BIOSYNTHESIS OF LEUPEPTIN. II

PURIFICATION AND PROPERTIES OF LEUPEPTIN ACID SYNTHETASE

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(Received for publication February 13, 1979)

An enzyme which condenses acetyl-L-leucyl-L-leucine and L-arginine into acetyl-L-leucyl-L-leucyl-L-arginine (leupeptin acid) was partially purified from a cell extract of *Streptomyces roseus* MA839-A1. With respect to this catalytic activity, the enzyme showed the following characteristics: ATP is essential; optimum pH is 9.5; the activity is inhibited either by EDTA or pyrophosphate or N-ethylmaleimide. The molecular weight of the enzyme is about 260,000 daltons. It also catalyzes some other extension reactions, such as, acetyl-L-leucine+L-leucine+L-arginine → leupeptin acid, and acetyl-L-leucine+L-leucine → acetyl-L-leucyl-L-leucine, but neither L-leucine+L-arginine → (L-leucyl)$_{1-2}$-L-arginine, nor acetyl-L-leucine+L-arginine → acetyl-L-leucyl-L-arginine. ATP-PPi exchange, catalyzed by this enzyme, proceeds with either acetyl-L-leucine, or acetyl-L-leucyl-L-leucine or L-leucine, but not with acetate or arginine.

Leupeptin, or acetyl-L-leucyl-L-leucyl-L-argininal, is an antitryptic compound produced by *Streptomyces roseus* MA839-A1 and some other strains[1]. There is a limited substitution in the structural components as in other small peptide antibiotics. Our previous study[2] on the biosynthesis of leupeptin showed that acetate, L-leucine and L-arginine were efficiently incorporated into the corresponding moieties of leupeptin under fermentation conditions and that a cell extract of the strain catalyzed the reactions yielding leupeptin acid from any of the following combinations of the substrates; sodium acetate, L-leucine and [^{14}C]-L-arginine (Reaction I), acetyl-L-leucine, L-leucine and [^{14}C]-L-arginine (Reaction II), and acetyl-L-leucyl-L-leucine and [^{14}C]-L-arginine (Reaction III). The rate of conversion of a given amount of [^{14}C]-L-arginine into [^{14}C]-leupeptin acid was in the increasing order of Reactions I, II and III. Only a trace of [^{14}C]-leupeptin was detected even with a fresh cell extract by Reaction III. It was thought that a presumptive enzyme responsible for reducing the carboxyl of the L-arginine residue to aldehyde should be unstable. As an extension o f these studies, the enzyme yielding leupeptin acid was partially purified and its properties were studied. Unlike other multienzyme systems synthesizing small peptide antibiotics[3], this enzyme activates the chain inter-

Abbreviations in Tables and Figures: AcONa (sodium acetate), AcCoA (acetyl coenzyme A), leu (L-leucine or L-leucyl), D-leu (D-leucine or D-leucyl), arg (L-arginine), D-arg (D-arginine), ile (L-isoleucine or L-isoleucyl), phe (L-phenylalanine or L-phenylalanyl), ac-leu (acetyl-L-leucine), ac-D-leu (acetyl-D-leucine), ac-leu-leu (acetyl-L-leucyl-L-leucine), ac-leu-D-leu (acetyl-L-leucyl-D-leucine), leu-leu (L-leucyl-L-leucine), leu-arg (L-leucyl-L-arginine), leu-leu-arg (L-leucyl-L-leucyl-L-arginine), PCMB (*p*-chloromercuribenzoate) and NEM (N-ethylmaleimide).

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mediates, namely, acetyl-L-leucine or acetyl-L-leucyl-L-leucine, and completes the formation of acetyl-L-leucyl-L-leucyl-L-arginine by the addition of the latter component amino acids. The enzyme is tentatively named leupeptin acid synthetase. These results are reported in this paper.

