C-Terminal His-Tagging Results in Substrate Specificity Changes of the Thioesterase I from *Escherichia coli*

Ya-Lin Lee[a], May-Shyuan Su[a], Tai-Huang Huang[b], and Jei-Fu Shaw[a,*]
[a]Institute of Botany and [b]Division of Structure Biology, Institute of Biomedical Sciences, Academia Sinica, Nankang, Taipei, Taiwan 11529, Republic of China

ABSTRACT: The biochemical properties of *Escherichia coli* thioesterase I, His-tagged (HT) on the C-terminal, were systematically analyzed and compared with that without the His-tag (WT). These two types of enzymes exhibit similar optimal temperature and pH dependence, but subtle differences were detected. Kinetic studies revealed that the k_{cat}/K_m values of the HT enzyme for the substrates palmitoyl-CoA and *p*-nitrophenyl dodecanoate were 36- and 10-fold lower than those of the WT, respectively. In contrast, HT had a fivefold increased catalytic efficiency for *p*-nitrophenyl acetate, and up to fourfold increases toward phenylalanine- and tyrosine-derived ester substrates, L-NBPNPE (*N*-carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester) and L-NBTNPE (*N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester), respectively. For L-NBPNPE and L-NBTNPE, the increases were attributed to the higher k_{cat} values with little changes in K_m, whereas the increase for *p*-nitrophenyl acetate was mainly attributed to the lower K_m value. It is concluded that addition of six hydrophilic histidine residues on the C-terminus resulted in a change in substrate specificity of *E. coli* thioesterase I toward more hydrophilic substrates.

Paper no. J9243 in *JAOCS 76*, 1113–1118 (October 1999).

KEY WORDS: *Escherichia coli*, His-tag, substrate specificity, thioesterase I.

Thioesterase I of *Escherichia coli* specifically hydrolyzes long-chain fatty acyl thioesters such as palmitoyl-CoA (1). This multifunctional enzyme catalyzes the hydrolysis of several aromatic esters as well as several amino acid-derived esters (2–4). The 183-amino acid-residue mature protein shares a high homologous sequence (51.7% identity) with a protein, arylesterase, from *Vibrio mimicus* (5). These two functionally similar proteins are expected to possess industrial potential such as stereospecific hydrolysis and synthesis of a wide variety of regiospecific esters (6,7). Both proteins are members of a new subfamily of lipolytic enzymes, which consists of eight proteins from different species (8). The amino acid sequences of proteins in this family are characterized by the presence of five consensus sequence blocks arranged in the same order. However, all proteins in this family share little sequence homology with known lipases, and they exhibit diverse substrate specificity. At present, the structure–function relationship of these proteins is not known. It would be a great benefit to protein engineering applications if the three-dimensional structures of these proteins were solved.

To facilitate structure-functional studies, we prepared an active recombinant *E. coli* thioesterase I with a hexahistidyl peptide (His-tag) extension attached to the C-terminus. The recombinant protein was purified by an immobilized Ni-resin column with high purity and high yield. The secondary structure and topology of the purified enzyme was established by nuclear magnetic resonance (NMR) (9). Although His-tagging engineering has been extensively implemented for facile protein purification, the effect of His-tagging on enzyme properties has not been systematically studied. In this work, we showed that the biochemical properties of the His-tagged *E. coli* thioesterase I are very similar to those of the wild-type enzyme. However, an enzyme kinetic study revealed that the engineered protein showed substantial differences in substrate specificity with preference shifted toward less hydrophobic substrates.

MATERIAL AND METHODS

Materials. Oligonucleotide primers were made by DNAFax Co. (Taipei, Taiwan), and Taq DNA polymerase and DNA sequencing kit were obtained from HT Biotechnology Ltd. (Cambridge, England) and US Biochemicals (Cleveland, OH), respectively. Isopropyl thio-β-D-galactoside (IPTG) was obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany). *p*-Nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl hexanoate, *p*-nitrophenyl dodecanoate, α-naphthyl butyrate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester (L-NBPNPE), *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (L-NBTNPE) and *N*-carbobenzoxy-L-tryptophan *p*-nitrophenyl ester (L-NBWNPE) were purchased from Sigma Co. (Saint Louis MO). Ni-NTA resin (6 × His-binding resin) was purchased from Novagene Co. (Madison, WI). DEAE Sepharose CL-6B and Superdex 75 HR10/30 were the products of Pharmacia Co. (Quarry Bay, Hong Kong). Protein molecular-weight markers were purchased from Novex Co. (San Diego, CA).

