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Expression in *Escherichia coli*, purification and characterization of two mammalian thioesterases involved in fatty acid synthesis

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Thioesterase I, a constituent domain of the multifunctional fatty acid synthase, and thioesterase II, an independent monofunctional protein, catalyse the chain-terminating reaction in fatty acid synthesis *de novo* at long and medium chain lengths respectively. The enzymes have been cloned and expressed in *Escherichia coli* under the control of the temperature-sensitive λ repressor. The recombinant proteins are full-length catalytically competent thioesterases with specificities indistinguishable from those of the natural enzymes.

INTRODUCTION

In animal tissues medium- and long-chain fatty acids are assembled *de novo* on the pantetheine thiol group of the multifunctional fatty acid synthase. Termination of growth of the acyl chain can be effected through the intervention of a thioesterase, which removes the acyl moiety from the pantetheine residue, releasing it as a non-esterified fatty acid. In most tissues it is the resident thioesterase I domain of the fatty acid synthase which releases the acyl chain once it has reached 16 carbon atoms in length [1]. However, in some specialized tissues, such as non-ruminant mammary glands [2,3] and avian uropygial glands [4], a discrete monofunctional monomeric protein, thioesterase II, catalyses the hydrolysis of medium-chain fatty acids from the fatty acid synthase. The amino acid sequences of both the thioesterase I domain of the rat fatty acid synthase [5] and the thioesterase II enzyme from lactating rat mammary gland [6,7] have been determined, and their cDNAs have been cloned. The two types of chain-terminating enzyme share some common structural features and appear to be distantly related [5]. In order to investigate the structural basis for the mechanism of action of these enzymes, particularly their different substrate specificities, we have sought to identify a suitable host/vector system that could be used conveniently to express the enzymes and their mutants.

MATERIALS AND METHODS

Materials

Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and used according to manufacturers' recommendations. A GeneAmp DNA amplification kit was purchased from Perkin Elmer Cetus (Norwalk, CT, U.S.A.), and DNA polymerase (Klenow fragment) was from United States Biochemical Corp. (Cleveland, OH, U.S.A.).

Plasmids and *Escherichia coli*

pUC120, a derivative of pUC12, was constructed by Dr. Jeffrey Vieira and obtained from Dr. Vic Knauf (Calgene, Davis, CA, U.S.A.). pJLA502 [8] was purchased from Medac (Hamburg, Germany) and DH5α cells were obtained from BRL (Gaithersburg, MD, U.S.A.).

Primers

Primers were synthesized on a DuPont Coder 300 and purified [9] by reversed-phase h.p.l.c. on a Vydac C4 column. Changes in the original sequence are italicized. (1) Thioesterase I 5′–3′:5′-GCC*ATGGA*A*T*C*A*AA*A*AATGATACATC*A*CT*T*AA-3′, corresponds to the coding strand of pFAS5 [5] (bases 339–371). (2) Thioesterase I 3′–5′:5′-GATGGGGATCGGAGCATCTC-3′, corresponds to the non-coding strand of pFAS5 (bases 1327–1308). (3) Thioesterase II 5′–3′:5′-*AATTCC*ATGGAGA-*C*C*G*C*T*GT*T*AA*C*GC-3′, corresponds to the coding strand of pTE2-512 [10] (bases −6 to 20). (4) Thioesterase II 3′–5′:5′-ACTCTAGAGGATCCCC-3′, corresponds to the non-coding strand in the multiple cloning site of pUC12 (bases 418–433).

