Purification and Characterization of Myristoyl-Acyl Carrier Protein Thioesterase from *Iris tectorum*

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The myristoyl-acyl carrier protein (ACP) specific thioesterase from *Iris tectorum* was purified to a considerable homogeneity and characterized. The enzyme was eluted with a considerable stability by double-gradients using Triton X-100 and low ionic KCl or Na-phosphate through DEAE-52, Octyl-Sepharose, Q-Sepharose, and hydroxyapatite chromatography. SDS-PAGE analysis showed a single band of 39 kDa. The native molecular weight was estimated to be 82 kDa by Sephacryl S-200 chromatography, indicating that the enzyme was a dimer. The thioesterase showed a chain-length specificity to myristoyl-ACP in preference to other-ACPs. The enzyme activity decreased by 1.0 mM myristate to about 27% of the original activity, whereas the re-maining activity with decanoate was about 90%. The purified thioesterase was inhibited by myristoyl-CoA more than by myristate, suggesting that the myristoyl-ACP thioesterase might be controlled by myristic acid and/or a subsequent product myristoyl-CoA. In addition, some biochemical characteristics of the enzyme were described.

Key words : Iris tectorum, *myristoyl-acyl carrier protein thioesterase.*

Fatty acid biosynthesis in plants is catalyzed by a group of discrete enzymes[1] including protein cofactor, ACP.[2] Of these, acyl-ACP thioesterases have been suggested to be involved in chain termination by catalyzing the hydrolysis of acyl-ACP thioesters. Free fatty acids produced thus in the chloroplast[3] may be used for the production of chloroplast lipids or synthesized as CoA esters.[4-6] Acyl-ACP thioesterases have been found in the animal,[7,8] plant[9,10] and bacterial[11,12] kingdoms. Despite their important role in fatty acid biosynthesis, complete purification of appropriate acyl-ACP thioesterases has not been common. The acyl-ACP thioesterases purified from plants are mostly specific to oleoyl-ACP.[10,13,14]

How biosynthesis of fatty acids with different chain-lengths can be controlled by different types of acyl-ACP thioesterases has been little understood. The active site of acyl-ACP thioesterases has been partly investigated,[9,15,16] showing a SH group in the active site. The molecular weights of plant acyl-ACP thioesterase were in the range from 70~80 kDa[9,10,14] to 42[15] or 28 kDa.[17]

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Abbreviations: ACP, acyl carrier protein; DTNB, 5,5'-dithio-*bis*-(2-nitro benzoic acid).

The great loss of activity[10] and the salt inhibition[17] have been reported for the acyl-ACP thioesterases. Through an observation, myristoyl-ACP thioesterase from iris roots used in this study was very unstable in relatively high salt concentrations. Due to the instability and resultant difficulty in purification, studies of biochemical properties and regulation mechanisms of acyl-ACP thioesterases might have been impeded. It has been found that *Iris tectorum* roots contained a relatively large proportion of myristic acids in the total fatty acid composition. The acyl-ACP thioesterases purified from plant materials were mostly from seeds[10,14] or cotyledons.[13,15] Myristoyl-ACP thioesterase from Iris tectorum roots was investigated and developed a purification procedure with a considerable enzyme stability.

Materials and Methods

Plant materials. *Iris tectorum* Maxim was grown in the fields with natural illumination. After harvest, roots were dissected as soon as possible and either directly used or stored at -70°C.

Preparation of substrates. [1-^{14}C]fatty acids (lauric, myristic, palmitic, stearic, and oleic acid) with specific radioactivities of 1.85~2.20 GBq mmol^{-1} were obtained

from Amersham (Amersham, UK) and ICN (Costa Mesa, USA). All [1-^{14}C]acyl-ACP substrates were prepared from radioactive fatty acids using ACP and acyl-ACP synthetase (both from *Escherichia coli*, Sigma) and then purified according to the method described by Rock and Garwin.[18] The acyl-ACPs were stored frozen at -20°C, with less than 1% hydrolysis over 4 months.

