Plant Physiol. (1981) 67, 499–502
0032-0889/81/67/0499/04/$00.50/0

Proteinases and Enzyme Stability in Crude Extracts of Castor Bean Endosperm[1]

Received for publication June 30, 1980 and in revised form October 14, 1980

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ABSTRACT

The stability of catalase, fumarase, and isocitrate lyase from deliberately broken organelles in crude extracts from endosperm tissue of castor bean seedlings has been examined. These enzymes are relatively stable at 2 C in extracts from endosperm of 2-day seedlings, but rapid losses of activity occur in extracts from older seedlings. These losses are shown to be brought about by the thiol-proteinase present in the extracts. The inclusion of 35% glycerol prevented the loss of catalase, fumarase, and isocitrate lysase activity, and various inhibitors of proteinases afforded limited protection. The most striking protectant was leupeptin, an inhibitor of serine and thiol-proteinases. Leupeptin completely inhibited the loss of activity of the three enzymes in crude extracts and improved yields when included in the grinding medium.

It is frequently observed that significant losses of enzyme activity occur in crude extracts from plants, even when the usual protectants are added and the temperature is maintained close to 0 C. Loss of particularly unstable enzymes may occur during extraction before an initial assay can be performed. One feature contributing to this loss is the fact that enzymes from the cytosol and organelles become exposed to proteinases released from vacuoles during grinding.

In this paper we show that, in crude extracts from endosperm of germinating castor bean seedlings, the major part of the selective loss of particular enzymes is due to proteinases. Various treatments which mitigate this loss are described, and the addition of leupeptin, a potent inhibitor of serine and thiol proteinases, is shown to be remarkably effective; it completely prevents the loss of sensitive enzymes when added at micromolar concentrations.

MATERIALS AND METHODS

Plant Material. Castor bean seeds (*Ricinus communis* L. cv. Hale) were soaked in running tap water and germinated in moist vermiculite at 30 C. The seedlings were harvested at the desired age and the endosperm tissue was collected for extraction.

Preparation of Endosperm Extract, Chemical Treatments, and Enzymatic Assays. Endosperm tissue from 10 seedlings was ground using a Virtis No. 45 homogenizer at full speed for 1 min in 10 ml 50 mM K-phosphate (pH 7.2) at 0 C. The crude homogenates were centrifuged at 10,000*g* for 20 min, and the supernatant solutions were assayed for enzyme activities. Changes of enzyme activities with time were followed when the supernatant solution was kept at 2 C or at room temperature (~21 C). Enzyme assays were those described in the literature as follows: catalase (3), fumarase (4), and isocitrate lyase (2). Trasylol was obtained from Mobay Chemical Corporation, New York. Leupeptin and Pepstatin from Sigma were added at concentrations indicated in the text. The various proteolytic assays were conducted after the methods of Tully and Beevers (5). When the ninhydrin method was used, the endosperm extract was desalted on Sephadex G-25. The effects of leupeptin on the thiol-proteinase were examined with a partially purified enzyme prepared by ion-exchange chromatography according to Tully and Beevers (5).

Sucrose Density Gradient Centrifugation. Endosperm tissue (10 g) from 5-day-old seedlings was chopped with razor blades for 7 min in 10 ml ice-cold medium: 13% w/w sucrose, 150 mM Tricine, 1 mM EDTA (pH 7.5). After straining through polyester cloth, the extract was divided in two parts (±10 μM leupeptin) and centrifuged at 270*g* for 20 min. The supernatant was loaded over a sucrose gradient [5 ml 15% sucrose, 24 ml linear 30 to 60% sucrose containing 1 mM EDTA (pH 7.5)] and centrifuged for 2 h at 21,000 rpm in a Beckman SW 25.2 rotor. After centrifugation, 1.2-ml fractions were collected on ISCO gradient fractionator, model 640.