Construction of expression plasmids

Changes in the thioesterase sequences were introduced by the polymerase chain reaction [11] using the pairs of primers described above. Polymerase chain reactions (100 μl) were run using 1 ng of plasmid pFAS5 or pTE2-512 and 500 ng of the appropriate primers. Amplification was performed with four cycles at 94 °C for 1 min, 28 °C for 1 min and 72 °C for 2 min, followed by 26 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. Reaction mixtures were then extracted with phenol and chloroform, the $MgCl_2$ concentration was adjusted to 10 mM and the ends of the DNA fragments were repaired by the action of Klenow polymerase (2 units) for 20 min at 20 °C. The reaction products were separated on 1.5% (w/v) agarose gels in TBE buffer (0.089 M-Tris/borate buffer (pH 8)/2 mM-EDTA), the appropriate ethidium bromide-stained bands were cut out and the DNA was electroeluted. The blunt-ended thioesterase I and thioesterase II fragments were then cloned into the *Sma*I site of pUC120. Cloned plasmids that contained the insert in the opposite orientation as the *lacZ* gene were digested with *Nco*I and *Eco*RI enzymes and their inserts were isolated as above. These fragments were finally cloned into *Nco*I/*Eco*RI-digested pJLA 502.

Expression and purification of recombinant thioesterases

Typically, a 500 ml culture of DH5α cells containing either the pJLA502/TE1 or pJLA502/TE2 construct was grown at 30 °C in TB medium [yeast extract (24 g/l), tryptone (12 g/l), K_2HPO_4 (16.43 g/l), KH_2PO_4 (2.3 g/l), glycerol (4 ml/l) and carbenicillin (50 mg/l)] to an A_{600} of approx. 1. The temperature was increased

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to 42 °C and growth was allowed to continue for 2.5 h (thioesterase II) or 4 h (thioesterase I).

The harvested cell pellet was lysed by treatment for 20 min at 0 °C with 3 vol. of 0.2 M-Tris/HCl (pH 8)/0.1 M-NaCl/10 mM-EDTA/1 mM-dithiothreitol/lysozyme (1 mg/ml), followed by 10 min with deoxycholic acid (0.4%, by wt., of cell mass) and 15 min with DNAaseI (10 μg/ml)/3.8 mM-$MgCl_2$. The cytosol was separated by centrifugation at 10000 *g* for 20 min and stored at −72 °C.

The cytosol was dialysed against cold 50 mM-Tris/HCl buffer (pH 7.2 for thioesterase II; pH 7.8 for thioesterase I)/1 mM-EDTA/1 mM-dithiothreitol, centrifuged and applied to a column (2.5 cm × 24 cm) of DE 53 (Whatman). The column was washed at a flow rate 200 ml/h with 0.5–1 litre of the buffer at 4 °C, and proteins were eluted with a 1.6 litre linear gradient of NaCl (0–0.2 M).

Fractions containing thioesterase activity were pooled and adjusted to 80% satn. with respect to $(NH_4)_2SO_4$, and the protein precipitate was collected at 4 °C. The precipitate was dissolved in 50 mM-Tris/HCl buffer (pH 7.2)/0.15 M-NaCl/1 mM-EDTA/1 mM-dithiothreitol, and the thioesterase was further purified by gel filtration on a column (2.5 cm × 90 cm) of AcA 54 (LKB) at a flow rate 20 ml/h at 4 °C. Fractions containing thioesterase activity were pooled and the purified enzyme was dialysed at 4 °C against 50 mM-potassium phosphate (pH 7)/1 mM-EDTA/1 mM-dithiothreitol and, after addition of glycerol to 20% (w/v), it was stored at −72 °C.

Thioesterase assays

Model substrates. Thioesterase activity with decanoyl- and dodecanoyl-CoA substrates was determined spectrophotometrically [3] in a 200 μl assay system containing 50 mM-potassium phosphate buffer, pH 8. Myristoyl- and palmitoyl-CoA hydrolysis was measured radiochemically [12] in a 120 μl assay system containing 25 mM-potassium phosphate buffer, pH 8. All assays were performed at 30 °C and contained 1 μg of BSA [from the enzyme diluent: 50 mM-potassium phosphate (pH 7)/1 mM-EDTA/1 mM-dithiothreitol/BSA (100 μg/ml)]. Stock solutions of myristoyl- and palmitoyl-CoA were made in 5 mM-ammonium acetate, pH 5.6. Parallel incubations without enzyme were conducted to determine background hydrolysis rates.

