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MAIZE LEAF PROTEASES AND THEIR EFFECT ON ENDOGENOUS INORGANIC PYROPHOSPHATASE*

PHILIP C. ANDREWS, FREDERICK A. SCHNEIDER, DAN LEBRYK and LARRY G. BUTLER
Department of Biochemistry, Purdue University, West Lafayette, IN 47907, U.S.A.

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Key Word Index—*Zea mays*; Gramineae; maize; proteolytic enzymes; proteases; pyrophosphatase; regulatory interactions.

Abstract—Several different proteolytic enzymes are present in leaf and root tissue of maize seedlings. The activity of these enzymes diminishes to a basal level by the time seedling height reaches 20–30 cm. We have partially characterized an endopeptidase with trypsin-like specificity and two aminopeptidases, all from leaf tissue, and compared them to previously reported proteases from maize. Both the endopeptidase and the aminopeptidases degrade the maize leaf enzyme, inorganic pyrophosphatase. Modification of the pyrophosphatase by the peptidases results in the formation of catalytically active, electrophoretically distinct products. The aminopeptidases have little effect on several other maize leaf enzymes, but also modify yeast inorganic pyrophosphatase.

INTRODUCTION

During the purification of maize leaf inorganic pyrophosphatase [1, 2], we observed apparent degradation of the enzyme by endogenous proteases on storage at 4°. Degradation caused lowering of the molecular weight and appearance of heterogeneity on polyacrylamide gel electrophoresis of what had appeared to be a homogeneous sample as judged by both SDS and discontinuous gel electrophoresis. Degradation can take place without loss of pyrophosphatase activity. Both exopeptidase and endopeptidase activities appear to be present. We report here on the properties of these proteases, some of which are different from those previously described from this source [3–5], and their effects on maize leaf pyrophosphatase, including a possible regulatory interaction.

RESULTS AND DISCUSSION

A series of amino acid β-naphthylamides were employed as protease substrates [6] for assays of crude maize leaf extracts, both in solution and on polyacrylamide gels after electrophoresis, to confirm the presence of aminopeptidases. Arginine- and alanine-β-naphthylamides were cleaved much more rapidly at pH 7 than those of tyrosine, isoleucine, glycine, lysine, serine, and leucine. However, arginine-β-naphthylamide was cleaved more rapidly than alanine-β-naphthylamide at pH 9. However, the activity towards both alanine- and arginine-β-naphthylamide migrated with the same R_f (0.54) on gel electrophoresis.

Table 1. Ratio of aminopeptidases from pH precipitation

	% Yield of ala-activity	% Yield of arg-activity	Ratio of ala-/arg-activity
Crude extract	100	100	0.77
pH 5.0 Supernatant	36	46	0.71
pH 5.0 Pellet	6	3	1.57

The extract was obtained from 23-day-old maize leaves as described in Experimental, except the pH was adjusted to 6.0 instead of 5.0. After a preliminary centrifugation, the pH was adjusted to 5.0 with 1.0 N HCl and centrifuged. The pellet was taken up in 2.5 ml 0.1 M Tris, pH 9.0, and centrifuged at 25000 *g* for 30 min. The supernatant from the pH 5.0 precipitation and the pellet extract were assayed immediately against arginine- and alanine-β-naphthylamide.

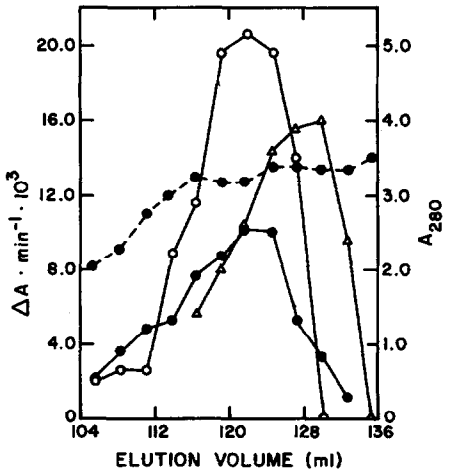

Fig. 1. Gel permeation chromatography of various peptidase activities in a concentrated extract of maize leaves on a Sephacryl S-200 column. ----, A_{280}; O——O, ala-specific activity; ●——●, arg-specific activity; △——△, endopeptidase activity.

