Gel Electrophoretic Profiles of Proteinases in Dark-Germinated Flax Seeds[1]

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ABSTRACT

The proteinases present in dark-germinated flax seeds (*Linum usitatissimum*) were studied as a function of germination at 25°C. A majority of activity was present in basic proteinases with an acidic pH optimum and a temperature optimum of 45°C in the digestion of hemoglobin. Electrophoresis in a sodium dodecyl sulfate-polyacrylamide mixture which had been polymerized with gelatin was used to separate proteins in extracts of seedlings. Subsequent activation of proteinases with Triton X-100 and resultant digestion of gelatin proved to be very reproducible and afforded detection and good quantification of various proteinase zones. An ethylenediaminetetraacetate-sensitive proteinase zone, P4 (about 60,000 daltons), appeared at day 3 after imbibition and attained maximum activity at day 4. This correlates with a rapid loss *in vivo* of the glyoxysomal enzyme, isocitrate lyase (EC 4.1.3.1). Ethylenediaminetetraacetate also slowed the loss of isocitrate lyase activity in extracts of 4-day seedlings in a dose-dependent manner. The addition of leupeptin, α-tolylsulfonyl fluoride, Pepstatin A, *p*-chloromercuribenzoate, or 1,10-phenanthroline prior to, during, or after exchange of Triton X-100 for sodium dodecyl sulfate had almost no inhibitory effect upon proteinases in 4-day seedlings.

Intracellular protein levels are determined by the rate of synthesis and degradation. In germinating seeds, a study of proteinases reveals complexity in terms of tissue specificity, hormonal control, targets of action, and other parameters (16). It is well established that the glyoxysomal enzyme, isocitrate lyase (EC 4.1.3.1), is synthesized *de novo* during the germination of several fat-rich seeds (3, 4, 7, 12). The enzyme activity attains a maximum, which is followed by a decline concomitant with the transition from glyoxysomes to leaf peroxisomes; isocitrate lyase is not found in peroxisomes. Previous reports on flax (10) and castor bean (1) seeds are consistent with proteolytic degradation as a mechanism for this decline. In sunflower seeds, the involvement of a proteinaceous factor was reported in the loss of isocitrate lyase from crude extracts (18). However, the mechanism of the proteolytic degradation of isocitrate lyase implied during later periods of seed germination is still unclear.

In working with flax seeds (*Linum usitatissimum*), we have consistently observed highly unstable preparations of isocitrate lyase in crude extracts (McFadden, unpublished observation). The present study was undertaken to follow changes in proteinases during the germination of flax seeds, and to elucidate any correlation between one or more proteinases and the loss in isocitrate lyase activity. Developmental changes in proteinases have been followed quantitatively by electrophoresis in SDS-containing polyacrylamide gels containing gelatin as the proteinase substrate.

MATERIALS AND METHODS

Materials. German flax seeds were obtained from Frau Musset of the Technisches Universität, München, and were harvested near Freising-Weihenstephan in October of 1980. Noble agar and skim milk were purchased from Difco and Cellagram II cellulose acetate strips from Shandon Southern. Pepstatin A, PCMB[3] α-tolylsulfonyl fluoride (also called phenylmethylsulfonyl fluoride or PMSF) 1,10-phenanthroline, leupeptin, Triton X-100, and bovine hemoglobin were from Sigma. Highly purified SDS (sequanal grade) was obtained from Pierce Chemical Co. All other reagents were of the highest grade available.

Seed Germination. Flax seeds were germinated at 25°C for the required time according to Khan *et al.* (10) except that plates were wrapped in aluminum and germinated in a dark incubator to insure total darkness.

Preparation of Seed Extract. For preparation of the crude extract, 25 seedlings were rapidly ground to a paste in the presence of broken Pasteur pipet tips with a prechilled mortar and pestle. To this paste, 2 ml of chilled extraction buffer (0.05 M Tris·Cl, pH 7.5, 25°C) were added and mixed into an even slurry. This paste was then centrifuged at 27,000 g for 20 min at 2°C and the supernatant defatted through glass wool yielding the 'crude extract'. As needed, the extract was diluted with extraction buffer.

