JOURNAL OF BACTERIOLOGY, Aug. 1988, p. 3650–3654
0021-9193/88/083650-05$02.00/0

Vol. 170, No. 8

A Novel Outer-Membrane-Associated Protease in *Escherichia coli*

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Received 31 December 1987/Accepted 23 May 1988

Human gamma interferon produced by recombinant *Escherichia coli* was degraded by endogenous protease after cell disruption. Specific cleavages took place at the center of two pairs of basic amino acids (Lys-131–Arg-132 and Arg-142–Arg-143) in the C-terminal region, giving rise to products with molecular weights of 17,500 and 16,000. The proteolytic activity was associated with the outer membrane of *E. coli*. It was insensitive to the protease inhibitors diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, tosyl-L-lysine chloromethyl ketone, EDTA, and *p*-chloromercuribenzoate. Benzamidine and the bivalent cations Zn^{2+} and Cu^{2+} inhibited the activity. Dynorphin A(1-13) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys) was a good substrate and was preferentially cleaved at the center of Arg-6–Arg-7. Neither the amino nor carboxyl sides of Arg-9 and Lys-11 were digested. These results indicate that the protease specifically cleaves the peptide bond between consecutive basic residues and therefore is different from the known membrane enzymes, proteases IV, V, and VI. We have designated this new enzyme protease VII.

It is known that *Escherichia coli* contains a variety of membrane-bound proteolytic enzymes. Several lines of evidence indicate that these enzymes may play an important role in bacterial cell physiology. Specific cleavage reactions, such as the cleavage of colicins Ia, A, and AI to inactive polypeptide fragments (1), the cleavage of plasminogen to plasmin (9), caseinolytic activity (20), nitrate reductase-solubilizing activity (11), and ferric enterobactin receptor-modifying activity (5), have been reported to be carried out by membrane preparations from *E. coli*. The purification and characterization of two membrane enzymes (protease IV and protease V) which are capable of degrading alkylated and high-molecular-weight cytoplasmic proteins have been reported by Pacaud (17). Protease IV has been identified by Ichihara et al. (6) as a signal peptide peptidase which degrades the signal peptide after its release from prolipoprotein by the signal peptidase during the maturation and export process. Additionally, Palmer and St. John have identified another membrane-associated protease termed protease VI (19). Protease VI is a serine protease located at the outer membrane of the cells and generates acid-soluble fragments from a mixture of *E. coli* membrane proteins. It may play a role in the turnover of *E. coli* membrane proteins, but the physiological role has not been identified.

In the course of our investigations into the production of human gamma inteferon (4) in *E. coli* by recombinant DNA technology, we found a novel proteolytic activity in *E. coli* lysate. The human gamma interferon accumulated to a level about 10 to 15% that of the total *E. coli* proteins at the end of cultivation and was found to have undergone proteolytic cleavage in the C-terminal region after the cells were harvested and disrupted. The localization, inhibitor profile, and substrate specificity of the proteolytic enzyme revealed that it is a novel protease. We report here the characterization of this enzyme and compare it with the previously studied membrane proteases.

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MATERIALS AND METHODS

Materials. Diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), and lipopolysaccharides from *E. coli* O111:B4 were purchased from Sigma Chemical Co., St. Louis, Mo. Antipain, chymostatin, leupeptin, dynorphin A(1-13), and MCA substrates were from Protein Institute Inc., Osaka, Japan. Casamino Acids and yeast extract were from Difco Laboratories, Detroit, Mich. Recombinant human gamma interferon (rHu IFN-γ) was produced in our laboratory. A solution containing 2.2 mg of rHu IFN-γ, 0.1% (vol/vol) 2-mercaptoethanol, and 0.3 M NaCl in 1 ml of 20 mM sodium phosphate buffer (pH 6.8) was used in all experiments.

Bacterial strain and plasmid. *E. coli* W3110 and plasmid pIN5T4, which has a synthetic gene for rHu IFN-γ (21) under the control of the *lpp* promoter of pIN IA-2 (15) inserted into the multiple-copy plasmid pBR322, were used in this study.

