Phytochemistry, Vol 27, No 5, pp 1303 1307, 1988
Printed in Great Britain

0031-9422/88 $3 00+0 00
Pergamon Press plc

PROTEOLYTIC ACTIVITY IN PURE PREPARATIONS OF *PINUS PINEA* ISOCITRATE LYASE

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(*Revised received* 23 *September* 1987)

Key Word Index—*Pinus pinea*, Pinaceae, isocitrate lyase; isocitrate lyase inactivation; *Pinus* proteinases

Abstract—A proteinase was copurified with *Pinus pinea* isocitrate lyase Its proteolytic action, consisting of isocitrate lyase irreversible inactivation and the appearance of enzyme forms with lower M_rs, became evident when Mg^{2+} was removed from pure preparations or when EDTA or SDS was added This action was greatly reduced by Mn^{2+} or oxalate, and partially by PMSF, but not by several other protease inhibitors. Some data suggest that the proteolytic activity may be adsorbed on isocitrate lyase. A model for isocitrate lyase inactivation, involving Mg^{2+} dissociation, is proposed.

INTRODUCTION

Isocitrate lyase (ICL†, *threo*-D_s-isocitrate-glyoxylate lyase, EC 4.1.3.1) becomes increasingly unstable in the course of purification [1, 2] and purified ICL preparations from various sources are highly unstable during storage [1–8] In general, the lower the storage temperature, the slower is the rate of enzyme inactivation [1, 2, 4, 8] ICL thermal sensitivity has been widely investigated [8–13]. In some cases Mg^{2+} [3, 13] and other divalent cations [3] protect against heat inactivation, while in others oxalate provides protection but magnesium does not [8, 10] ICL instability in crude extracts [14, 15] as well as its decline *in vivo* [13, 16] have been attributed to proteolysis. There is direct evidence that proteinases are responsible for the more [17, 18] or less [19] selective inactivation of ICL in crude extracts of oil-rich seeds.

Proteases have been found to contaminate highly purified preparations of several enzymes [20] and are sometimes absorbed on the target enzyme [21, 22]. We have previously reported that the use of PMSF during the purification procedure and the shortening of the time of germination eliminate both the fragmentaion of the ICL elution pattern from Sephadex G-150 [7] and the appearance of multiple enzyme forms differing in electric charge and heat stability [13].

This paper provides the first evidence for proteolytic activity copurified with *Pinus pinea* ICL. Repeated treatment with PMSF during purification, though it can abolish proteolytic artifacts, was evidently not sufficient to prevent the subsequent appearance of PMSF-sensitive proteolytic contaminants, as has also pointed out in ref. [20]. Our data suggest that the proteolytic activity may be tightly adsorbed to ICL and thus may copurify with it in an inactive, PMSF-resistant complex. These findings may explain ICL's instability during storage as well as its selective decline *in vivo*. If so, the proteinase would play a major role in ICL's regulation and Mg^{2+} dissociation might be directly involved in this process

RESULTS AND DISCUSSION

ICL inactivation and reactivation

ICL was always completely stable in the presence of 6 mM Mg^{2+} at temperatures up to 45° but became markedly unstable following Mg^{2+} removal (Fig. 1) The addition of 6 mM $MgCl_2$ after various incubation times only partially reactivated the enzyme (Table 1) but stop-

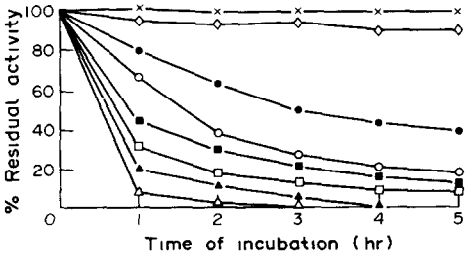

Fig 1 Stability of *Pinus* ICL at 45° after various treatments Two different preparations from the final purification steps were desalted with 50 mM Tris–HCl, pH 7 5 (see Experimental) and various reagents were added Sephacryl S-300 pool plus· ×6 mM $MgCl_2$, ◇ 6 mM oxalate, □ 5 mM EDTA (molar ratio ICL to EDTA = 1 3000), △ 0 02% (w/v) SDS (molar ratio ICL to SDS = 1 400), ○ no addition. Octyl Sepharose pool plus· ■ 5 mM EDTA (molar ratio = 1 3000), ▲ 0 02% SDS (molar ratio = 1 400), ● no addition For clarity, only the control (6 mM $MgCl_2$) of Sephacryl S-300 pool (×) is shown Activity is expressed as per cent of zero-time activity of each sample