Enzyme and Protein Assays. Isocitrate lyase was assayed by the discontinuous method of Roche *et al.* (14) with some modifications (9).

The proteinase activity in crude extract was measured against dialyzed 2% bovine hemoglobin or gelatin as substrate. Prior to the assay, the crude extract was dialyzed against extraction buffer to remove free amino acids. In a total volume of 1.5 ml, 0.5 ml each of the substrate and crude extract were incubated in 0.2 M buffer for 1 h at the required temperature. The reaction was stopped with 0.5 ml of 20% (w/v) chilled TCA and the tubes kept on ice for 1 h. The solution was filtered through a Whatman No. 1 filter and 0.2 ml of the filtrate then assayed for ninhydrin-positive material according to Rosen (15) using leucine as a standard. The control was treated identically, except that TCA was added prior to the crude extract. One unit of activity is defined as 1 μmol eq leucine released/h under conditions of the assay. For pH studies conducted at 45°C, buffers used were succinate (3.0–6.5), MOPS (6.5–7.9), borate (8.5–10.0), and CAPS (10.0–11.0). In some studies, crude extracts from 4-d seeds were either dialyzed at 2°C against three changes of 250 volumes

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[3] Abbreviations: PCMB, *p*-chloromercuribenzoate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

of: (a) H_2O or (b) 0.1% SDS or, alternatively, the extract adjusted to 0.1% SDS and dialyzed as described against: (a) H_2O or (b) 2.5% Triton X-100. Samples within the dialysis membrane were then incubated with hemoglobin at 37°C (pH 5.0) for 60 min and the amino acids liberated were measured as described.

Protein was estimated according to Lowry et al. (13).

Proteinase Inhibitions. Various proteinase inhibitors were prepared as 10-fold concentrated stock solutions as follows: 1,10-phenanthroline, α-tolylsulfonyl fluoride, Pepstatin A, and leupeptin in ethanol, PCMB in 0.5 M glycylglycine (pH 8.5) and EDTA in water. The inhibitor stock was mixed with 9 volumes of crude extract and incubated on ice for 30 min. This was followed by estimation of proteinase activity against hemoglobin or by electrophoretic detection of proteinases. For studies after gel electrophoresis, proteinase inhibitors were added at the required concentration to the Triton X-100 wash as well as the succinate buffer. When isocitrate lyase activity measurements were made in the presence of proteinase inhibitors, a suitable aliquot from this reaction mixture was assayed as mentioned earlier. Appropriate inhibitor-less controls were done as required.

Detection of Proteinases after Electrophoresis. Cellulose acetate electrophoresis and visualization on milk-agar substrate plates was done according to Cohen (2) with a few modifications. Briefly, 1 to 3 μl extract was spotted in the center of a 11 × 5 cm cellulose acetate strip (cellagram II) and subjected to electrophoresis at 22°C and 170 v for 40 min in 0.25 M Tris-borate buffer, pH 9.2 (25°C). This strip was then laid over a 2% milk-agar plate preequilibrated with 0.1 M succinate (pH 5.0) and incubated at 45°C for 3 h in a moist chamber. Clear zones on milk-agar reflected positions of proteinase activity.

Electrophoresis was also carried out in 0.1% SDS-polyacrylamide-gelatin gels according to Heussen and Dowdle (6), except that plasminogen was omitted. After electrophoresis, SDS was exchanged with Triton X-100 for 1 h at 22°C, and the gel was incubated for 2 h (unless otherwise specified) at 45°C in 0.1 M succinate, pH 5.0. Proteinases were localized as lighter regions on a dark blue background after staining with amido black as described (6). For quantification of the proteinase zones, the gels were scanned in a densitometer (Helena Labs) and trough areas traced on Hammermill 10 M paper, cut out, and weighed. All quantifications were done on duplicate samples (at a minimum) which had been subjected to electrophoresis identically but separately. Non-SDS-containing gels were made and processed similarly, except that the acrylamide concentration prior to polymerization was 6% instead of 11% as used for gels containing SDS.

