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PURIFICATION AND PROPERTIES OF AN ENZYME REDUCING LEUPEPTIN ACID TO LEUPEPTIN

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An enzyme catalyzing the reduction of leupeptin acid to leupeptin was partially purified from a cell extract of *Streptomyces roseus* MA839-A1, a leupeptin producer. The enzyme was tentatively named leupeptin acid reductase. The molecular weight was estimated to be 320 000 by chromatography on Sepharose 6B. The reductase eluted with leupeptin acid synthetase both in molecular sieve chromatography and in affinity chromatography. The main properties of the reductase were: (1) ATP and NADPH were required for activity. ATP could not be replaced by GTP, ADP or AMP. NADPH could not be replaced by NADH. (2) Michaelis constants for ATP and NADPH were $4.2 \cdot 10^{-5}$ M and $1.3 \cdot 10^{-6}$ M, respectively. (3) The enzyme was inhibited by leupeptin, the reaction product, and antipain. Both inhibitors have an L-argininal residue at the C-terminal structure. (4) The enzyme did not catalyze the conversion of leupeptin to leupeptin acid. Leupeptin acid reductase and leupeptin acid synthetase were found in the 10 000 × *g* pellet of the cell homogenate. The reductase was not released as readily from the pellet as the synthetase either by washing or by repeated freeze-thawing. Synthesis of leupeptin from acetyl-CoA, L-leucine and L-arginine in vitro was accomplished by combining leucine acyltransferase and the enzyme complex consisting of leupeptin acid synthetase and leupeptin acid reductase.

Introduction

Two enzymes involved in the biosynthesis of leupeptin, leucine acyltransferase (E_1) and leupeptin acid synthetase (E_2) have been partially purified from cell extracts of *Streptomyces roseus* MA839-A1, a leupeptin producer [1–3]. The reactions catalyzed by these enzymes are as follows:

$$\text{Acetyl-CoA} + \text{L-leucine} \xrightarrow{E_1} \text{acetyl-L-leucine}$$

(Reaction 1) [2]

$$\text{Acetyl-L-leucine} + \text{L-leucine} \xrightarrow[\text{ATP}]{E_2} \text{acetyl-L-leucyl-L-leucine}$$

(Reaction 2a) [1]

$$\text{Acetyl-L-leucyl-L-leucine} + \text{L-arginine} \xrightarrow[\text{ATP}]{E_2} \text{acetyl-L-leucyl-L-leucyl-L-arginine ;}$$

leupeptin acid (Reaction 2b) [1]

As an extension of these studies, we searched for an enzyme (E_3) which would reduce leupeptin acid (acetyl-L-Leu-L-Leu-L-Arg) to leupeptin (acetyl-L-Leu-L-Leu-L-argininal) (Reaction 3). The present communication describes some of the properties of this enzyme. The synthesis of leupeptin from acetyl-CoA, L-leucine and L-arginine was also achieved and the sequence of reactions for biosynthesis of leupeptin was confirmed.

Materials and Methods

Preparation of [^{14}C]leupeptin acid. The reaction mixture (62.5 ml) contained the following components at their indicated concentrations: 100 mM Tris-HCl (pH 9.0)/3 mM acetyl-L-leucyl-L-leucine 1.1 μM L-[U-^{14}C]arginine (351 mCi/mmol)/2 mM ATP/2 mM $MgCl_2$/2 mM dithiothreitol/50 U/ml leupeptin acid synthetase [1] (Sepharose 6B-fraction [2]; 490 U/mg protein). The reaction proceeded at 27°C for 2 h and was terminated by addition of 1.25 ml 5 N HCl. [^{14}C]Leupeptin acid was extracted twice with butanol from the reaction mixture. The butanol extract (140 ml) was dried in vacuo and the residue dissolved in 200 ml water. The solution was applied to a column of Dowex 50W-X8 (H^+ type, 50–100 mesh, 1 × 7.6 cm). The column was eluted with 1 N NH_4OH. Radioactive fractions (33 ml) were combined and dried in vacuo and the residue was dissolved in 4 ml water. The specific activity and the concentration of the [^{14}C]leupeptin acid were 351 mCi/mmol and 4 μCi/ml, respectively. Paper electrophoresis, performed on a 2 μl sample of the above solution together with 50 μg leupeptin acid and 200 μg arginine as carriers, revealed that 98% of the radioactivity resided in the leupeptin acid locus.