Materials and Methods

Enzyme reactions

A reaction mixture for synthesis of leupeptin acid or its possible congeners with L-arginine residue at the C-terminal position consisted of 250 μl, 0.1 M Tris·HCl, pH 9.0, 2 mM ATP, 50 mμCi of [^{14}C]-L-arginine (300 mCi/mmole), N-terminal component(s) (see legends), 2 mM $MgCl_2$, 2 mM dithiothreitol, 600 μg/ml leupeptin and an indicated amount of the enzyme. The mixture was incubated at 27°C for 15 minutes and the reaction was terminated by quick mixing with 1.5 ml of butanol-saturated water and 1.75 ml of water-saturated butanol. The reaction conditions and the components of the reaction mixture were modified in specific experiments (see legends). The butanol-treated mixture was centrifuged at 1,000 *g* for 10 minutes and a sample (1.2 ml) of the butanol layer was washed with 0.5 ml of butanol-saturated water by mixing and centrifugation (for extraction efficiencies of various reactants and products into the butanol layer, see below) and a 1.0 ml sample was taken for a radioactivity measurement with a scintillation solution (0.27% Omnifluor in a 2:1 mixture of toluene - triton X-100) in a liquid scintillation counter. When identification of reaction products was necessary, the radioactive measurement was done with a 0.2-ml sample, while from the remainder 0.8 ml was concentrated to dryness *in vacuo* below 50°C and the resulting residue, together with about 10 μg of an appropriate carrier compound (leupeptin acid, *etc.*), was dissolved in a minimum volume of methanol and submitted to identification of reaction products, as described below. For identification of possible products remaining in the aqueous layer, a portion of the aqueous layer was dried *in vacuo* below 50°C and the resulting residue was treated as above.

A reaction mixture for synthesis of acetyl-L-leucyl-L-leucine consisted of in 1.0 ml, 0.1 M Tris·HCl, pH 7.5, 2 mM ATP, 3 mM acetyl-L-leucine, 0.4 μCi of [^{3}H]-L-leucine (1 Ci/mmole), 2 mM $MgCl_2$, 2 mM dithiothreitol, and 148 μg of the enzyme (DEAE-cellulose fraction). After incubation at 27°C for 30 minutes, the mixture was chilled, combined with 250 μl of bovine serum albumin solution (40 mg/ml), applied to a column of Sephadex G-25 (1.5×23 cm) which had been equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The column was eluted with the same buffer and fractions in a radioactive peak were combined and submitted to identification of the product(s).

A reaction mixture for the ATP-PPi exchange reaction consisted of 250 μl, 0.1 M Tris·HCl, pH 9.0, 2 mM ATP, 2 mM $Na_4{}^{32}P_2O_7$ (0.3 μCi), 5 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM KF, a test compound at 10 mM, and 29 μg of the enzyme (DEAE-cellulose fraction). After incubation at 27°C for 30 minutes, the reaction was terminated by mixing with 250 μl of cold 10% trichloroacetic acid. To the mixture, 0.1 ml of 7.5% (w/v) suspension of Norit A was added and, after standing at room temperature for 15 minutes, Norit A was filtered on a Whatman GF/C disc (2.5 cm diameter), washed with 5 ml water 5 times, dried and determined for radioactivity with a scintillation solution (0.4% Omnifluor in toluene) in a liquid scintillation counter. For an ATP-Pi exchange reaction, 2 mM $Na_4{}^{32}P_2O_7$ was replaced by 2 mM $Na_2H^{32}PO_4$ (0.05 μCi) and otherwise the same as above.

Identification of reaction products

Efficiencies of butanol extraction at pH 3[(a)] and pH 9[(b)], expressed as % recoveries in the butanol layer, were 100[a] and 24[b] (ac-leu-leu), 95[a] and 3[b] (ac-leu), 61[a] and 44[b] (leupeptin acid), 6[a] and 4[b] (leu), 0[a] and 0[b] (arg).

