BIOSYNTHESIS OF LEUPEPTIN. IV

IS PROTEIN TURNOVER IN LEUPEPTIN PRODUCER CELLS AFFECTED BY LEUPEPTIN?

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There was no significant difference between the rates of protein degradation in cells of a leupeptin-producing strain and that of a leupeptin-nonproducing strain, the latter being derived from the former by mutation. Protein autodigestion in a cell homogenate of the leupeptin producer was sensitive to EDTA and chymotrypsin and less sensitive to leupeptin. On the contrary, protein degradation caused by exogenous trypsin in a similar homogenate was highly sensitive to leupeptin. A labeling experiment with [^{14}C]-arginine of a culture of the leupeptin producer strain revealed that leupeptin was accumulated mostly in the medium and only slightly in the cells; the ratio between the amount in the medium and that in the cells was about 250: 1. In contrast, leupeptin acid, the proximal intermediate having no antiplasmin activity, showed a ratio of 5: 1.

Leupeptin, or acetyl-L-leucyl-L-leucyl-L-argininal, was isolated from several strains of *Streptomycetes* on the basis of its antiplasmin activity[1]. It also inhibits trypsin and some other serine proteases[1]. From *Streptomyces roseus* MA839-Al, a leupeptin producer, we partially purified 2 enzymes which should be involved in biosynthesis of leupeptin; leucine acyltransferase catalyzing acetyl-CoA + L-leucine → acetyl-L-leucine and leupeptin acid synthetase catalyzing acetyl-L-leucine + L-leucine + L-arginine → acetyl-L-leucyl-L-leucyl-L-arginine (leupeptin acid).[2~4] An enzyme responsible for the reduction of leupeptin acid to leupeptin (leupeptin acid reductase) is currently being studied. Leupeptin acid, the proximal precursor of leupeptin, has no antiprotease activity[5].

Protein turnover, a cycle of biosynthesis and enzymatic degradation of proteins, is observed in cells of a variety of organisms and the degradation process (proteolysis) is thought to be essential for life[6]. Some serine proteases are evidently involved in proteolysis[7]. In view of the inhibitory effect of leupeptin on a group of serine proteases, we wondered if proteolysis in leupeptin-producing cells is affected by leupeptin, the cells' own metabolite. Generally, a microorganism producing an antibiotic is not affected by its own antibiotic[8]. It is interesting to examine if this rule also applies to a microbial secondary metabolite with a different type of biological activity, such as leupeptin.

Materials and Methods

Determination of protein degradation in intact cells

The leupeptin producer or nonproducer[9] strain was cultured in 11 ml of LAG medium[10] in a L-

shaped tube, with 1% inoculum of a seed culture, at 27°C for one day with shaking. In the case of the leupeptin producer, 0.7 mg/ml leupeptin was found in the culture fluid at this stage. To the culture of either the producer or nonproducer, 0.1 ml of [^{14}C]-phenylalanine (472 mCi/mmole, 5 μCi/ml) was added and incubation was continued for 2 hours. The culture was chilled rapidly, centrifuged at 4,000 g for 10 minutes and the labeled cells were separated from the medium by decantation. The labeled cells were washed in cold conditioned medium* by 2 cycles of suspension and centrifugation. The washed cells were suspended in 11 ml of cold conditioned medium supplemented with unlabeled phenylalanine and chloramphenicol, both at 100 μg/ml. The cell suspension was incubated at 27°C with shaking ("proteolysis" step). At the times indicated, 2 ml samples were taken, mixed with 10 μl of a solution of bovine serum albumin (10 mg/ml), then mixed with 0.3 ml of 50% trichloroacetic acid (TCA) and centrifuged. Of the supernatant, a 0.5 ml portion was mixed with Omnifluor-toluene-Triton scintillation solution and the radioactivity was determined (acid-soluble radioactivity). The precipitate was suspended in 4 ml of 10% TCA, heated at 90°C for 15 minutes, washed sequentially in 4 ml of 10% TCA and 4 ml of ethanol, heated at 50°C for 15 minutes in 4 ml of ethanol-ether (1/1 by volume), washed in 4 ml of ether, and oxidized in a Packard Tri-Carb 306 Sample Oxidizer. The [^{14}C]-CO_2 sample was mixed with the scintillation solution and the radioactivity was determined (acid-insoluble radioactivity). The extent of proteolysis is expressed as % release of radioactivity into an acid-soluble fraction from the original acid-insoluble radioactivity observed at hour 0 of the "proteolysis" step.