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† Abbreviations employed are: βNA, β-naphthylamide; EDTA, ethylenediaminetetraacetic acid; IAA, iodacetic acid; NEM, *N*-ethylmaleimide; pHMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethane sulfonylfluoride; pNA, *p*-nitroanilide; SDS, sodium dodecyl sulfate.

N-Benzoyl-arginine-*p*-nitroanilide, an endopeptidase substrate, was not hydrolysed by these aminopeptidases, but was hydrolysed by another enzyme which lost its activity rapidly in solution. A very weak carboxypeptidase activity was observed in the crude extract using hippuryl-L-phenylalanine as a substrate at pH 8.0, but was not characterized further.

The alanine- and arginine-specific aminopeptidases could be partially separated by the use of a pH precipitation procedure (Table 1). Gel permeation chromatography of the pH 5 supernatant on Sephacryl S-200 gave the results shown in Fig. 1. The profiles of the arginine- and alanine-specific exopeptidases are similar but not identical. Only the arginine-specific aminopeptidase activity was detectable in the 130 ml volume fraction. The shoulder in the alanine-specific activity occurring at 113 ml appears to indicate a minor high molecular weight aminopeptidase. This shoulder was more noticeable in other preparations, especially from younger plants. The endopeptidase activity peak does not coincide with the aminopeptidase peaks. The aminopeptidases slowly lost activity when stored at 4°. The high A_{280} values are due to the presence of residual polyphenolic pigments.

The molecular weight of the two aminopeptidases, as determined by standardization of the above gel permeation chromatography column, is 53000 whereas that for the endopeptidase is 40000. If the shoulder in the alanine-specific peak represents a high molecular weight peptidase, then the molecular weight for that enzyme would be 77000. The combined fractions of the aminopeptidase peak were used in the characterization studies.

The activity as a function of pH of the two major aminopeptidases eluted from the gel permeation column is shown in Fig. 2. The alanine-specific enzyme has a sharp optimum near pH 7; the optimum for the arginine-specific enzyme is near pH 9. The broad pH optimum observed for the arginine-specific enzyme may represent a contribution by the alanine-specific enzyme at lower pH. A different alanine-specific aminopeptidase with an acid pH optimum is also illustrated. This enzyme was observed only when the isolation procedure did not include the pH 5.0 precipitation step. The acidic pH optimum aminopeptidase had a higher molecular weight than the other aminopeptidases as judged by its elution volume from a Sephacryl S-200 column, and was well separated from those aminopeptidases.

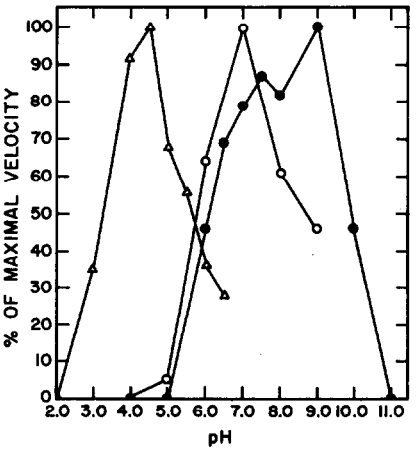

Fig. 2. pH Optima of arginine- and alanine-β-naphthylamidase activities. Buffers were pH 2.0, 7.0, and 7.5, phosphate; pH 3.0–6.5, citrate; pH 8.0–11.0, borate. △——△, High MW aminopeptidase; ○——○, ala-specific activity; ●——●, arg-specific activity.