Marker enzymes fumarase, catalase, and isocitrate lyase were assayed as already mentioned; NADPH-Cyt *c* reductase was measured as described (1).

RESULTS AND DISCUSSION

Loss of Enzyme Activity from Crude Extracts Prepared from Seedlings of Different Ages. Crude extracts containing enzymes released from all cellular compartments were prepared as described from 2-, 4-, and 6-day-old seedlings and maintained at 2 C throughout. Initial enzyme assays for three enzymes, fumarase, catalase, and isocitrate lyase, were performed immediately after centrifuging the extract at 10,000*g* and at intervals thereafter (Fig. 1). The results in Figure 1, from 2-day-old seedlings, show that the activities of catalase and isocitrate lyase are maintained unchanged for 24 h before declining, whereas that of fumarase had declined by some 20% during the first 18 h. In extracts from the older seedlings (Fig. 1), all of the enzyme activities declined sharply from time zero.

It is known that the activities of various proteolytic enzymes in the endosperm tissue increase strikingly during the 2- to 6-day period of growth (5) and, inasmuch as it was also observed that the changes described in Figure 1 occurred much more rapidly when the extracts were maintained at 21 C, we suspected that degradation of the measured enzymes by proteinases was responsible for the pattern of enzyme loss shown in Figure 1. This was confirmed for fumarase (Fig. 2). Extracts from the endosperm of 6-day seedlings, known to have high proteolytic activity (5), were incubated at 21 C for 4 h. During this period, the fumarase activity disappeared completely. Boiled and unboiled portions of this

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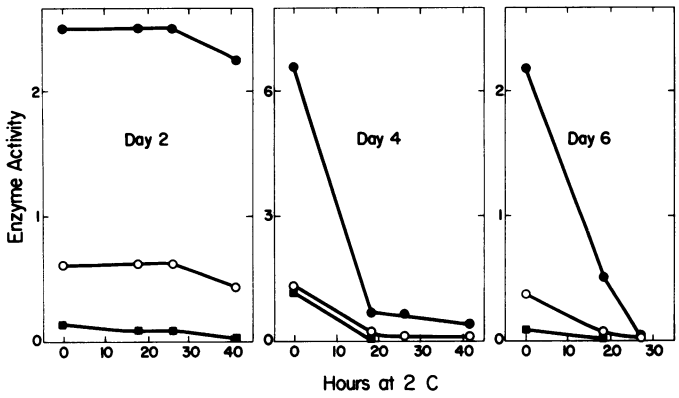

FIG. 1. Stability of enzymes in crude extracts from endosperm of seedlings of different ages. Extracts prepared as described under "Materials and Methods" were assayed for catalase (●) (mmol/min·ml extract), fumarase (■) (μmol/min·ml extract), and isocitrate lyase (○) (μmol/min·ml extract) immediately after preparation and at intervals while the extracts were maintained at 2 C.

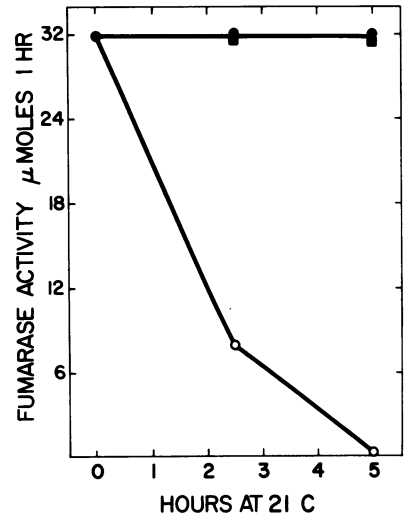

FIG. 2. The effect of 6-day extract on the stability of fumarase in the 2-day extract. Fumarase activity in the 2-day extract (●) remained constant. The addition of a 6-day extract which had lost its fumarase activity during incubation at 21 C for 4 h resulted in rapid loss of fumarase activity from the 2-day sample (○). Boiled 6-day extract was without effect (■).