Protein degradation in cell homogenates

Leupeptin producer cells were cultured in 8 L-shaped tubes, labeled with [^{14}C]-phenylalanine and collected as described above. The cells were washed 3 times with 10 ml of borate buffer (0.14 M H_3BO_3 - 0.0175 M NaCl - 0.015 M $Na_2B_4O_7$, pH 8.0) and suspended in 30 ml of either LAG(−GS+CP) medium** (LAG medium minus glucose and starch plus ^{12}C-phenylalanine and chloramphenicol, both at 100 μg/ml) or of borate (+CP) buffer** (borate buffer plus ^{12}C-phenylalanine and chloramphenicol, both at 100 μg/ml), and passed through a French pressure cell at 10,000 psi (cell homogenate). A reaction mixture consisted of 1.7 ml of the cell homogenate and 0.3 ml of other components, if indicated, and was incubated at 27°C for 6 hours. To the mixture, 0.3 ml of 50% TCA was added and acid-insoluble radioactivity (for the sample of hour 0) or acid-soluble radioactivity (for the samples of hour 6) was measured and protein degradation (%) was obtained as in the experiment with intact cells.

Determination of the amounts of leupeptin and leupeptin acid accumulated in medium and in cells

From a seed culture of the leupeptin producer in LAG medium, 1 ml portions were inoculated into 4 flasks each containing 125 ml of the same medium. To one flask, 40 μCi of [^{14}C]-arginine (341 mCi/mmole) was added. All were incubated at 27°C with shaking. At 24, 48 and 72 hours of incubation, 125 ml samples of the unlabeled culture (1 flask at a time) and 30 ml samples of the labeled culture were taken. Each of the unlabeled samples was centrifuged at 6,000 g for 30 minutes and the supernatant (unlabeled medium) was separated from the precipitate (unlabeled cells). The unlabeled cells (as carrier) were mixed with the 30 ml sample of the labeled culture which had been taken at the corresponding time and the mixture was centrifuged at 10,000 g for 20 minutes. The supernatant (labeled medium) was separated from the precipitate (a mixture of labeled and unlabeled cells). The precipitate, after washing with about 100 ml of the unlabeled medium, was suspended in 40 ml of buffer (0.1 M Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol), passed twice through a French pressure cell (10,000 psi), mixed with 0.2 mg of DNase I (in 0.4 ml water), left standing at 0°C for 30 minutes and centrifuged at 10,000 g for 20 minutes. From the supernatant, 10 ml was taken, mixed with 10 ml of water-saturated butanol for 5 minutes, and centrifuged. The butanol layer (0.9 ml) was dried *in vacuo* and the residue was dissolved in 1 ml of methanol together with 50 μg each of leupeptin and leupeptin acid. The methanol solution was submitted to paper electrophoresis and the leupeptin and leupeptin acid loci were cut out and their radioactivities were determined[3]. From the labeled medium, 2 ml was taken and

* The conditioned medium was the supernatant of the cultures prepared likewise except that no [^{14}C]-phenylalanine was added. The conditioned medium derived from the cultures of the leupeptin producer strain was used for the labeled cells of the same strain and *vice versa*.

** See legends.

mixed with 2 ml of water-saturated butanol for 30 seconds. After centrifugation, the butanol layer (1.5 ml) was taken and mixed with 1.0 ml of butanol-saturated water for 15 seconds and the mixture was centrifuged. The washed butanol layer (1.0 ml) was dried *in vacuo* and the residue was analyzed for leupeptin and leupeptin acid, as described above.

Results and Discussion

We first examined if there was any difference between the rate of proteolysis in cells of the leupeptin producer and that of a leupeptin nonproducer, *Streptomyces roseus* MA839-A1 LN-S, which had been derived from the producer strain by treatment with acriflavin[9]. A preliminary experiment showed that both strains incorporated [^{14}C]-phenylalanine into their protein fractions at similar rates in a culture medium which was suitable for leupeptin production by the producer strain (data not shown). Proteins degraded also at similar rates in cells of both strains, as Fig. 1 shows, indicating that proteases of the leupeptin producer cells were not affected by leupeptin which was produced during the experiment. A question arose as to whether the leupeptin producer lacked proteases sensitive to leupeptin. To answer this, the rate of protein degradation in a cell homogenate (protein autodigestion) of the producer strain was determined in the presence and absence of leupeptin. Other protease inhibitors were also tested for comparison. As Table 1 shows, the autodigestion was inhibited only by high concentrations of leupeptin as opposed to strong inhibition by low concentrations of chymostatin and EDTA. The result indicates that the leupeptin producer does not lack proteases sensitive to leupeptin, although they are more sensitive to chymostatin and EDTA. Some chymotrypsin type of endoprotease (s)[11] of metal requiring peptidase(s)[12] should be important for autodigestion in these cells. Protein degradation caused by exogenous trypsin in a similar homogenate was strongly inhibited by low concentrations of leupeptin, as shown in Fig. 2, indicating that a trypsin type of protease is not involved in autodigestion.

