J. Biochem. Mol. Biol. Vol. 29, No. 5 pp. 436~441 September 30, 1996

Biochemical Characteristics of a Palmitoyl Acyl Carrier Protein Thioesterase Purified from *Iris pseudoacorus*

Han-Chul Kang* and **Young-Soo Hwang**

Division of Biochemistry, National Agricultural Science and Technology Institute, Rural Development Administration, Suwon 441-707, Korea

(Received May 15, 1996)

Abstract: The palmitoyl acyl carrier protein (ACP) specific thioesterase (EC 3.1.2.14) from *Iris pseudoacorus* was purified and characterized. The thioesterase which was very unstable in relatively high salt concentrations was eluted using a co-gradient of Triton X-100 and low concentration of KCl or Na-phosphate from Q-Sepharose, DEAE-Sepharose, and hydroxyapatite chromatography. SDS-PAGE analysis showed a single band with a molecular weight of 35,000. The native molecular weight of approximately 37,000 was estimated by Sephacryl S-200 chromatography, indicating that the enzyme is a monomer. The thioesterase activity was inhibited about 75% and 50% by N-ethylmaleimide (2 mM) and phenylmethylsulfonyl fluoride (2 mM), respectively. The N-ethylmaleimide-inactivation was protected by sodium palmitate but the inactivation with phenylmethylsulfonyl fluoride was not protected. Oxidation of thiols by 2 mM 5,5′-dithio-*bis*-(2-nitrobenzoic acid) resulted in 65% inactivation of the enzyme. These results suggest that a cysteinyl residue is essential to the catalytic reaction of the enzyme. The enzyme activity was increased by sodium citrate and also by Cu^{2+}

Key words: active site, co-gradient protein elution, palmitoyl acyl carrier protein thioesterase.

Thioesterases are involved in various biochemical reactions including the biosynthesis of fatty acids (De Renobales *et al.*, 1980; Voelker *et al.*, 1992). Acyl-ACP thioesterases have been suggested to be involved in chain termination during fatty acid biosynthesis by catalysing the hydrolysis of acyl-ACP thioesters to free fatty acid and ACP. It is believed that free fatty acid is transferred to the cytoplasm for complex lipids biosynthesis (Ohlrogge *et al.*, 1978). Acyl thioesterases have been found in the animal (Lin and Smith, 1978; Rogers *et al.*, 1982), plant (Ohlrogge *et al.*, 1978; Mckeon and Stumpf, 1982) and bacterial (Barnes 1975; Seay and Lueking, 1986) kingdoms. Despite their important role in fatty acid biosynthesis, complete purifications of appropriate acyl-ACP thioesterases have not been common. Furthermore, some acyl-ACP thioesterases purified from plants are mostly oleoyl-ACP specific enzymes (Mckeon and Stumpf, 1982; Davies *et al.*, 1991; Amanda *et al.*, 1992).

By reason of insufficient purification and instability of acyl-ACP thioesterases, studies of the biochemical properties and regulation mechanisms have been difficult. Also, there are many differences in the molecular weights of plant acyl-ACP thioesterases. These values are in the range of 70,000~80,000 for avocado (Ohlrogge *et al.*, 1978), safflower (Mckeon and Stumpf, 1982), and oil seed rape (Amanda *et al.*, 1992). But the molecular weight of the 12:0-ACP thioesterase from *Umbellularia californica* was reported to be 42,000 with a monomer (Davies *et al.*, 1991) and for the medium chain acyl-ACP thioesterase from Cuphea seeds to be a value of 28,000 (Dormann *et al.*, 1993). The medium chain thioesterase from *Umbellularia californica* was inhibited by N-ethylmaleimide (Pollard *et al.*, 1991), however, on the whole, much is also unknown on the active site of acyl-ACP thioesterases. In this paper, we describe the purification of 16:0-ACP thioesterase from iris as well some biochemical properties.

Materials and Methods

Plant material

Iris pseudoacorus L. was grown in fields with natural illumination. After harvest, roots were dissected as soon as possible and directly used or stored at −70℃.