RESULTS

pH and Temperature Optima of Proteinases. Extracts from 4-d-old flax cotyledons were assayed for total proteinase activity against 2% bovine hemoglobin as a substrate. Most proteinase activity was found at pH values below 7 using an assay temperature of 45°C (Fig. 1a). No precipitation was observed at any pH tested. Figure 1b shows that the overall temperature optimum was 45°C at pH 5 under the assay conditions. An identical pH-activity profile was obtained when gelatin was used as a substrate (not shown).

Electrophoretic Characterization of Proteinases. Electrophoresis on cellulose acetate at pH 9.2 followed by detection on milk-agar plates at pH 5.0 revealed that the major portion of proteinase activity in crude extracts was in basic proteins that moved towards the cathode even at this high pH (Fig. 2). At least three distinct proteinase bands could be detected towards the cathode in these crude extracts from 4-d-old seedlings in addition to a broad zone between the origin and the first band. Although this method served as a good qualitative tool, quantification proved to be difficult so the SDS-polyacrylamide-gelatin electro-
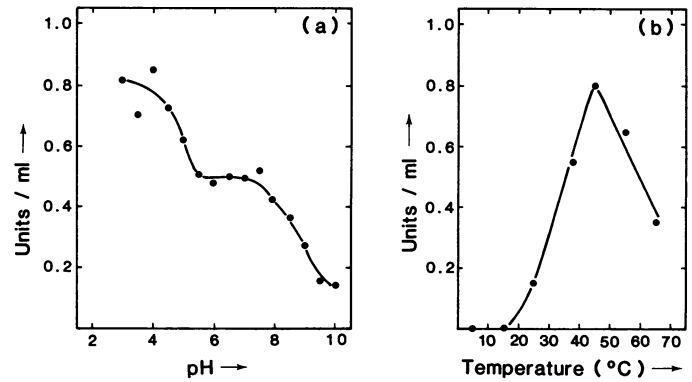

FIG. 1. Effect of (a) pH (at 45°C) and (b) temperature (using succinate buffer, pH 5.0) on proteolytic activity of a crude extract from 4-d seedlings. Assays in both cases were performed as described in the text using 2% bovine hemoglobin.
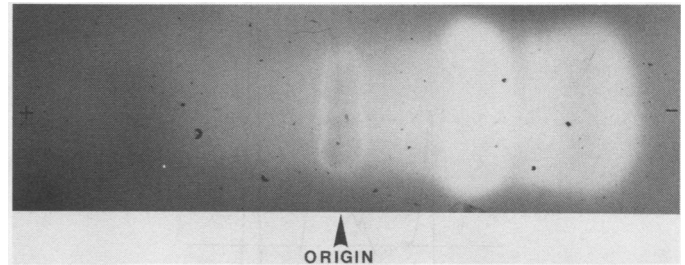

FIG. 2. Electrophoresis on cellulose acetate of a crude extract from 4-d-old seedlings followed by visualization on 2% milk-agar substrate plates. Clearing indicates zones of proteolytic activity towards the cathode.
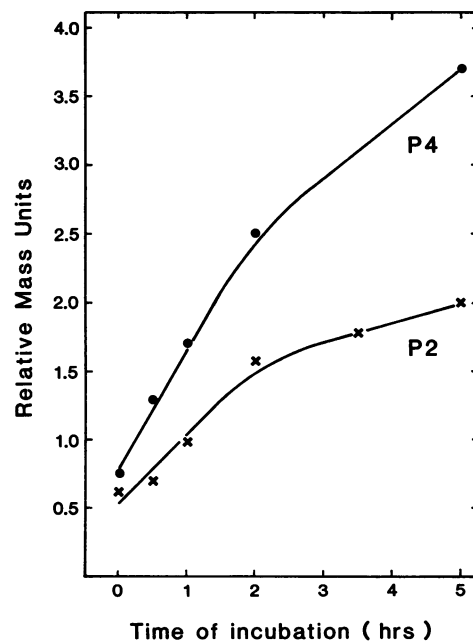