For paper electrophoresis, samples were applied to paper (Toyo No. 51A 10×40 cm) and electrophoresed at 800 volts for 2 hours with 0.1 M potassium phosphate, pH 7.0. Relative mobilities were +7.75 (ac-leu), +3.56 (ac-leu-leu), −1.00 (leu), −1.06 (leupeptin acid), and −3.31 (leupeptin); plus and minus indicate the direction towards anode and cathode, respectively.

For thin-layer chromatography, samples were applied to silica gel plates (20×20 cm Art, 11798, Merck) and developed with butanol - CH_3COOH - H_2O (60:15:25, in volume). Rf values were

0.724 (ac-leu-leu), 0.671 (ac-leu), 0.632 (leupeptin), 0.519 (leupeptin acid), 0.445 (leu), 0.269 (leu-arg), 0.090 (arg).

On chromatograms and electrophoretograms, L-leucine, L-arginine and L-leucyl-L-arginine were localized by the ninhydrin color reaction while leupeptin acid and its congeners by the RYDON-SMITH color reaction.

Sources of chemicals

Leupeptin acid, a by-product on fermentation of *Streptomyces roseus* MA839-A1, was supplied from Nippon Kayaku Co., Ltd. Acetyl-L-leucine, acetyl-L-leucyl-L-leucine and L-leucyl-L-arginine were synthesized in the authors' laboratory. Catalase (Calibration proteins, Combithek kit Size II) was purchased from Boehringer Mannheim GmbH, and ferritin (Type I) and β-galactosidase (Grade VI) were pruchased from Sigma Chemical Co.

Results

Purification of Leupeptin Acid Synthetase

The leupeptin-producing strain was cultured in an enriched medium as described[1]. Mycelia were harvested at the peak of leupeptin synthesis (day 1 or 2 of cultivation), washed twice with cold buffer A (100 mM Tris·HCl buffer, pH 8, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol), and stored at −85°C until use. Purification of the enzyme was performed below 10°C. In an experiment, 25 g mycelia were suspended in 108 ml of buffer A dissolving deoxyribonuclease at 5 μg/ml and disrupted by twice-repeated passage through a French pressure cell at 10,000 psi. The homogenate was centrifuged at 10,000 *g* for 20 minutes and the supernatant was taken (S 10 fraction, 106 ml). The S 10 fraction was made 20% saturated with $(NH_4)_2SO_4$ and the mixture was centrifuged at 10,000 *g* for 20 minutes. The supernatant was made 40% saturated with $(NH_4)_2SO_4$ and centrifuged as above. The pellet was dissolved in 7 ml of the same buffer (ammonium sulfate fraction, 7.9 ml). This fraction was applied to a Sephadex G-200 column (1.5 × 85 cm) equilibrated with the same buffer. Active fractions were pooled (Sephadex G-200 fraction, 27.5 ml). The Sephadex G-200 fraction was applied to a DEAE-cellulose column (1.5 × 23 cm) equilibrated with buffer A. After a wash with 80 ml of 0.1 M NaCl in buffer A, the column was developed with a 200 ml linear gradient of 0.1 ~ 0.5 M NaCl in buffer A. Active fractions (0.27 ~ 0.31 M NaCl) were combined (40 ml) and were made 80% saturated with $(NH_4)_2SO_4$. The mixture was centrifuged and the precipitate was dissolved in 2 ml of buffer A (DEAE-cellulose fraction). As Table 1 shows, these procedures achieved about 80-fold purification of the enzyme on the basis of the catalytic activity yielding leupeptin acid from acetyl-L-leucyl-L-

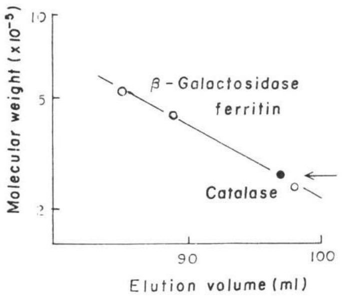
Table 1. Summary of purification

	Total protein, mg	Total unit	Purification index
10,000 *g*, sup	498.2 (100)	1,265 × 10^3 (100)	1
$(NH_4)_2SO_4$, 20 ~ 40%	150.1 (30.1)	1,319 (104.3)	4.4
Sephadex G-200	66 (13.3)	1,925 (152.1)	14.6
DEAE-cellulose	3.8 (0.76)	604 (47.8)	79.7

The enzyme was followed by determining the activity synthesizing leupeptin acid from ac-leu-leu and arg* (see below). One unit of the enzyme activity was arbitrarily defined as the activity yielding 1.5 pmoles of leupeptin acid in 15 minutes of incubation under conditions described under "Methods." In parenthesis, % recoveries are given.

