Therefore RSIP might be the major protease involved in the degradation of cellular proteins because it is the major vacuolar endopeptidase (80% of the total activity *in vitro*). This point of view is reinforced by the low specificity of RSIP. Indeed, because the frequency of occurrence of hydrophobic dipeptide sequences is relatively high in proteins, extensive fragmentation of these proteins during glucose starvation would be expected in the presence of RSIP, and the carboxypeptidase activities, which increase during starvation [13], could act concomitantly with the endopeptidase to complete the hydrolysis of peptide chains.

RSIP was shown to be markedly induced in glucose-starved roots. The role of sugars in the regulation of gene expression has been extensively described in prokaryotic and yeast systems (see [50,51] and references therein). In plants, such studies are more recent [52], and some work has shown that the gene expression of enzymes involved in vegetative storage protein [53] or sucrose [16] metabolism, glyoxylic acid cycle [54] and photosynthesis [55] can be regulated by sugars and/or their derivatives (ester phosphates). In good agreement with our previous results [13], RSIP expression could also be regulated by intracellular sugar/phospho-sugar content. This hypothesis is currently under investigation in our laboratory.

We thank Dr. Michael Burnet for helpful discussions and a critical reading of the manuscript.

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