Crude extraction All the purification procedures including crude extraction were carried out at 4°C. Iris roots (500 g) were shredded and homogenized for 2 min in 1 liter of 40 mM potassium phosphate buffer (pH 7.2) supplemented with 2 mM benzamidine hydrochloride, 0.02 mM leupeptin, 1 mM polyvinyl polypyrrolidone, 1 mM dithiothreitol (DTT), 1 mM EDTA, 2 mM KF, 5 mM MgSO₄, 0.1% Triton X-100, and 5% glycerol. The resulting homogenate was clarified by filtration through 3 layers of cheese cloth. After centrifugation at 8,000 g for 20 min, the supernatant was further clarified by filtration using 2 layers of nylon cloth.

DEAE-52 chromatography. The above filtrate (300 ml) was dialyzed in 25 mM sodium phosphate buffer (pH 7.8) comprising 2 mM benzamidine hydrochloride, 1 mM DTT, 0.4 mM EDTA, 2 mM KF, and 5% glycerol (buffer A) and then loaded on a DEAE-52 column (4× 25 cm). The column was eluted with buffer A containing linear double-gradients of Triton X-100 (0~0.4%) and KCl (0~200 mM).

Octyl-Sepharose CL-4B chromatography. The active fractions from DEAE-52 chromatography were dialyzed with buffer A and loaded onto an Octyl-Sepharose CL-4B column equilibrated with the same buffer. After rinsing the column, proteins were eluted with increasing concentrations of Triton X-100 (0~0.4%) and KCl (0~200 mM). Protein fractions were collected and myristoyl-ACP thioesterase activity was measured. The DEAE-52 and Octyl-Sepharose chromatography were repeated twice to obtain sufficient proteins for further purification step.

Biogel P-100 chromatography. Gel chromatography was performed with the Octyl-Sepharose CL-4B pooled-fractions using Biogel P-100 column (5×60 cm). Proteins were eluted with 25 mM sodium phosphate buffer (pH 7.6) plus 1 mM DTT, 0.4 mM EDTA, 1 mM KF, and 6% glycerol (buffer B). The fractions containing the thioesterase activity were collected.

Q-Sepharose chromatography. The active fractions from Biogel P-100 chromatography were applied to a Q-Sepharose column (2×10 cm) equilibrated with buffer B. After rinsing the column with the same buffer, proteins were eluted with 200 ml of the same buffer containing linear double-gradients of Triton X-100 (0~0.5%) and KCl (0~200 mM).

Hydroxyapatite chromatography. The fractions obtained from Q-Sepharose chromatography was dialyzed in 25 mM sodium phosphate buffer (pH 7.0) supplement-

ed with 1 mM DTT, 0.2 mM EDTA, 1 mM KF, and 6% glycerol for 4 hours and then applied to a hydroxyapatite column (1×10 cm) equilibrated with the same buffer. Proteins were eluted using linear double-gradients of Triton X-100 (0~0.5%) and sodium phosphate (25~200 mM, pH 7.0).

Thioesterase assay. The standard acyl-ACP thioesterase assay was performed in the reaction mixture (final volume 100 µ*l*) containing 50 mM Tris/HCl (pH 8.0) and 0.5 µM [1-^{14}C]myristoyl-ACP in microtubes according to the method of Ohlrogge *et al.*[9] Enzymatic reaction was initiated by an addition of enzyme and incubated for 5 min at 37°C. The reaction was stopped by an addition of 300 µ*l* of acetic acid (1 M) in isopropanol. The liberated radio-labelled fatty acid was partitioned into 1 ml of diethyl ether and radioactivity was counted with liquid scintillation counter (Beckman). A correction was made for the small amount of fatty acid liberated in the absence of enzyme preparation. One unit of activity was defined as the production of 1 nmol of liberated fatty acid per minute.