Table 2. Amino acid inhibition of aminopeptidases

Amino acid	% Inhibition Ala-βNA	% Inhibition Arg-βNA	Ratio of ala/arg inhibition
L-Ala	19.6	6.3	3.1
D-Ala	9.0	6.8	1.3
L-Arg	22.6	16.8	1.3
L-Gly	7.6	2.7	2.8
L-Leu	55.2	11.4	4.8
L-Trp	21.4	3.2	6.7

Substrate concentration, 0.95 mM; inhibitor concentration, 0.48 mM. Inhibition of alanine-β-naphthylamidase was measured at pH 7.0, and inhibition of arginine-β-naphthylamidase was measured at pH 9.0.

Specificity and amino acid inhibition

Inhibition of the two major aminopeptidases by selected amino acids is shown in Table 2. The amino acid which causes the greatest inhibition is L-leucine, which inhibits the alanine-specific activity to a five-fold greater extent than the arginine-specific activity. In general, the alanine-specific enzyme is inhibited to a greater extent by amino acids than the arginine-specific activity.

The relative activities of the aminopeptidases against several synthetic substrates at two pH values are presented in Table 3. The aminopeptidases appear to hydrolyse the less bulky, more hydrophilic *p*-nitroanilide substrates faster than the β-naphthylamide substrates. The D-amino acid-β-naphthylamides apparently do not inhibit the aminopeptidases since there is little difference between the rates of hydrolysis of the L-amino acid-β-naphthylamides and the corresponding D,L mixtures. No activity towards succinyl-L-phenylalanyl-*p*-nitroanilide

Table 3. Relative substrate specificities at pH 7.0 and 9.0

Substrate	% Activity pH 7.0	% Activity pH 9.0
L-Ala-βNA	100	100
L-Lys-βNA	61	107
L-Met-βNA	55	29
L-Leu-βNA	41	ND
L-Ser-βNA	36	121
L-Pro-βNA	13	64
L-His-βNA	11	0
DL-Ala-βNA	100	87
L-Arg-βNA	22	207
Gly-pNA	84	272
L-Ala-pNA	254	1210

The substrate solutions were made from 100 mM stock solutions in 50% Me_2CO–H_2O. Final concentration of substrate is 10 mM. All rates are corrected for blank values. The enzyme was the combined aminopeptidase activity from the gel permeation column. The % activity at pH 7.0 and 9.0 is normalized to L-alanine-β-naphthylamide (L-ala-βNA).

Table 4. Effect of various reagents on the aminopeptidase activities

Reagent	Activity (% of control) Ala-specific activity	Arg-specific activity
0.91 mM NEM	53	94
0.91 mM IAA	75	118
0.24 mM pHMB	0	0
1.2 mM $HgCl_2$	0	0
Saturated PMSF	98	100
9.1 mM EDTA	102	197
Control	100	100

Incubation conditions were 0.1 M Tris, pH 8.0, at 25° for 12 hr; a 50 µl aliquot was assayed. The alanine-specific activity was assayed at pH 7.0 and the arginine-specific activity was assayed at pH 9.0.

was observed, nor was protease activity detectable using hide powder azure as a substrate [7]. Benzoyl-L-arginine-*p*-nitroanilide was not a substrate for the aminopeptidase.

Mercurials are the only reagents found to completely inactivate the aminopeptidases (Table 4). Other sulfhydryl reagents only partially inactivated the alanine-specific activity. $HgCl_2$ at 1.0 mM completely inactivated the endopeptidases. EDTA did not affect the alanine-specific enzyme, but produced a two-fold activation of the arginine-specific enzyme.

The addition of 0.25 mM Mg^{2+}, Ca^{2+}, Mn^{2+}, or Zn^{2+} did not affect the activity of either aminopeptidase. Neither puromycin, an inhibitor of arylamidases [8], penicillin, nor bacitracin [9] at 1.0 µg/ml has an effect upon the activities. Tris is not an inhibitor in concentrations up to 100 mM.

