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Proteolytic activity associated with the cell wall

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SUMMARY

As extracellular soluble proteins decreased during hypocotyl elongation significant changes in polypeptide pattern in the same protein fraction were observed, suggesting the presence of cell-wall associated protease(s) Extracellular fluid (EF), containing water-soluble and cell wall-ionically bound proteins, was extracted from etiolated bean hypocotyls, and protease activity was measured at different pHs. Significant endopeptidase activity was observed at acid pH, becoming maximal at pH 4 5 Moreover, an endopeptidase (EP) with similar pH optimum, was isolated from EF by affinity chromatography on haemoglobin-agarose gel The isolated enzyme showed high thermal and temporal stability and dependence on metal cofactors. EP seems to be a monomeric enzyme with apparent mol mass of 52 kDa and it could hydrolyze several extracellular soluble polypeptides Acidic EP activity increased during hypocotyl growth, reaching its highest value during the exponential phase of the elongation, and then decreased It is proposed that the EP could be involved in the regulation of cell wall elongation

Key words: Bean, cell wall proteins, extracellular endopeptidase, *Phaseolus vulgaris* L , proteolytic activity

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RESUMEN

El fluido extracelular (EF), conteniendo proteínas solubles y unidas iónicamente a la pared celular, fue extraído de hipocótilos etiolados de poroto Se observó que, durante el alargamiento de los hipocótilos, disminuyó la cantidad de proteínas y se produjeron cambios significativos en el patrón polipeptídico del EF, sugiriendo la presencia de proteasas asociadas a la pared celular. Se determinó a diferentes pHs la actividad proteolítica presente en el mismo, observándose una actividad endopeptidasa acídica, con máximo a pH 4,5 Dicha endopeptidasa fue aislada del resto de las proteínas presentes en el EF por cromatografía de afinidad, en un gel de hemoglobina agarosa La enzima aislada presentó alta estabilidad térmica y temporal, además de dependencia de cofactores metálicos La endopeptidasa parece ser una enzima monomérica, con una masa molecular aproximada de 52 kDa, que tiene como sustrato a numerosas proteínas extracelulares. La actividad endopeptidasa aumentó durante el crecimiento de los hipocótilos hasta alcanzar su máximo valor durante la fase exponencial de elongación Posteriormente, la actividad disminuyó sugiriendo alguna función en la

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ción. Posteriormente, la actividad disminuyó sugiriendo alguna función en la regulación del alargamiento.

Palabras clave: Poroto, proteínas de pared celular, endopeptidasa extracelular, *Phaseolus vulgaris* L., actividad proteolítica

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INTRODUCTION

Polysaccharides and proteins are the main components of the primary cell wall of both monocotyledonous and dicotyledonous species (Albersheim, P., 1965). Polypeptides constitute 10 to 15 percent of the cell wall dry weight, and they are mostly formed by hydroxyproline rich proteins with structural functions (Lamport and Catt, 1981). A number of cell wall associated soluble proteins were found in the apoplast of expanded leaves, many of which have been implicated in defense against microorganisms (Van Loon, L., 1989; Van Loon and Gerritsen, 1989). Some of these extracellular proteins are proteases, as was demonstrated in bean (Van der Wilden *et al.*, 1983), oat (Van der Valk and Van Loon, 1988), tomato (Rodrigo *et al.*, 1989) and tobacco leaves (Rodrigo *et al.*, 1991)

The cell walls of growing tissues also contain soluble proteins, some of which seem to be glycoproteins with lectin-like functions (Hatfield and Nevins, 1988). The findings that at least some of those proteins exhibit enzymic activities, such as endo and exoglucanase(s) (Hoson and Nevins, 1989), β-galactosidase (Dopico *et.al.*, 1990a) and peroxidases (Sanchez *et al.*, 1989), argue in favor of a possible role of the extracellular soluble proteins in the regulation of the cell growth. However, the latter possibility is still obscure, since reports from different authors are far from agreement. While antibodies raised against cell wall proteins of maize coleoptiles inhibited the auxin- and H^+-induced growth (Hoson and Nevins, 1989), Melan and Cosgrove (1988), using a similar experimental approach, could not prevent auxin-promoted elongation in sections of legume stems. Different specificities of the antibodies may account for those apparent contradictory results. Besides, it is important to remark in the Melan and Cosgrove work (1988) that the treatment with an exogenous protease inhibited the elongation of isolated cell walls. However, the possible role of another group of extracellular proteins, such as proteolytic enzymes, in the regulation of cell elongation is an infrequent topic in the current literature. This may be due to the lack of experimental evidence of cell wall-associated proteases in tissues usually employed in studies of cell growth (gramineous coleoptyls or legume stems).