Analytical methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli.[19] Slab gel consisted of 12.5% acrylamide running gel and 5% stacking gel was used. Gels were stained with Coomassie brilliant blue (Sigma) or by the silver staining kits (Bio-Rad) according to the manufacturer's instruction. Protein concentration was determined by the method of Bradford[20] using bovine serum albumin as a standard.

Native molecular weight determination. Native molecular weight of the purified thioesterase was determined by gel permeation chromatography using Sephacryl S-200. The column (2×90 cm) was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 7.2) containing 0.2 mM EDTA, 0.01% Triton X-100, and 5% glycerol. The following standard proteins were used for the calibration; aldolase (M$_r$=158,000), phosphorylase b (94,000), bovine serum albumin (66,000), ovalbumin (43,000), and cytochrome c (12,000). The column was eluted with the same buffer described above and the thioesterase activity was measured.

Chemical modification. The DTT-free enzyme solution was reacted with chemical modification reagents in 100 µ*l* (final volume) sodium phosphate buffer (25 mM, pH 7.0) or sodium carbonate buffer (25 mM, pH 8.5) at 25°C. After incubation for 20 min, the reaction mixtures were measured for residual activity under standard assay conditions. In order to test the protection of the thioesterase against chemical modification, the enzyme was pre-incubated with appropriate concentrations of myristate for 5 min. All chemical reagents were prepared just before use. Stock solutions of *N*-ethylmaleimide, phenylmethylsulfonyl fluoride, and 2-(2'-nitrophenylsulfenyl)-3-

methyl-3-bromoindolenine (BNPS-skatole) were prepared by dissolving in 70% dimethylformamide.

Results and Discussion

Enzyme stability and purification of the thioesterase. Based on the instability of other acyl-ACP thioesterases, the stability of myristoyl-ACP thioesterase from iris roots was tested in a preliminary experiment prior to enzyme purification. Ammonium sulfate fractionation of the crude extract resulted in a considerable protein precipitation but the thioesterase activity drastically decreased as saturation percentage was increased. A preliminary experiment was performed in order to test the potential of Triton X-100 and KCl to elute proteins from DEAE-52 chromatography (Table 1). The thioesterase was bound to the column and eluted by several elution conditions described. The advantage of eluting the enzyme by Triton X-100 plus 0.1 M KCl was the considerable retention of the thioesterase stability. The elutions using 0.2~1.0 M KCl were not appropriate to elute the enzyme since the recoveries were much less. The thioesterase activity of the crude extract was not decreased by adding Triton X-100 up to 1.0% (v/v) (data not shown).

The elution of proteins from DEAE-52 column was, therefore, carried out using a double-gradients of Triton X-100 and KCl. The resulting active fractions were then applied to an Octyl-Sepharose column and eluted with increasing concentrations of Triton X-100 and KCl. By

this chromatography, the thioesterase was also eluted with a considerable stability (Table 2). Similarly, the elutions of thioesterase from Q-Sepharose and hydroxyapatite columns (Fig. 1) were performed by double-gradients of Triton X-100/KCl or Triton X-100/Na-phosphate, respectively.

The thioesterase was purified to a considerable homogeneity, showing about 680-fold of purification and 25% yield by the chromatographic steps (Table 2). The purified enzyme migrated as a single band with a molecular weight of 39 kDa from analysis on SDS-PAGE (Fig. 2).

Substrate specificity of the thioesterase. The substrate specificities of the purified enzyme and of the crude extract were tested using various acyl-ACPs (Table 3). The thioesterase showed strong preference for myristoyl-ACP rather than for other fatty acyl-ACPs. The enzyme barely hydrolyzed lauroyl-ACP and stearoyl-ACP. The K_m values for the acyl-ACPs were in the range from 1.30 to 3.32 μM, but V_{max} was apparently high for myristoyl-ACP. The chain-length specific activity of the purified enzyme compared with the broad activity of the crude extract suggested that acyl-ACPs hydrolysis activities found in *Iris tectorum* were not associated with one protein. The enzyme showed a similar range of K_m value but somewhat difference of V_{max} value amongn other substrates. This is due to a broad range of substrate binding site but a distinct catalytic site, in the enzyme.