Media and culture conditions. For rHu IFN-γ production, W3110 (pIN5T4) was grown in a medium which consisted of 20 g of glycerol, 30 g of Casamino Acids, 2 g of yeast extract, 1 g of $MgSO_4 \cdot 7H_2O$, 5 g of potassium phosphate, 0.6 g of NaOH, and 20 mg of tetracycline in 1 liter of deionized water (GC medium). Cells were grown to the stationary phase in a 3-liter fermentor containing 2 liters of medium under the following conditions: temperature, 30°C; pH 6.5 controlled with 20% NaOH; aeration, 1 liter/min; and agitation, 800 rpm. For subcellular fractionation and preparation of envelope fractions, W3110 was cultured in LB medium (12) at 37°C with shaking in 1-liter Erlenmeyer flasks each containing 400 ml of medium. Cells were harvested at the logarithmic phase at an optical density at 600 nm of about 0.7.

Membrane preparation and separation. Membrane preparation and fractionation into inner and outer membranes by centrifugation on a discontinuous sucrose density gradient were performed as described by Osborn et al. (16).

Protein concentration determination. The method of Lowry et al. (10) was used with bovine serum albumin as a standard.

Detection of rHu IFN-γ-degrading activity. Degradation of rHu IFN-γ was assayed as follows. *E. coli* W3110 (pIN5T4) cells (1 g) cultured in GC medium were suspended in 10 ml of

50 mM Tris hydrochloride buffer (pH 7.5) and disrupted with a French press at a pressure of 20,000 lb/in^2 below 10°C. After centrifugation at 10,000 × *g* for 10 min at 4°C, the supernatant was incubated at 37°C for 0, 15, 30, and 60 min. Aliquots (500 μl) were added to 250 μl of 3 × sodium dodecyl sulfate (SDS) sample buffer (300 mg of glycerol, 150 mg of 2-mercaptoethanol, and 69 mg of SDS in 1 ml of 187.5 mM Tris hydrochloride buffer [pH 6.8]), heated at 100°C for 5 min, and submitted to SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie brilliant blue R-250.

Enzyme assays. NADH oxidase was estimated by the method of Osborn et al. (16).

The assay of rHu IFN-γ-degrading activity was performed as follows. rHu IFN-γ solution (10 μl, 22 μg of protein), 10 μl of an appropriately diluted enzyme fraction, and 80 μl of 50 mM Tris hydrochloride buffer (pH 8.0) were mixed and incubated at 37°C for 30 min. After the reaction was stopped by the addition of 50 μl of 3× SDS sample buffer, aliquots were heated at 100°C for 5 min and submitted to SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie brilliant blue R-250, and the amount of the degradation product was determined with a Shimazu CS-9000 gel scanner. One unit of rHu IFN-γ-degrading activity was expressed as one nanomole of rHu IFN-γ degraded per minute.

Measurements of rHu IFN-γ-degrading activity in the presence of various inhibitors or cations were obtained as follows. An outer membrane fraction (0.2 μg of protein) was added to 90 μl of 50 mM Tris hydrochloride buffer (pH 7.5) containing a protease inhibitor or cation. After 30 min of incubation at 37°C, the reaction mixture was added to 10 μl of rHu IFN-γ solution (22 μg of protein) and incubated for a further 30 min. The degradation activity was determined as described above.

Hydrolysis of MCA substrates was assayed as follows. Protein (20 μg) from the outer membrane fraction was added to each reaction mixture containing 10 μg of substrate in 1 ml of 50 mM Tris hydrochloride buffer (pH 7.5) at 37°C. The increase in the relative fluorescence (percent) with excitation at 380 nm and emission at 460 nm was monitored with a Hitachi F-4000 fluorescence spectrophotometer.

Electrophoresis. Electrophoresis with SDS was carried out on 15% polyacrylamide gels by the method of Laemmli (8).

Analysis of cleavage sites. The site(s) of cleavage of rHu IFN-γ by protease VII was analyzed as follows. A reaction mixture containing 10 μl of rHu IFN-γ solution (22 μg of protein), 10 μl of outer membrane preparation (1 μg of protein), and 80 μl of 0.1 M Tris hydrochloride buffer (pH 8.0) was incubated at 37°C for 5 to 60 min. After the reaction was stopped by the addition of 25 μl of 50% trichloroacetic acid (TCA), insoluble materials were precipitated by centrifugation. The supernatant (25 μl) was applied to a column of YMC A-302 ODS (4.6 by 150 mm), and elution was carried out with a linear gradient of 0 to 48% CH_3CN in 0.1% trifluoroacetic acid solution at a flow rate of 1 ml/min. The effluent was monitored by measuring the A_{210}. The material in the respective peptide peaks was collected, hydrolyzed with 6 M HCl at 110°C for 24 h, and analyzed on an amino acid analyzer (Hitachi 835).