*To whom correspondence should be addressed.
E-mail: boplshaw@gate.sinica.edu.tw

Copyright © 1999 by AOCS Press

Construction of the recombinant thioesterase I gene. Recombinant thioesterase I gene was synthesized from a plasmid containing the *tesA* gene (4) by a two-primer polymerase chain reaction (PCR) method (10). The 5′ end primer of 5′-ACCTTCCGTGCCCATATGGCGGACACGTTA-3′, and 3′ end primer of 5′-CTCCGTCTCGAGTGAGTCATGATTTAC-3′, were designed for digestion by restriction enzymes *Nde*I (CA/TATG) and *Xho*I (C/TCGAG), respectively. After the restriction enzyme treatment, the PCR products were ligated into a 3.7 kb *Nde*I/*Xho*I-restricted pET-20b(+) vector (Novagene Co.). The constructed plasmid was cloned from *E. coli* HB101 (Promega Co., Madison, WI), and was then transformed into *E. coli* BL21(DE3) (Novagene Co.) for target protein overexpression. The DNA sequence of the recombinant enzyme was confirmed from plasmid isolated from HB101.

Cell growth condition. Escherichia coli BL21(DE3) cells harboring the desired plasmid were grown at 37°C in LB broth which consisted of 1% (wt/vol) Bacto tryptone, 0.5% (wt/vol) Bacto yeast extract, and 1% (wt/vol) NaCl (pH value was adjusted to 7.0). For plasmid selection, ampicillin was added to a final concentration of 50 μg/mL. The cultures for protein overexpression were induced by adding IPTG to a final concentration of 2.5 mM when the absorbance value of OD_{600} was approximately 0.6. Cells were harvested 4 h after IPTG induction at 30°C.

Enzyme extraction and purification. His-tagged thioesterase I was purified by affinity chromatography with Ni-NTA resin as previously described (11). The protein bound to the column was eluted with 1.0 M of imidazole. For the wild-type thioesterase I the harvested cell pellet (from 500 mL of LB broth culture) was suspended in 40 mL of 50 mM Tris buffer (pH 8.0, containing 2 mM EDTA) and then frozen at –70°C for 20 min. After thawing, the suspension was sonicated and then centrifuged. The supernatant of the crude extract was separated on a DEAE Sepharose CL-6B column (2.6 × 20 cm) equilibrated in 25 mM sodium phosphate buffer, pH 7.0. Elution was carried out with a linear gradient of equilibrating buffer containing 0.2 to 0.3 M NaCl. The fractions with enzyme activity were pooled and concentrated for the next purification by gel filtration. The gel filtration was performed with a Superdex 75 column (HR10/30) equilibrated in 25 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.02% (wt/vol) sodium azide. Elution was carried out with the equilibrating buffer at a flow rate of 0.5 mL/min. The protein purity was monitored by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in every step of purification. Both purified proteins were dialyzed against 25 mM sodium phosphate buffer, pH 7.0.

SDS-PAGE analysis of thioesterase I. SDS-polyacrylamide gel (15% gel, 1% SDS) was prepared for analysis. Samples were made of equal volumes of protein solution and SDS-PAGE loading dye which contained 2% (wt/vol) SDS and 200 mM dithiothreitol (DTT) in 100 mM Tris buffer, pH 6.8. Samples were then boiled for 5 min, and then held on ice for 10 min. After that, electrophoresis-separated protein gels were stained by Coomassie brilliant blue and by esterase activity stain. Before activity stain, SDS in the gel was removed by submersion in 25% (vol/vol) isopropanol for 30 min twice, then in 0.1 M sodium phosphate buffer, pH 7.0, for 30 min once (12). Esterase activity stain was performed by the method described by Vallejos (13).