Activity against pyrophosphatases

The treatment of both yeast and maize leaf inorganic pyrophosphatases with the aminopeptidases resulted in loss of activity (Fig. 3). When a mixture of the two purified

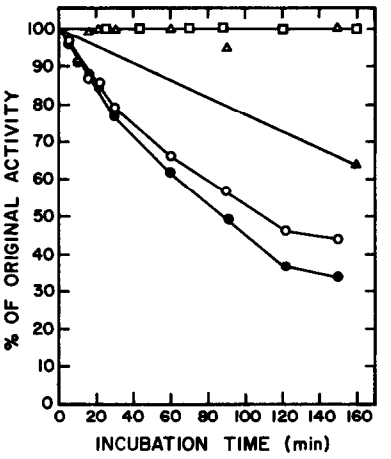

Fig. 3. Proteolysis of yeast and maize leaf inorganic pyrophosphatase. Conditions were as described in the text. △——△, Maize leaf pyrophosphatase control; ○——○, maize leaf pyrophosphatase + 0.19 unit of mixed proteases; ●——●, maize leaf pyrophosphatase + 0.39 unit of mixed proteases; ▲——▲, yeast pyrophosphatase + 0.06 units of mixed proteases; □——□, yeast pyrophosphatase control.

maize leaf pyrophosphatase isoenzymes (R_f values of 0.54 and 0.60 on discontinuous polyacrylamide gel electrophoresis) was incubated with the aminopeptidases (free of endopeptidase activity), two new bands of pyrophosphatase activity with R_f values of 0.69 and 0.80 appeared in amounts that increased with increasing incubation time. When treated with the maize leaf endopeptidase (containing some alanine-specific aminopeptidase), the maize leaf pyrophosphatase showed a broadening of the higher-R_f pyrophosphatase isoenzyme activity band.

After treatment with the aminopeptidases, electrophoretically homogeneous yeast pyrophosphatase could

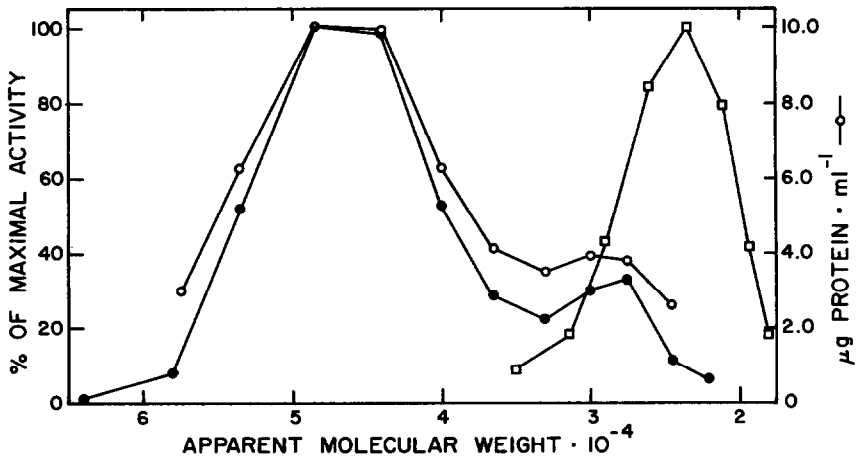

Fig. 4. Apparent molecular weight of maize leaf inorganic pyrophosphatase. The 2.5 × 59 cm column of Sephacryl S-200 was equilibrated with 0.05 M Tris, pH 8.0, containing 300 mM NaCl. Inorganic pyrophosphatase, 300 units, either homogeneous or partially degraded by endogenous proteases, in 0.5 ml 0.05 M imidazole, pH 7.0, containing 10% ethylene glycol and 25 mM $MgCl_2$ was applied. Fraction volume was 4.2 ml, and flow rate was 31.5 ml/hr. The column was calibrated using standard proteins. ○——○, Protein concentration; ●——●, homogeneous inorganic pyrophosphatase; □——□, partially degraded pyrophosphatase.