Natural substrate. The fatty acid synthase core (20 μM), from which the thioesterase domains had been removed by trypsin treatment, was first incubated for 5 min at 37 °C with acetyl-CoA (110 μM), [2-^{14}C]malonyl-CoA (180 μM, 55 Ci/mol) and NADPH (360 μM) in 0.1 M-potassium phosphate buffer, pH 6.6, to form the *S*-[^{14}C]acyl-fatty acid synthase substrate [12]. It was then re-isolated by gel filtration on a column (10 cm × 1 cm) of Bio-Gel P-6DG (Bio-Rad, Richmond, CA, U.S.A.) equilibrated with 0.1 M-potassium phosphate buffer (pH 8)/1 mM-EDTA. Since the interaction of rat thioesterase II with fatty acid synthase is dependent on the presence of acyl chains on the synthase [13], the *S*-acyl-fatty acid synthase core concentration was calculated from the total radioactivity, assuming an average acyl chain length of 18 carbon atoms [14]. Typically, 0.5–0.6 long-chain acyl moieties were present per core subunit. Hydrolysis of the *S*-[^{14}C]acyl-enzyme by thioesterase was measured in the presence of 100 μg of BSA/ml at 30 °C for 1 min, and the fatty acid product was isolated [12].

Physico-chemical characterization

SDS/PAGE was carried out on 12% (w/v) polyacrylamide gels [15]. Protein staining was done with ISS ProBlue (Integrated Separation Systems, Hyde Park, MA, U.S.A.) and Western immunoblotting was performed on nitrocellulose using the ABC immunoperoxidase staining technique [16]. Average molecular mass was estimated by electrospray ionization m.s. [17].

RESULTS AND DISCUSSION

The strategy for production of recombinant thioesterases basically involved creation of unique *Nco*I sites positioned to encode the ATG start codon for the proteins in the correct reading frame, and modification of the codon usage to maximize expression (Fig. 1). Whereas, in the case of thioesterase II, minimal changes in the cDNA sequence and no changes in the amino acid sequence were involved, design of the thioesterase I