The site(s) of cleavage of dynorphin A(1-13) by protease VII was analyzed as follows. Dynorphin A(1-13) (10 μg) was incubated at 37°C for 15 min with 0.2 μg of protein from the outer membrane preparation in 100 μl of 0.1 M Tris hydrochloride buffer (pH 8.0). TCA precipitation, high-pressure liquid chromatography (HPLC), and amino acid analysis were performed as described above.

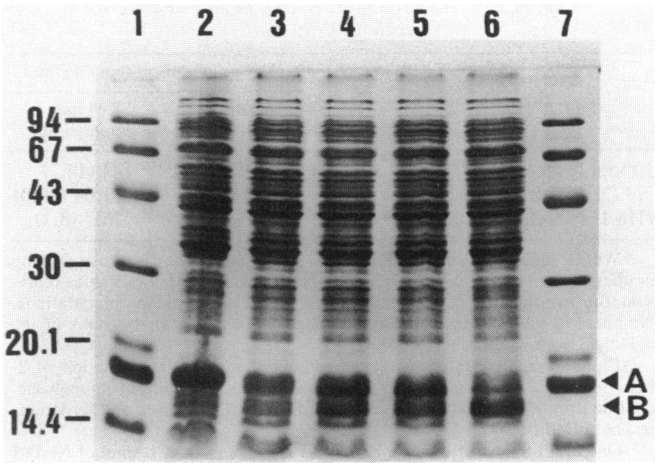

FIG. 1. Degradation of rHu IFN-γ by *E. coli* protease(s) after cell disruption. *E. coli* W3110 (pIN5T4) cells were cultured in GC medium, harvested, and disrupted with a French press. After centrifugation, the supernatant was incubated at 37°C for various times and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Lanes 1 and 7, Molecular weight standards (in thousands); 2, total proteins of the cells before disruption; 3 to 6, supernatant of the cell extract incubated for 0, 15, 30, and 60 min, respectively. Arrowheads A and B indicate intact rHu IFN-γ and the 16k degradation product, respectively. The 17.5k product migrated slightly ahead of intact rHu IFN-γ.

RESULTS

Detection of a proteolytic enzyme which cleaves rHu IFN-γ specifically. *E. coli* W3110 transformed by the vector of pIN5T4 accumulated rHu IFN-γ to a level about 10 to 15% that of the total *E. coli* proteins at the end of cultivation (Fig. 1, lane 2). About one-third of the expressed rHu IFN-γ remained in a soluble state, and the rest formed inclusion bodies in the cells. When the cells were disrupted with a French press cell disruptor to extract rHu IFN-γ, degradation of the soluble form of rHu IFN-γ was detected by SDS-polyacrylamide gel electrophoresis (Fig. 1, lanes 3 to 6). Fifty percent of the intact molecules migrating at a molecular weight of 18,000 (18k) were transferred within 15 min to the faster-moving band having an apparent molecular weight of 17.5k. One-hundred percent conversion to a further-degraded product of 16k was exhibited within 1 h (Fig. 1, lanes 3 to 6), but no more degradation was observed at 2 h (data not shown). The observations and results described below suggested the existence of a specific protease in the *E. coli* lysate. Similar specific cleavages of rHu IFN-γ were observed in another *E. coli* strain, WA802 (data not shown), suggesting the general existence of this proteolytic activity in *E. coli*.