pH profile of enzyme activity. Good's buffer is composed of four equal quantities of solutions including Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine], CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], sodium acetate and Bis-Tris propane {1,3-bis(tris[hydroxymethyl]methylamino) propane} (50 mM, each). Buffer pH values from 3 to 10 were adjusted by HCl and NaOH solutions (14). Substrate solutions were 2.64 mM *p*-nitrophenyl butyrate dissolved in 2.1% (vol/vol) Triton X-100 and various pH values of 50 mM Good's buffers. Ten microliters of purified enzyme was added to a preheated 190-μL reaction solution at 37°C. The reaction was terminated by adding 400 μL of acetone to the reaction mixture. The enzyme activity was measured by the increase of absorbance at 346 nm, characteristic of *p*-nitrophenol production. One unit is defined as the enzyme activity that catalyzes the hydrolysis of 1 μmol of substrate to produce 1 μmol of *p*-nitrophenol per minute (15).

Temperature profile of enzyme activity. Substrate solution was 2.64 mM of *p*-nitrophenyl butyrate dissolved in 50 mM sodium phosphate buffer, pH 7.0, containing 2.1% (vol/vol) Triton X-100. The enzyme reactions were carried out at temperatures from 0 to 75°C.

Kinetic study of the HT and WT proteins. Purified proteins were used for kinetic study with different substrates at pH 7.0 and at 37°C. Esterase activity assayed with substrates *p*-nitrophenyl acetate, *p*-nitrophenyl hexanoate, and *p*-nitrophenyl dodecanoate was determined by the increase of absorbance at 346 nm as described (15). Thioesterase activity with palmitoyl-CoA as substrate was determined by the increase of absorbance at 412 nm with a coupled reaction with DTNB as described (1). Chymotrypsin-like activity using L-NBPNPE, L-NBTNPE, and L-NBWNPE as substrates was determined by the increase of absorbance at 400 nm as described (3). The values of K_m and k_{cat} (kinetic parameters) were calculated from triplicate experiments by nonlinear regression plots of the Michaelis-Menten equation.

RESULTS AND DISCUSSION

Purification of WT and HT proteins. WT and HT proteins were purified by traditional and Ni-resin affinity chromatographies, respectively. Both proteins were purified to homogeneity and exhibited esterase activities on SDS-polyacrylamide gel (Fig. 1). The purified HT has been analyzed with heteronuclear multidimensional NMR for structure determination and was shown to consist of four β-strands and seven α-helices (9).

His-tagging the C-terminal end of the thioesterase I. Direct addition of His-tag to the C-terminus of the thioesterase I unexpectedly resulted in a functional protein. The same man-

FIG. 1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of His-tagged and wild-type thioesterase I. The His-tagged enzyme was purified by Ni-resin column. The wild type was purified by ion exchange and gel filtration. Lane M, protein molecular weight markers; lane 1, His-tagged thioesterase I; lane 2, wild-type thioesterase I. Part A, Coomassie brilliant blue staining; part B, esterase activity staining.

ufactured protein of *V. mimicus* arylesterase, which shares a high amino acid sequence identity (51.7%) with the thioesterase I (Fig. 2), fails to exhibit enzyme activity (11). Previous work revealed that a hexadecapeptide spacer between the His-tag and C-terminus is needed to restore the enzyme functions of arylesterase (11). The recombinant thioesterase I constructed with the same spacer between the His-tag and the C-terminus resulted in an opposite result, i.e., this recombinant has no enzyme activity (data not shown).

Sequence alignment (Fig. 2) of the two highly homologous proteins shows that there is a four-amino-acid-residue extension at the C-terminus of the thioesterase I. The four extended amino acid residues presumably have a protective effect on the integral protein structure from the detrimental effect exerted by His-tag. The resolved secondary structure of thioesterase I revealed that the last helix (α-7) is located in the range of Phe[165]-Leu[174] (9). Thus, the last octapeptide behind this α-helix may serve to prevent the His-tag from affecting the structure of the protein.

pH and temperature profiles. With *p*-nitrophenyl butyrate as substrate, HT and WT enzymes showed the same pH optima at 8.0 with very similar profiles on pH effect (Fig. 3). Although the imidazole ring of histidine has a pK_a value of 6.3 under normal conditions (16), the His-tag did not affect the pH profile of HT protein. This result indicated that the His-tag did not directly interact with the protein structure involved in the catalysis. HT and WT also exhibited very similar temperature profiles, with the same optimal temperatures of 45°C (Fig. 4). The HT protein exhibited higher specific activity than that of the WT under the assay condition. For less hydrophobic residues HT possesses higher specific activity than does the WT enzyme (discussed below).