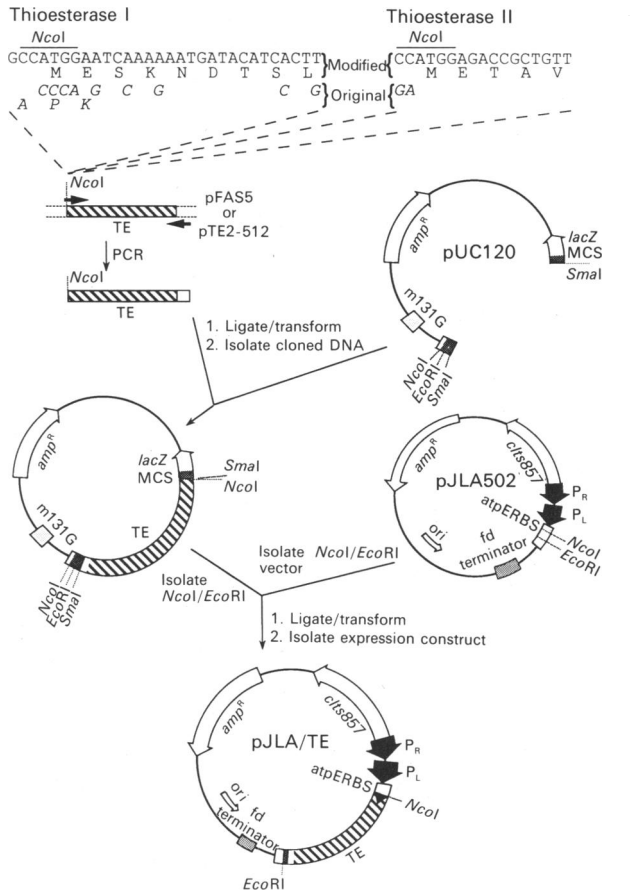

Fig. 1. Construction of the thioesterase expression plasmids

The polymerase chain reaction was used to modify the *N*-terminal sequences of thioesterase I and thioesterase II to include *Nco*I sites at the start codon and runs of As and a TTAA motif, said to increase translational efficiency [18]. The modified (upper) and original (lower) DNA and amino acid sequences at the 5′-ends and *N*-termini of the thioesterases are shown. After blunt-end cloning of the polymerase chain reaction products, the modified thioesterase inserts were cut out with *Nco*I and *Eco*RI and cloned into the expression plasmid pJLA502. This plasmid carries the translation initiation sequences of the *atpE* gene under the control of the strong λ promoters [8]. Expression at low temperatures is efficiently prevented by the presence of the constitutively expressed temperature-sensitive λ repressor on the same plasmid. Expression of the thioesterases is induced by increasing the temperature of the bacterial culture. Abbreviations: P_L and P_R, major leftward and rightward bacteriophage λ promoters, respectively; RBS, ribosome-binding site; fd terminator, bacteriophage fd transcription terminator; *cIts*857, bacteriophage λ temperature-sensitive repressor gene; amp^R, ampicillin-resistance gene; ori, origin of replication; MCS, multiple cloning site; TE, thioesterase.

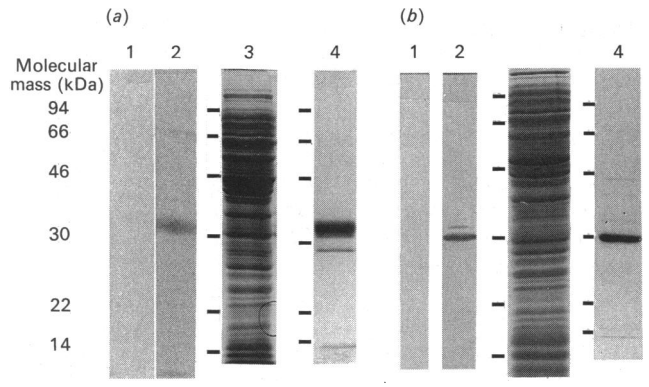

Fig. 2. SDS/PAGE of recombinant thioesterases

Bacterial cytosols [2.5–5 μg for Western immunoblotting (lanes 1 and 2) and 25–30 μg for IS ProBlue stain (lanes 3 and 4)] and purified enzymes (0.7–0.8 μg) were analysed on 12% (w/v) gels. Rabbit anti-(rat fatty acid synthase) and anti-(rat thioesterase II) antibodies were used to detect thioesterases I (*a*) and II (*b*) respectively. Positions of molecular size markers are indicated. Lanes 1–3, cytosol samples; lane 4, purified sample. Induction was carried out for 4 h (*a*, lanes 2–4) or 2–3 h (*b*, lanes 2–4), or was not carried out.

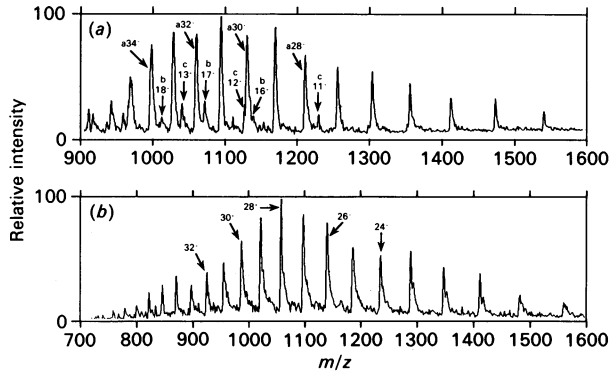

Fig. 3. Electrospray ionization m.s. analysis of the recombinant thioesterases

(*a*) Thioesterase I, (*b*) thioesterase II. Samples were dialysed exhaustively against either 5 mM-ammonium acetate (*a*) or 0.2 M-acetic acid (*b*) and lyophilized prior to redissolution in acetic acid/water/methanol (1:19:20, by vol.) for analysis. Both thioesterases produced characteristic bell-shaped distributions of multiply charged ions, each adjacent peak in the series differing by one charge. The molecular masses (means ± S.D. for three different preparations of each recombinant thioesterase) are: (*a*) a = 33779.4 ± 4.6 Da, b = 18161.7 ± 3.5 Da, c = 13478.9 ± 1.9 Da; (*b*) 29475.0 ± 2.9 Da. The spectrum shown for thioesterase I was produced with the highest purity preparation obtained; nevertheless, two minor impurities, b and c, were also detected.