The marked preference and relatively high V_{max} value for myristoyl-ACP suggested that the thioesterase from *Iris tectorum* roots played an important role in the selectivity of fatty acyl groups during fatty acid biosynthesis. The enzyme in this work was distinct from the long-chain acyl-ACP specific thioesterases found in *Cucurbita moschata*,[13] *Brassica napus*,[14] *Carthamus tinctorius*,[10]

Table 1. Comparative analysis of the elution potential and of the enzyme stability by different elution conditions from DEAE-52 chromatography.

Elution	Total protein eluted (mg)	Total activity[a] (units)	Total activity[b] (units)
1) Triton X-100 (0.4%) plus KCl 0.1 M	32.6	80.1	76.5
2) KCl 0.2 M	24.3	30.8	20.2
3) KCl 0.5 M	34.2	41.5	14.3
4) KCl 1.0 M	40.7	12.4	4.2

Each 10 ml of the crude extract was loaded onto four separate DEAE-52 columns. Proteins were eluted separately with 6 ml of the same buffer containing Triton X-100 or different concentrations of KCl. After eluting proteins, myristoyl-ACP thioesterase activity of the samples was measured immediately (a) and after 12 h of storage at 4°C (b).

Table 2. Purification of myristoyl-ACP thioesterase from *Iris tectorum*.

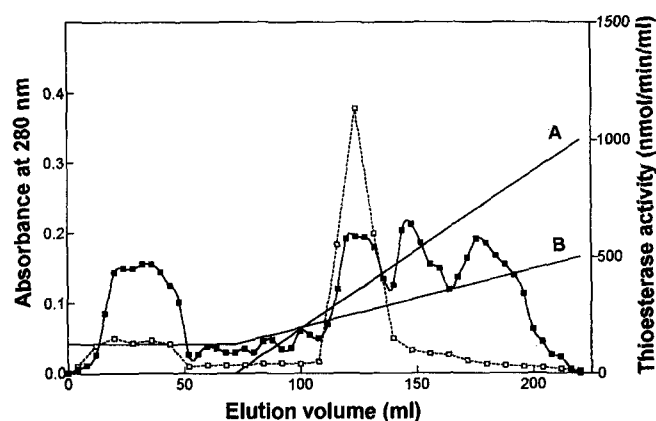| Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)
Crude extract	3100	7984	2.57	1.0
DEAE-52	952	6402	6.73	2.6
Octyl-Sepharos	210	4850	23.10	9.0
Biogel P-100	38	4105	108.30	42.0
Q-Sepharose	9	3051	339.00	131.9
Hydroxyapatite	1.2	2102	1751.67	681.6

Fig. 1. Elution profile of the myristoyl-ACP thioesterase from hydroxyapatite chromatography. The active fractions from Q-Sepharose chromatography was applied to a hydroxyapatite column. Proteins were eluted with the buffer containing a double-gradient of Triton X-100 (0~0.5%, line A) and sodium phosphate buffer (pH 7.0, 25~100 mM, line B). Absorbance at 280 nm (filled rectangle) and the thioesterase activity (open rectangle) were measured.
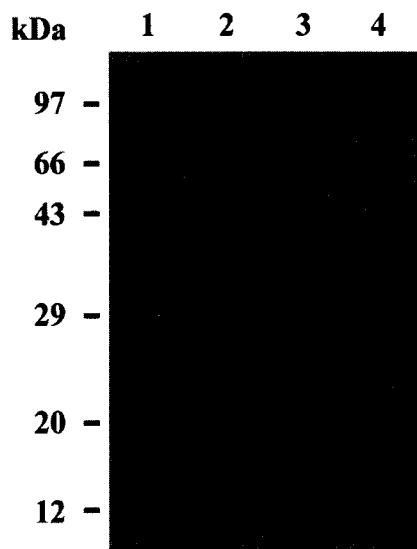
Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified myristoyl-ACP specific thioesterase from iris roots. Lane 1; reference proteins, lane 2; the fraction from Biogel P-100 chromatography, lane 3; the fraction from Q-Sepharose chromatography, lane 4; the purified thioesterase from hydroxyapatite chromatography. The purified enzyme (lane 4) was concentrated about 3-fold for better resolution. Reference proteins were phosphorylase b (M_r=97,000), BSA (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), trypsin inhibitor (20,100), and cytochrome c (12,000). The gel was stained with Coomassie brilliant blue and then with silver nitrate.