* On DEAE-cellulose chromatography, this activity was superimposed by another catalytic activity synthesizing leupeptin acid from ac-leu, leu and arg.

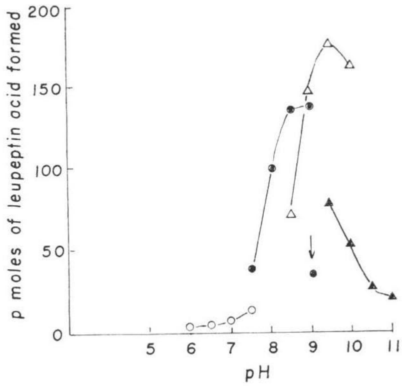
Fig. 1. Determination of molecular weight by Sephadex CL-6B gel filtration.



Leupeptin acid synthetase (Sepharose CL-6B fraction*, 7.4 mg) and marker proteins (5 mg each) were applied to a column of Sepharose CL-6B (1.6×90 cm) equilibrated with 0.1 M Tris·HCl, pH 8.0. 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 ml fractions, with which the enzyme activity yielding leupeptin acid from ac-leu-leu and arg was determined. The arrow indicates the elution of leupeptin acid synthetase.

* An enzyme fraction used for this study was prepared as follows; the S10 fraction was made free from most UV-absorbing contaminants by a preliminary column chromatography with Sepharose CL-6B, which was performed in a similar manner as described above and active fractions were combined.

Fig. 2. Synthesis of leupeptin acid at various pH.



The S10 fraction (100 μg/reaction) was used and the enzyme activity synthesizing leupeptin acid from ac-leu-leu and arg was determined. Tris·HCl at 100 mM in the standard reaction mixture (pH 7.5~9.0) was replaced by 100 mM imidazole·HCl (pH 6.0~7.5), 100 mM glycine·NaOH (pH 8.5~10.0), or $NaHCO_3 \cdot Na_2CO_3$ (pH 9.5~11.0).

Incubation time was extended to 1 hour. The arrow indicates that 0.1 M potassium phosphate (pH 9.0), added into the Tris·HCl system, inhibited the enzyme activity by 81%.

leucine and L-arginine. The purified enzyme appeared unstable; a fresh eluate from the DEAE-cellulose column showed 220-fold purification but the specific activity decreased on storage (see "Stability of the enzyme"). Because of the instability of the purified enzyme, some experiments were conducted with enzyme preparations of lower purity (see legends).

Determination of the Molecular Weight of the Enzyme

The molecular weight of this enzyme was calculated to be about 260,000 from the elution from a Sepharose CL-6B column measured in 0.1 M Tris·HCl, pH 8.0, as shown in Fig. 1.

Optimum pH

The optimum pH of this enzyme was 9.5 in glycine - NaOH buffer (Fig. 2). However, Tris·HCl (9.0) was chosen for general use considering the results of other buffer systems.

Synthesis of Leupeptin Acid and its Possible Congeners Containing the C-Terminal L-Arginine Residue

Characteristics of the enzyme in various reactions for peptide chain extension were studied (Table 2). The results show the following points. (a) Acetyl-L-leucyl-L-leucine reacted with L-arginine more efficiently than a combination of acetyl-L-leucine and L-leucine did. This fact indicates that acetyl-L-leucyl-L-leucine was linked to L-arginine without prior degradation into its components. (b) Since no leupeptin acid was formed by the reaction of sodium acetate+2L-leucine+L-arginine as

Table 2. Synthesis of leupeptin acid and its possible congeners with C-terminal arginine.