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†*Abbreviations used*· DTT, dithiothreitol, ICL, isocitrate lyase, NEM, *N*-ethylmaleimide, PMSF, phenylmethylsulphonylfluoride, *p*OHMB, *p*-hydroxymercuribenzoate; T I soybean trypsin inhibitor.

Table 1 ICL reactivation by $MgCl_2$

Time of incubation at 45° (hr)	Enzyme activity (% control)			
	Sephacryl S-300		Octyl-Sepharose	
	Control (○ in Fig 1)	$+MgCl_2$	Control (● in Fig 1)	$+MgCl_2$
0	100 0	157 3	100 0	155 6
0 5	77 0	116 7	87 0	133 3
1	66 7	88 9	80 6	94 4
2	38 9	69 4	63 9	88 9
3	27 8	61 1	50 0	83 3
4	22 2	57 0	44 4	81 0

After different times of incubation at 45°, 6 mM $MgCl_2$ was added to controls, from which $MgCl_2$ was removed by gel-filtration, and their activities were measured after 10 min of further incubation. The activity of the reactivated enzyme remained unchanged from 5 min up to 5 hr after $MgCl_2$ addition

ped the inactivation process The degree of reactivation was time dependent and purer preparations were reactivated to a greater extent (Table 1) EDTA, an Mg^{2+} sequestering agent, produced the same result as ion removal by gel filtration (Fig 1), the effect was proportional to EDTA concentration

Under these conditions, the ICL subunit was cleaved into at least two smaller peptides (Figs 2 b, c and 3 b, c), implying proteolytic breakdown The higher temperature enhanced both inactivation and proteolysis The degree of inactivation also followed the enzyme elution-pattern from S-300, suggesting so that the proteinase may be adsorbed on the enzyme, rather than being copurified with it because of a similarity of their M_rs Comparable

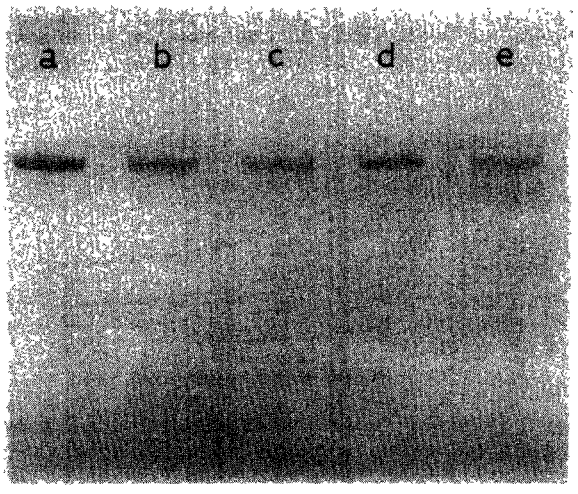


Fig 2 Results of SDS-PAGE of Sephacryl S-300 fractions after 5 hr treatments as in Fig 1 (a) control (6 mM $MgCl_2$ × in Fig 1), (b) no addition (○ in Fig 1), (c) 5 mM EDTA (□ in Fig 1), (d) 6 mM oxalate (◇ in Fig 1), (e) 0 02% SDS (△ in Fig 1) The gel is shown with its electrophoretic origin at the top of the figure SDS-PAGE was performed as described under Experimental and developed with silver staining

results obtained with ICL re-chromatographed on Sephacryl S-300 serve to support this supposition. The rate of inactivation was not affected by 0 1 to 1 mM DTT, but was 50% faster in the presence of 0.1 to 1% Triton X-100 Mixing the stable Mg-enzyme with unstable fractions yielded stable fractions with average activities ICL inactivation was faster after prolonged storage of the purified enzyme (e g 15–30 days) On storage there might be reactivation of a PMSF-sensitive proteinase and/or some slow (proteolytic?) event which leads to a 'metastable' form of ICL Finally, both inactivation rate and proteolysis were enhanced at advanced times (14–19 days) of seed germination.