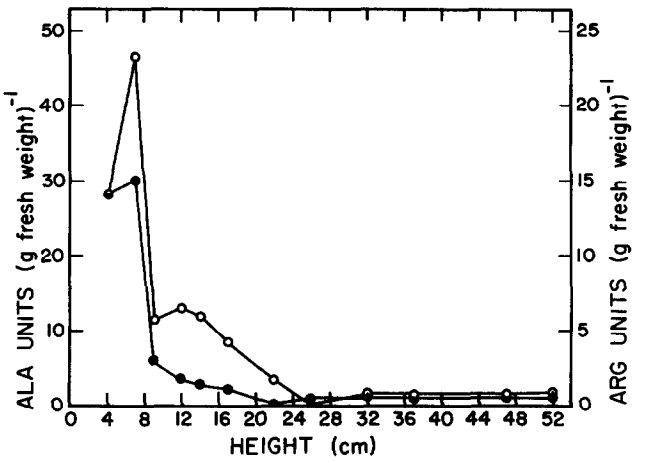

Fig. 5. Protease levels in maize plants as a function of maturity (plotted as height in cm). ●——●, Hydrolysis of arginine-β-naphthylamide, 0.1 M borate, pH 9.0; ○——○, hydrolysis of alanine-*p*-nitroanilide, 0.1 M imidazole, pH 7.0.

be resolved into two bands of activity with the new band having a higher R_f value (0.52 vs 0.42). The yeast pyrophosphatase gave two additional bands having R_f values of 0.58 and 0.81 when treated with the mixture of endopeptidase and alanine-specific aminopeptidase.

When a mercurial treatment was included in the purification procedure for maize leaf inorganic pyrophosphatase, modification was no longer observed. The resulting inorganic pyrophosphatase had slightly higher monomeric molecular weight by both gel permeation chromatography (Fig. 4) and SDS gel electrophoresis than enzyme isolated without the mercurial treatment. Moreover, the unmodified inorganic pyrophosphatase occurs as a weakly associated dimer (Fig. 4). The dimeric form of this enzyme is active and its quaternary structure may be affected by protein concentration and by various solutes (Andrews, P. C. and Butler, L. G., unpublished results).

Other enzymes, including acid phosphatase, 5′-nucleotide phosphodiesterase, and a 50000 MW peroxidase, were partially purified from the maize leaf extracts and incubated with the mixture of maize leaf aminopeptidases under similar conditions, only with the omission of 1,3-butanediol. In each case there was no detectable loss of activity after 18 hr, in contrast to the loss observed with pyrophosphatase.

The levels of the two aminopeptidase activities vary as a function of maturity (Fig. 5). Their levels are very high initially, but at about the time the first leaf unfurls, the levels drop rapidly until they reach a low basal level. The level of the arginine-specific enzyme drops at a greater rate than that of the alanine-specific enzyme. The levels of the proteases in the roots of the maize sprouts were comparable to those in the stem and leaves (Table 5).

Table 5. Levels of proteases in maize seedlings

Extract	Arg-specific nmol/min/g	Ala-specific nmol/min/g
1 cm plant	96	64
2 cm plant	58	34
3 cm plant	65	46
4 cm plant	75	46
1 cm root	85	57
3 cm root	53	29

The plant tissue was extracted and assayed as described in Experimental.

Discussion and conclusions

Care is seldom taken to prevent proteolysis during the purification of plant enzymes. We have shown that during the isolation of inorganic pyrophosphatase from maize leaves, the action of endogenous plant proteases may result in the appearance of new electrophoretically-distinct, catalytically-active forms of the enzyme, and eventual loss of pyrophosphatase activity.

The arginine- and alanine-specific aminopeptidase activities of maize leaves have similar, but not identical, elution patterns on the Sephacryl S-200 column and can be partially resolved by precipitation at pH 5.0. The two activities differ in their susceptibility to inhibition by amino acids. The ratio of the two aminopeptidase activities as a function of maturity also varies. It is likely that the two activities represent different enzymes.