In the present work, the presence of proteolytic activity(ies) associated with the cell walls of etiolated bean hypocotyls was detected. An endopeptidase was isolated and partially characterized. Its possible "in vivo" relevance was preliminarly studied by analyzing the natural substrates (among the extracellular soluble proteins), and the pattern of protease activity during the elongation of the hypocotyls.

Abbreviations. EF, extracellular fluid, EP, endopeptidase; N-EM, n-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride, TLCK, N`-p-tosyl-L-Lysine chloromethyl ketone.

MATERIAL AND METHODS

Plant material

Uniform seeds of *Phaseolus vulgaris* L. cv "Balin de Albenga" were germinated and grown in moist vermiculite over 86 h at 30°C in darkness.

Isolation of the extracellular fluid

Stem segments between 1 and 2 cm from the top were excised in each etiolated plantlet, and used for the extracellular fluid (EF) extraction. The EF, containing water soluble and ionicall-bound proteins of the cell wall, was extracted from segments of hypocotyls employing a modification of the method described by Klement (1965). The segments were longitudinally cut into halves and extensively washed with distilled water to remove the contents of damaged cells, and then vacuum infiltrated (13 sec at −60 mm Hg) with 50 mM sodium acetate buffer pH 5.0 containing 3.5% NaCl. After washing the segments with the buffer, they were gently dried with

filter paper and centrifuged for 40 sec at 188 xg in bottom perforated centrifuge tubes placed in non-perforated ones. This procedure allowed EF to be obtained with less than 1.5 % cytoplasmic and 1.4 % vacuolar contamination. The presence of cytosolic and vacuolar cotaminants was estimated by measuring the activities of marker enzymes: glucose 6-P dehydrogenase (EC 1.1.1.49, Kuby and Noltman, 1966) and acid phosphatase (EC 3.1.3.2, Boller and Kende, 1979). The whole extraction procedure was carried out at 4°C. In preliminary experiments, the effects of different concentrations of NaCl in 50 mM citrate-phosphate and 50 mM sodium acetate buffers at pH 5.0 were tested for the best extraction condition. Besides, incipient plasmolysis caused by 3.5% NaCl seemed to be necessary to obtain EF with low cytosolic and vacuolar contamination, since lower or higher salt concentrations increased the activity of marker enzymes (data not shown).

Protease activity in EF

In order to detect the presence of protease activity associated with the cell wall, EFs were incubated in a pH range from 3.5 to 8.0. Reaction mixtures contained 30 μg of acid-denatured haemoglobin (Sigma) as substrate, dissolved in either 100 mM sodium acetate buffer (from pH 3.5 to 6.0), 100 mM potassium phosphate buffer (from pH 6.5 to 7.0) or 100 mM Tris-HCl buffer (from pH 7.5 to 8.0), plus 15 ml of EF (7 μg total protein), in a total volume of 45 μl. After 0 and 60 min of incubation at 39°C, reactions were stopped with 3 ml of Comassie Brilliant Blue G- 250 (which stains undigested proteins), according to Saleemudin *et al.* (1980).

Proteolytic activity of EF was further assayed against two widely used commercial substrates for proteases: azocasein and azoalbumin, under the same conditions. However, the highest proteolytic rate was observed in reaction media containing acid-denatured haemoglobin as substrate (data not shown). Carboxypeptidase activity (Cheng and Kao, 1984) could not be detected.

