BIOSYNTHESIS OF LEUPEPTIN. III

ISOLATION AND PROPERTIES OF AN ENZYME SYNTHESIZING ACETYL-L-LEUCINE

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An enzyme which catalyzes acetylation of L-leucine with acetyl-CoA was partially purified from a cell extract of *Streptomyces roseus* MA839-A1, a leupeptin producer. The molecular weight of this enzyme is about 27,000 daltons. The enzyme has fairly broad specificity for acyl donors (I) and acceptors (II): as for I, propionyl-CoA was 1/10 as active as acetyl-CoA with L-leucine as the acceptor; as for II, L-leucyl-D-leucine, L-leucyl-L-leucine, L-arginine, L-leucyl-L-leucyl-L-leucine, L-phenylalanine, L-methionine, L-leucine, glycyl-L-phenylalanine and L-valine were acetylated in the decreasing order, as opposed to no or slight reactivity of D-phenylalanine, D-leucine, L-histidine, glycine, L-proline and L-glutamic acid. The Michaelis constants of acetyl-CoA and L-leucine were about 5×10^{-6} M and 6×10^{-5} M, respectively. The enzyme is stimulated by Fe^{++}. *p*-Chloromercuribenzoic acid (PCMB), N-ethylmaleimide, Mg^{++} and Mn^{++} were inhibitory. A leupeptin-nonproducing mutant, *Streptomyces roseus* MA839-A1 LN-S, derived from the producer strain by acriflavine treatment, also produced this enzyme.

As previously reported, a multienzyme synthesizing leupeptin acid (acetyl-L-leucyl-L-leucyl-L-arginine) from acetyl-L-leucine, L-leucine and L-arginine was found in a cell extract of *Streptomyces roseus* MA839-A1, a leupeptin producer, and was partially purified[1,2]. The enzyme was named leupeptin acid synthetase. In contrast to the gramicidin S and typocidin synthesizing systems[3], leupeptin acid synthetase is unique in that the intermediate, acetyl-L-leucyl-L-leucine, is spontaneously released from the enzyme, then activated at the expense of ATP, and reacts with L-arginine, the C-terminal amino acid. Little activity of leupeptin acid synthetase was detected in a leupeptin-non-producing mutant strain, derived from the producer strain by the acriflavine treatment[2]. Leupeptin acid synthetase requires preformed acetyl-L-leucine as the initiator of the reaction. Studies were extended to search for an enzyme synthesizing acetyl-L-leucine in cell extracts of the leupeptin producer strain and we were successful in partially purifying the enzyme synthesizing acetyl-L-leucine from acetyl-CoA and L-leucine and studied its properties. The present paper deals with these problems. This enzyme is tentatively referred to as leucine acyltransferase in this paper.

Materials and Methods

Enzyme Reaction

A reaction mixture for synthesis of acetyl-L-leucine contained, in 250 μl, 0.1 M Tris·HCl, pH 9.0, 50 mμCi [^{14}C]-acetyl-CoA (48.3 mCi/mmole), 3 mM L-leucine, 2 mM dithiothreitol, 0.2 mM $FeSO_4$

and an indicated amount of the enzyme. The mixture was incubated at 27°C for 2 hours and the reaction was terminated by quick mixing with 1 ml of cold 0.1 M glycine - 0.1 N HCl buffer (buffer B, pH 1.5) saturated with ethylacetate. The mixture then received 1.25 ml of ethylacetate saturated with buffer B and was mixed and centrifuged at 1,000 *g* for 10 minutes. From the ethylacetate layer, 0.9 ml was taken, mixed with 0.5 ml of buffer B saturated with ethylacetate and was centrifuged as above. A sample (0.75 ml) of the ethylacetate layer was dried *in vacuo* and the residue was dissolved in 0.4 ml of 50% methanol containing 50 μg of acetyl-CoA and 100 μg of acetyl-L-leucine and the solution was applied to paper (Toyo No. 514, 10×40 cm) as a 2-cm streak and electrophoresed in 100 mM phosphate buffer (pH 7.0) at 800 volts for 1 hour. Acetyl-CoA and acetyl-L-leucine were located on the electrophoretogram by UV absorption and by the RYDON-SMITH color reaction, respectively. Relative mobilities were +10.9 (acetyl-CoA), +7.8 (acetyl-L-leucine) and −1.0 (L-leucine)[2]; plus and minus denote migration towards cathode and anode, respectively. The reaction conditions and the components of the enzyme reaction mixture were modified in specific experiments (see legends).