Endopeptidases in maize have been previously reported, both in leaf [3, 5] and endosperm [3, 4]. A leaf endopeptidase has been partially purified and characterized [3, 5, 10]. The endopeptidase we report is similar to the one reported by Melville and Scandalios [3] with the exception that the molecular weight they found was 53000. We found a molecular weight of 40000.

Four aminopeptidase isoenzymes, designated isoenzymes A through D, were found in the immature endosperm of maize [11]; isoenzymes A and B were reported to be the major isoenzymes of maize leaf [12]. Isoenzymes A and D appeared to optimally hydrolyse arginine- and alanine-β-naphthylamides, whereas the derivatives of non-polar amino acids appear to be better substrates for isoenzymes B and C. The molecular weights found were 71500 and 63500 for each of the two groups of the isoenzymes, in contrast to the value of 53000 which we observed. Because EDTA does not inhibit these aminopeptidases (Table 3), they are different from isoenzymes A and D of Ott and Scandalios [11]. Our observation of

EDTA activation of the arginine-specific aminopeptidase, which was not previously reported, may represent removal of inhibition of heavy metal ions picked up during the course of the purification. For the aminopeptidase isoenzymes D and C [11], leucine-β-naphthylamide is a better substrate than the alanine-β-naphthylamide. This is not the case with the maize leaf aminopeptidases we describe here, which differ in several respects from those present in the immature maize endosperm [11].

The capacity of the maize leaf proteases to modify maize leaf pyrophosphatase, in contrast to their apparent lack of activity towards several other maize leaf enzymes, suggests a role for these proteases in the *in vivo* control of maize leaf pyrophosphatase levels. The activity of monomeric (no steps taken to prevent proteolysis) pyrophosphatase apparently is not regulated by metabolite levels [2]. It is not known whether intact, dimeric enzyme is regulated in this manner. The degradation of specific catalase isoenzyme in maize has been reported [13], although the protease responsible has not been identified. A physiological role for regulation by a specific protease has been proposed for yeast uridine nucleosidase [14].

Isoenzymes, as defined electrophoretically, are quite common in the higher plants. While in many cases these isoenzymes are separate gene products, we have shown that in the case of maize leaf pyrophosphatase some of these 'isoenzymes' are artifacts caused by proteolytic degradation. Although the level of protease activity is many-fold lower in older plants than in sprouts, this work was done on the enzymes from older plants; this demonstrates how serious the problem of proteolysis can be in isolation of plant enzymes.

It should be noted that our pyrophosphatase degradation studies were carried out with partially purified aminopeptidases. The aminopeptidase activities we detected and measured using artificial substrates may not represent the activity responsible for the degradation of pyrophosphatase.

EXPERIMENTAL

Leaf tissue of *Zea mays* (M-5040, Midwest Seed Growers Assn. Inc., Mitchell, IN 47446) greenhouse-grown plants was collected at 4–23 days and stored frozen until used.

Extraction and partial purification. Maize leaves were ground 2 min in a Waring blender at 4° in 2.5 ml 0.05 M Tris–HCl buffer, pH 8.0, per g fr. wt of leaf tissue. A typical purification used 500 g of leaf tissue from plants 16–23 days old. The crude homogenate was filtered through cheesecloth and the filtrate was titrated to pH 5.0 with HOAc. The precipitated Group I proteins [15] were removed by centrifugation at 10000 *g* for 15 min at 4°. The supernatant layer was titrated to pH 8.0 with dil NaOH and the extract was treated with Dowex AG 1 × 8 anion exchange resin (1 g per g tissue) in order to remove phenolic materials. The resulting extract was concd to 3 ml on a 500 ml stirred cell Diaflo apparatus (Amicon Corporation, Lexington, MA) using an XM-50 membrane. The concentrate was centrifuged at 10000 *g* for 15 min at 4° and the supernatant layer was applied to a 2 × 40 cm Sephacryl S-200 gel permeation column equilibrated with 2 M NaCl in 0.05 M Tris–HCl, pH 8.0. The column was run at 4° with a flow rate of 16.2 ml/hr. The fraction volume was 2.7 ml.