Reaction conditions for determining the activity of leupeptin acid reductase. The reaction mixture (250 μl) contained the following components at their indicated concentrations: 100 mM Tris-HCl (pH 9.0)/440 nM [^{14}C]leupeptin acid (351 mCi/mmol)/2 mM ATP/0.2 mM NADPH/2 mM $MgCl_2$/2 mM dithiothreitol and various amounts of leupeptin acid reductase. The reaction proceeded at 27°C for 2 h and was terminated by addition of 1 ml cold water saturated with butanol. An additional 1.25 ml butanol saturated with water was added to the reaction mixtures which were then agitated twice for periods of 15 s each. The reaction mixtures were then centrifuged at 1 000 × *g* for 10 min, 0.9 ml of the butanol layer was removed and washed with 0.5 ml water saturated with butanol. 0.75 ml was dried in vacuo and the residue dissolved in 0.4 ml 50% methanol. Leupeptin and leupeptin acid (50 μg each) were added as carriers and the sample was analyzed for radioactive leupeptin and leupeptin acid by paper electrophoresis [1].

Purification of leupeptin acid reductase. The leupeptin producing strain was cultured, mycelia were harvested, washed and disrupted, and the cell extract (the 10 000 × *g* supernatant; S10 fraction) was prepared as described in previous studies [1]. Purification of the enzyme was performed below 10°C. 14 ml of S10 fraction were brought to 20% saturation with $(NH_4)_2SO_4$ and centrifuged at 10 000 × *g* for 20 min. The supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$ and centrifuged as above. The pellet was dissolved in 1.5 ml buffer A ($(NH_4)_2SO_4$ fraction, 2.2 ml) and 0.8 ml was applied to a Sepharose 6B column (1.5 × 85 cm) equilibrated with the same buffer. The column was eluted with buffer A, 1-ml fractions were collected and the absorbance at 280 nm was monitored. Leupeptin acid reductase and leupeptin acid synthetase activities of these fractions were determined [1] (Fig. 1). Another portion of the $(NH_4)_2SO_4$ fraction (1 ml) was diluted with 10 ml buffer A and applied to a column of acetyl-leucyl-Sepharose (1 × 2 cm), which had been equilibrated with the same buffer. The column was eluted, in a stepwise manner, with 14 ml buffer A, 32 ml 0.3 M NaCl, 36 ml 0.6 M NaCl and 50 ml 1 M NaCl, all in buffer A. The eluant was divided into 1 ml fractions and the absorbance at 280 nm was monitored as above. The activities of leupeptin acid reductase, leupeptin acid synthetase [1] and leucine acyltransferase [2] were determined (Fig. 2).

Preparation of membrane fractions. Mycelia, 1.7 g, were harvested, washed, suspended in 8.5 ml buffer A (100 mM Tris-HCl, pH 8.0/2 mM $MgCl_2$/5 mM 2-mercaptoethanol), disrupted and treated with DNAase I as reported [1]. The homogenate was centrifuged at 1 000 × *g* for 10 min and the resulting supernatant centrifuged at 10 000 × *g* for 20 min. The pellet was suspended in 4.85 ml buffer A (P10 fraction). A 2 ml portion of P10 fraction was subjected to five cycles of freeze-thawing and then centrifuged at 10 000 × *g* for 20 min. The supernatant (fraction 1) was separated from the pellet which was suspended in 1 ml buffer A (fraction 2). Another 2 ml portion of P10 fraction was gently agitated on ice for 5 min, instead of freeze-thawing, and centrifuged yielding supernatant (fraction 3) and pellet (fraction 4).