The moderate inhibition of the protein autodigestion by leupeptin (Table 1) should not be overlooked considering the large production of leupeptin by the strain (sometimes as high as 2 mg leupeptin/ml culture broth). Since production of leupeptin did not affect protein degradation in intact cells of the producer strain (Fig. 1), we wondered if the producer cells have some excre-

Fig. 1. Protein degradation in cells of the leupeptin producer and nonproducer.

The experiment was performed as described under Methods.

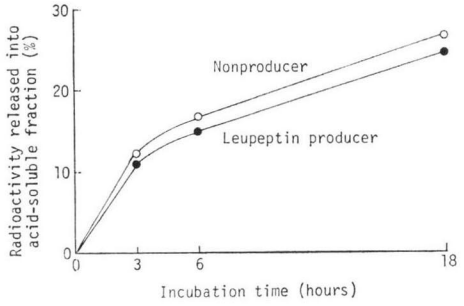

Fig. 2. Effect of leupeptin on the protein degradation in the cell homogenate of leupeptin producer with or without addition of trypsin.

The labeled cells were suspended in borate (+CP) buffer and broken. To 1.7 ml cell homogenate, 0.2 ml of leupeptin (in water) with or without 0.1 ml trypsin (50 μg/ml of 0.01 N HCl) were added, as indicated. Each reaction mixture was made up to 2.0 ml with borate(+CP) buffer. Other conditions were as given under Methods.

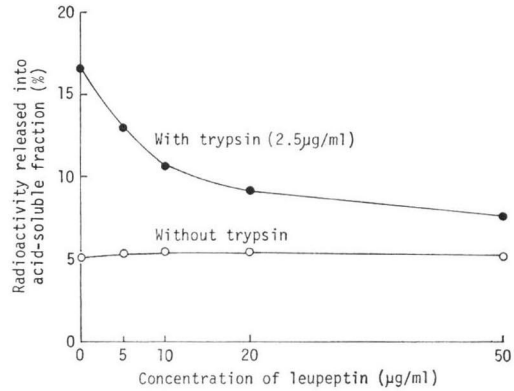

Table 1. Effect of leupeptin and other protease inhibitors on protein autodigestion in cell homogenate of leupeptin-producer.

Additions		Radioactivity released into acid soluble fraction	
None (control)		4,200 dpm	(100%)
+Leupeptin	10 μg/ml	3,601	(86)
	50	3,390	(81)
	100	3,289	(78)
	500	1,978	(47)
+Chymostatin			
	50	1,118	(27)
+EDTA	2900	524	(12)

The labeled cells were suspended in LAG(−GS +CP) medium. To 1.7 ml cell homogenate, 0.2 ml of leupeptin (in water) or chymostatin (in dimethylsulfoxide) or EDTA (in water) was added, if indicated. Each reaction mixture was made up to 2.0 ml with LAG(−GS+CP) medium. Other conditions are given under Methods.

Table 2. Localization of leupeptin and leupeptin acid in the medium and in the cells.

		Labeling time (hours)		
		24	48	72
Leupeptin	(a)	3,090	8,960	11,900
	(b)	18	36	138
	(a)/(b)	171	248	86
Leupeptin acid	(a)	460	670	570
	(b)	83	127	380
	(a)/(b)	5.5	5.3	1.5

The experiment was conducted as described under Methods.

(a): dpm in the medium derived from a 30 ml culture broth $\times 10^{-3}$

(b): dpm in the cells derived from the same culture broth $\times 10^{-3}$

(a)/(b): the ratio of "in the medium/in the cells".

tion mechanism for leupeptin, a harmful metabolite. For this purpose, we labeled a culture of the leupeptin producer with [^{14}C]-arginine and determined the radioactivity of the intra- and extracellular [^{14}C]-leupeptin and, for comparison, its proximal precursor, [^{14}C]-leupeptin acid. It should be remembered that the precursor has no antiplasmin activity. As Table 2 shows, the index of "in the medium/in the cells" of leupeptin was much larger than that of leupeptin acid throughout the incubation period, indicating that the cells actively pumped out leupeptin but probably not leupeptin acid.

We conclude that the protein degradation system of the leupeptin producer cells is not affected by leupeptin production because of the limited contribution of leupeptin-sensitive protease(s) to the process, or the active removal of leupeptin from the cells, or both.

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