Chemicals

Q-Sepharose, DEAE-Sepharose, and Sephacryl S-200 were purchased from Pharmacia (Uppsala, Swe-

*To whom correspondence should be addressed.
Tel: 82-331-290-0365, Fax: 82-331-290-0307.

den). Hydroxyapatite and Biogel P-100 were obtained from Bio-rad (Richmond, USA). N-ethylmaleimide, phenylmethylsulfonyl fluoride, and 5,5'-dithio-*bis*-(2-nitrobenzoic acid) were from Sigma (St. Louis, USA). All other chemicals used were of reagent grade or the best available purity.

Preparation of substrates

(1-^{14}C) fatty acids (lauric, myristic, palmitic, stearic, and linoleic acid) with specific radioactivities of 1.85~2.20 GBq mmol^{-1} were obtained from Amersham and ICN. Acyl carrier protein and acyl-ACP synthetase were from Sigma. All (1-^{14}C) acyl-ACP substrates were synthesized and purified according to the method described by Rock and Garwin (1979). The acyl-ACPs were stored frozen at −20℃ with less than 1% hydrolysis over 4 months.

Crude extraction

All the following procedures including purification steps were carried out at 4℃. Iris roots (1 kg) were homogenized in 2 liter of 40 mM potassium phosphate buffer (pH 7.0) supplemented with 2 mM benzamidine hydrochloride, 1 mM polyvinyl polypyrrolidone, 1 mM dithiothreitol, 1 mM EDTA, 2 mM KF, 0.5% Triton X-100, and 15% glycerol. The resulting homogenate was clarified by filtration through 4 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.

Chromatography on Biogel P-100

The crude extract was directly loaded on a Biogel P-100 column (5 cm×60 cm) which was equilibrated with 20 mM potassium phosphate buffer (pH 7.6) containing 0.4 mM EDTA, 2 mM KF, and 10% glycerol (buffer A). The column was eluted with the same buffer and the 16:0-ACP thioesterase activity was measured.

Chromatography on Q-Sepharose

The protein fractions from Biogel p-100 chromatography were loaded on a Q-Sepharose column (2 cm×10 cm) equilibrated with buffer A. Proteins were eluted with the same buffer containing linear co-gradient of Triton X-100 (from 0 to 1.0%) and KCl (from 0 to 100 mM) and the 16:0-ACP thioesterase activity was monitored. This step and the above Biogel P-100 chromatography were repeated two times to obtain sufficient proteins for further purification steps.

Gel filtration on Sephacryl S-200

The pooled fraction containing the thioesterase activity from Q-Sepharose chromatography was loaded onto a Sephacryl S-200 column (5 cm×60 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 0.2 mM EDTA and 8% glycerol. Proteins were eluted by the same buffer at a flow rate of 8 ml/h and the thioesterase activity was monitored.

Chromatography on DEAE-Sepharose

The thioesterase containing fractions from above chromatography were applied to DEAE-Sepharose chromatography (1 cm×10 cm) which was equilibrated with buffer A. After washing the column, proteins were eluted with the same buffer containing linear co-gradient of Triton X-100 (from 0 to 1.0%) and KCl (from 0 to 100 mM).

Chromatography on Hydroxyapatite

The active fractions from DEAE-Sepharose chromatography were dialyzed with 25 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM EDTA, and 8% glycerol then applied to a hydroxyapatite column (1 cm×10 cm) equilibrated with the same buffer. After washing the column, proteins were eluted with 200 ml of the same buffer containing linear co-gradient of Triton X-100 (from 0.01 to 1.5%) and sodium phosphate buffer pH 7.0 (from 25 to 100 mM).

Thioesterase assay

The thioesterase assay was performed in a reaction mixture (final volume, 100 μl) containing 50 mM Tris/HCl (pH 8.0), 0.5 μM (1-^{14}C) palmitoyl-ACP in microtubes. Enzymatic reaction was initiated by the addition of enzyme and incubated for 15 min at 37℃. The reaction was stopped by addition of 300 μl of acetic acid (1 M) in isopropanol. The liberated radiolabelled fatty acid was partitioned into 1 ml of diethyl ether and radioactivity was counted with liquid scintillation counter (Beckman).

Analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). Slab gel consisted of 12.5% acrylamide running gel and 5% stacking gel was used. Gels were stained with Coomassie Brilliant Blue (Sigma). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard protein.