Soluble proteins and protease activity during elongation

At selected times of hypocotyl elongation, the protein patterns of EF were analysed by SDS-PAGE (11% acrylamide; Laemmli, 1970) and "acid" protease activity was determined. Endopeptidase activity was measured as described above. Soluble proteins were measured using Coomassie Brilliant Blue G-250 and bovine serum albumin (Sigma) as standard (Sedmark and Grossberg, 1977)

Purification of the protease

The isolation procedure consisted of an affinity chromatography on haemoglobin-agarose gel. Thirty ml of EF, obtained from 90 h old plants, were loaded on an haemoglobin-agarose (Sigma) column (1.2 x 4.5 cm) previously equilibrated with 100 mM sodium acetate buffer pH 5.0 containing 100 mM NaCl. After loading, the column was washed with the same buffer to remove non-specifically-bound proteins (A_{280} = 0). The protease was eluted with 1 % (v/v) acetic acid (pH 3.0), and the pH of fractions was shifted rapidly to 5.0 with 2 M sodium acetate. Fractions of 2 ml were collected. The whole procedure was carried out at 4°C

Partial characterization of the isolated protease

In order to study the effect of temperature, time and pH on the activity of the isolated EP, the enzyme was assayed as described, in a temperature range of 25 to 45°C, incubation times from 0 to 120 min, and pH from 3.5 to 8.0. In the last case buffer systems were identical to those used for detection of proteolytic activity in EF

The molecular mass of EP was determined by column chromatogrphy on Sephacryl S-300 (1 x 70 cm) equilibrated with 100 mM sodium acetate buffer pH 5.0. After loading 2 ml of EF, elution was performed with sodium acetate buffer at pH 5.0. The flow rate was 1.0 mL/min, and 2 mL fractions were collected. The column was precalibrated with the following standards: α-amylase (200,000), alcohol dehydrogenase (150,000), bovin serum albumin (66,000), carbonic anhydrase (29,000), and Cytochrome c (12,400).

Effect of inhibitors on protease activity

The influence of some protease inhibitors on the activity of the "acid" EP was studied through digestion experiments against one endogenous substrate, an extracellular polypeptide with a molecular mass of about 75 kDa. This polypeptide was obtained as follows: 300 μg of EF proteins were separated by electrophoresis under denaturing conditions (SDS-PAGE, 11% acrylamide). After running the gel, the corresponding band was carefully sliced (4 mm width) from the gel with a scalpel and then cut in sections of 7 mm in length. Samples of the purified protease (3-5 μg protein) were pre-incubated with inhibitors during 60 min at 4°C, and then incubated with one polyacrylamide slice containing the 75 kDa polypeptide band, in the presence of 100 mM sodium acetate buffer pH 4.5 for 90 min at 39°C. Reactions were stopped with 150 mM Tris-HCl, 3.3% SDS, 70mM EDTA, 7% glycerol and 0.01% Bro-

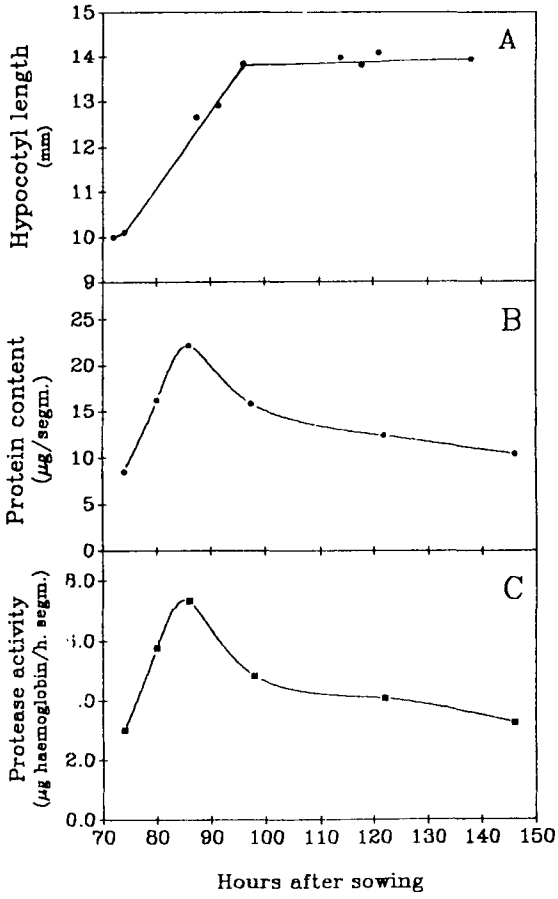

Fig. 1. Time course of elongation (A), protein content (B) and acidic protease activity (C) in etiolated bean hypocotyls Immediately after the emergence of the plumular hook a section of 10 mm length was carefully labeled and the change in its length was recorded At different times EF was extracted from those plants and used for protein and protease activity determination, as described in Material and methods