Proteinase inhibitors

Of the protease inhibitors, chymostatin, pepstatin A, T.I, E-64, and leupeptin failed to prevent both ICL inactivation and proteolysis *p*OHMB and NEM quickly inactivated ICL (a-SH enzyme) and had no effect on enzyme breakdown. PMSF partially slowed down the rate of ICL inactivation but its effect on proteolysis was not clear It is to be noted that PMSF must be dissolved in ethanol or *iso*-propanol and both of these compounds were found to accelerate (25–30%) the rate of ICL inactivation Moreover, the degree of reactivation of Mg^{2+} addition was unaffected by the presence of these inhibitors Combining the proteinase inhibitors in various ways had no effect

ICL proteolysis

Proteolysis was quite limited (Figs 2 b, c and 3 b, c) and rapidly reached a plateau (same electrophoretic patterns after 5 or 24 hr), suggesting the generation of a protein which cannot be further degraded. Gel chromatography on Sephadex G-50 of ICL treated as in Fig 2b or c showed (in addition to a main poorly active peak) the appearance of two low $-M_r$ peptides, of *ca* M_r 5500 and 2500, whose amounts were *ca* 7–14% and 10–22% of total recovered proteins, respectively On the other hand, the same chromatography of 'contol' ICL (treated as in Fig 2a) yielded a single ICL-active peak

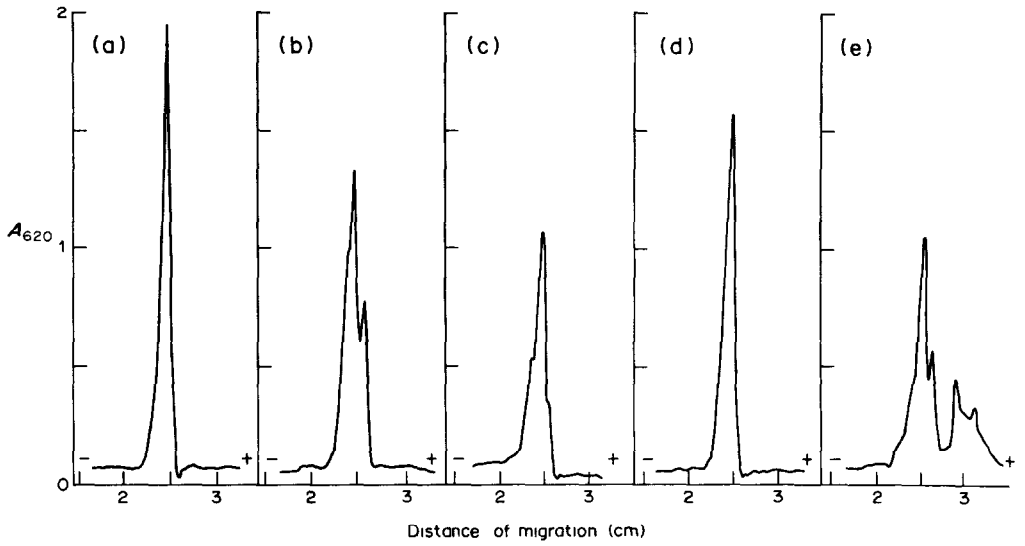


Fig 3 SDS-PAGE patterns obtained by scanning at 620 nm the gel of Fig 2 (in the same order), stained with Coomassie Brilliant Blue R-250 In (b) and (c), the same three enzyme forms appeared; i.e the native subunit (M_r 66 000) and two minor peptides (*ca* M_r 63 500 and 60 000), in (e) there are also two additional forms (*ca* M_r 45 000 and 33 000). M_r were determined by comparison with standards of known M_r as reported elsewhere [27] The abscissa refers to the distance of protein migration from the top (—) of the gel (9 cm total length)