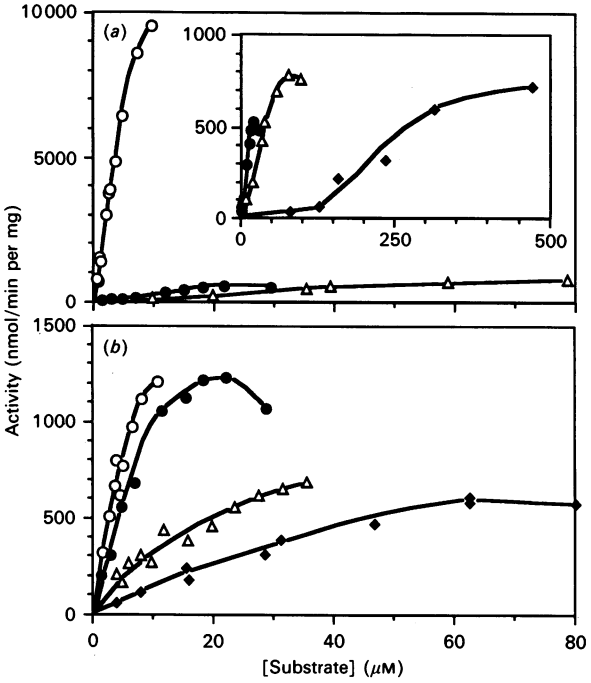

Fig. 4. Kinetic studies on recombinant thioesterases

(*a*) Thioesterase I assay systems contained 14 ng (C_{16} substrate, ○), 46 ng (C_{14}, ●) or 415 ng [C_{12} (△) and C_{10} (◆)] of enzyme. The insert shows activities towards C_{10}, C_{12} and C_{14} over a wider range of concentrations. (*b*) Thioesterase II assay systems contained either 60 ng (C_{16} and C_{14}) or 512 ng (C_{12} and C_{10}) of enzyme (symbols as in *a*).

construct presented a unique problem. Since this enzyme is the *C*-terminal domain of the multifunctional fatty acid synthase, it has no clearly defined *N*-terminus. We therefore selected for introduction of the *N*-terminus a location adjacent to residue Lys-2199, the tryptic cleavage site, which results in separation of the thioesterase I domain from the core protein [5]. Insertion of the *Nco*I site in the coding sequence at this point introduced two new amino acids (Met-Glu-). However, since the hinge regions of multifunctional proteins are often poorly conserved, we guessed that small sequence changes might have no ill-effect. The inducible pJLA expression vector was selected primarily because at the outset we were unsure whether constitutive expression of the mammalian chain-terminating enzymes might affect growth of *E. coli* cells. In fact, the cells continued to grow well at the induction temperature, even though substantial expression of the thioesterases was achieved, and it does not appear that these enzymes disrupt the normal pathways of lipid metabolism in the bacterial cells. The presence of enzymically active recombinant thioesterases in the *E. coli* extracts was detected by elevated thioesterase activity and identification by Western blotting (Fig. 2). The level of expression of the recombinant thioesterases in induced cells amounted to 2–3% of the soluble proteins, as estimated from densitometric scanning of the cytosol electrophoretograms stained with ProBlue and from the thioesterase activities associated with the crude extracts and purified enzymes; additionally, some of the recombinant protein was sequestered into inclusion bodies (results not shown). No thioesterase was detectable immunochemically in uninduced cells (Fig. 2).