Assay procedures. The continuous assay procedure was similar to that of ref. [16] for the β-naphthylamides. The concn of aminoacyl β-naphthylamides was 1 mM in 0.1 M borate, pH 9.0. Assays utilizing the *p*-nitroanilides of amino acids as substrates were run using 2.0 mM substrate in pH 7.0 phosphate or pH 9.0 borate buffer. The change in absorbance was measured at 410 nm. The discontinuous assay for aminopeptidase activity was that of Goldbarg and Rutenberg [17]. The aminopeptidases were assayed using the same buffers used in the continuous assay. A control containing no substrate was run for each enzyme sample. The endopeptidase activity was followed using 1 mM benzoyl-arginine-*p*-nitroanilide as substrate in pH 7.0, 0.1 M phosphate buffer. One unit of protease activity is defined as 1.0 nmol substrate hydrolysed/min. The peroxidase activity was followed at 470 nm using 10^{-9} M H_2O_2 and 1.7 mM guaiacol as substrates in 3.3 mM phosphate, pH 7.0 [18]. Acid phosphatase activity was followed in 0.10 M acetate buffer, pH 5.0, using 1.0 ml 1.3 mM *p*-nitrophenyl phosphate as substrate. The reaction was stopped by the addition of 2.0 ml of a soln containing 1.0 M $NaHCO_3$, 0.25 M Na_3PO_4, and 0.01 M disodium EDTA, and *p*-nitrophenol absorbance was measured at 400 nm. 5'-Nucleotide phosphodiesterase activity was measured using the method of ref. [19]. Pyrophosphatase activity was assayed by a modified Fiske–SubbaRow method [1]. Carboxypeptidase activity was measured using hippuryl-L-phenylalanine (*N*-benzoyl-glycyl-L-phenylalanine) as a substrate [20]. Protease activity was detected on polyacrylamide gels by incubation in pH 9.0, 0.1 M Tris buffer, 1 mM amino acid-β-naphthylamide, 1 mg/ml fast garnet.

Discontinuous polyacrylamide gel electrophoresis was run using the method of Davis [21]. Protein concn was estimated by A_{280} or by the Coomassie blue dye binding assay of Bradford [22].

Digestion of enzymes by maize leaf proteases. Homogeneous maize leaf pyrophosphatase, 0.8 unit (sp. act. 1000 units/mg) in 0.4 ml of a buffer containing 0.10 M Tris, pH 8.0, and 10% 1,3-butanediol, or purified yeast pyrophosphatase, 0.6 unit (sp. act. 473 units/mg [23]) in 0.15 ml 0.10 M Tris buffer, pH 8.0, was incubated at 30 and 35°, respectively, with 0.08 unit of endopeptidase-free aminopeptidase and assayed at intervals for pyrophosphatase activity. Effects of endopeptidase were determined by incubating 1.0 unit of maize leaf or yeast pyrophosphatase and 0.2 unit of protease at 30° in 300 μl of 0.1 M Tris buffer, pH 8.0, for 3 hr. After incubation, one third of the mixture was applied to 0.3 × 10 cm polyacrylamide gels and, after electrophoresis, assayed for pyrophosphatase activity on the gels [24].

Maize growth conditions. Greenhouse-grown maize was used in the age dependence study of protease levels in corn of 4–25 cm height. The entire plant was harvested at the first node and frozen. The tissue was mixed with sand and ground with 2.5 ml 0.1 M Tris, pH 8.0, per g of maize at 0° with a mortar and pestle. The extract was centrifuged and assayed directly. The 1–4 cm sheathed maize shoots and roots were grown at 30° on blotting paper under ambient laboratory lighting conditions. The height reported with the roots is that of the corresponding shoot.

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