Cellular localization of the proteolytic activity. The expressed rHu IFN-γ accumulated in the cytoplasm of recombinant *E. coli* W3110, and no degradation in the cells was observed for over 12 h in a pulse-chase experiment with [^{35}S]methionine (data not shown). Despite the stability in the cells, rHu IFN-γ recovered in the soluble fraction of the extracts was degraded during and after cell disruption. Three possibilities were considered to explain these phenomena. (i) The proteolytic activity was masked in the cells and activated by a factor such as another protease or mechanical

TABLE 1. Localization of rHu IFN-γ-degrading activity in *E. coli*

Component	U or μg/mg of protein (%)[a] in:	
	Outer membrane	Inner membrane
Lipopolysaccharide[b]	838 (85.3)	144 (14.7)
NADH oxidase[c]	0.01 (1.0)	1.04 (99.0)
rHu IFN-γ-degrading enzyme[d]	179 (91.9)	15.7 (8.1)

[a] Whole inner and outer membranes were isolated by sucrose density gradient centrifugation as described in Materials and Methods. Values represent the averages of determinations with three independent preparations. Numbers in parentheses represent average percentages of the sums of the values in both membranes.

[b] Lipopolysaccharide content was estimated by the determination of 2-keto-3-deoxyoctonate as described by Osborn et al. (16) with the lipopolysaccharide from *E. coli* O111:B4 as a standard and was expressed as micrograms instead of units.

[c] One unit of NADH oxidase activity was expressed as 1 nmol of NADH hydrolyzed per min.

[d] One unit of rHu IFN-γ-degrading activity was expressed as 1 nmol of rHu IFN-γ degraded per min.

stress during cell disruption. (ii) An endogenous protease inhibitor like Ecotin (2) exists in the cytoplasm of *E. coli* and interfered with the rHu IFN-γ-degrading activity in the intact cells. (iii) The proteolytic activity was localized outside of the cytoplasm and could not be in contact with the produced rHu IFN-γ without cell disruption. To examine these possibilities, we first investigated the localization of the proteolytic activity. *E. coli* cells were disrupted and fractionated into membrane and other fractions. The proteolytic activity was examined with purified rHu IFN-γ as a substrate. Only the membrane fraction showed the degradation activity (data not shown). We further fractionated the membrane preparation into inner and outer membranes by centrifugation on a discontinuous sucrose density gradient. 2-Keto-3-deoxyoctonate from lipopolysaccharide was used as a marker component of the outer membrane, and NADH oxidase was used as that of the inner membrane. More than 90% of the rHu IFN-γ-degrading activity existed in the outer membrane fraction of *E. coli* (Table 1), suggesting that the last possibility is the case.

Effect of inhibitors and bivalent cations. Table 2 summarizes the effect of protease inhibitors and a metal chelator on the rHu IFN-γ-degrading activity. The serine protease inhibitors DFP and PMSF, the thiol protease inhibitor *p*-chloromercuribenzoate (PCMB), and the metalloprotease inhibitor EDTA did not inactivate the protease activity. A reagent which alkylates the active-site histidine residue of trypsin, tosyl-L-lysine chloromethyl ketone (TLCK), did not affect the rHu IFN-γ-degrading activity; a reagent which alkylates that of chymotrypsin, tosyl-L-phenylalanine chloromethyl ketone (TPCK), inactivated the activity by 28%. Benzamidine, an inhibitor of trypsinlike proteases, inhibited the protease activity by 68%. The effect of bivalent cations was also examined, and the activity was significantly decreased with a low concentration of $CuCl_2$ (0.5 mM) and completely inhibited by $ZnCl_2$ (0.5 mM). The bacterial enzyme inhibitors antipain (inhibitor of papain and cathepsins A and B), chymostatin (inhibitor of chymotrypsin, papain, and cathepsins A, B, and D), and leupeptin (inhibitor to trypsin, plasmin, papain, and cathepsin B) had no effect on the protease activity at concentrations of up to 10 μg/ml.

TABLE 2. Effect of various protease inhibitors and bivalent cations[a]

Inhibitor or cation (concn)	Relative activity (%)[b]
None (5 mM)	110
DFP (5 mM)	102
PMSF (5 mM)	107
PCMB (5 mM)	110
EDTA (5 mM)	107
Benzamidine (5 mM)	32
TLCK (1 mM)	112
TPCK (1 mM)	72
Leupeptin (10 μg/ml)	108
Antipain (10 μg/ml)	108
Chymostatin (10 μg/ml)	98
$ZnCl_2$ (0.5 mM)	0
$CuCl_2$ (0.5 mM)	15

[a] rHu IFN-γ-degrading activity was measured in the absence or presence of a protease inhibitor or cation as described in Materials and Methods.

[b] Values represent the averages of duplicate assays.