Native molecular weight determination

Native molecular weight of the purified 16:0-ACP specific thioesterase was determined by gel permeation chromatography using Sephacryl S-200. The column (2 cm×90 cm) was equilibrated with 50 mM potassium

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), phospholylase b (94,000), bovine serum albumin (66,000), ovalbumin (43,000), and cytochrome c (12,000). The column was eluted with above buffer and 16:0-ACP thioesterase activity was located.

Chemical modification

The purified enzyme (20 μM) was reacted with chemical modification reagents in 40 mM sodium carbonate buffer (pH 8.0) at 25℃. Aliquots were withdrawn from the reaction mixture and measured for residual activity under standard assay conditions. Stock solutions of phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide, and 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) were prepared just before use by dissolving in 80% dimethyl sulfoxide. This dimethylsulfoxide in the reaction mixtures was kept at less than 4% so that it could not affect the enzyme activity.

Results and Discussion

Enzyme stability and purification

Based on the instability of other acyl-ACP thioesterases (Mckeon and Stumpf, 1982; Rogers *et al.*, 1982; Dormann *et al.*, 1993), the stability of 16:0-ACP thioesterase from iris was tested in a preliminary experiment prior to enzyme purification. Without glycerol, more than 70% of the original activity from the crude extract was lost within 24 h at 4℃. Ammonium sulfate fractionation of the crude extract resulted in considerable protein precipitation but the thioesterase activity was drastically decreased as long as saturation percent is increased (data not shown). Therefore ammonium sulfate fractionation was not adopted for the thioesterase purification. The enzyme activity of the crude extract or the active fraction from Biogel P-100 chromatography was also considerably decreased by the addition of 0.4 M NaCl and about 40% of initial activity was lost even though the sample contained 10~15% glycerol.

The fraction containing 16:0-ACP thioesterase from Sephacryl S-200 chromatography (Fig. 1) was applied to DEAE-Sepharose chromatography (Fig. 2). A coupling gradient of Triton X-100 with relatively low concentration of KCl was used to elute the thioesterase from this chromatography. The thioesterase was bound to the column and eluted with considerable stability. Similarly, the thioesterase elutions from hydroxyapatite chromatography (Fig. 3) was performed by co-gradient elution consisted of Triton X-100 with Na-phosphate, and the thioesterase was also eluted stably from this column. Exclusive salt gradient using such as NaCl or KCl was not adequate to elute the thioesterase from ion exchange or hydroxyapatite columns because relatively high ionic strength for the enzyme elution accelerated irreversibly the activity loss even in the presence of 10% glycerol. The detergent, Triton X-100 could substitute for high ionic strength buffers for thioesterase elution on various columns without affecting the thioesterase stability.

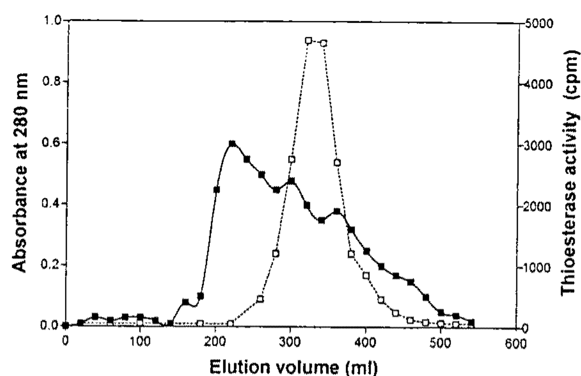

Fig. 1. Elution profile of the 16:0-ACP thioesterase from Sephacryl S-200 chromatography. The active fractions from Q-Sepharose chromatography was applied to a Sephacryl S-200 column. Proteins were eluted (filled rectangle) and the thioesterase activity was measured (open rectangle) as described in materials and methods.