This confirmed the results of SDS-PAGE and suggested that only few peptide bonds were cleaved by the proteinase

Proteinase assay

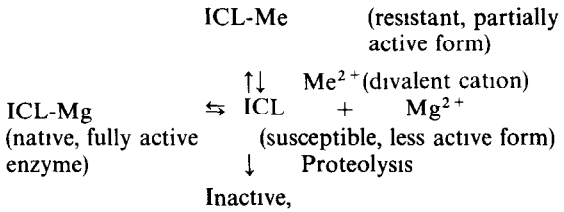
The amount of the proteolytic contaminant was very low It could not be measured with the usual assays for proteinases and was below the sensitivity of silver staining after SDS-PAGE By using immobilized β-glucosidase-labelled casein as substrate, we found that the activity of the proteolytic contaminant, in 50 mM Tris pH 7.5, was the same as 2–3 ng trypsin or 15–20 ng papain under the same conditions. The weight ratio between trypsin-like activity and ICL was approx 1:400 000. This acitivity was not affected by proteinase inhibitors (alone or in various combinations), 1 mM EDTA, 6 mM $MgCl_2$, or 6 mM $MnCl_2$, $NiCl_2$, $CaCl_2$, $BaCl_2$. It was about 50% lower in the presence of 1 mM PMSF and 30% higher in 1% Triton X-100 or 0 02% SDS.

Enzyme denaturation

We also found that after treatment with 0.02–0 05% SDS, ICL underwent a more extensive proteolysis, even in the presence of Mg^{2+}. The enzyme was rapidly inactivated (Fig. 1) due to denaturation and at least three secondary forms, whose amounts continued to increase for at least 24 hr, were detected by SDS-PAGE (Figs 2e and 3e), starting 5 hr after incubation at 45°. In this case, the appearance of secondary forms was completely abolished by 1 mM PMSF

Model for degradation

ICL requires Mg^{2+} for activity [23]. As is the case for other enzymes from which ions [24] or coenzyme [25] have been removed, Mg^{2+} removal may alter ICL's conformation, yielding a protein more susceptible to proteolysis as suggested by the following model:



As in other metallo-proteins [26], divalent ions might maintain in a 'tight' state a loop between two domains of ICL, while ion dissociation would render a specific peptide bond accessible to the proteinase, in line with the limited proteolysis observed under such conditions. On the other hand, ICL unfolding by SDS would render other peptide bonds susceptible to proteolysis.

Thus, Mg^{2+} dissociation would be the rate-limiting step in the irreversible inactivation of ICL and the enzyme's stability may depend on both the strength with which the ion is retained and the concentration of Mg^{2+} in the medium In this view, the apparent saturation of proteolysis might also reflect the presence of ICL forms which bind Mg^{2+} with different affinities. Proteolysis was more marked in the presence of EDTA (Figs 2c and 3c), probably because EDTA is more effective than gel filtration in removing Mg^{2+} from ICL. The model is also supported by the finding that Mn^{2+}, Ni^{2+}, Ca^{2+}, and Ba^{2+}, by replacing Mg^{2+}, reduced the degree of ICL inactivation with efficiencies (Table 2) proportional to their affinities towards ICL [7] and that oxalate (a glyoxylate analogue) also provided protection (Figs 2d, 3d and Table 2), probably by maintaining ICL in the native stable conformation

Table 2 ICL stability in the presence of various compounds

Reagent added	Apparent K_i (mM)	% residual activity after 5 hr at 45°
$MnCl_2$	0 4	88
$NiCl_2$	2 0	81
$CaCl_2$	8 0	78
$BaCl_2$	--	63
Oxalate	0 02	91

Reagents were added to Sephacryl S-300 fractions immediately after $MgCl_2$ removal by gel-filtration, at a final concentration of 6 mM ICL activity is expressed as % of that of the respective controls measured immediately after ion or oxalate addition K_i values are taken from ref [7] and [27] Ba^{2+} does not inhibit ICL