	Substrates			dpm	−(none) dpm
Exp. I	3 mM AcONa	+6 mM Leu	+[^{14}C]-Arg	3,739	−348
	3 mM Ac-leu	+3 mM Leu	+ 〃	7,411	3,324
	3 mM Ac-leu-leu		+ 〃	10,183	6,096
		none	+ 〃	4,087*	0
Exp. II	3 mM Ac-leu	+3 mM Leu	+[^{14}C]-Arg	21,100	12,176
	3 mM Ac-leu	+3 mM D-Leu	+ 〃	15,802	6,878
	3 mM Ac-leu	+3 mM Ile	+ 〃	12,138	3,214
	3 mM Ac-leu	+3 mM Phe	+ 〃	7,137	−1,787
	3 mM Ac-leu		+ 〃	5,402	−3,522
	3 mM Ac-D-leu	+3 mM Leu	+ 〃	7,454	−1,470
	6 mM Leu		+ 〃	6,357	−2,567
		none	+ 〃	8,924*	0
Exp. III	0.03 mM Ac-leu-leu		+[^{14}C]-Arg	9,101**	4,694
	3 mM Leu		+ 〃	4,623	216
	0.03 mM Ac-leu-leu	+3 mM Leu	+ 〃	9,057	4,650
		none	+ 〃	4,407*	0

Only D-configuration is indicated in this table, otherwise L-configuration. The radioactive products formed in the absence of N-terminal components (Exp. I, II and III)* were identified as leupeptin acid which was thought to be formed at the expense of enzyme-bound components. However, preincubation of the enzyme with unlabeled arg, ATP, *etc*., in an attempt to wash out possible enzyme-bound constituents, failed to lower these background values. The reaction velocity was a linear function of ac-leu-leu concentration as high as 0.03 mM (Exp. III)**. The enzyme preparations, their amounts in a reaction mixture (250 μl), and incubation periods: 6 μg of the $(NH_4)_2SO_4$ fraction for Exp. I; less than 1 μg of the DEAE-cellulose fraction and 1 hour of incubation time for Exp. II; and 9 μg of the $(NH_4)_2SO_4$ fraction and 1 hour of incubation time for Exp. III.

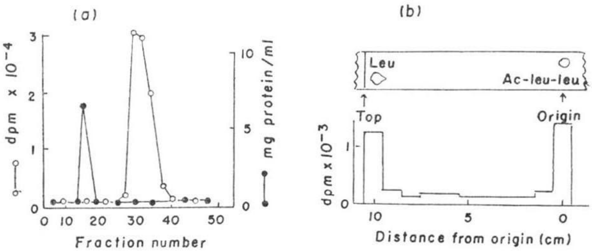
opposed to that of acetyl-L-leucine + L-leucine + L-arginine or of acetyl-L-leucyl-L-leucine + L-arginine, this enzyme failed to synthesize acetyl-L-leucine but was capable of extending a chain with preformed acetyl-L-leucine. Acetyl coenzyme A in place of sodium acetate was ineffective, (data not shown). (c) In the reaction yielding leupeptin acid from acetyl-L-leucine, L-leucine and L-arginine, the L-leucine could partially be replaced by L-isoleucine or D-leucine but not by L-phenylalanine. Since there are minor leupeptins which contain the L-isoleucine residue in place of L-leucine residue[1], the incorporation of L-isoleucine but not L-phenylalanine seems reasonable. On the other hand, the incorporation of D-leucine is difficult to be interpreted because all the amino acid residues of leupeptin are in L-configuration[4]. A possible explanation would be that D-leucine concentration in cells may be much lower than that of L-leucine. Alternatively, acetyl-L-leucyl-D-leucine although once formed would fail to react with L-arginine. In this respect, it was interesting to find that acetyl-D-leucine could not replace acetyl-L-leucine in the reaction yielding leupeptin acid from acetyl-L-leucine, L-leucine and L-arginine. (d) Reaction products, such as acetyl-L-leucyl-L-arginine, L-leucyl-L-arginine and L-leucyl-L-leucyl-L-arginine were not formed indicating that the chain extended in one direction in a stepwise manner; acetyl-L-leucine was linked with L-leucine and L-arginine in this order. (e) L-Leucine at 100 times higher concentration of acetyl-L-leucyl-L-leucine had no effect on the latter to react with L-arginine indicating that L-leucine and acetyl-L-leucyl-L-leucine did not share the same binding site on the enzyme.