Results and Discussion

Molecular weight of leupeptin acid reductase. The reductase eluted with the more rapidly eluting part of

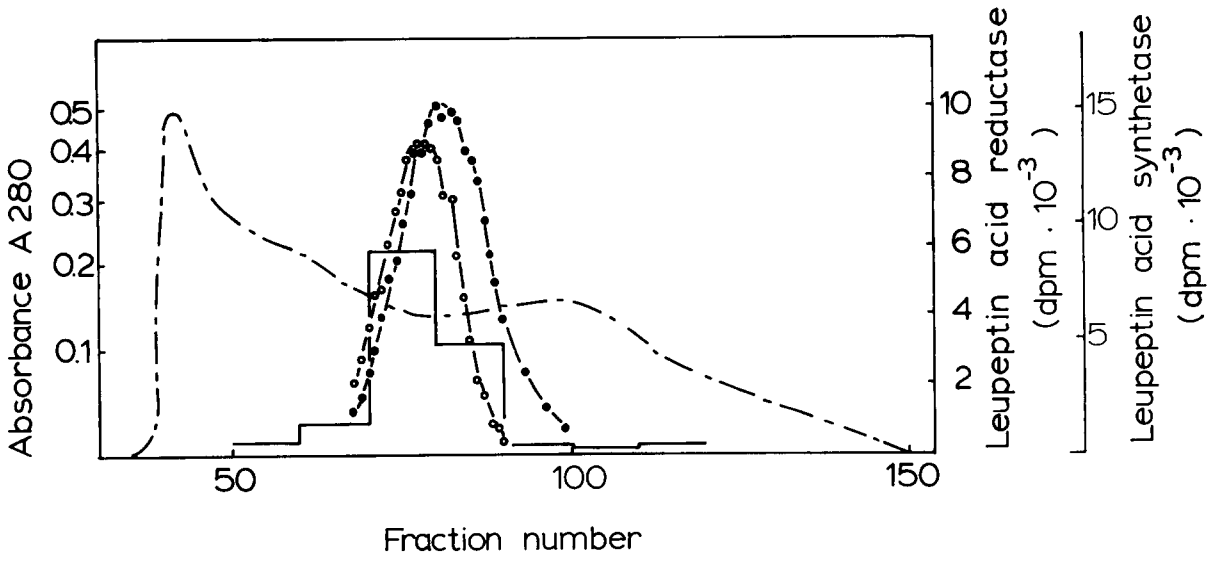

Fig. 1. Sepharose 6B column chromatography of leupeptin acid reductase (o———o) and leupeptin acid synthetase (•———•). Leupeptin acid synthetase was determined based on the activity catalyzing Reaction 2b. · — · — ·, absorbance.