Isolation of the recombinant thioesterases from *E. coli* extracts presented no particular problems, and both enzymes were readily purified to near-homogeneity by conventional procedures. Thioesterase I, which previously had only been isolated from a limited digest of the fatty acid synthase by gel filtration, bound loosely to the DE 53 column and was eluted at 45–80 mM-NaCl. The exact molecular masses of the recombinant proteins were determined by electrospray ionization m.s. as 33779.4 Da for thioesterase I and 29475.0 Da for thioesterase II (Fig. 3). The value for thioesterase I is very close to the expected molecular mass of the *N*-formyl full-length enzyme, i.e. 33776.4 Da. Indeed, an attempt to sequence the *N*-terminal region of the recombinant thioesterase I was unsuccessful, consistent with the presence of a blocked *N*-terminus. The observed molecular mass for recombinant thioesterase II was close to the value predicted for the *N*-terminally unblocked enzyme, i.e. 29470.9 Da, and in this case *N*-terminal sequencing gave the residues Met-Glu-Thr-Ala-Val-Asn, in good yield. The reason for the differential *N*-terminal

Table 1. Kinetic parameters for natural and recombinant thioesterase II

Values are the means ± S.D. for calculations by Lineweaver–Burk, Hanes and Cornish-Bowden methods. *Units of activity for the *S*-acyl-FAS (fatty acid synthase) have been converted to nmol of fatty acyl moieties released/min per mg of protein, assuming an average acyl chain length of about 16 carbon atoms. Units for the acyl-CoA thioesters are nmol/min per mg of protein.

Enzyme	Substrate	$V_{max.}$* (nmol/min per mg)	K_m (μM)
Natural	*S*-Acyl-FAS	1220 ± 106	3.3 ± 0.3
Recombinant	*S*-Acyl-FAS	1540 ± 93	3.8 ± 0.4
Recombinant	C_{16}-CoA	2320 ± 580	10.1 ± 3.4
Recombinant	C_{14}-CoA	2160 ± 78	15.7 ± 0.6
Recombinant	C_{12}-CoA	1160 ± 140	20.9 ± 3.0
Recombinant	C_{10}-CoA	1270 ± 111	81.4 ± 8.0

processing of the two thioesterases is unclear, but presumably results from their different *N*-terminal sequences. The recombinant thioesterase II differs from the form isolated from mammary glands in two respects. First, the *N*-terminus of the recombinant enzyme is unblocked, whereas that of the natural enzyme is acetylated. Secondly, whereas the mammary gland-derived enzyme is commonly truncated at the *C*-terminus (possibly an artifact introduced during isolation [17]), the recombinant enzyme is exclusively the full-length polypeptide.

The specific activity of recombinant thioesterase I (9.5 μmol/min per mg), assessed with palmitoyl-CoA as substrate, was higher than that of preparations of the 'natural' form of the enzyme obtained by limited proteolysis of the fatty acid synthase (3–6 μmol/min per mg [1]). The difference can be attributed to the presence, in preparations of the natural enzyme, of variable amounts of nicked polypeptide species, typically 40–60% of the total species, the specific activity of which is much lower than that of the intact enzyme [19]. Use of the recombinant strategy circumvents this limitation, which is inherent in the original procedure. As suspected, inclusion of two new residues, Met-Glu-, at the *N*-terminus had no adverse effect on catalytic activity. The specific activities of the natural and recombinant thioesterase II enzymes, assayed with decanoyl-CoA as substrate, were very similar (580 and 600 nmol/min per mg of protein respectively). A detailed study of the kinetic properties of the recombinant thioesterases was undertaken (Fig. 4). Recombinant thioesterase I exhibited the highest affinity and highest activity for the C_{16}-CoA thioester, as had been found with the natural enzyme [1]. Appreciable activity with shorter acyl-CoA thioesters could be detected if the substrate concentration was raised by one or two orders of magnitude; this property had gone unnoticed in earlier studies with the natural thioesterase I. Under normal circumstances the major product of the fatty acid synthase is the C_{16} fatty acid. However, when the rate of elongation of the enzyme-bound intermediates is slowed by limiting malonyl-CoA availability, shorter-chain-length products are released [20], consistent with the chain-terminating thioesterase I enzyme having some activity toward these thioesters. Because of the sigmoidal substrate–velocity curves typically found with thioesterase I and acyl-CoA substrates [1], it was not possible to calculate K_m values. The substrate concentration curves for the various chain-length acyl-CoA substrates obtained with recombinant thioesterase II were grouped more closely together, as found with the natural enzyme, consistent with the ability of this enzyme to release a broader range of chain-length products from the fatty acid synthase [3]. The substrate–velocity curves in this case were not sigmoidal and kinetic parameters were calculated (Table 1). The activities of the recombinant and natural enzymes toward their natural substrate, *S*-acyl-fatty acid synthase, were also compared. Activity of the recombinant thioesterase II was slightly higher than that of the natural enzyme, possibly because the natural enzyme is partially truncated at the *C*-terminus [17]. Neither the natural nor the recombinant form of thioesterase I exhibited significant activity toward the *S*-acyl-fatty acid synthase substrate. It appears that, unlike thioesterase II, thioesterase I relies entirely on its covalent linkage to the core of the complex for accessing the *S*-acyl-pantetheine thioester and lacks the necessary sites for effective non-covalent association with other domains of the multifunctional protein.