mophenol Blue (final concentration). Substrate degradation was analyzed by electrophoresis on SDS-PAGE (11% acrylamide) and subsequent densitometrical determination. In other experiments, the effect of protease inhibitors was estimated by measuring the activity of the pretreated EP (see above) in the standard assay (haemoglobin as substrate) Inhibitors were prepared in 100 mM sodium acetate buffer pH 5 0, except for PMSF, TLCK and leupeptin, which were previously dissolved in 1% dimethyl sulfoxide This concentration of dimethyl sulfoxide showed no effect on EP activity

Endogenous substrates of the protease

Endogenous substrates of the protease were detected by analyzing the degradation of EF polypeptides after digestion experiments in the presence or absence of isolated EP EF proteins (50 µg) were incubated with 0 and 4 µg of the isolated protease for 0 and 2 h at 39°C. Reactions were stopped with SDS-Buffer solution. After boiling for 5 min and centrifugation (10 min at 14500 xg), polypeptide patterns were analyzed by SDS-PAGE (11% acrylamide) according to Laemmli (1970).

Experiments were repeated at least three times. Representative data are given

RESULTS AND DISCUSSION

Soluble proteins and proteolytic activity in EF

The amount of soluble proteins in the apoplast, both water-soluble and ionically-bound to the cell wall, increased up to the middle of the exponential growth phase of the hypocotyl and then progressively decreased (Fig 1 A and B) Not only quantitative but also qualitative variations did occur in the extracellular soluble proteins of growing hypocotyls, as can be seen by changes in polypeptide patterns (Fig 2) When protease activity of EF was assayed,

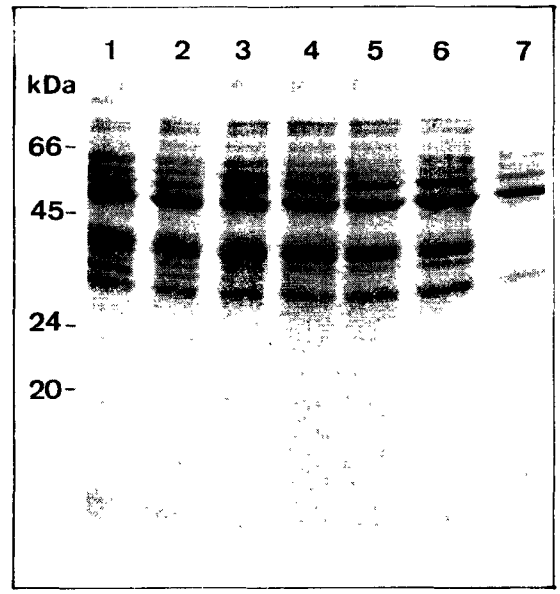

Fig. 2. Changes in extracellular polypeptide pattern during growth of etiolated bean hypocotyls At 70 (1), 75 (2), 88 (3), 96 (4), 113 (5), 120 (6) and 146 hours after sowing (7), hypocotyl segments were harvested and EF was extracted Polypeptide patterns were obtained by subjecting 30-70 µ of EF (15 mg protein) to SDS-PAGE (11% acrylamide) Peptide staining was performed with Coomassie Brilliant Blue