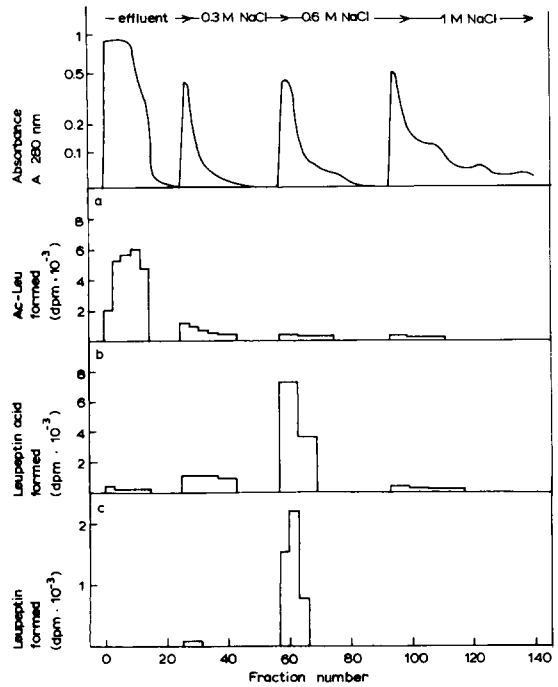

Fig. 2. Affinity chromatography using Ac-Leu-AH-Sepharose. Leupeptin acid was formed by Reaction 2b. 5 g AH-Sepharose 4B (Pharmacia Fine Chemicals) were washed with 1 l of 0.5 M NaCl and 500 ml water. To the gel, 3 ml of an aqueous solution of Ac-Leu (70 mg/ml) and 2 ml of an aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (75 mg/ml, Merck) were added and the mixture was kept at 24°C for 18 h with gentle shaking. The Ac-Leu-AH-Sepharose thus obtained was washed with 1 l water and 500 ml buffer A and stored at 10°C until use.

the broad peak of the synthetase on molecular sieve chromatography with Sepharose 6B (Fig. 1). This broad peak had shoulders on both sides, the more rapidly eluting portion being a shoulder which coincided with the peak of the reductase. Hence, we suspected that an enzyme complex between the reductase and the synthetase was eluted first followed by free synthetase. In order to test for such a putative enzyme complex, we investigated possible co-elution of both activities on affinity chromatography with acetyl-leucyl-Sepharose. The reductase again co-eluted with most of the synthetase (Fig. 2). The ratio between the activities of these two enzymes in the peak fractions (fractions 58–66; 18720 dpm leupeptin acid formed/2325 dpm leupeptin formed) was similar to that of the peak of the reductase on Sepharose 6B chromatography (Fig. 1, fraction 69; 25900 dpm leupeptin acid formed/2566 dpm leupeptin formed). These results suggest that the enzymes exist as a complex.

Additional experiments will be conducted to conclusively prove the reductase-synthetase complex. However, we tentatively refer to these fractions as a

TABLE I

CHARACTERISTICS OF THE REACTION CATALYZED BY LEUPEPTIN ACID REDUCTASE

ATP, ADP, AMP and GTP were tested at 2 mM and NADPH, NADH, $FeSO_4$, leupeptin, antipain and elastatinal at 0.2 mM. The Sepharose 6B fraction of the enzyme was used at 2.2 U (9.1 μg)/reaction. The complete reaction mixture contained [^{14}C]leupeptin acid + ATP + NADPH, yielding 1698 dpm leupeptin. 1 unit of leupeptin acid reductase activity was tentatively defined as the activity yielding 1 pmol leupeptin by Reaction 3.

Reaction mixture		Leupeptin formed (dpm)
minus	plus	
ATP		80
ATP	ADP	113
ATP	AMP	0
ATP	GTP	50
NADPH	NADH	28
NADPH	$FeSO_4$	0
	Leupeptin	81
	Antipain	983
	Elastatinal	1 790

reductase-synthetase complex in this paper. The molecular weight of the reductase-synthetase complex, 320 000, was determined by comparison of the elution peak of the reductase (Fig. 1) with the elution of marker proteins on the same column.

An enzyme resembling carboxypeptidase B was found in the cell extract. Since this enzyme also degraded leupeptin acid, it was necessary to separate this enzyme (M_r 50 000) from leupeptin acid reductase. This was accomplished by column chromatography on Sepharose 6B.

The characteristics of the leupeptin acid reductase reaction. Leupeptin acid reductase required ATP and NADPH (Table I). ATP could not be replaced by ADP, AMP or GTP, while NADPH could not be replaced by NADH. Leupeptin, the product of the reaction, was a strong inhibitor of the reaction, as was antipain, which like leupeptin has an L-argininal residue at the C terminal. In contrast, elastatinal whose C terminal is L-alaninal was not an inhibitor. These observations suggest that enyzme binding may require an arginine residue as the C terminal structure. The apparent Michaelis constants of this enzyme for ATP and NADPH were $4.2 \cdot 10^{-5}$ M and $1.3 \cdot 10^{-6}$ M, respectively, as determined by the Lineweaver-Burk plot.