Kinetic study of HT and WT enzymes. *Escherichia coli* thioesterase I possesses hydrolytic activity toward several kinds of esters including long-chain acyl-CoA, aromatic esters, and amino acid-derived esters (1–4). Enzyme kinetic parameters of HT and WT were measured with substrates of palmitoyl-CoA, *p*-nitrophenyl esters, and aromatic amino acid-derived esters (L-NBPNPE, L-NBTNPE and L-NB-

```
                Gap Weight:      12       Average Match:  2.912
             Length Weight:       4    Average Mismatch: -2.003

                   Quality:     470              Length:    185
                     Ratio:   2.597                Gaps:      1
        Percent Similarity:  60.674    Percent Identity:  51.685

         Match display thresholds for the alignment:
          | = IDENTITY       : =    2        . =    1

E. coli        1 ADTLLILGDSLSAGYRMSASAAWPALLND...KWQSKTSVVNASISGDTS 47
                 .: ||:|||||||||.|    .||.|| |   .     .|:| ||||||.
V. mimicus     1 SEKLLVLGDSLSAGYQMPIEKSWPSLLPDALLEHGQDVTVINGSISGDTT 50

E. coli       48 QQGLARLPALLKQHQPRWVLVELGGNDGLRGFQPQQTEQTLRQILQDVKA 97
                 |||||| || || |  ||:||| ||||||| |.     | ..:  :|
V. mimicus    51 GNGLARLPQLLDQHTPDLVLIELGANDGLRGFPPKVITSNLSKMISLIKD 100

E. coli       98 ANAEPLLMQIRLPANYGRRYNEAFSAIYPKLAKEFDVPLLPFFMEEVYLK 147
                 . |  .:||||.| |||:||.: |  ||||||.   | |:|||:| |  |
V. mimicus   101 SGANVVMMQIRVPPNYGKRYSDMFYDIYPKLAEHQQVQLMPFFLEHVITK 150

E. coli      148 PQWMQDDGIHPNRDAQPFIADWMAKQLQPLVNHDS 182
                 |:|| |||:||  :|||.||:..|.:|   .
V. mimicus   151 PEWMMDDGLHPKPEAQPWIAEFVAQELVKHL.... 181
```

FIG. 2. Sequence comparison between mature proteins of *Escherichia coli* thioesterase I and *Vibrio mimicus* arylesterase. Identical residues are indicated as "|"; conservation amino acid replacements are indicated as ":"; less conservation amino acid residues are indicated as ".".

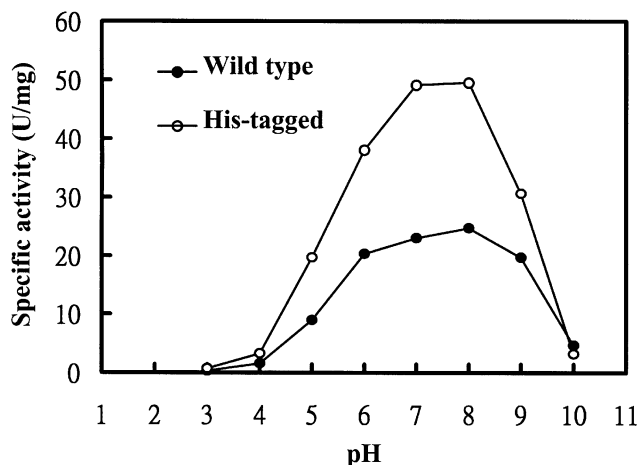

FIG. 3. pH effects. Activity was measured at 37°C in 50 mM Good's buffer (pH 3.0–10.0) containing 2.1% (vol/vol) Triton X-100, using 2.64 mM *p*-nitrophenyl butyrate as substrate. Both His-tagged and wild-type thioesterase I purified to homogeneity as shown on SDS-polyacrylamide gel were used in this study. Each value is calculated from triplicate experiments. For abbreviation see Figure 1.