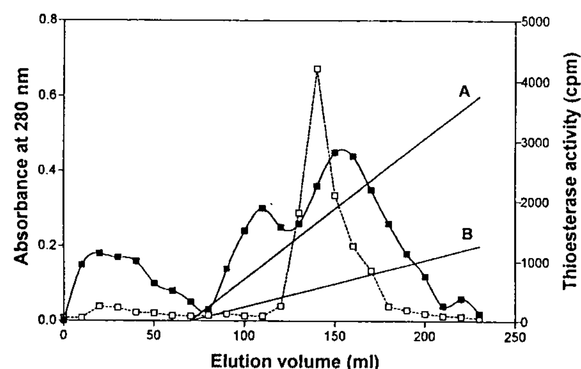

Fig. 2. Elution profile of the 16:0-ACP thioesterase from DEAE-Sepharose chromatography. The active fraction from Sephacryl S-200 chromatography was applied to a DEAE-Sepharose chromatography. Proteins were eluted with 20 mM potassium phosphate pH 7.4 plus 0.2 mM EDTA and 8% glycerol containing linear co-gradients of Triton X-100 (from 0 to 1.0%, line A) and KCl (from 0 to 100 mM, line B). Absorbance at 280 nm (filled rectangle) and the thioesterase activity (open rectangle) were measured.

Based on SDS-polyacrylamide gel electrophoresis, the thioesterase has been purified to homogeneity (Fig. 4 and Table 1). The purified enzyme migrated as a single band which corresponds to a monomeric molecular weight of 35 kDa. The purified enzyme could be

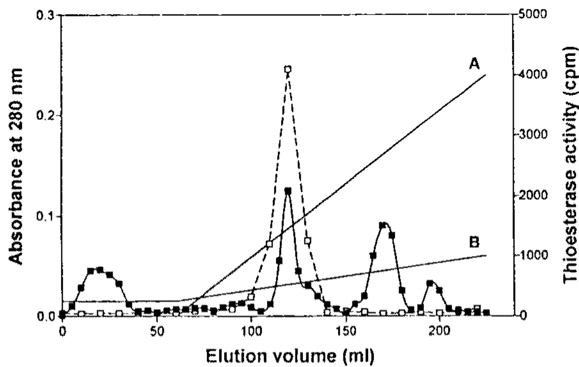

Fig. 3. Elution profile of the 16:0-ACP thioesterase from hydroxyapatite chromatography. The pooled active fraction from DEAE-Sepharose chromatography was dialyzed as described in materials and methods, then applied to a hydroxyapatite column. Proteins were eluted with the buffer containing linear co-gradients of Triton X-100 (from 0 to 1.5%, line A) and Na-phosphate buffer (from 25 to 100 mM, line B). Absorbance at 280 nm (filled rectangle) and the thioesterase activity (open rectangle) were measured.

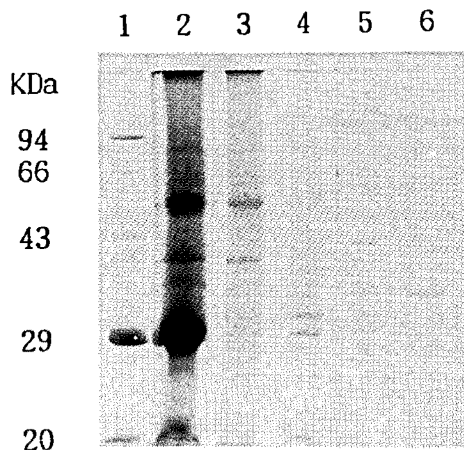

Fig. 4. SDS-polyacrylamide gel electrophoresis of the purified 16:0-ACP thioesterase from iris. Lane 1: reference proteins; lane 2: the active fraction from Biogel P-100 chromatography; lane 3: the active fraction from Q-Sepharose chromatography; lane 4: the active fraction from Sephacryl S-200 chromatography; lane 5: the active fraction from DEAE-Sepharose chromatography, and lane 6: the purified thioesterase from Hydroxyapatite chromatography. The purified thioesterase was concentrated for better resolution about 3 folds by Centricon-10.

stored at −20℃ with 20% glycerol without considerable activity loss up to 3 months.

Substrate specificity

The substrate specificity of the purified enzyme using 12:0, 14:0, 16:0, 18:0, and 18:2-ACP was tested (Table 2). The thioesterase showed strong preference for palmitoyl-ACP rather than for other fatty acyl-ACPs. This result suggest that the 16:0-ACP thioesterase from iris roots plays an important role in the selectivity of fatty acyl groups during lipid biosynthesis.