Enzymes are known to reduce carboxyl to aldehyde through a variety of reaction mechanisms [4–6]. Among them, aryl-aldehyde: $NADP^+$ oxidoreductase (ATP-forming) (EC 1.2.1.30) [4] and an enzyme reducing long fatty acids to the corresponding aldehydes [5] seem to resemble leupeptin acid reductase in that these enzymes both require ATP and NADPH for reduction. Leupeptin acid reductase is the first enzyme reported to reduce the carboxyl group of an aminoacyl residue to an aldehyde.

The enzyme did not catalyze the oxidation of leupeptin to leupeptin acid when appropriate substrates were present. Possible oxidation of leupeptin to leupeptin acid by this enzyme was determined in the presence of $NADP^+$ and either ADP + orthophosphate or AMP + pyrophosphate. No oxidation of leupeptin occurred under any of these conditions.

The optimum temperature for the leupeptin acid reductase reaction was 22°C. No significant effect of pH on the reductase reaction was observed in the pH range 7–9. The enzyme lost 40% of its activity on storage at −180°C for 45 days and 20% of its activity following five cycles of freeze-thawing.

Lack of leupeptin acid reductase in a leupeptin non-producer mutant. Enzyme activity was not detectable in *S. roseus* MA839-A1 LN-S, a leupeptin non-producer which had been derived from the producer strain by treatment with acriflavine [7]. Leupeptin acid synthetase was also greatly reduced in the non-producer; the activities catalyzing Reactions 2a and 2b were one-third and one-eighth those of the producer strain, respectively (data not shown). The various decreases in these catalytic activities may not be due to independent mutations. As described above, no reductase fraction free of synthetase activity has so far been detected suggesting that the reductase was in the form of a complex with the synthetase and that free reductase is inactive and/or unstable. Therefore, if a structural alteration of the synthetase is caused by a mutation in such a way that affects formation of the synthetase-reductase complex, then no reductase activity may be found in the organism.

Subcellular localization of leupeptin acid reductase. We have reported previously that the leupeptin producer exports leupeptin into the medium and

TABLE II

ACTIVITIES OF LEUPEPTIN ACID SYNTHETASE AND LEUPEPTIN ACID REDUCTASE IN THE SOLUBILIZED FRACTION AND IN THE MEMBRANE-BOUND FRACTION

The enzyme activities were determined with 40 μl portions of each fraction. Leupeptin acid synthetase was determined by Reaction 2b. Fractions 1 and 3 are solubilized fractions and fractions 2 and 4 are membrane-bound fractions prepared either by freeze-thawing or by washing.

Enzyme reaction	Fraction					
	1	2	(1 : 2)	3	4	(3 : 4)
LPTA synthetase	10120	2370	(81 : 19)	9307	2362	(80 : 20)
LPTA reductase	3015	4291	(41 : 59)	1760	4870	(27 : 73)

dpm and (ratio)

thereby prevents it from accumulating in cells [8] and interfering with normal protein turnover. In contrast to leupeptin, leupeptin acid, the penultimate product of leupeptin biosynthesis, has no antiprotease activity. Although leupeptin acid reductase was found principally in S10 fraction some activity was also found in P10 fraction. It seemed possible that the enzyme is normally bound to the cell membrane and has a role in establishing a concentration gradient of leupeptin across the membrane. To test this possibility, we attempted to characterize the enzyme localized in the membrane fraction. We first examined how firmly the enzyme was bound to the membrane. For comparison, a parallel experiment was performed with leupeptin acid synthetase which was also partially located in P10 fraction. As can be seen in Table II, about 80% of the activity of leupeptin acid synthetase, originally localized in P10 fraction, was released into the supernatant fraction either by washing or by freeze-thawing. In contrast, only 30–40% of reductase was solubilized under similar conditions. The results indicated that reductase was bound to the membrane more firmly than synthetase and suggested that leupeptin acid reductase may be naturally bound to the cell membrane. Since the reductase-synthetase complex (putative) and free leupeptin acid synthetase, but not free reductase, were found in S10 fraction, the complex of membrane-reductase-synthetase may exist in a state which allows it to dissociate either into membrane + reductase-synthetase or into membrane-reductase + synthetase.