Table 3. Substrate specificity and kinetic data of the purified thioesterase.

Substrates	Activity[a] (cpm)	Activity[b] (cpm)	K_m (μM)	V_{max} (pmol·min⁻¹·μg⁻¹)
Lauroyl-ACP	2250	486	1.30	6
Myristoyl-ACP	6905	3510	2.90	41
Palmitoyl-ACP	7504	1201	3.32	14
Stearoyl-ACP	3845	260	1.54	5
Oleoyl-ACP	4121	694	2.54	10

Substrate specificities were tested for the crude extract (a) and the purified thioesterase (b). Activity (a and b) was expressed in cpm values when concentration of all substrates was 0.5 μM. K_m and V_{max} values were obtained from Lineweaver-Burk plots.

and from the lauroyl-ACP specific enzyme of *Umbellularia californica*.[15]

Product inhibition of the thioesterase. Product inhibition of the thioesterase was investigated by measuring activity at varying concentrations of different fatty acids (Fig. 3). It was interesting to note that the inhibition was specific to the chain-length and the enzyme activity was decreased by myristate the most among the fatty acids. The activity was decreased to 27% of the original activity by 1.0 mM myristate, indicating a 7-fold decrease compared with that by 1.0 mM decanoate. The inhibition of enzyme activity was dependent on the fatty acid concentration (Fig. 3-A). The thioesterase was also inhibited by fatty acyl-CoAs (Fig 3-B). The CoA thioester of myr-
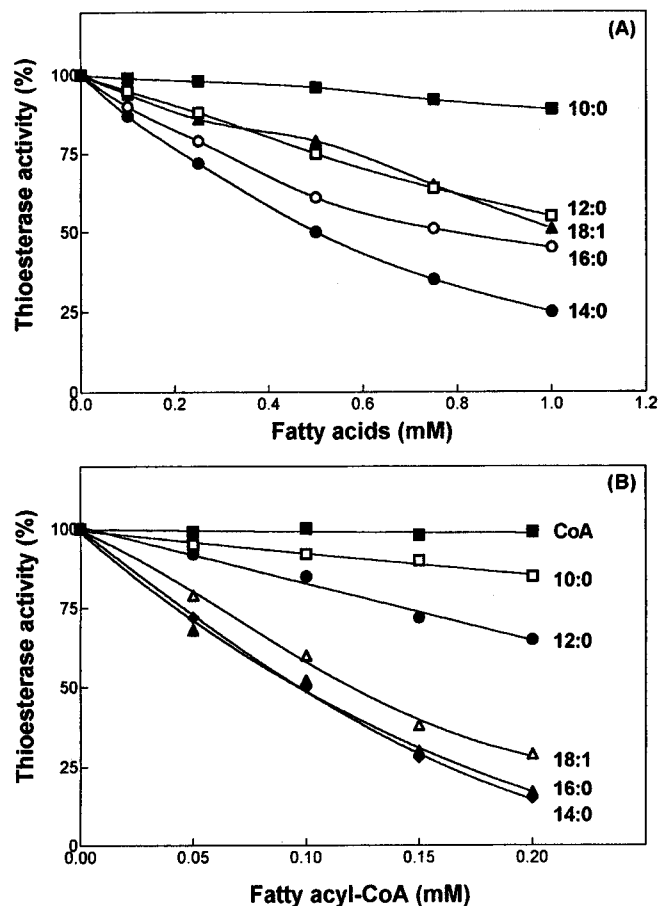