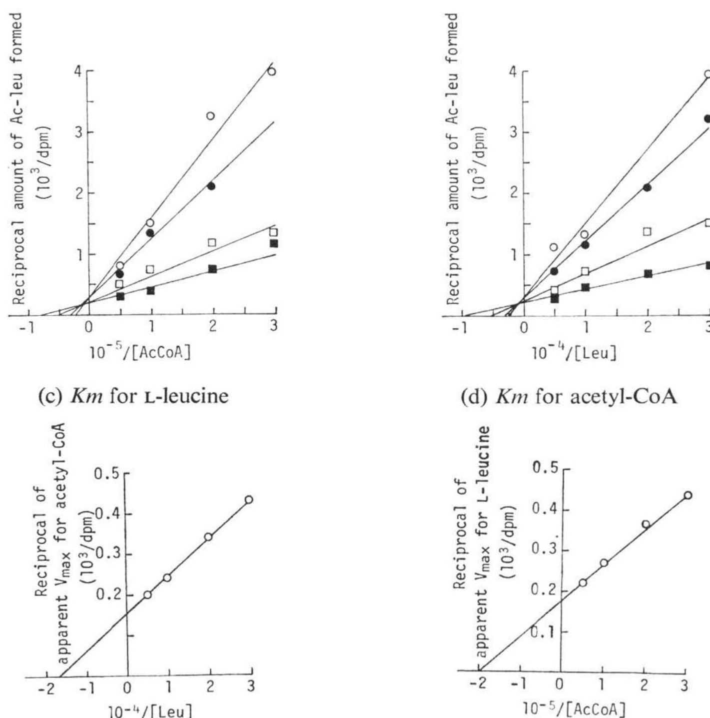
Results and Discussion

Initial Velocity Studies

Radioassay of the leucine acyltransferase can be performed with either acetyl-CoA or L-leucine as

Fig. 1. *Km*'s for acetyl-CoA and L-leucine.

(a) Double reciprocal plot of initial velocity against acetyl-CoA concentration. (b) Double reciprocal plot of initial velocity against L-leucine concentration.



A reaction mixture contained, in 250 μl, 0.1 M Tris·HCl (pH 9.0), 0.2 mM $FeSO_4$, 2 mM dithiothreitol, desired amounts of [^{14}C]-acetyl-CoA and L-leucine, and 0.01 mg of the enzyme (Sepharose 6B fraction).

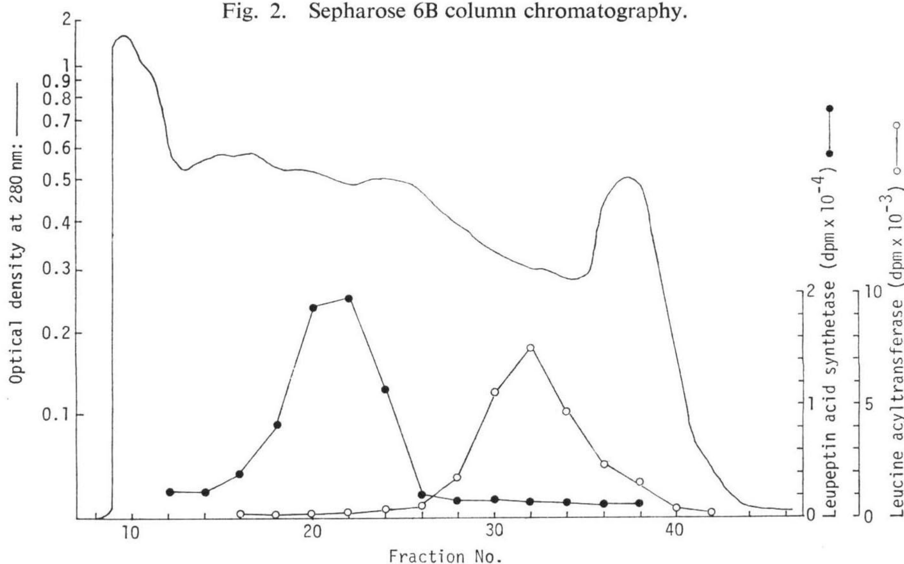
The reaction and analysis of the products were performed as described under "Methods". (a) L-Leucine concentrations were held constant at 3.3×10^{-5} M (○), 5×10^{-5} M (●), 1×10^{-4} M (□), and 2×10^{-4} M (■). (b) Acetyl-CoA concentrations were held constant at 3.3×10^{-6} M (○), 5×10^{-6} M (●), 1×10^{-5} M (□), and 2×10^{-5} M (■). Secondary plot of the apparent V_{max}'s obtained from the Y axes of (a) and (b), was made against reciprocals of molar concentrations of L-leucine (c) and acetyl-CoA (d).