FIG. 3. Effect of incubation time at 45°C on clearing in SDS-polyacrylamide gels containing gelatin. Crude extract from 4-d seedlings was subjected to electrophoresis and the gel incubated with Triton X-100 for 1 h, followed by 0.1 M succinate, pH 5.0, for times shown in the plot. Duplicate lanes were densitometrically traced and regions corresponding to P2 and P4 (see Fig. 6) quantified as described. Results are an average of triplicate measurements. One relative mass unit equals 10 mg of paper.
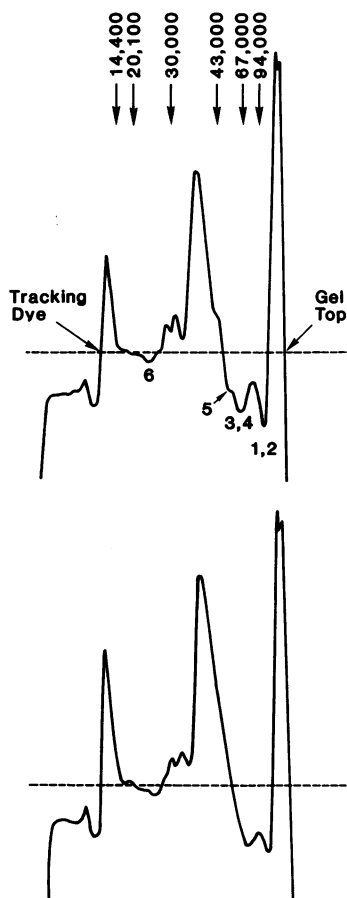
FIG. 4. Electrophoresis in SDS-polyacrylamide gelatin of crude extract from 4-d seedlings. Separate electrophoretic experiments were run on the same extract and each gel traced densitometrically; (- - -), background staining. The total peak areas were calculated to be 11.9 and 12.6 relative mass units and the total trough areas to be 3.35 and 3.27 relative mass units for the upper and lower traces, respectively. One relative mass unit equals 10 mg of paper. Results are an average of duplicate measurements. Regions (P1–P6) corresponding to those which are better resolved in the longer gel depicted in Figure 6 are identified above.
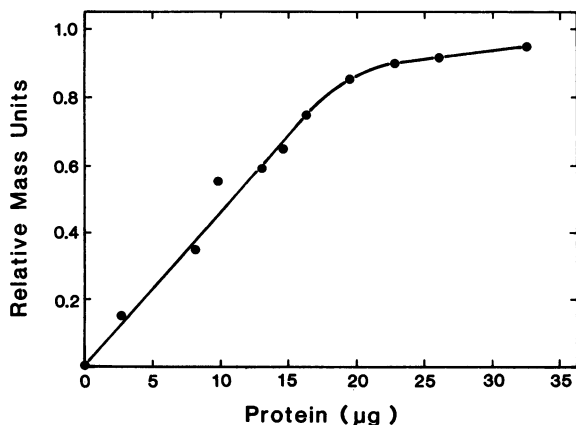

FIG. 5. Effect of protein content (in 10 μl) of crude extract on clearing in SDS-polyacrylamide gels containing gelatin after activation by Triton X-100. Electrophoresis, staining, and quantification were done as described in the text. Results are an average of duplicate measurements.

phoretic technique was employed for all subsequent studies.

When gels were incubated at pH 5 and 45°C for different times following activation of proteinases by the addition of Triton X-100, a biphasic time dependence of activity was found (Fig. 3) for two representative proteinase zones in the gel. For all subsequent gel assays, therefore, the development time was kept in the linear range at 1.5 to 2 h.

The SDS-polyacrylamide-gelatin electrophoretic method was highly reproducible when repeated on a given extract under identical conditions as shown in Figure 4 which represents densitometric scans of two gel lanes. Moreover, there was linearity between gelatin digestion and the protein concentration of flax extracts up to 17 μg/10 μl (Fig. 5). The plateau observed at higher levels of protein was presumably due to depletion of the substrate gelatin. In all experiments presently described, the protein concentration of the extract was adjusted into the linear range (Fig. 5).