Table 1. Purification of a palmitoyl-acyl carrier protein thioesterase from iris

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)
Crude extract	1450	2400	1.65	100
Biogel P-100	580	1850	3.18	77
Q-Sepharose	92	1104	12.00	46
Sephacryl S-200	40	720	18.00	30
DEAE-Sepharose	8	452	56.00	19
Hydroxyapatite	3	360	120.00	15

Table 2. Substrate specificity of the purified thioesterase from iris

Substrates	Relative activity
Lauroyl-ACP	175
Myristoyl-ACP	245
Palmitoyl-ACP	1850
Stearoyl-ACP	320
Linolenoyl-ACP	100

Enzymatic reaction was carried out as described under Materials and Methods. Activity was expressed as relative values when all substrates concentrations were 0.5 μM. Thioesterase activity with linolenoyl-ACP was considered as 100.

Molecular weight determination

Due to the inconsistency of published thioesterase molecular weights, the native molecular weight of the 16:0-ACP thioesterase from iris was determined by Sephacryl S-200 chromatography (Fig. 5). The elution position of the thioesterase activity was equivalent to a molecular weight of 37,000, indicating that the thioesterase is a monomer. This molecular weight and a monomer are similar to the 12:0-ACP specific thioesterase from *Umbellularia californica* (Davies *et al.*, 1991).

Active site investigation

The thioesterase was preincubated with serine specific reagent, PMSF (Fig. 6-A) or thiol specific reagent, N-ethylmaleimide (Fig. 6-B) and assayed for its residual activity. More than 50% of the initial activity was lost by addition of 2 mM PMSF or N-ethylmaleimide. Addition of 5 mM sodium palmitate protected most of the thioesterase inactivation mediated by N-ethylmaleimide (Fig. 6-B). But the substrate did not confer any protection against the PMSF mediated inactivation (Fig. 6-A). In an attempt to test an eventual activation by reducing agents, the thioesterase was assayed after preincubation with dithiothreitol or glutathione (reduced), but any es-

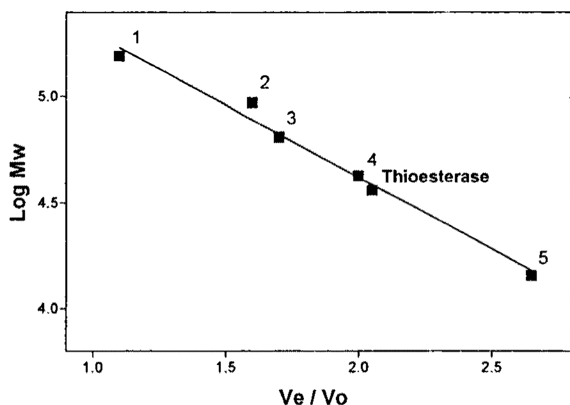

Fig. 5. Molecular weight estimation of the palmitoyl acyl carrier protein thioesterase by Sephacryl S-200 chromatography. Number 1 to 5 represents aldolase (M_r, 158,000), phospholylase b (94,000), bovine serum albumin (66,000), ovalbumin (43,000), and cytochrome c (12,000), serially.

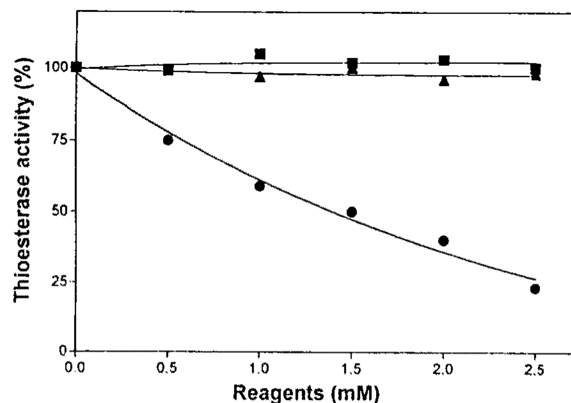

Fig. 7. Reduction and oxidation on the 16:0-ACP thioesterase activity. The enzyme was reduced with dithiothreitol (rectangle) or glutathion (reduced form) (triangle), then measured for its residual activity as described in materials and methods. Oxidation of cysteinyl residues was carried out with different concentration of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) for 10 min (circle). The enzyme activity was represented as % of control activity.