Fig. 3. Inhibition of the myristoyl-ACP thioesterase by fatty acids and by fatty acyl-CoAs. The enzyme was preincubated with fatty acids (A) or fatty acyl-CoAs (B) for 10 min and myristoyl-ACP hydrolysing activity was measured. Even though acyl-CoAs were well solubilized, the same concentration of DMSO was also added to the reaction mixture of acyl-CoAs-inhibition for impartial comparison. 100% activity was equivalent to 2812 cpm.

istate was more effective to inhibit the thioesterase than myristate. The extent of inhibition by 1.0 mM myristate was equivalent to that by 0.16 mM myristoyl-CoA. The inhibition by acyl-CoA was roughly specific to chain-length. These results suggested that the myristoyl-ACP thioesterase from *Iris tectorum* might be controlled *via* specific inhibition by the thioesterase product, myristic acid and/or a subsequent product, myristoyl-CoA.

Only one specific acyl-ACP thioesterase might not act exclusively during fatty acid biosynthesis and a proper control mechanism might be exerted between different acyl-ACP thioesterases. So, the activities of acyl-ACP thioesterases in fatty acid biosynthesis of various chain-lengths could be controlled by product inhibition.

Other characteristics. The thioesterase was treated with various chemical modification reagents to search the active site(s) of the enzyme. As shown in Table 4, the activity was considerably decreased by treatment with acylation reagents including acetic anhydride, *N*-ethylmaleim-

Table 4. Effects of various modifying reagents on the myristoyl-ACP thioesterase.

Reagents	Relative activity (%)	
	pH 7.0	pH 8.5
Control	100	100
Acetic anhydride	25	28
Succinic anhydride	54	56
Citraconic anhydride	50	60
Iodoacetamide	70	80
Sulfo-NHS-acetate	126	85
N-Ethylmaleimide	50	40
BNPS-skatole	92	114
2-Iminothiolane-HCl	117	123
p-Hydroxyphenylglyoxal	91	75
2,3-Butanedione	107	101
Phenylmethylsulfonyl fluoride	52	55

The enzyme was incubated with 1.0 mM of the various modification reagents in 100 µl (final volume) of 25 mM sodium phosphate buffer (pH 7.0) or sodium carbonate buffer (pH 8.5) at 25°C for 20 min. Enzymatic activity was presented as activity in percentage compared with the control activities which were equivalent to 3245 and 3011 cpm at pH 7.0 and pH 8.5, respectively. All values were the means of two experiments.

ide, and phenylmethylsulfonyl fluoride which were specific to amino, sulfhydryl, and hydroxyl group, respectively. Acylation reagents such as acetic, succinic, and citraconic anhydride reacted with sulfhydryl groups in addition to amino groups. In this respect, the thioesterase activity was tested at varying concentrations of reduced-glutathion, N-ethylmaleimide, or DTNB (Fig. 4). The activity remained relatively constant with increasing concentration of glutathione, whereas the activity seriously decreased along with increasing concentration of N-ethylmaleimide or DTNB. The enzyme was incubated with each 0.2 mM of either phenylmethylsulfonyl fluoride, N-ethylmaleimide, or acetic anhydride in the presence and absence of 1.0 mM myristate and assayed for residual ac-
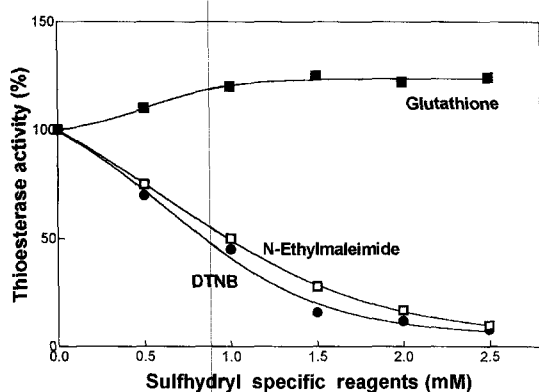