Since electrophoresis was carried out in the presence of SDS, which was then replaced during proteinase reactivation by Triton X-100, it became crucial for our quantification to estimate the extent of proteinase reactivation following replacement of SDS. To do this, we subjected aliquots from the same crude extract (4-d seedlings) to brief electrophoresis in SDS and non-SDS gels both containing gelatin. After incubation of both gels in Triton X-100 and succinate buffer (pH 5) at 45°C for identical times and staining, the lanes were densitometrically scanned for quantification. The results showed greater than 90% recovery of proteinase activity following SDS removal and excellent reproducibility. Parallel studies of extracts of 4-d seeds showed that there was no gelatin digestion when water-treatment of the gel was substituted for Triton X-100 treatment after SDS-PAGE.

In additional studies of extracts of 4-d seedlings, it was found that extracts dialyzed against 0.1% SDS or adjusted to 0.1% SDS and dialyzed against H_2O gave specific proteinase activities that were 29% and 12% of that for a H_2O control (0.107 μmol amino acid liberated/mg protein·h). In contrast, an extract treated with 0.1% SDS and dialyzed against 2.5% Triton X-100 had a specific proteinase activity that was 81% of that for the H_2O control.

Developmental Changes in Proteinases. Gelatin-containing SDS polyacrylamide gels revealed six distinct proteinase zones (P1 to P6) between 1 and 5 d of germination (Fig. 6). The darkly stained regions especially evident in extracts of 1- and 2-d seedlings were due to seed storage proteins. This entire developmental pattern was reproducible. P1 and P2 were present as early as day 1, P3 and P6 appeared at day 3 and increased until day 5, and P4 and P5 appeared at day 3 with a peak at day 4. The mol wt of protein standards is also presented in Figure 6. When seeds were germinated in light, there were significant differences in the developmental profiles. However, since patterns were not highly reproducible, the data are not shown.

Effect of Proteinase Inhibitors. Crude extracts from 4-d-old seedlings were treated with the indicated concentrations of various proteinase inhibitors and subjected to electrophoresis as described (Fig. 7). The results indicate that P4 and P5 are EDTA-sensitive proteinase zones. Partial inhibition of P5 was also observed with Pepstatin A and α-tolylsulfonyl fluoride. The apparent inhibition of the minor proteinase(s) in P6 by α-tolylsulfonyl fluoride (lane 6) was no greater than that observed in the ethanol control (lane 2), but there may have been inhibition by PCMB of the proteinase(s) in P6. Leupeptin appeared to enhance proteinase activities not seen otherwise. When run separately in a control gel, leupeptin showed no nonspecific clearing (data not shown). The inclusion of putative proteinase inhibitors in the Triton X-100 wash solution or the incubation buffer (succinate, pH 5.0) did not show any significant changes in the proteinase bands. This ruled out the possibility that proteinase-inhibitor complexes such as those expected with Pep-
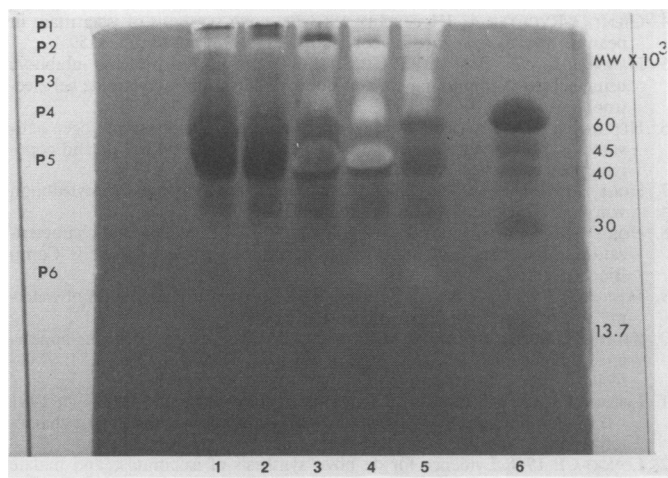
FIG. 6. Electrophoretic profiles of proteinases from flax seeds germinated in dark for 1 to 5 d (lanes 1–5, respectively). P1 to P6 indicate proteinase zones in terms of increasing mobility (decreasing mol wt) towards the anode. Mol wt of standard proteins (from top to bottom: catalase, ovalbumin, aldolase, carbonic anhydrase, and ribonuclease) are listed on the right.
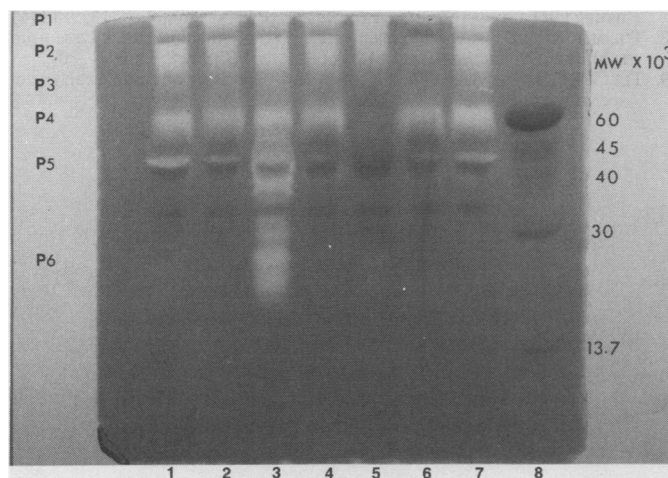