Some data suggest the existence of interactions (probably hydrophobic) between ICL and proteinase. In fact: (i) both the degree of inactivation and proteolysis were milder with ICL purified by octyl-Sepharose chromatography than with the enzyme re-chromatographed on Sephacryl S-300 or on DEAE-Sephacel. (In hydrophobic chromatography ICL elution is preceded by exhaustive washing with 30–40% ethylene glycol [27].) (ii) In the presence of 1% Triton X-100 ICL inactivation was faster and the proteolytic activity was slightly higher (iii) PMSF had a clear effect only when proteolysis was promoted by ICL denaturation with SDS The ICL-proteinase interactions might 'mask', at least partially, the proteinase active-site which would become easily accessible to the inhibitor following the action of the anionic detergent

EXPERIMENTAL

Materials Chemicals were obtained as reported elsewhere [27] PMSF was supplied by Boehringer and Sigma, Chymostatin, pepstatin A, soybean trypsin inhibitor, leupeptin hemisulphate (synthetic), *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, casein (purified powder), β-glucosidase (EC 3 2 1 21), *p*-nitrophenyl-β-D-glucopyranoside were obtained from Sigma, E-64 protease inhibitor, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine was obtained from Boehringer, CNBr-Sepharose and Sephadex G-50 Superfine from Pharmacia

Enzyme purification. ICL was purified as previously described [27], usually at the day 8 of seed germination, in the presence of 1 mM PMSF and 6 mM $MgCl_2$ The last two steps of the purification procedure (gel filtration on Sephacryl S-300 and hydrophobic-interaction chromatography on octyl-Sepharose) yielded fractions with purities about 90–95% and higher than 95%, respectively [27], ICL and proteins were assayed as described elsewhere [27]

Enzyme inactivation Mg^{2+} was removed by gel filtration on Sephadex G-25 disposable columns (Pharmacia) equilibrated and eluted with 50 mM Tris–HCl, pH 7 5 Instead of dialysis, we used gel filtration because we needed a fast, efficient desalting EDTA was added directly to the samples (desalted with 50 mM Tris–HCl, pH 7 5 plus 6 mM $MgCl_2$) from a stock soln (100 mM) For the inactivation studies, protein concentration was adjusted by dilution with 50 mM Tris–HCl, pH 7 5 as needed Protease inhibitors were added (from stock soln 100 × concentrated) about 20 min before Mg^{2+} removal or EDTA addition, at the following final concentration PMSF and *p*OHMB 1 mM, NEM, T1, and E-64- 0 1 mM, chymostatin, pepstatin A, and leupeptin 0 01 mM.

Sephadex G-50 chromatography Gel chromatography was carried out on a 1 6 × 54 cm Sephadex G-50 Superfine column equilibriated and eluted with 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, 0 1 mM EDTA, at a flow rate of 15 ml/hr The effluent proteins were monitored at 280 nm Applied sample vols were about 1 ml

Proteinase assay Proteolytic activity was assayed by the method with Sepharose-coupled casein labelled with β-glucosidase, according to ref [28], 0 2 g immobilized substrate protein were incubated with 1 ml sample or standard solns (trypsin or papain) or buffer (blanks), at 45 for 1 hr and then filtered 1 ml of filtrate was assayed for β-glucosidase activity by mixing with 1 ml (2 mg/ml) *p*-nitrophenyl-β-D-glucopyranoside in 1 M Na-Pi buffer, pH 5 0, incubating at 37° for 1 hr, and reading absorbance at 410 nm Appropriate blanks (1 ml sample incubated without the substrate complex) were assayed for β-glucosidase in the same way Proteolytic activity is expressed by reference to known amounts of trypsin or papain

Electrophoresis SDS-PAGE was carried out in 0 1 M imidazole buffer (pH 7 0), 0 1% SDS, according to ref [29], 4–20% acrylamide gradient gels were used All samples were prepared by incubation with 1% SDS and 1% 2-mercaptoethanol, at 100° for 4 min Gels were stained with Coomassie Brilliant Blue R-250 or by silver staining according to ref [30]

Acknowledgements—This research was supported by grants of the Italian Ministero della Pubblica Instruzione Thanks are due to Professor G Ramponi for his continuing interest in our work

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