Fig. 4. Reduction and oxidation on the myristoyl-ACP thioesterase activity. The enzyme was preincubated with reduced-glutathion for 10 min and then measured for residual activity. Oxidation of cysteinyl residues was carried out with different concentration of 5,5'-dithio-bis-(2-nitrobenzoic acid). Alternatively, cysteines were blocked by covalent bonds using N-ethylmaleimide. The enzyme activity was represented as control activity in percentage which was equal to 3412 cpm.

tivity. Addition of myristate protected partly (about 36%) against the N-ethylmaleimide-mediated inactivation, while any essential protection was not observed from phenylmethylsulfonyl fluoride- or acetic anhydride-inactivation (data not shown). Collectively, these results suggested that cysteinyl residue might be located at the active site of the thioesterase. The thioesterase from *Umbellularia californica*[16] and avocado mesocarp[9] were considerably inactivated by reagents which attacked primarily sulfhydryl groups.

The native molecular weight of the thioesterase was determined by Sephacryl S-200 chromatography. The elution position of the thioesterase activity was equivalent to a molecular weight of 82,000, indicating that the thioesterase was a dimer.

The effect of pH on the thioesterase activity was tested over a pH range from 4 to 11 (Fig. 5). The enzyme activity showed a pH optimum of 7.8 and decreased rapidly as the pH was changed in either direction. The activity at pH 6.5 and 10.0 were 40 and 18% of the maximal, respectively, showing strong pH dependency.
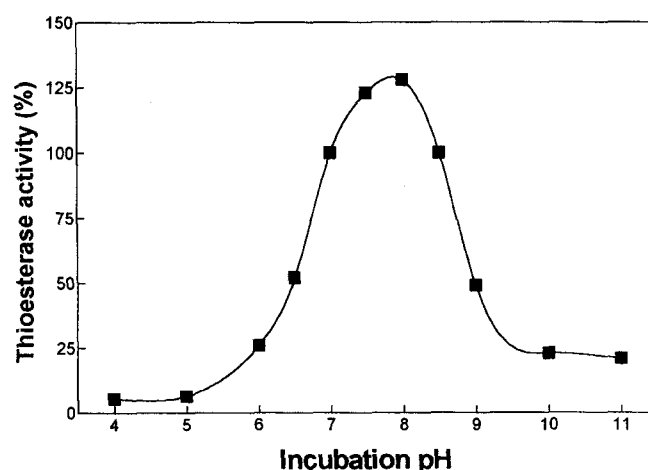

Fig. 5. Effect of pH on the myristoyl-ACP thioesterase activity. Enzymatic reactions were performed using the following buffers: pH 4-5, Na-acetate; pH 6, K-phosphate; pH 7-11, Tris-HCl. The activity at pH 7.0 was considered 100% which was equivalent to 3250 cpm.

Table 5. Effect of divalent cations on the myristoyl-ACP thioesterase activity.

Divalent ions	Relative activity (%)
Control	100
Mg^{2+}	140
Mn^{2+}	97
Cu^{2+}	6
Cd^{2+}	0
Co^{2+}	3
Zn^{2+}	1

The thioesterase was incubated in the presence of each cation individually. All cations were added as Cl⁻ salts (1 mM). EDTA in the sample was eliminated by ultrafiltration using Centricon-10. Activities were represented as activity in percentage compared with the control activity which was equivalent to 3328 cpm.

Effect of various divalent cations on the thioesterase activity was tested (Table 5). The presence of 1 mM Mg^{2+} increased the activity by 40%. This might not be attributed merely to ionic strength since Mg2+ concentration was low (1 mM). Other cations Cu^{2+}, Cd^{2+}, Co^{2+}, and Zn^{2+} (each 1 mM) were severely inhibitory to the activity.