FIG. 7. Effect of inhibitors on the proteinase profile of 4-d dark-germinated flax seeds. Lanes contained: 1, H_2O; 2, ethanol (10%); 3, leupeptin (10 μM); 4, Pepstatin A (1 mM); 5, EDTA (30 mM); 6, α-tolylsulfonyl fluoride (2 mM); 7, PCMB (2.5 mM); 8, mol wt markers.

statin A or leupeptin had dissociated during electrophoresis. It was noted that the inclusion of α-tolylsulfonyl fluoride in the incubation buffer interfered with subsequent staining of the gel with amido black.

The effect of various proteinase inhibitors on the decay of isocitrate lyase in crude extracts from 4-d-old seedlings was also estimated. Ethylenediaminetetraacetate protected against the decay of enzyme activity at 37°C; increasing concentrations of EDTA revealed increased protection, the effect saturating at around 50 mM EDTA (Fig. 8). Leupeptin, α-tolylsulfonyl fluoride, PCMB, and Pepstatin A did not show any protection at the concentrations tested (see legend for Fig. 7). 1,10-Phenanthroline at 1 mM also showed no protection. The protection of isocitrate lyase activity by EDTA is of significance when viewed in terms of the proteinase pattern of 4-d-old seedlings. This correlation suggests that proteinase(s) in P4 and/or P5 degrade isocitrate lyase.
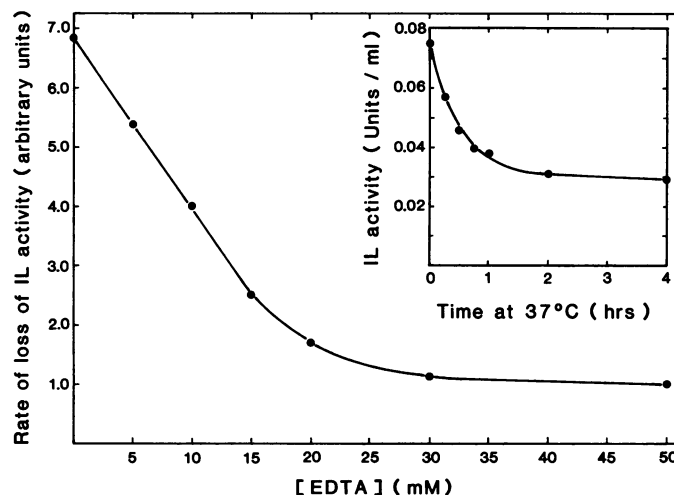