a labeled precursor. However, the substrate with a smaller MICHAELIS constant (*Km*) should be chosen, if the labeled product is readily separated from the precursor. Double reciprocal plots of initial velocity against the concentration of one substrate at a series of fixed concentrations of the second substrate are presented in Fig. 1(a) and (b). The apparent V_{max}'s of the figures were replotted for true *Km*'s in Fig. 1(c) and (d). From the results, the *Km*'s of acetyl-CoA and L-leucine in the presence of saturating amount of the second substrate were calculated to be about 5×10^{-6} M and 6×10^{-5} M, respectively. Therefore, [^{14}C]-acetyl-CoA was used as the radioactive substrate throughout these studies unless otherwise specified.

Purification of the Leucine Acyltransferase

A cell extract (S10 fraction) of the leupeptin producer was prepared as reported[2]. The S10 fraction (40 ml) was made 30% saturated with $(NH_4)_2SO_4$, left standing at 0°C for 1 hour, and centrifuged at 10,000 *g* for 20 minutes. The supernatant was then saturated to 60% with $(NH_4)_2SO_4$ and centrifuged as above. The precipitate was suspended in 2 ml of 100 mM Tris·HCl (pH 8.0) - 2 mM $MgCl_2$ - 5 mM 2-mercaptoethanol (buffer A), mixed and centrifuged at 1,000 *g* for 10 minutes. The supernatant (ammonium sulfate fraction, 4.0 ml) was applied to a Sepharose 6B column (1.6×100 cm) previously equilibrated with buffer A and was eluted with the same buffer under monitoring UV absorption (280 nm). The eluate was cut into 5-ml fractions with which the leucine acyltransferase activity was determined as described in Methods. As Fig. 2 shows, this enzyme was eluted in a peak ranging from fraction 30 to 35 (pooled as Sepharose 6B fraction), being clearly separated from leupeptin acid synthetase which was eluted in the other peak ranging from fraction 19 to 24. The purification index and the recovery of the former enzyme, from S10 to Sepharose 6B fraction, were calculated to be 130 fold and 650%, respectively. These values were obviously overestimated ones, probably caused by removal of endogenous acetyl-CoA and/or some contaminating acetyl-hydrolase during the purification procedures.

Fig. 2. Sepharose 6B column chromatography.



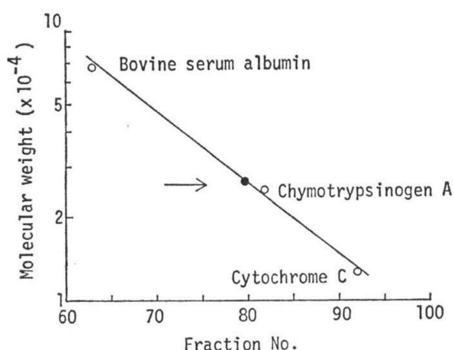
The experiment was conducted as described in the text.

The activity of leupeptin acid synthetase (●) was determined based on the reaction of acetyl-L-leucyl-L-leucine+L-arginine→leupeptin acid[2].