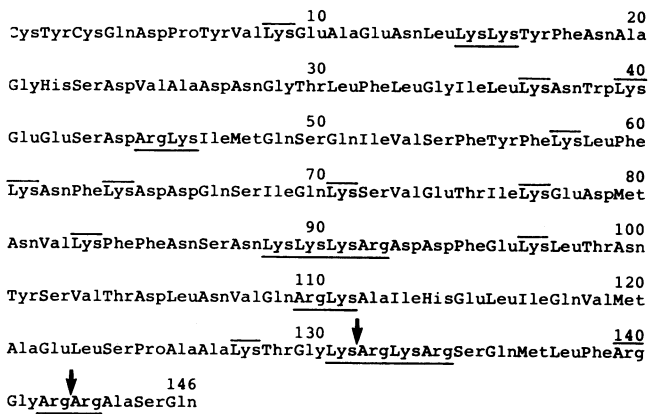

FIG. 2. Amino acid sequence of rHu IFN-γ (4). Arrows indicate the sites of cleavage by protease VII. Single and consecutive basic residues are indicated by overlining and underlining, respectively.

Specific sites of cleavage of rHu IFN-γ. Intact rHu IFN-γ with a molecular weight of 18k was purified from cells disrupted in the presence of 1 mM $ZnCl_2$. The amino acid sequence of rHu IFN-γ is shown in Fig. 2. We also purified the degradation products of rHu IFN-γ in the absence of $ZnCl_2$; these had apparent molecular weights of 17.5 and 16k on a 15% SDS-polyacrylamide gel. Amino acid sequence analyses revealed that the 17.5 and 16k molecules were composed of amino acids 1 to 142 and 1 to 131 of rHu IFN-γ, respectively (Fig. 2). To elucidate whether the outer membrane fraction of *E. coli* specifically cleaved the center of the peptide bonds between Lys-131–Arg-132 and Arg-142–Arg-143 and which site was the first step in cleavage, we analyzed the small peptides released from rHu IFN-γ after incubation with the isolated outer membrane. HPLC of TCA-soluble reaction products after 5 min of incubation at 37°C resolved two peptide peaks (Fig. 3B) which, on the basis of amino acid analyses and comparison with the amino acid sequence of rHu IFN-γ, were identified as Arg-143–Ala–Ser–Gln-146 (peak 1) and Arg-132–Lys–Arg–Ser–Gln–Met–Leu–Phe–Arg–Gly–Arg-142 (peak 2). This result revealed that the outer membrane of *E. coli* has a proteolytic enzyme which preferentially cleaves rHu IFN-γ at Arg-142–Arg-143 and Lys-131–Arg-132 specifically. Additionally, the ratio of peak 2 to peak 1 at 60 min increased to 2.3 times that at 5 min (Fig. 3), indicating that the peptide bond of Arg-142–Arg-143 was more susceptible to the specific proteolytic enzyme than was that of Lys-131–Arg-132.

Substrate specificity of the rHu IFN-γ-degrading protease.

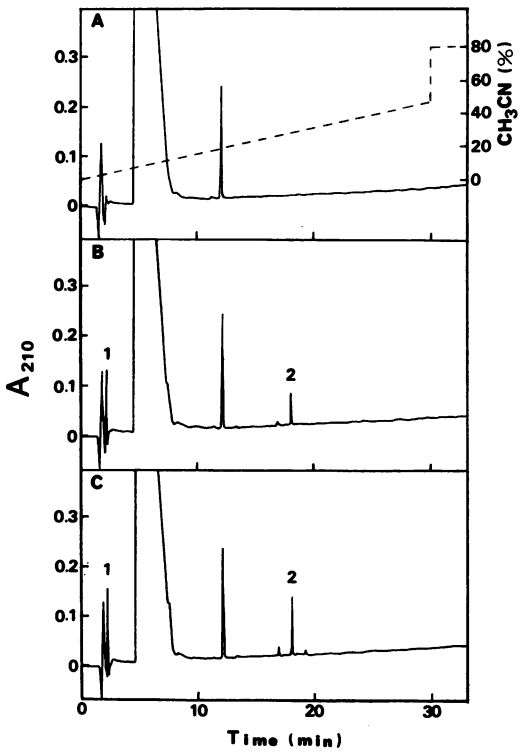

FIG. 3. HPLC patterns of the TCA-soluble reaction products from rHu IFN-γ. rHu IFN-γ was incubated at 37°C in the absence or presence of an outer membrane preparation. TCA-soluble materials were fractionated by HPLC and analyzed as described in Materials and Methods. (A) Incubation for 60 min in the absence of the outer membrane preparation. (B and C) Incubation for 5 and 60 min, respectively, in the presence of the outer membrane preparation. The peptide peaks were identified as follows: peak 1, Arg-Ala-Ser-Gln; and peak 2, Arg-Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly-Arg. Other peaks were not identified as peptides.