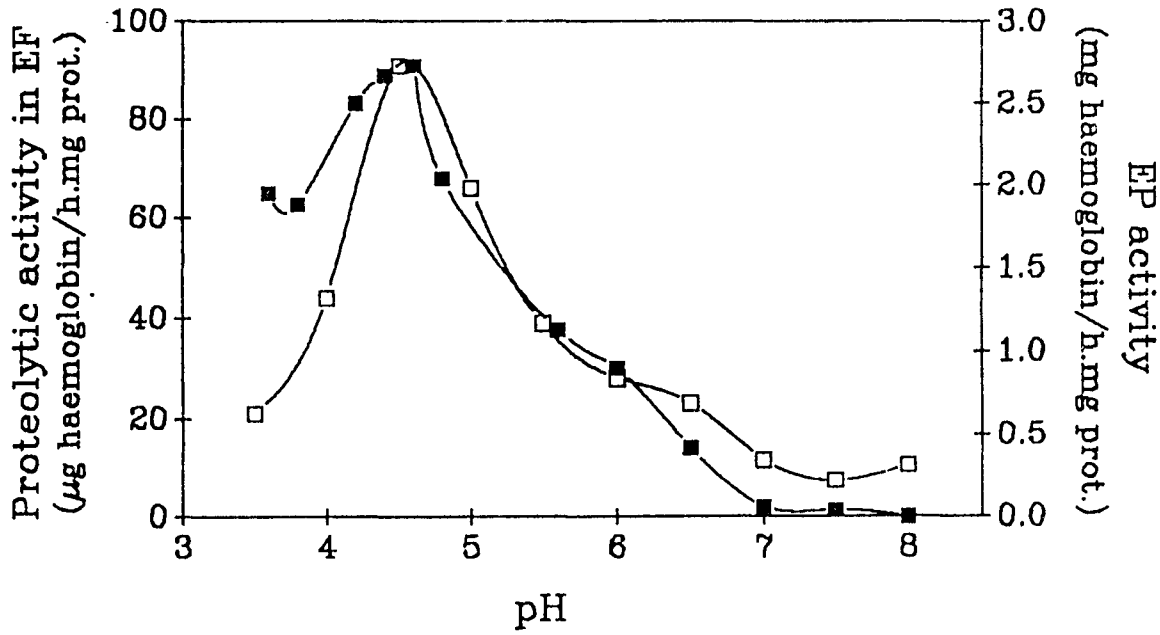

Fig. 3. Effect of pH on endopeptidase activity in EF and in isolated EP. Fifteen μl of EF (□) and isolated enzyme (■) were incubated at indicated pH values with 30 μg of haemoglobin as substrate, for 60 min at 39°C. For details see Material and methods.

employing a procedure for endopeptidases detection (Saleemudin *et al.*, 1980), the optimal level was at pH 4.5 (Fig. 3). No carboxypeptidase activity could be found under the same conditions. These results strongly indicate the presence of at least one cell wall-associated EP, active at acid pH.

In the cell walls of mature leaves of bean, and using a similar procedure for isolating EF, Van der Wilden *et al.* (1983), found an azocoll- digesting proteinase with an optimum at basic pH. Van der Valk and Van Loon (1988) reported that EF of oat leaves showed an important acidic protease activity. However, at present we have not found reports of extracellular proteolytic activity in growing tissues.

Protease Isolation

Some characteristic features of the purification of the assumed endopeptidase within the extracellular fluid are shown in Table 1. Soluble protein fraction was extracted from the apoplast of bean hypocotyls by EF isolation and then subjected to affinity chromatography on haemoglobin-agarose gel (Fig. 4 A). A single peak of EP activity was obtained (Fig. 4 A) and peak fractions were pooled. SDS-PAGE of this preparation stained with Coomassie Brilliant Blue showed a single band indicating an homogeneus enzyme (Fig. 5).

Table 1. Some characteristics of the protein fraction containing endopeptidase in the extracellular fluid (EF) of bean hypocotyls before and after its affinity-gel filtration.

Step of Purification	Protein (mg)	Activity (A_{595}/h)	Specific activity (A_{595}/h mg)	Enrichment of specific enzyme activity
EF	18.4	616.0	33.5	–
Haemoglobin-agarose	0.2	207.7	1038.5	31