Membrane-bound leupeptin acid reductase shared various characteristics with solubilized leupeptin acid reductase. As shown in Table III, catalytic activity required ATP and NADPH and was inhibited by leupeptin but not by elastatinal. With this enzyme preparation, ATP could be partially replaced by ADP which contrasted with results obtained with the solubilized enzyme (Table I). The possible presence of an ATP-regenerating system in the membrane preparation could explain this apparent inconsistency. The K_m of NADPH of the membrane-bound leupeptin acid reductase was $1.3 \cdot 10^{-6}$ M, a value similar to that of the solubilized enzyme, suggesting

TABLE III

SUBSTRATE SPECIFICITY OF MEMBRANE-BOUND LEUPEPTIN ACID REDUCTASE

The washed P10 fraction (equivalent to 80 mg wet cells/reaction) was used as leupeptin acid reductase. ATP, ADP and GTP were at 2 mM while NADPH, NADH, leupeptin and elastatinal were at 0.2 mM. The complete reaction contained [^{14}C]leupeptin acid + ATP + NADPH, and yielded 6931 dpm leupeptin.

Reaction mixture		Leupeptin formed (dpm)
minus	plus	
ATP		128
ATP	ADP	2926
ATP	GTP	316
NADPH		293
NADPH	NADH	835
	Leupeptin	945
	Elastatinal	7266

TABLE IV

ENZYMATIC SYNTHESIS OF LEUPEPTIN AND ITS INTERMEDIATES

E_1, leucine acyltransferase; E_2-E_3, enzyme complex between leupeptin acid synthetase and leupeptin acid reductase; E_2-(E_3), leupeptin acid synthetase-leupeptin acid reductase under conditions where leupeptin acid reductase was not active because of the presence of leupeptin and absence of NADPH; neg. for 'negligible count'; and n.d., not determined. The enzyme preparations were Sepharose 6B fractions. Radioactive substrates were 50 mμCi/reaction of [1-^{14}C]acetyl-CoA (48.3 mCi/mmol) and 50 mμCi/reaction of L-[U-^{14}C]arginine (340 mCi/mmol). Final concentrations of the unlabeled substrates were 3 mM acetyl-CoA; 6 mM Leu; 3 mM Ac-Leu-Leu; and 3 mM Arg. Reaction 1: the reaction determining leucine acyltransferase activity was performed as reported [2] except that arginine was added and incubation time was extended to 3 h. Leucine acyltransferase was used at 50 U (1 unit leucine acyltransferase activity was tentatively defined as the activity yielding 1 pmol Ac-Leu under conditions as described in the previous paper [2]) (16 μg)/reaction. Radioactive products were analyzed as reported [1,2]. Reaction 2: In Step I, arginine was removed and the incubation time was shortened to 2 h, otherwise conditions were the same as in Reaction 1. In Step II, the reaction mixture resulting from Step I was mixed with 250 μl of buffer 1 (100 mM Tris-HCl, pH 9.0/2 mM ATP/2 mM $MgCl_2$/2 mM dithiothreitol) in which 150 μg leupeptin and 72 U [1] (21 μg) of leupeptin acid synthetase-leupeptin acid reductase has been dissolved. The solution was incubated at 27°C for 1 h. For analysis of products, twice as much butanol as in Reaction 1 was used for extraction. Reaction 3: Arginine was added in Step II, otherwise it was the same as Reaction 2. Reaction 4: Radioactive substrate was L-[^{14}C]arginine, otherwise the same as Reaction 3. Reaction 5: Conditions were as Reaction 3 except that, in Step II, 0.2 mM NADPH and 18 U (see Table I) (12 μg) leupeptin acid synthetase-leupeptin acid reductase were added while leupeptin was removed and the incubation time was extended to 3 h. Reaction 6: Radioactive substrate was L[^{14}C]-arginine, otherwise the same as Reaction 5. Reaction 7: A reaction mixture contained in 250 μl buffer 1 (see Reaction 2), Ac-Leu-Leu, L-[^{14}C]arginine, NADPH and 12 U (see Table I) (8 μg) of leupeptin acid synthetase-leupeptin acid reductase and was incubated at 27°C for 3 h.