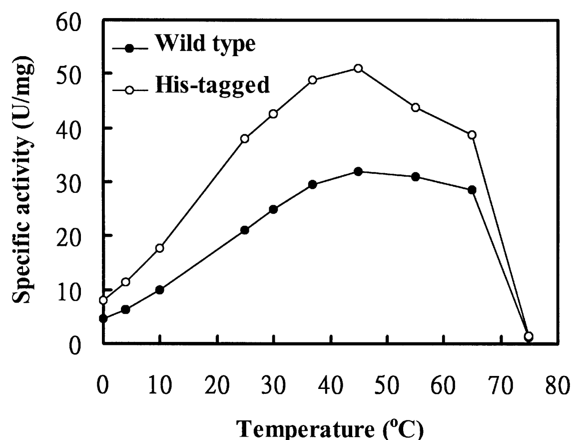

FIG. 4. Temperature effects. Activity was measured with *p*-nitrophenyl butyrate as substrate. Both His-tagged and wild-type thioesterase I were purified to homogeneity as shown on SDS-polyacrylamide gel were used in this study. Each value is calculated from triplicate experiments. For abbreviation see Figure 1.

WNPE) to study the effect of the His-tag on the enzyme substrate specificity. *p*-Nitrophenyl ester substrates with 2-carbon, 6-carbon, and 12-carbon acyl chains were used for investigating chain-length preference of these two enzymes.

Table 1 summarizes the kinetic data of HT and WT toward seven substrates. Interestingly, HT shows more preference toward short acyl chain substrates than does the WT enzyme. Thus, the only two substrates, for which HT has lower k_{cat}/K_m than WT were the two long acyl chain substrates, palmitoyl-CoA and *p*-nitrophenyl dodecanoate. The k_{cat}/K_m values of HT for these two substrates, palmitoyl-CoA and *p*-nitrophenyl dodecanoate, are 36- and 10-fold lower than those of WT, respectively. Both decreased catalytic efficiencies (k_{cat}/K_m) resulted from the reduced k_{cat} (catalytic rate constant) values. The corresponding values of $\Delta\Delta G^*$ (the change in the "free energies of transition-state stabilization") signifi-

TABLE 1
Kinetic Analysis of HT and WT Thioesterase I with Esters, Thioesters, and Amino Acid-Derived Esters as Substrates[a]

Substrate	WT K_m (μM)	WT k_{cat} (s^{-1})	WT k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	HT K_m (μM)	HT k_{cat} (s^{-1})	HT k_{cat}/K_m ($s^{-1}\mu M^{-1}$)	$\Delta\Delta G^{*b}$ (kJ mol^{-1})
Thioester							
Palmitoyl-CoA	6.38	19.1	2.990	5.95	2.46	0.083	9.25
				(0.93	0.13	0.03)[c]	
Ester							
p-Nitrophenyl acetate	2411.1	25.2	0.010	751.7	40.6	0.054	−4.35
				(0.31	1.61	5.4)[c]	
p-Nitrophenyl hexanoate	1360.3	30.5	0.022	1690.0	50.2	0.030	−0.80
				(1.24	1.65	1.36)[c]	
p-Nitrophenyl dodecanoate	1448.6	10.2	0.007	1312.3	0.9	0.0007	5.93
				(0.91	0.09	0.10)[c]	
Amino acid derivative							
L-NBPNPE	4.39	5.93	1.351	5.16	26.68	5.171	−3.46
				(1.18	4.50	3.83)[c]	
L-NBTNPE	10.43	17.20	1.649	11.32	66.38	5.863	−3.27
				(1.09	3.86	3.56)[c]	
L-NBWNPE	10.51	0.58	0.056	12.17	0.78	0.064	−0.34
				(1.16	1.34	1.14)[c]	