Treatment of the leupeptin-producing strain with acriflavin gave various nonproducing mutant strains[5]. The enzyme activity yielding leupeptin acid from any combination of the substrates was not found in the S10 fraction prepared from these nonproducers indicating that there is close correlation between biosyntheses of leupeptin and leupeptin acid (data not shown). The search for an enzyme which will synthesize acetyl-L-leucine is in progress.

Spontaneous Release of Acetyl-L-leucyl-L-leucine from the Enzyme

In biosynthesis of small peptide antibiotics, such as gramicidin and tyrocidin, intermediate peptides are thioesterified to the enzymes until chains are completed[3]. Release of intermediate peptides is reported for mycobacillin[6]. However, it is not known if the released intermediates are incorporated into mycobacillin. In this respect, it was an interesting finding that this enzyme could use preformed chain intermediates, namely, free acetyl-L-leucine or free acetyl-L-leucyl-L-leucine as substrate, and complete the chain by adding the latter components, as shown above. This mechanism suggested that these intermediates, once formed, would leave the enzyme and stand by for the next extension reactions. To prove this, a reaction yielding acetyl-L-leucyl-[^{14}C]-L-leucine from acetyl-L-leucine and [^{14}C]-L-leucine was performed and the reaction mixture, without any treatment which would denature the enzyme, was applied to a column of Sephadex G-25. Radioactivity was found only in the low molecular weight fractions of the eluate, as shown in Fig. 3a, and about 61% of the radioactivity was found to be acetyl-L-leucyl-L-leucine, as shown in Fig. 3b. These results showed that acetyl-L-leucyl-L-leucine, an intermediate for leupeptin acid synthesis, was spontaneously released from the enzyme.

Fig. 3. Spontaneous release of ac-leu-leu from the enzyme.



(a) Enzyme reaction synthesizing ac-leu-leu from ac-leu and [^{3}H]-leu and fractionation of the products by Sephadex G-25 column chromatography were performed as described under Methods.

(b) Radioactive fractions (28～33, (a)) were combined and dried *in vacuo*. The residue was dissolved in 50 μl of methanol and a 20 μl portion of the solution was applied to paper (Toyo No. 514) and chromatographed with 20% methanol as a developing solvent. On the chromatogram, leu and ac-leu-leu were located by the ninhydrin and the RYDON-SMITH color reactions, respectively, and the paper strip was cut into pieces of 1 cm in length whose radioactivities were determined with a sample oxidizer and a liquid scintillation counter.

ATP-PPi Exchange Reaction

In biosynthesis of most peptide antibiotics, their component amino acids are activated by the enzymes at the expense of ATP, yielding amino acyl·AMP·enzyme complexes. This activation step can be demonstrated by the ATP-PPi exchange reaction dependent on each amino acid. On the other hand, the ATP required for biosynthesis of glutathione at each step of condensation is hydrolyzed to ADP+Pi[7]. We wondered if the ATP required for biosynthesis of leupeptin acid was hydrolyzed into AMP and PPi or into ADP and Pi. Another question was if acetyl-L-leucine and acetyl-L-

Table 3. ATP-PPi exchange reaction.

Substrate	dpm
none	177
AcONa	157
AcCoA	164
Leu	595
Ac-leu	6252
Ac-leu-leu	1453
Arg	114
Leu-leu	408
Ile	80
Phe	110

The experiment was conducted as described under "Methods".