In summary, the host/vector system adopted has facilitated the production of recombinant thioesterases in a catalytically active form and appears well suited for future mutagenesis studies.

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REFERENCES

1. Lin, C. Y. & Smith, S. (1978) J. Biol. Chem. **253**, 1954–1963
2. Knudsen, J., Clark, S. & Dils, R. (1976) Biochem. J. **160**, 683–691
3. Libertini, L. & Smith, S. (1978) J. Biol. Chem. **253**, 1393–1401
4. De Renobales, M., Rogers, L. & Kolattukudy, P. E. (1980) Arch. Biochem. Biophys. **205**, 464–477
5. Naggert, J., Witkowski, A., Mikkelsen, J. & Smith, S. (1988) J. Biol. Chem. **263**, 1146–1150
6. Safford, R., de Silva, J., Lucas, C., Windust, J. H. C., Shedden, J., James, C. M., Sidebottom, C. M., Slabas, A., Tombs, M. P. & Hughes, S. G. (1987) Biochemistry **26**, 1358–1364
7. Randhawa, Z. I. & Smith, S. (1987) Biochemistry **26**, 1365–1373
8. Schauder, B., Blöcker, H., Frank, R. & McCarthy, E. G. (1987) Gene **52**, 279–283
9. Becker, C. R., Efcavitch, J. W., Heiner, C. R. & Kaiser, N. F. (1985) J. Chromatogr. **326**, 293–299
10. Naggert, J., Williams, B., Cashman, D. P. & Smith, S. (1987) Biochem. J. **243**, 597–601
11. Scharf, S. J., Horn, G. T. & Erlich, H. A. (1986) Science **233**, 1076–1078
12. Smith, S. (1981) Methods Enzymol. **71**, 188–200
13. Mikkelsen, J., Witkowski, A. & Smith, S. (1987) J. Biol. Chem. **262**, 1570–1574
14. Libertini, J. L. & Smith, S. (1979) Arch. Biochem. Biophys. **192**, 47–60
15. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
16. Hsu, S.-M., Raine, L. & Fanger, H. (1981) J. Histochem. Cytochem. **29**, 577–580
17. Witkowska, H. E., Green, B. N. & Smith, S. (1990) J. Biol. Chem. **265**, 5662–5665
18. Stormo, G. D. (1986) in Maximizing Gene Expression (Reznikoff, W. & Gold, L., eds.), pp. 195–224, Butterworths, Boston
19. Dileepan, K. N., Lin, C. Y. & Smith, S. (1978) Biochem. J. **175**, 199–206
20. Smith, S. & Abraham, S. (1970) J. Biol. Chem. **245**, 3209–3217

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