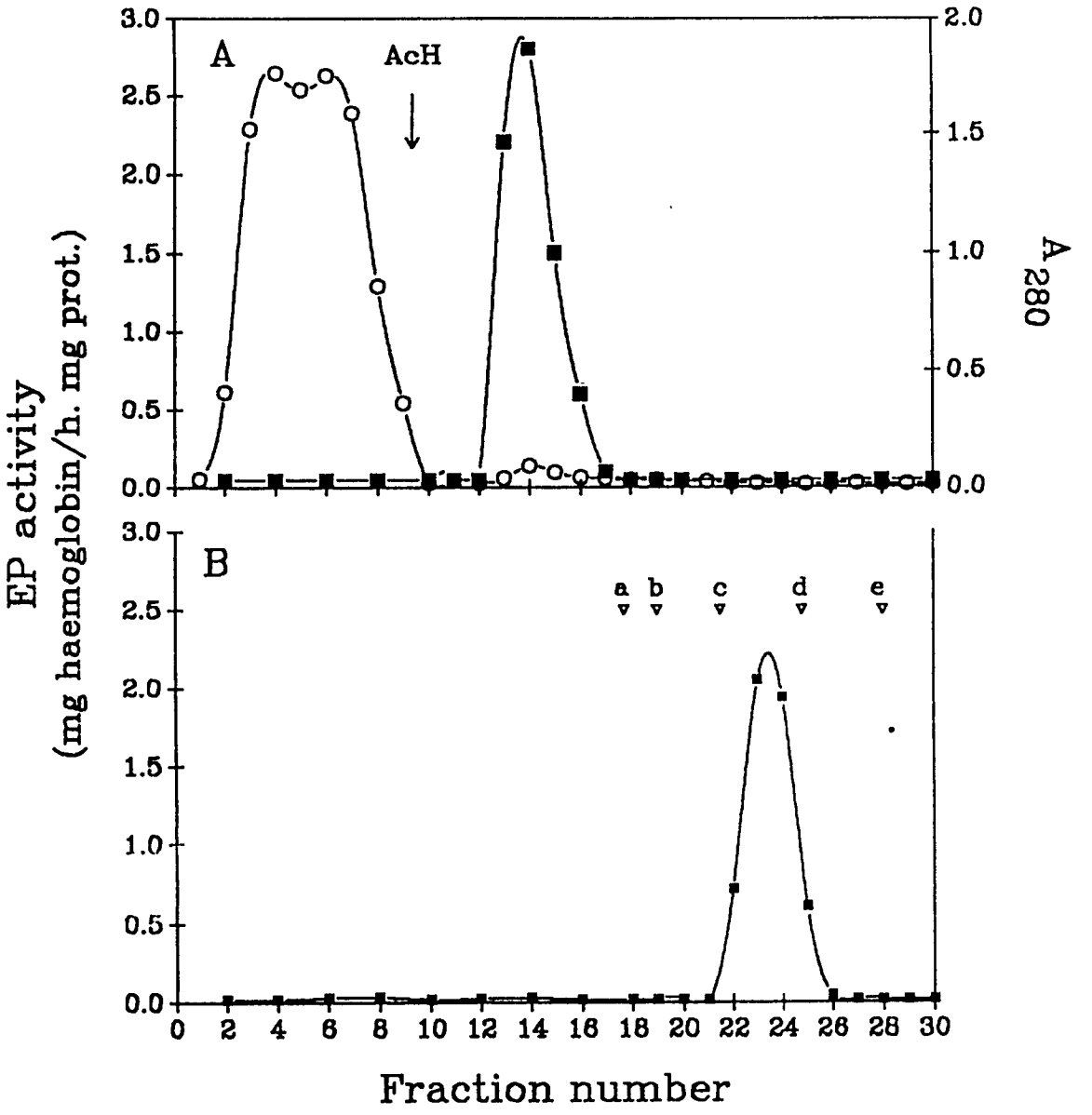

Fig. 4. Purification and mol. mass estimation of the cell wall- associated EP. The EP was isolated from EF by affinity chromatography on haemoglobin-agarose gel (A). After washing the column, the enzyme was eluted with 1% (v/v) acetic acid. Mol mass of EP activity was estimated by EF filtration through a precalibrated column of Sephacryl S-300 (B), as described in Material and methods. Mol mass standards were. α-amylase (200,000, a), alcohol dehydrogenase (150,000, b), bovin serum albumin (66,000, c), carbonic anhydrase (29,000, d), and Cytochrome c (12,400, e). Open circles represent protein profile (A 280 nm) and closed squares, EP activity.