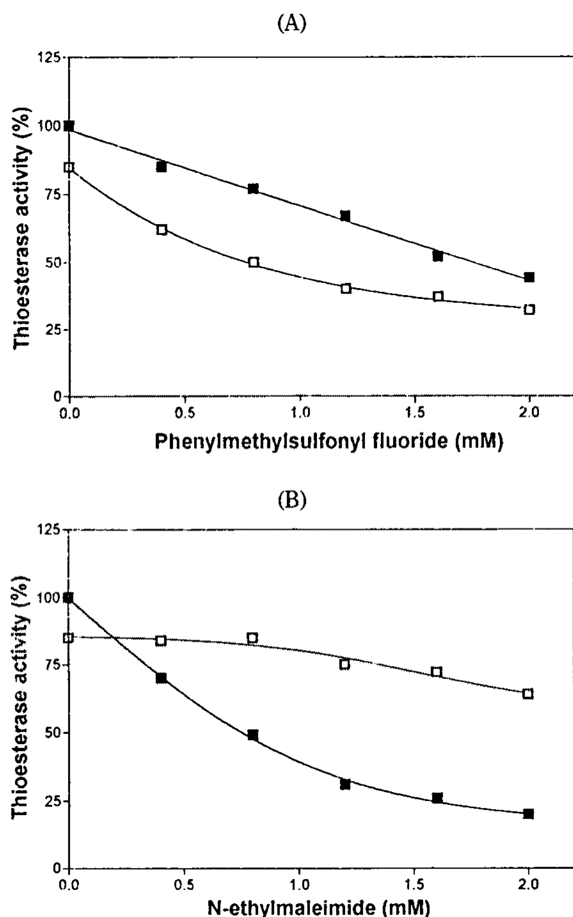

Fig. 6. Inactivation and protection of the 16:0-ACP thioesterase. The enzyme was reacted with different concentration of phenylmethylsulfonyl fluoride in the absence (Panel A, filled rectangle) and presence of sodium palmitate (Panel A, open rectangle) for 10 min and measured for its residual activity as described in materials and methods. The enzyme inactivation by N-ethylmaleimide was performed in the same way without (Panel B, filled rectangle) and with 5 mM sodium palmitate (Panel B, open rectangle). The enzyme activity without any reagents was considered as 100 %.

sential activation or inactivation was not observed (Fig. 7). Sulfhydryl groups of the thioesterase were oxidized by incubation with DTNB, then the residual enzyme activity was measured (Fig. 7). About 75% of the initial activity was lost by oxidation of sulfhydryl groups with 2.5 mM DTNB.

The protection of palmitate against N-ethylmaleimide-inactivation and the thiol oxidation-mediated inactivation of the 16:0-ACP thioesterase from iris suggest that a cysteinyl residue is located at the active site of the thioesterase and is essential for the catalytic reaction. Similarly, the dodecanoyl and lauroyl-ACP thioesterase from Umbellularia californica have been shown to be inhibited by N-ethylmaleimide (Pollard *et al.*, 1991) or diethylpyrocarbonate (Davies *et al.*, 1991). The rat mammary thioesterase II was inhibited by diisopropylphosphorofluoridate and diethylpyrocarbonate (Smith *et al.*, 1986; Randhawa *et al.*, 1987). S-acyl fatty acid synthase thioester hydrolase from uropygial gland of mallard was severely inhibited by the thiol specific reagents N-ethylmaleimide and *p*-hydroxymercuribenzoate and also by the serine-directed reagent phenylmethylsulfonyl fluoride (Rogers *et al.*, 1982).

Other characteristics

The activity change of the thioesterase in the presence of various divalent cations was tested with an EDTA-free sample (Table 3). It is interesting that activity of the thioesterase was increased by Cu^{2+} and this Cu^{2+}-mediated activity increase was reproducible. Ca^{2+} and other divalent cations such as Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+} (each 2 mM) were inhibitory to the thioesterase activity.