FIG. 8. Effect of increasing EDTA concentrations on the rate of loss of isocitrate lyase (IL) activity in crude extracts (4-d-old seeds) at 37°C. Extracts were prepared by grinding 30 seedlings to a paste and adding 8 ml of homogenization buffer. Nine volumes were mixed with one volume of test compound (or with the appropriate solvent in the case of the control. Rates were normalized to the rate of loss of isocitrate lyase at 50 mM EDTA, taken as 1 arbitrary unit. The inset shows the loss of isocitrate lyase (IL) as a function of time in the absence of EDTA or presence of leupeptin, Pepstatin A, α-tolylsulfonyl fluoride, PCMB, or 1,10-phenanthroline at concentrations indicated in the legend for Figure 7.

DISCUSSION

In this report, a novel method to study changes in proteinases during seed germination is presented. Previously, this technique has been used to visualize plasminogen activators (6), epidermal proteinases (8), and proteinase-inhibitor polypeptides (5) in animal cells. We have demonstrated the reproducibility of this method as well as the quantitative reactivation of proteinases in the polyacrylamide-gelatin gel after exchange of SDS with Triton X-100.

Electrophoretic analysis of crude extracts on cellulose acetate at pH 9.2 shows at least four active proteinase zones towards the cathode. Unusually basic proteinases have also been found in fungi (2, 11). These proteinases in 4-d flax seedlings are most active at acidic pH values and 45°C. In germinating seeds, the endopeptidases and carboxypeptidases involved primarily in storage protein degradation also have acidic pH optima supporting a lysosomal digestive process (16).

Studies on proteinases from castor bean endosperm (19) show the presence of a hemoglobin-degrading activity with an acidic pH optimum (3.5–4.0). This activity was strongly inhibited by the thiol reagents, iodoacetamide, N-ethyl maleimide, and leupeptin. Leupeptin also stabilized the activity of catalase, fumarase, and isocitrate lyase in crude extracts of castor beans (1). We, however, see no effect of leupeptin or another thiol-directed reagent, PCMB, on the stability of isocitrate lyase in crude extracts from germinating flax seeds. This finding is not necessarily in conflict with the earlier report, as the two tissues are different. In castor beans, isocitrate lyase is found in the endosperm whereas in flax it is present in the cotyledons.

The metalloproteinase inhibitor, 1,10-phenanthroline at a concentration of 1 mM, did not inhibit any of the flax acid proteinases and did not stabilize isocitrate lyase activity in crude extracts in contrast to EDTA. The former reagent complexes especially strongly with transition metal ions whereas EDTA is a more general metal chelating agent. It is therefore somewhat puzzling that in a study of jojoba seeds (17), 1 mM 1,10-phenanthroline completely inhibited the endopeptidase activity of crude extracts

at pH 4 whereas EDTA at a similar concentration had no effect. This may only reflect once again the diversity of proteinases in oil-rich seeds.

Isocitrate lyase activity during flax seed germination peaks at day 3 and then decays to a negligible level by day 5 (10). In previous research, an α-tolylsulfonyl fluoride-sensitive proteinaceous isocitrate lyase-degrading factor was partially characterized from 4-d seedlings (10). We cannot account for the discrepancy between these prior findings and the observations reported both here and by another investigator in this laboratory (M. W. Carrington, unpublished) that degradation of isocitrate lyase is unaffected by α-tolylsulfonyl fluoride. On the basis of the present results, it is interesting to note that proteinase(s) in P4 (mol wt ≅ 60,000) starts appearing around day 3 of germination and peaks at day 4 when the rate of loss of isocitrate lyase activity is at its maximum. This proteinase zone is inhibited by high concentrations of EDTA which also considerably stabilizes isocitrate lyase activity in extracts from 4-d-old seedlings. Whether P4 contributes to the disappearance of isocitrate lyase *in vivo* remains to be seen. Efforts are currently underway to purify the proteinase P4. This may help to elucidate the nature of isocitrate lyase decay during later periods of germination.

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