Dynorphin A(1-13), a biologically active peptide composed of Tyr-1–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys-13, carries a dibasic residue, Arg-6–Arg-7, and other basic amino acids, such as Arg-9, Lys-11, and Lys-13. Whether it could be cleaved only at the center of the dibasic residue or at the peptide bonds between other basic amino acids was examined. Figure 4 shows the HPLC patterns of reaction products of dynorphin A(1-13) incubated with the isolated other membrane of *E. coli*. Only two peptide peaks were detected. Based on the amino acid composition and a comparison with the amino acid sequence of dynorphin A(1-13), the peptide peaks were identified as Arg-7–Ile–Arg–Pro–Lys–Leu–Lys-13 (peak 2) and Tyr-1–Gly–Gly–Phe–Leu–Arg-6 (peak 3). Hydrolysis of small synthetic substrates was also examined. The synthetic substrates for trypsin (benzoyl-Arg-MCA) and urokinase (glutalyl-Gly-Arg-MCA) and all other substrates examined (*t*-butyloxycarbonyl-Leu-Gly-Arg-MCA, *t*-butyloxycarbonyl-Ile-Glu-Gly-Arg-MCA, and *t*-butyloxycarbonyl-Val-Leu-Lys-MCA) were not hydrolyzed by the isolated outer membrane. These results indicated that the enzyme is a novel endoprotease which specifically cleaves the peptide bond between consecutive basic residues.

DISCUSSION

The studies reported here have focused on the detection and characterization in *E. coli* of a novel membrane-associated protease which we designate protease VII.

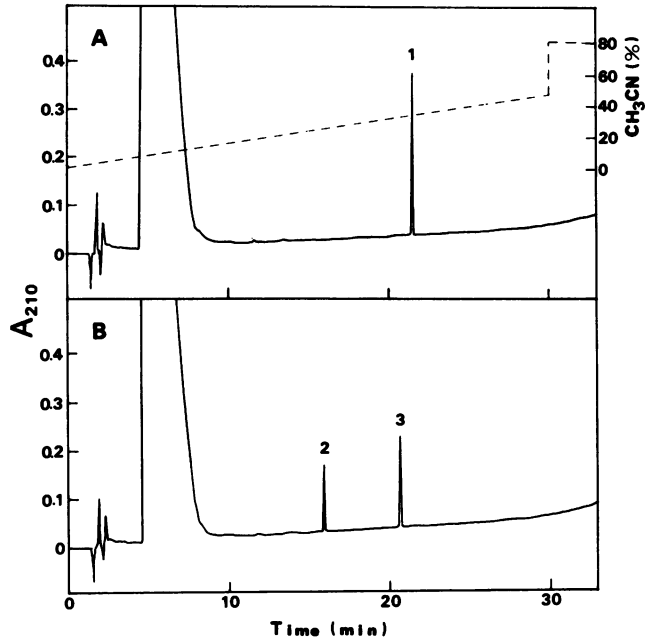

FIG. 4. HPLC patterns of the TCA-soluble reaction products from dynorphin A(1-13). Dynorphin A(1-13) was incubated at 37°C for 15 min with (B) or without (A) the outer membrane preparation. TCA-soluble degradation products were fractionated by HPLC and analyzed as described in Materials and Methods. The peptide peaks were identified as follows: peak 1, dynorphin A(1-13); peak 2, Arg-7–Ile–Arg–Pro–Lys–Leu–Lys-13; and peak 3, Tyr-1–Gly–Gly–Phe–Leu–Arg-6.