Table 4. Inhibitors of leupeptin acid synthetase.

Substrates and inhibitors	Leupeptin acid produced
Ac-leu-leu + [^{14}C]-Arg (complete)	20,497 dpm
None + [^{14}C]-Arg (background)	3,945
Complete + 10 mM EDTA	5,525
〃 + 10 mM EDTA + 10 mM Mg^{2+}	15,547*
〃 + 40 mM NEM	4,942
〃 + 20 mM PCMB	126

The DEAE-cellulose fraction (less than 1 μg/reaction) was used and incubation time was extended to 1 hour. A reaction mixture* (200 μl) containing 2.5 mM ATP, 125 mM Tris·HCl, pH 9.0, 3.75 mM ac-leu-leu, 62.5 mμCi [^{14}C]-L-arginine (300 mCi/mmole), 12.5 mM EDTA, 2.5 mM DTT, 150 μg leupeptin was first incubated at 0°C for 10 minutes, mixed with 50 μl of 50 mM $MgCl_2$ and further incubated at 27°C for 1 hour. Other conditions were as described under "Methods".

leucyl-L-leucine, which served as the substrates for further extension reactions (see above), were also activated. Results of these studies are shown in Table 3. The rate of ATP-PPi exchange was in the decreasing order with acetyl-L-leucine, acetyl-L-leucyl-L-leucine, L-leucine and L-leucyl-L-leucine. No or slight activity was observed with sodium acetate, acetyl coenzyme A or L-arginine. None of the substrates supported ATP-Pi exchange reaction examined in parallel (data not shown). The activation of acetyl-L-leucine, acetyl-L-leucyl-L-leucine and L-leucine was as expected because this enzyme could use any of them as a substrate for synthesis of leupeptin acid. The lack of activation of sodium acetate was also consistent with the above observation that this enzyme required preformed acetyl-L-leucine as an initiator for the extension reactions. Formation of the peptide bond between the L-leucine and L-arginine residues must proceed in a unique manner because acetyl-L-leucyl-L-leucine was activated but L-arginine was not. A possible mechanism would be that enzyme-bound, probably thioesterified, acetyl-L-leucyl-L-leucine reacts with free L-arginine.

Inhibitors

By the reaction yielding leupeptin acid from acetyl-L-leucyl-L-leucine and L-arginine, possible effect of various compounds on this enzyme was determined. EDTA strongly inhibited the reaction probably by sequestering Mg^{2+} which is believed to co-operate with ATP. Mg^{2+}, at equimolar amount of EDTA, partially recovered the enzyme activity. *p*-Chloromercuribenzoate and N-ethylmaleimide at 20 mM and 40 mM, respectively, strongly inhibited the reaction suggesting importance of some thiol groups of the enzyme. The reaction was not inhibited by any of excess D-arginine, agmatin, canavanine and ornithine indicating the specificity for L-arginine as the C-terminal (data not shown).

Stability of the Enzyme

With enzyme preparations of various purification stages, the stability of the enzyme was examined by use of the reaction yielding leupeptin acid from acetyl-L-leucyl-L-leucine and L-arginine. The S10 fraction lost its 10% and 60% activities after storage for 2 weeks at −180°C and −20°C, respectively. The $(NH_4)_2SO_4$ fraction lost its 16% and 27% activities after storage at −180°C for a month and after incubation at 27°C for 24 hours, respectively. The DEAE-cellulose fraction lost its 50% and 80% activities after storage at −180°C for 10 days and after storage at 0°C for 4 days, respectively. But the same enzyme preparation showed no loss of activity on 5-repeated freezings and thawings. Various attempts to stabilize this enzyme preparation were made; addition of 50% glycerol, 0.1 M $(NH_4)_2SO_4$, 5 mg/ml of leupeptin, 5 mg/ml of bovine serum albumin, 5 mM ATP or 2 mM Mg^{2+}. None of these attempts was successful.

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