extract then were added in a 3:1 ratio to extracts from 2-day seedlings and to a control, a similar amount of buffer. The fumarase activity of the 2-day extract (control) remained essentially unchanged for 5 h (Fig. 2). The inclusion of the unboiled 6-day extract resulted in a precipitous loss of fumarase activity, but the corresponding boiled extract was without effect. The 6-day extract still retained some catalase and isocitrate lyase activity after 4 h at 21 C but, when this was added to the 2-day extract, an accelerated loss of these enzymes occurred; again, the boiled 6-day extract was without effect (results not shown). We concluded that hydrolytic enzyme activities present in the 6-day extract were at least partially responsible for the loss of the measured enzyme activities, and the greater stability in the 2-day extracts was ascribed to the lower hydrolytic activities in these extracts.

Effect of Additives on Stability of Enzymes in 6-day Extract. In an attempt to stabilize the fumarase, isocitrate lyase, and catalase activities in 6-day extracts, various additives were examined. As shown in Table I, some protection was afforded by the inclusion of iodoacetamide, *p*-chloromercuribenzoate, and phenylmethylsulfonyl fluoride, but the inclusion of glycerol at 35% was completely effective. EDTA, DTT, iodoacetate, and *N*-ethylmaleimide were ineffective (results not shown). Trasylol (also called aprotinin) inhibits a variety of proteinases (trypsin, chymotrypsin, plasmin, etc.) which have a serine residue in their active center (8). The inclusion of this inhibitor had only a small protective effect on the stability of the three measured enzyme activities (Table I).

Effect of Inhibitors on Proteinase Activities. Two of the various hydrolases that could be responsible for loss of enzyme activity are carboxypeptidase and the thiol-proteinase (5). The effects of the inhibitory reagents on these enzymes are shown in Table II. Clearly, none of the reagents is completely inhibitory to either of the hydrolyases, although the effects on protection of the measured enzyme activities in Table I can probably be ascribed to the inhibitors of the hydrolases shown in Table II. The effects of two additional proteinase inhibitors on the hydrolases are shown in Table III. Pepstatin is known to inhibit carboxylendopeptidases such as pepsin and cathepsin D. Leupeptin, a tripeptide with mol wt 503.7, isolated from *Streptomyces* sp., has been shown to inhibit several serine proteinases and is particularly effective on thiol-endopeptidases; it does not inhibit pepsin (6).

Pepstatin had only small inhibitory effects on carboxypeptidase and thiol-proteinase. However, whereas leupeptin had only a small effect on the carboxypeptidase, it completely inhibited the hydrolysis of hemoglobin at a concentration of 10 μM. From the curve showing dosage response (Fig. 3), it can be seen that 50% inhibition of enzyme activity at pH 4.0 was obtained at 1 μM leupeptin. At a concentration of 1 mM, leupeptin was without effect on two additional hydrolases in the extracts L-leucyl-β-naphthylamidase and L-prolyl-β-naphthylamidase but reduced α-N-benzoyl-DL-arginine-β-naphthylamidase activity by 50%.

When leupeptin was added to 6-day extracts, the results were very striking. Whereas, in the controls held for 4.5 h at 21 C, fumarase activity was completely lost and isocitrate lyase had declined to 7% of its original activity, these losses were completely prevented by the inclusion of leupeptin at concentrations as low as 1 μM. Pepstatin afforded only minor protection even at a concentration of 1 mM.

The effectiveness of leupeptin in preventing enzyme losses prompted us to investigate its effects when added to the grinding medium. Duplicate samples (10 g) of endosperm tissue were extracted, one in the standard medium and one on the standard medium containing 10 μM leupeptin. After centrifugation for 30 min at 10,000*g*, the enzyme activities in the supernatant solution were measured (time zero). Both extracts were held at 2 C for 5 h and changes in enzyme activities measured at intervals (Table IV). The inclusion of leupeptin completely prevented loss of enzyme activity, as before, but additionally, prevented loss of activity during grinding and centrifugation since the time zero values, particularly for fumarase, were considerably higher when leupeptin was included.