References

1. Shimakata, T. and Stumpf, P. K. (1982) The procaryotic nature of the fatty acid synthetase of developing *Carthornus tinctorius* L. (safflower) seeds. *Arch. Biochem. Biophys.* **217**, 144-154

2. Harwood, J. L. (1988) Fatty acid metabolism. *Annu. Rev. Plant Physiol. Mol. Biol.* **39**, 101-138

3. Ohlrogge, J. B., Kuhn, D. N. and Stumpf, P. K. (1979) Subcellular localization of acyl carrier protein in leaf protoplasts of Spinacia oleracea. *Proc. Natl. Acad. Sci. USA* **76**, 1194-1198.

4. Joyard, J. and Stumpf, P. K. (1981) Synthesis of long-chain acyl-CoA in chloroplast envelope membranes. *Plant Physiol.* **67**, 250-256.

5. Roughan, P. G. and Slack, C. R. (1977) Long chain acyl-coenzyme A synthetase activity of spinach chloroplast is concentrated in the envelope. *Biochem. J.* **162**, 457-459.

6. Joyard, J. and Douce, R. (1977) Site of synthesis of phosphatidic acid and diacylglycerol in spinach chloroplasts. *Biochim. Biophys. Acta* **486**, 273-285.

7. Lin, C. Y. and Smith, S. (1978) Properties of the thioesterase component obtained by limited trypsinization of the fatty acid synthetase multienzyme complex. *J. Biol. Chem.* **253**, 1954-1962.

8. Rogers, L., Kolattukudy, P. E. and de Renobales, M. (1982) Purification and characterization of S-acyl fatty acid synthase thioester hydrolase which modifies the product specificity of fatty acid synthase in the uropugial gland of mallard. *J. Biol. Chem.* **257**, 880-886.

9. Ohlrogge, J. B., Shine, W. E. and Stumpf, P. K. (1978) Fat metabolism in higher plants. *Arch. Biochem. Biophys.* **189**, 382-391.

10. Mckeon, T. A. and Stumpf, P. K. (1982) Purification and characterization of the stearoylacyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower. *J. Biol. Chem.* **257**, 12141-12147.

11. Barnes, E. M. (1975) Long-chain fatty acyl thioesterase I and II from *Escherichia coli. Meth. Enzymol.* **35**, 102-109.

12. Seay, T. and Lueking, D. R. (1986) Purification and properties of acyl coenzyme A thioesterase II from *Rhodopseudomonas sphaeriodes. Biochemistry* **25**, 2480-2485.

13. Imai, H., Nishida, I. and Murata, N. (1992) Acyl-(acyl-carrier protein) hydrolase from squash cotyledons specific to long-chain fatty acids: purification and characterization. *Plant Mol. Biol.* **20**, 199-206.

14. Hellyer, A., Leadlay, P. F. and Slabas, A. R. (1992) Induction, purification and characterization of acyl-ACP thioesterase from developing seeds of oil seed rape (*Brassica napus*). *Plant Mol. Biol.* **20**, 763-780.

15. Davies, H. M., Anderson, L., Fan, C. and Hawkins, D. J. (1991) Developmental induction, purification, and further characterization of 12:0-ACP thioesterase from immature cotyledons of *Umbellularia californica. Arch. Biochem. Biophys.* **290**, 37-45.

16. Pollard, M. R., Anderson, L., Fan, C., Hawkins, D. J. and Davies, H. M. (1991) A specific acyl-ACP thioesterase implicated in medium-chain fatty acid production in immature cotyledons of *Umbellularia californica. Arch. Biochem. Biophys.* **284**, 306-312.

17. Dormann, P., Spencer, F. and Ohlrogge, J. B. (1993) Characterization of two acyl-acyl carrier protein thioesterases from developing *Cuphea* seeds specific for mediumchain- and oleoyl-acyl carrier protein. *Planta* **189**, 425-432.

18. Rock, C. O. and Garwin, J. L. (1979) Preparative enzymatic synthesis and hydrophobic chromatography of acyl-acyl carrier protein. *J. Biol. Chem.* **254**, 7123-7128.

19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* **227**, 680-685.

20. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.