Reaction	Radioactivity of products (dpm)			
	Ac-Leu	Ac-Leu-Leu	Leupeptin acid	Leupeptin
1. [^{14}C]Ac-CoA + 2 Leu + Arg $\xrightarrow{E_1}$	5 898	neg.	neg.	neg.
2. [^{14}C]Ac-CoA + 2 Leu $\xrightarrow{E_1\ E_2\text{-}(E_3)}$	1 684	12 091	729	neg.
3. [^{14}C]Ac-CoA + 2 Leu $\xrightarrow{E_1}$ + Arg $\xrightarrow{E_2\text{-}(E_3)}$	2 084	13 267	1 572	neg.
4. Ac-CoA + 2 Leu $\xrightarrow{E_1}$ + [^{14}C]Arg $\xrightarrow{E_2\text{-}(E_3)}$	n.d.	n.d.	34 620	neg.
5. [^{14}C]Ac-CoA + 2 Leu $\xrightarrow{E_1}$ + Arg $\xrightarrow{E_2\text{-}E_3}$	1 178	6 743	2 420	403
6. Ac-CoA + 2 Leu $\xrightarrow{E_1}$ + [^{14}C]Arg $\xrightarrow{E_2\text{-}E_3}$	n.d.	n.d.	2 845	481
7. Ac-Leu-Leu + [^{14}C]Arg $\xrightarrow{E_2\text{-}E_3}$	n.d.	n.d.	23 400	2 183

that the two forms of leupeptin acid reductase are identical.

Synthesis of leupeptin from the structural units by a combination of the enzymes involved in biosynthesis of leupeptin. Synthesis of leupeptin from acetyl-CoA, L-leucine and L-arginine by a combination of leucine acyltransferase and leupeptin acid synthetase-leupeptin acid reductase was accomplished (Table IV). Leupeptin, when present, inhibits leupeptin acid reductase (Expt. 3 and 4) and thereby inhibits leupeptin synthesis.

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References

1 Suzukake, K., Fujiyama, T., Hayashi, H., Hori, M. and Umezawa, H. (1979) J. Antibiot. 32, 523–530
2 Suzukake, K., Hayashi, H., Hori, M. and Umezawa, H. (1980) J. Antibiot. 33, 857–862

3 Hori, M., Hemmi, H., Suzukake, K., Hayashi, H., Uehara, Y., Takeuchi, T. and Umezawa, H. (1978) J. Antibiot. 31, 95–98
4 Gross, G.G. and Zenk, M.H. (1969) Eur. J. Biochem. 8, 413–419
5 Meighen, E.A. (1979) Biochem. Biophys. Res. Commun. 87, 1080–1086
6 Morita, H., Yamamoto, K., Tani, Y., Ogawa, K. and Yamada, H. (1979) Agric. Biol. Chem. 43, 317–323
7 Umezawa, H., Okami, Y. and Hotta, K. (1978) J. Antibiot. 31, 99–102
8 Suzukake, K., Takada, M., Hori, M. and Umezawa, H. (1980) J. Antibiot. 33, 1172–1176