Table 3. Effect of divalent cations on the 16:0-ACP thioesterase activity

Divalent cations (2 mM)	Relative activity
Control	100
Mg^{2+}	110
Ca^{2+}	75
Mn^{2+}	44
Cu^{2+}	151
Cd^{2+}	55
Co^{2+}	23
Zn^{2+}	47

All cations were added as Cl^- salts. All other conditions were as indicated for the standard assay conditions. Activities were presented as % activity compared with the control activity which was not added any divalent ions. EDTA which was already contained in the sample during purification procedure, was eliminated by ultrafiltration using centricon-10.

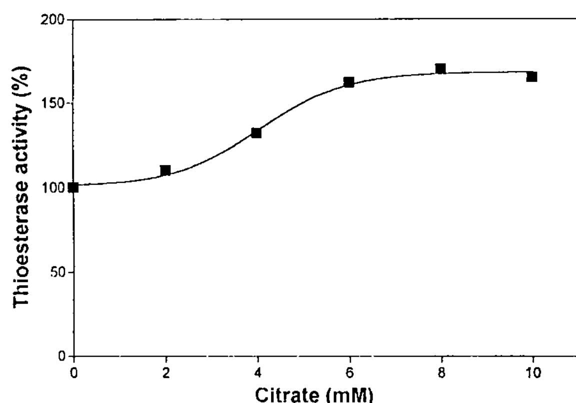

Fig. 8. Activation of the 16:0-ACP thioesterase by citrate. The enzyme was incubated with different concentration of sodium citrate in Tris/HCl, pH 8.0 (50 mM) for 10 min at 25℃ and assayed for its residual activity as described in the text.

Citrate has been recognized as a potential enzyme regulator *in vivo*. Considering this, the eventual activity change by citrate of the thioesterase was tested (Fig. 8). The enzyme activity was increased about 60~70% by 5~10 mM citrate under the conditions described.

Thermal stability of the thioesterase was tested with an active fraction from DEAE-Sepharose chromatography. Over 70% of the original activity was lost by 20 min incubation at 50℃. This thermal instability of the 16:0-ACP thioesterase is similar to the thiesterase from oil seed rape (Amanda *et al.*, 1992).

Acknowledgement

This work was supported by a grant (post doctoral fellowship) from Korea Science and Engineering Foundation (1996).

References

Amanda, H., Peter, F. L. and Antoni, R. S. (1992) *Plant Mol. Biol.* **20**, 763.

Barnes, E. M. (1975) *Method Enzymol.* **35**, 102.

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.

Davies, H. M., Anderson, L., Fan, C. and Hawkins, D. J. (1991) *Arch. Biochem. Biophys.* **290**, 37.

De Renobales, M., Rogers, L. and Kolattukudy, P. (1980) *Arch. Biochem. Biophys.* **205**, 464.

Dormann, P., Spencer, F. and Ohlrogge, J. B. (1993) *Planta* **189**, 425.

Laemmli, U. K. (1970) *Nature* **227**, 680.

Lin, C. Y. and Smith, S. (1978) *J. Biol. Chem.* **253**, 1954.

Mckeon, T. A. and Stumpf, P. K. (1982) *J. Biol. Chem.* **257**, 12141.

Ohlrogge, J. B., Shine, W. E. and Stumpf, P. K. (1978) *Arch. Biochem. Biophys.* **189**, 382.

Pollard, M. R., Anderson, L., Fan, C., Hawkins, D. J. and Davies, H. M. (1991) *Arch. Biochem. Biophys.* **284**, 306.

Randhawa, Z. I., Naggert, J., Blacher, R. W. and Smith, S. (1987) *Eur. J. Biochem.* **162**, 577.

Rock, C. O. and Garwin, J. L. (1979) *J. Biol. Chem.* **254**, 7123.

Rogers, L., Kolattukudy, P. E. and de Renobales, M. (1982) *J. Biol. Chem.* **257**, 880.

Seay, T. and Lueking, D. R. (1986) *Biochemistry* **25**, 2480.

Smith, S., Mikkelsen, J., Witkowski, A. and Libertini, L. J. (1986) *Biochem. Soc. Trans.* **14**, 583.

Voelker, T. A., Norrell, A. C., Anderson, L., Bleibaum, J., Fan, C., Hankins, D. J., Radke, S. E. and Davies, H. M. (1992) *Science* **257**, 72.