[a]Kinetic analysis is calculated from Michaelis-Menten equation and nonlinear regression plots.
[b]$\Delta\Delta G^*$ is the change of free energy of transition state stabilization. $\Delta\Delta G^* = -RT \ln[(k_{cat}/K_m)_{HT}/(k_{cat}/K_m)_{WT}]$.
[c]The values in parentheses are the constants of HT enzyme divided by the corresponding constants of WT enzyme. Abbreviations: HT, His-tagged; WT, without His-lag; L-NBPNPE, *N*-carbobenzoxy-L-phenylalanine *p*-nitrophenylester; L-NBTNPE, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenylester; L-NBWNPE, *N*-carbobenzoxy-L-tryptophan *p*-nitrophenylester.

cantly increased by 9.25 and 5.93 kJ/mol for the hydrolysis of palmitoyl-CoA and *p*-nitrophenyl dodecanoate, respectively, as also listed on Table 1.

The catalytic efficiencies of HT and WT toward the substrate *p*-nitrophenyl hexanoate were about the same. On the other hand, for the shortest acyl chain substrate, *p*-nitrophenyl acetate, HT showed a five fold higher catalytic efficiency, mainly due to the decrease in K_m value.

The kinetic study thus revealed that the HT enzyme was less active toward longer acyl chain-length esters with decreased k_{cat} values, especially for the longest acyl chain ester, palmitoyl-CoA. The diminished k_{cat} values and increased free energies indicated that the extended His-tag destabilized the transition state of these substrates. On the other hand, His-tag improved the activity for shortest chain-length substrate, *p*-nitrophenyl acetate, by increasing substrate affinity (K_m value decreased). These results indirectly illustrate why the HT enzyme exhibited higher activity than the WT enzyme in the pH-dependence and temperature-effect experiments, in which the substrate with four-carbon acyl chain, *p*-nitrophenyl butyrate, was used to assay the enzyme activity.

For amino acid-derived substrates, HT showed approximate fourfold increases in the catalytic efficiencies for phenylalanine- and tyrosine-derived substrates, L-NBPNPE and L-NBTNPE, respectively. For the tryptophan-derived substrate, L-NBWNPE, both WT and HT exhibited very low activities, therefore, L-NBWNPE was demonstrated to be an unfavorable substrate for this enzyme.

The increased activity in the aromatic-amino acid-derived substrates was attributed to the increase in k_{cat} with little change in K_m values. The higher k_{cat} values as well as the lower free energies ($\Delta\Delta G^*$) may indicate that the HT has lower transition-state barriers for catalyzing the aromatic-amino acid-derived substrates than the WT.

The C-terminal His-tag is speculated to be located on the protein surface owing to its high hydrophilicity. Our biochemical characterization revealed that HT and WT enzymes exhibit the same pH and temperature profiles, strongly suggesting that the His-tag does not seriously perturb the overall structural integrity of the enzyme, whereas subtle changes in the active-site geometry did occur, resulting in changes in substrate specificity and catalytic activity. The moderate changes in substrate specificity for some substrates suggest that the C-terminus is probably located near the active site such that added His-tag is close enough to exert some effects on the substrate specificity.

The effect of the His-tag on protein function varies considerably from system to system. In the extreme case, such as observed in *V. mimicus* arylesterase, directly adding His-tag in its C-terminus leads to a complete loss of enzymatic activity. Although *E. coli* thioesterase I and *V. mimicus* arylesterase are highly homologous, the His-tag attached to the C-terminus of *E. coli* thioesterase I exerts only moderate effect and the protein is still very well-behaved, suitable for further extensive structure-functional characterization (9). The variation and unpredictable consequences of the effect of His-tag suggest that it is necessary to study the effect in each individual case. On the other hand, the increase of enzyme activity and change in enzyme specificity by C-terminal His-tagging in the present work suggest that C-terminal extension such as His-tagging could be used as an alternative method for engineering enzyme properties for biotechnological applications.

ACKNOWLEDGMENT

We thank David Chow for editing the English of this manuscript. This research was supported by a National Science Council, Republic of China grant (NSC 85-2321-B001-017-A18) to J.F. Shaw.