Protease characterization

The enzymatic activity of the EP increased linearly with the increase of the temperature from 25 to 45°C, and hyperbolically with the increase in the reaction time (Fig. 6 A and B, respectively). As in other proteases reported (Drivdahl and Thimann, 1977), this enzyme is characterized by high thermal and temporal stability, as well The activity of this enzyme shows a pH optimal (4 5) similar to that observed for protease activity in EF (Fig. 3)

The apparent mol. mass of the isolated EP, determined by SDS-PAGE, was about 52 kDa (Fig. 5) Gel chromatography of EF on Sephacryl S-300 yielded in protease activity as a single peak at about 50 kDa

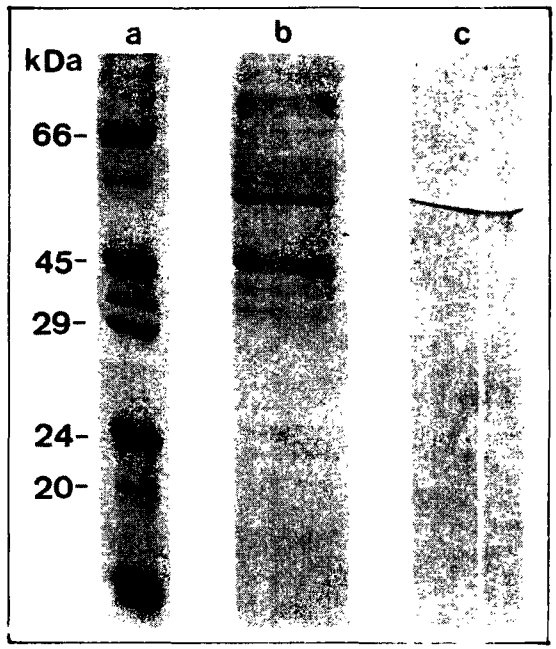

Fig. 5. Electroforetic separation (SDS-PAGE) of the polipeptides present in the peak fractions eluted from the affinity column during the purification of the cell wall EP (a), mol mass markers, (b) EF and (C), pooled peak fractions after the affinity chromatography (Fig 4,B)

(Fig. 4B) Both results indicated that the EP is a monomeric enzyme

The effect of protease inhibitors on the activity of EP was analyzed by studying the degradation of both, a 75 kDa polypeptide present in EF (see below) and acid-denatured haemoglobin with the pretreated enzyme. Some inhibition with PMSF was observed (Table 2), but EDTA was the only inhibitor that prevented almost completely the breakdown of both the 75 kDa polypeptide and haemoglobin at the same time, showing that this protease contains essential metal group(s). Moreover, the lack of effect of pepstatin, an aspartyl protease inhibitor, points out that the extracellular acidic aspartyl proteases isolated from tomato (Rodrigo *et al*, 1989) and tobacco leaves (Rodrigo *et al.*, 1991) are different from the EP of bean hypocotyls described in this work

Endogenous substrates of the protease

To study the physiological relevance of the EP from bean hypocotyls, possible endogenous substrates of the enzyme and the pattern of acidic proteolytic activity in EF during growth were determined.

When EF proteins were incubated at pH 4 5 and with or without isolated EP, several polypeptide bands were degraded (Fig. 7). This pattern of degra-

Table 2. Effect of inhibitors on the extracellular EP. After treatments with inhibitors, EP was assayed against either 75 kDa polypeptide (A) or acid-denatured haemoglobin (B) at pH 4.5 and 39°C. Hydrolysis of both substrates was estimated as described in Material and methods

Inhibitor	Concentration	Inhibition (% of control)	
		A	B
PMSF	1.0 mM	25	28
TLCK	1.0 mM	5	2
Pepstatin	0.1 mg/ml	6	1
Leupeptin	0 1 mg/ml	7	5
ZnCl2	1.0 mM	10	0
Iodoacetamide	1.0 mM	0	0
N-EM	1.0 mM	0	0
EDTA	5.0 mM	92	89