Molecular Weight of the Leucine Acyltransferase

The molecular weight of the leucine acyltransferase was calculated to be about 27,000 from the elution from a Sephacryl S-200 superfine column, as shown in Fig. 3.

Fig. 3. Determination of molecular weight by Sephacryl S-200 gel filtration.



The leucine acyltransferase (Sepharose 6B fraction, 0.12 mg), bovine serum albumin (5 mg), chymotrypsinogen A (2 mg) and cytochrome C (2 mg) were dissolved in 2 ml of buffer A and applied to a column (1.5×90 cm) of Sephacryl S-200 superfine previously equilibrated with buffer A. The column was eluted with the same buffer under monitoring the optical density of the eluate at 280 nm. The eluate was cut into 1.2-ml fractions, with which the leucine acyltransferase activity was determined. The arrow indicates the elution of the leucine acyltransferase.

Substrate Specificity

In the structure of leupeptin, there are limited substitutions of the components, *i.e.* between acetyl and propionyl and between leucyl and valyl or isoleucyl. We examined if these substitutions could be reflected by the substrate specificity of the leucine acyltransferase. As shown in Table 1 (Exp. I), the enzyme-catalyzed synthesis of propionyl-L-leucine about 1/10 as fast as that of acetyl-L-leucine. As for acyl acceptors, specificity of the enzyme was broader than expected (Table 1 (Exp. II, III)); the enzyme catalyzes acetylation of not only L-leucine and L-valine but also unrelated amino acids, such as L-arginine, L-phenylalanine and L-methionine and even peptides, such as L-leucyl-D- or -L-

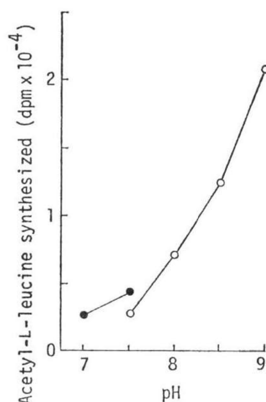
Table 1. Substrate specificity of the leucine acyltransferase.

Exp. I	^{14}C-Acetyl-CoA	+Leu	100
	^{14}C-Propionyl-CoA	+Leu	10*
Exp. II	^{14}C-Acetyl-CoA	+Leu	100
		+D-Leu	12
		+Val	50
		+Phe	179
		+D-Phe	18
		+Met	118
		+His	7
		+Glu	0
		+Leu-leu	246
		+Leu-D-leu	393
		+Gly-Phe	80
		+Leu-leu-leu	191
		+Kanamycin	0
Exp. III	^{14}C-Acetyl-CoA	+Leu	100
		+Gly	2
		+Pro	2
		+Arg	197

In Experiment I, [^{14}C]-acetyl-CoA was replaced by 50 mμCi [^{14}C]-propionyl-CoA (42 mCi/mmole)*.

In Experiments II and III, L-leucine was replaced by a possible acetyl acceptor (3 mM, all were of L-configuration unless specified). Leu stands for L-leucine or L-leucyl. Other amino acids (or amino acyls) are also shortened likewise. The relative value 100 of Experiments I, II and III corresponds 19,523 dpm, 20,201 dpm and 24,491 dpm, respectively. Sepharose 6B fraction (0.014 mg/assay) was used as the enzyme. Reaction products were analyzed as described under Methods except acetylglycine, -L-proline and -L-arginine which were treated as follows. From the incubated mixtures which possibly included these compounds, 200 μl samples were taken, mixed with 200 μl of conc. NH_4OH, left standing overnight at room temperature, and dried *in vacuo*. The residues were dissolved in 20 μl 5 N HCl, dried *in vacuo* and the residues were dissolved in 1 ml of 50% methanol and submitted to electrophoresis (see Methods). Relative electrophoretic mobilities; +10.9 (propionyl-CoA), +8.1 (propionyl-leu), −1.0 (val), −1.0 (phe), −0.2 (gly), −0.8 (met), −0.4 (pro), −2.6 (his), −11.2 (arg), +13.4 (glu), −1.0 (leu-leu), +0.4 (gly-phe), −1.0 (leu-leu-leu), −0.9 (kanamycin), +8.5 (acetyl-val), +8.2 (acetyl-phe), +7.6 (acetyl-gly), +8.0 (acetyl-met), +8.9 (acetyl-pro), +7.8 (acetyl-his), −4.1 (acetyl-arg), +13.2 (acetyl-glu), +5.8 (acetyl-leu-leu), +5.7 (acetyl-gly-phe), +4.4 (acetyl-leu-leu-leu), +1.0 (acetyl-kanamycin).