REFERENCES

1. Cho, H., and J.E. Cronan Jr., *Escherichia coli* Thioesterase I, Molecular Cloning and Sequencing of the Structural Gene and Identification as a Periplasmic Enzyme, *J. Biol. Chem. 268*: 9238–9245 (1993).
2. Ichihara, S., Y. Kato, C. Matsubara, K. Akasaka, and S. Mizushima, Molecular Cloning, Sequencing, and Mapping of the Gene Encoding Protease I and Characterization of Protease and Protease-Defective *Escherichia coli* Mutants, *J. Bacteriol. 175*:1032–1037 (1993).
3. Cho, H., and J.E., Cronan Jr., Protease I of *Escherichia coli* Functions as a Thioesterase *in vivo*, *Ibid. 176*:1793–1795 (1994).
4. Lee, Y.L., J.C. Chen, and J.F. Shaw, The Thioesterase I of *Escherichia coli* Has Arylesterase Activity and Shows Stereospecificity for Protease Substrates, *Biochem. Biophys. Res. Comm. 231*:452–456 (1997).
5. Shaw, J.F., R.C. Chang, K.H. Chuang, Y.T. Yen, Y.J. Wang, and F.G. Wang, Nucleotide Sequence of a Novel Arylesterase Gene from *Vibrio mimicus* and Characterization of the Enzyme Expressed in *Escherichia coli, Biochem. J. 298*:675–680 (1994).
6. Aragozzini, F., M. Valenti, E. Santaniello, P. Ferraboschi, and P. Grisenti, Biocatalytic, Enantioselective Preparations of (*R*)- and (*S*)-Ethyl 4-Chloro-3-hydroxybutanoate, a Useful Chiral Synthon, *Biocatalysis 5*:325–332 (1992).
7. Cambillau, C., S. Longhi, A. Nicolas, and C. Matinez, Acyl Glycerol Hydrolases: Inhibitors, Interface and Catalysis, *Current Opinion Struct. Biol. 6*:449–455 (1996).
8. Upton, C., and J.T. Buckley, A New Family of Lipolytic Enzymes, *Trend Biochem. Sci. 20*:178–179 (1995).
9. Lin, T.H., C. Chen, R.F. Huang, Y.L. Lee, J.F. Shaw, and T.H. Huang, Multinuclear NMR Resonance Assignments and the Secondary Structure of *Escherichia coli* Thioesterase I/Protease I—A Member of a New Subclass of Lipolytic Enzymes, *J. Biomol. NMR 11*:363–380 (1998).
10. Sharrocks, A.D., and P.E. Shaw, Improved Primer Designed for PCR-Based, Site-Directed Mutagenesis, *Nucleic Acids Res. 20*:1147 (1992).
11. Lee, Y.L., R.C. Chang, and J.F. Shaw, Facile Purification of a C-Terminal Extended His-Tagged *Vibrio mimicus* Arylesterase and Characterization of the Purified Enzyme, *J. Am. Oil Chem. Soc. 74*:1371–1376 (1997).
12. Blank, A., R.H. Sugiyama, and C. Dekker, Activity Staining of Nucleolytic Enzymes After Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis: Use of Aqueous Isopropanol to Remove Detergent from Gels, *Anal. Biochem. 120*:267–275 (1982).
13. Vallejos, C.E., Enzyme Activity Staining, in *Isozymes in Plant Genetics and Breeding, Part A*, edited by S.D. Tanksley, and T.J. Orton, Elsevier Science Publishers, Amsterdam, 1983, pp. 500–501.

14. Shaw, J.F., R.S. Pan, and W.H. Hsu, Influence of pH on the Inactivation Kinetics of Isoamylase, *Bot. Bull. Acad. Sinica 30*:155–159 (1989).
15. Rua, M.L., T. Diaz-Maurino, V.M. Fernandez, C. Otero, and A. Ballesteros, Purification and Characterization of Two Distinct Lipases from *Candida cylindracea, Biochim. Biophys. Acta 1156*:181–189 (1993).
16. Antosiewicz, J., J.A. McCammon, and M.K. Gilson, Prediction of pH-Dependent Properties of Proteins, *J. Mol. Biol. 238*:415–436 (1994).

[Received May 17, 1999; accepted July 21, 1999]