The foregoing experiments were all carried out on crude extracts made under conditions where the various organelles were deliberately broken by vigorous homogenization in a medium without osmotic protection. When preparations were made under conditions where the organelle integrity was maintained, fumarase, isocitrate lyase, and catalase activities in the organelles did not decline with time, and the inclusion of leupeptin in the grinding medium did not affect the yield or stability of the enzymes within the organelles.

The experiments with crude extracts show that the presence of proteinases can markedly influence the recovery and stability of other exposed enzymes even at 2 C and that leupeptin in small amounts can offset these losses. The major proteinase responsible for enzyme breakdown in the endosperm extracts appears to be the thiol-proteinase (5). Although this particular enzyme has an acid pH optimum (5), there is still detectable activity at pH 7.0.

Table I. *Effect of Chemical Treatments on Enzyme Stability*

Samples of a crude extract from 5- to 6-day endosperm were incubated at 2 C with the indicated additions.

Treatment	Enzyme Activity at Various Times											
	Catalase				Fumarase				Isocitrate lyase			
	0 h	4 h	8 h	20 h	0 h	4 h	8 h	20 h	0 h	4 h	8 h	20 h
	mmol/min·ml				*μmol/min·ml*							
Control	7.00	6.15	5.13	3.87	0.32	0.06	0.02	0.00	1.00	0.54	0.33	0.11
Glycerol, 35%	7.00	7.00	7.00	7.00	0.32	0.38	0.40	0.40	1.00	1.10	1.15	1.15
p-Cl-mercuribenzoate, 1 mM	7.00	6.10	5.11	3.15	0.32	0.19	0.10	0.04	1.00	1.05	0.88	0.88
Iodoacetamide, 1 mM	7.00	6.35	6.00	6.09	0.32	0.33	0.16	0.08	1.00	0.76	0.47	0.41
Phenylmethylsulfonyl fluoride, 1 mM	7.00	6.45	5.87	4.12	0.32	0.09	0.06	0.00	1.00	1.00	0.40	0.23
Trasylol, 0.02 mM	7.00	6.58	5.93	3.95	0.32	0.09	0.06	0.00	1.00	1.00	0.40	0.23

Table II. *Effect of Inhibitors on Carboxypeptidase and Thiol-proteinase*

Reagents were added as indicated to a crude, desalted extract from 5- to 6-day old endosperm and enzyme activities measured after 21 h at 2 C.

Treatment	Enzyme Activity	
	Carboxypeptidase	Thiol-proteinase
	% control	
Control	100	100
Phenylmethylsulfonyl fluoride, 1 mM	27	101
Iodoacetamide, 1 mM	95	21
p-Cl-mercuribenzoate, 1 mM	92	66
Trasylol, 0.002 mM	83	104
Trasylol, 0.02 mM	78	98
Trasylol, 0.2 mM	78	98

Table III. *Effect of Leupeptin and Pepstatin on Carboxypeptidase and Thiol-Proteinase*

Fifty μl of a desalted crude extract of endosperm from 5- to 6-day seedlings was incubated for 30 min with various additions and assayed for carboxypeptidase and acid proteinase (5). For carboxypeptidase, the substrate was 1 mM *N*-carbobenzoxyphenylalaninalanine in 0.1 M K-acetate at pH 4.5; for thiol-proteinase, the substrate was 1% hemoglobin in 0.1 M K-malate (pH 3.5).