Fig. 4. Effect of pH on the leucine acyltransferase.



Buffered solutions were 100 mM potassium phosphate (pH 7~7.5, ●) and 100 mM Tris·HCl (pH 7.5~9.0, ○). Sepharose 6B fraction (0.02 mg/reaction mixture) was used as the enzyme. Other conditions are given under Methods.

Table 2. Stimulators and inhibitors of the leucine acyltransferase.

	dpm
^{14}C-Acetyl-CoA+leucine (Complete)	12,893
Complete +2 mM ATP	12,802
+2 mM ADP	11,872
+2 mM GTP	12,773
+2 mM K_2HPO_4	13,580
+10 mM $Na_4P_2O_7$	9,804
+2 mM $MgCl_2$	8,526
+0.2 mM $MnCl_2$	9,531
+0.2 mM $FeSO_4$	26,430
+0.2 mM $CuSO_4$	14,925
+5 mM PCMB	1,909
+10 mM N-Ethylmaleimide	2,394

The complete system included the components of the reaction mixture described under Methods except 0.2 mM $FeSO_4$. Sepharose 6B fraction (0.018 mg/reaction mixture) was used as the enzyme.

leucine, glycyl-L-phenylalanine and L-leucyl-L-leucyl-L-leucine. It should be noted that acetylation of some unrelated amino acids and peptides, especially L-leucyl-D-leucine, proceeded faster than that of L-leucine. On the other hand, D-amino acids seem to be acetylated more slowly than L-isomers; D-leucine against L-leucine at the ratio of 12:100 and D-phenylalanine against L-phenylalanine 18:179. L-Leucyl-L-leucine was more rapidly acetylated than L-leucine or L-leucyl-L-leucyl-L-leucine, suggesting that this enzyme prefers dipeptides to free amino acids or longer peptides. Possible acetylation of kanamycin was tested in view of the recent finding of aminoglycoside N-acetyltransferase in some strains of *Streptomyces*[4]. The answer was negative. Although this enzyme should be important for biosynthesis of leupeptin, the physiological function of this enzyme is difficult to assess because of its broad substrate-specificity and because of the finding that the leupeptin-nonproducing mutant, *Streptomyces roseus* MA839-A1 LN-S, also had this enzyme; the identity was confirmed by elution profile of Sepharose 6B column chromatography and by the substrate specificity (data not shown). When [^{14}C]-phenylalanine was added to a culture of *Streptomyces roseus* MA839-A1, about 6% of the total radioactivity was recovered as acetyl-[^{14}C]-phenylalanine from the medium. This observation suggests that this enzyme is acetylating some free amino acids in living cells. This enzyme may be analogous to acetyl-CoA-L-phenylalanine α-N-acetyltransferase found in *E. coli*.[5]

Effect of pH

On increasing pH from 7 to 9, the leucine acyltransferase activity increased as shown in Fig. 4. Because of the instability of acetyl-CoA to alkali, all the experiments in this paper were conducted with Tris·HCl (pH 9) unless otherwise stated.

Stimulators and Inhibitors

As shown in Table 2, $FeSO_4$ strongly stimulated this enzyme in contrast to the slight effect of $CuSO_4$ indicating that Fe^{++} but not SO_4^{--} is responsible for the stimulatory effect. ATP and some other nucleotides had no effect. PCMB and N-ethylmaleimide were strongly inhibitory.

Stability

This enzyme (Sepharose 6B fraction) lost about 30% activity during storage at −180°C for 3 months. Addition of neither 10% sucrose, nor 10% glycerol, nor 5 mg/ml of bovine serum albumin stabilized the enzyme significantly.

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