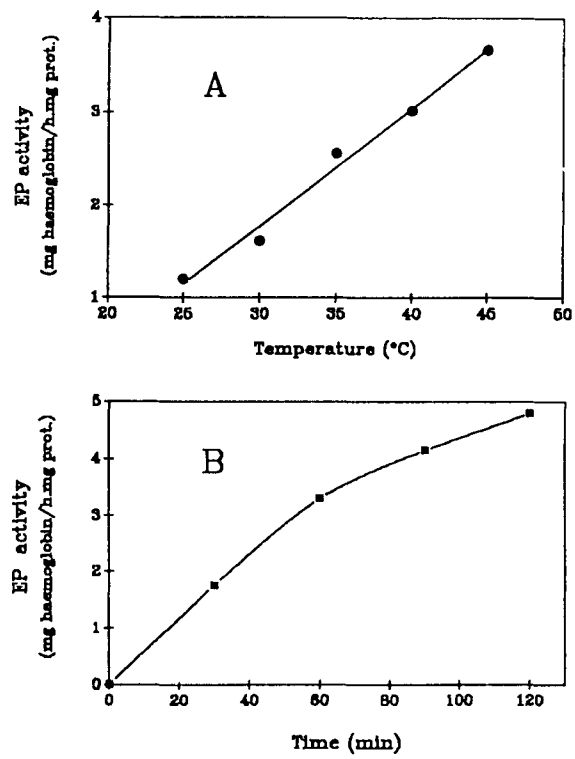

Fig. 6. The enzymatic activity of the isolated cell wall-associated EP in dependence on the temperature (A) and incubation time (B)

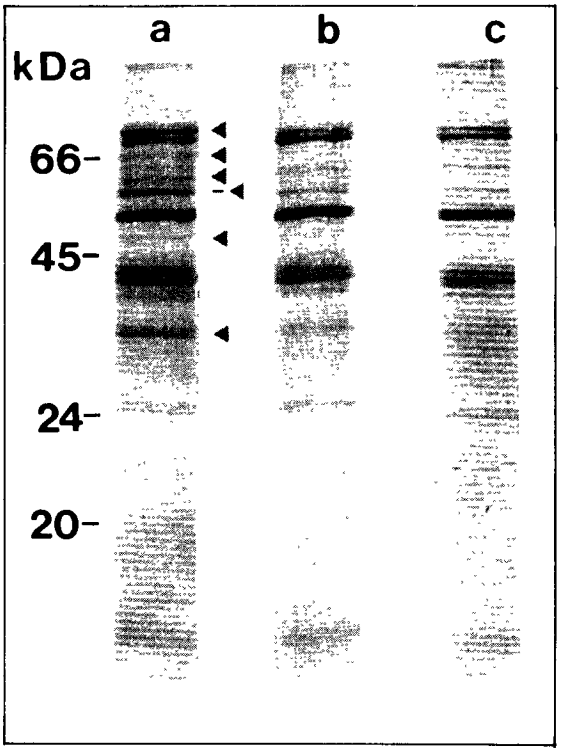

Fig. 7. Cell wall-associated substrates of the EP SDS-PAGE of extracellular proteins incubated at pH4 5 and 39°C for 0 (a) and 2 h in the absence (b) or presence of the purified enzyme (c). Polypeptidic bands were stained with Coomassie Brilliant Blue Triangles indicate possible substrates of EP

dation was only quantitatively enhanced but not qualitatively modified upon the addition of the purified EP (Fig. 7). Therefore, at least six extracellular poplypeptides with mol. mass ranging from 75 to 35 kDa could be endogenous substrates for the protease.

On the other hand, the fact that optimum pH, mol. mass and endogenous substrates for the protease activity in both EF and isolated EP were coincidental, indicates that the studied enzyme may account, at least partially, for proteolysis found in the apoplastic fluid of bean hypocotyls

Acidic protease activity during hypocotyl elongation

The hypocotyls emerged approximately 72 h after sowing. Then, the section below the plumular hook grew up linearly from 10 to 14 mm (100h, Fig 1A). The acidic EP activity increased up to the middle of the exponential growth phase of the hypocotyl, and then it decreased (Fig. 1 C).

The results show that soluble (non-structural) extracellular proteins undergo quantitative and qualitative changes during hypocotyl growth. The activity of an acidic EP located in the same compartment could partially be involved in these changes Since the highest EP activity takes place when elongation rate (expressed as cm/h) decreases, its possible role in cell wall elongation remains to be studied.

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