Treatment	Enzyme Activity	
	Carboxypeptidase	Thiol-proteinase
	% control	
Control	0	100
Pepstatin, 0.1 mM	87	100
Pepstatin, 1.0 mM	84	100
Leupeptin, 0.001 mM	84	2
Leupeptin, 0.01 mM	81	0

The importance of this is seen when extracts are adjusted to pH levels from pH 5.5 to 7.5, and the free amino acid content measured at intervals thereafter. At pH 5.5, the amount of free amino acid in the extract doubled (from 0.27 to 0.58 μmol/ml) within 1 h at 25 C. At pH 6.5, the level increased to 0.48 μmol and even at pH 7.5 there was a small increase, to 0.30 μmol. In such extracts, there are many proteins in addition to the enzymes we have been concerned with, and the susceptibility of individual proteins to attack by the proteinases at different pH levels can be expected to differ. Thus, we observed that a concentration of 10 μM leupeptin, which prevented the loss of fumarase activity from extracts of pH 6.5, did not completely block the increase of free amino acids in the extract.

In the tissues we have used, the thiol-proteinase activity changes

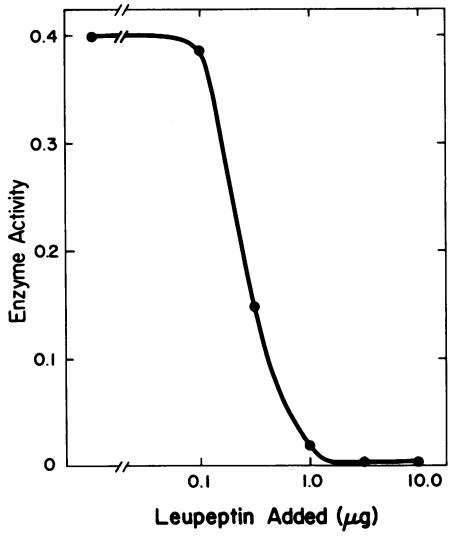

FIG. 3. Inhibition of thiol-proteinase by leupeptin. Purified enzyme was incubated with hemoglobin in 1 ml reaction medium for 30 min and the increase in *A* at 570 nm in the ninhydrin assay was measured at different leupeptin concentrations.

Table IV. *Effect of Adding Leupeptin to Grinding Medium on Enzyme Recovery and Stability at 2 C*

Two samples of endosperm tissue (5- to 6-day seedlings) were extracted in the usual medium, except that 10 μM leupeptin was added to one sample. Enzyme activities were assayed at time zero (immediately after centrifugation) and at intervals thereafter.

Time	Enzyme Activity					
	Isocitrate Lyase		Fumarase		Catalase	
	−Leupeptin	+Leupeptin	−Leupeptin	+Leupeptin	−Leupeptin	+Leupeptin
h	*μmol/min*				*mmol/min*	
0	1.06	1.21	0.10	0.33	7.17	7.52
2	0.46	1.21	0.08	0.35	2.87	7.52
4	0.46	1.37	0.02	0.38	2.87	7.52
6	0.38	1.37	0.00	0.40	1.43	7.17

with developmental age, increasing strikingly at 3 days, and the stability of fumarase, isocitrate lyase, and catalase in crude extracts decreases in consequence. In other tissues with low proteinase activity, this may present no problem, particularly if the pH is maintained above pH 7.5. In addition, as we have shown, enzymes which are susceptible to breakdown by proteinases when they are released from organelles are protected from attack when the organelle integrity is maintained. Nevertheless, crude total extracts are frequently prepared under pH conditions where proteolytic attack becomes a hazard. Leupeptin may be a useful general protectant in these circumstances.

Note Added in Revision. One of the reviewers drew attention to the frequent but inappropriate use of the term "acid proteinase" by plant physiologists (including ourselves) for proteinases with pH optimum of 3 to 5. The accepted biochemical nomenclature recommends that this term be restricted to those enzymes, such as pepsin, with pH optimum 1 to 2 and other proteinases described on the basis of their mechanism of action (7). Accordingly, the enzyme in castor bean extracts hydrolyzing hemoglobin is referred to here as a thiol-proteinase (5).

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