Protease VII was resistant to the serine protease inhibitors DFP and PMSF. It was also insensitive to a thiol protease inhibitor, PCMB, and a metal chelator, EDTA. TLCK, which alkylates the active-site histidine residue of trypsin, had no effect on protease VII activity. However, benzamidine, a trypsinlike protease inhibitor, reduced protease VII activity. Furthermore, the bivalent cations Zn^{2+} and Cu^{2+} strongly inhibited protease VII activity. From these observations, it can be concluded that protease VII apparently is not a serine, thiol, or metal protease. Because all our experiments were performed with an outer membrane preparation, it was possible that protease VII interacted with other outer membrane components, preventing interaction with various protease inhibitors. Further investigation is needed to clarify this point by purifying protease VII.

Protease VII is characterized by its unusual substrate specificity in hydrolyzing peptides or proteins only at the center of consecutive basic residues. rHu IFN-γ was cleaved at Lys-131–Arg-132 and Arg-142–Arg-143 of the C-terminal region. Dynorphin A(1-13) was cleaved only at Arg-6–Arg-7. Products digested at the other sites were not detected in our experiments. Neither the amino nor the carboxyl side of a solitary basic amino acid in the molecule was cleaved. Despite the presence of other proteolytic enzymes, such as proteases V and VI, in the outer membrane preparation, degradation products yielded by these proteases were not detected in our experiments.

rHu IFN-γ seems to be degraded stepwise from 18 to 17.5k and from 17.5 to 16k on SDS-polyacrylamide gels (Fig. 1). Degradation products analyzed by HPLC were Arg-143–Ala–Ser–Gln-146 and Arg-132–Lys–Arg–Ser–Gln–Met–Leu–Phe–Arg–Gly–Arg-142 (Fig. 3). The ratio of the latter to the former increased about twofold from 5 to 60 min of incuba-

tion. These observations suggest that the peptide bond of Arg-Arg may more susceptible to protease VII than that of Lys-Arg, although more detailed studies with other substrates should be performed to determine this.

Although rHu IFN-γ, composed of 146 amino acids (Fig. 2), contains several pairs of dibasic amino acids, only the C-terminal region was attacked by protease VII. This observation is explained by the conformation study of rHu IFN-γ with CADS (Computer Aided Drug-design System). The N-terminal region of rHu IFN-γ seems to assume a rigid structure which may prevent interaction with protease VII; however, the C-terminal region of about 20 amino acids seems to be relaxed and may be easily attacked by protease VII (unpublished observations). The reason why the cleavages at Arg-132–Lys-133 or Lys-133–Arg-134 were not shown to take place is unknown at this stage.

Protease VII is distinct from protease II, a trypsinlike protease catalyzing the hydrolysis of α-amino-substituted lysine and arginine (18). Protease II is a cytoplasmic protease, but protease VII is an outer-membrane-associated protease which does not hydrolyze benzoyl-Arg-MCA.

The characteristics of several membrane-associated proteases have been reported. Protease IV (17) is an inner membrane enzyme, and its substrate specificity and localization are distinct from those of protease VII. Protease V (17), which is localized in both the inner and outer membranes, is mainly active on phenylalanine derivatives, and its inhibitor profile is different from that of protease VII. Another outer membrane enzyme, protease VI, has been reported by Palmer and St. John, who used a complex protein mixture of *E. coli* membranes as a substrate for assaying its activity (19). Its sensitivity to benzamidine is similar to that of protease VII; however, protease VII is strongly inhibited by Zn^{2+}, while protease VI is not (19). Protein a, which is responsible for the modification of the ferric enterobactin receptor (5), is insensitive to DFP and PMSF and inhibited by benzamidine. These characteristics are similar to those found for protease VII, but the substrate specificity of protein a is as yet unknown. Our recent analysis of the N-terminal amino acid sequence of purified protease VII distinguished it from protein a, whose N-terminal sequence was reported by Gordon et al. (3) (manuscript in preparation).

Similar proteases which recognize and cleave consecutive basic residues specifically have been reported in *Saccharomyces cerevisiae* (7, 13, 14). One is located in the cytoplasm and cleaves peptide bonds between consecutive basic residues (13). The other is located at the membrane and cleaves the carboxyl side of paired basic residues (7, 14). The cleavage sites of protease VII reported in this paper are the same as those of the former, and the localization and Zn^{2+} sensitivity are similar to those of the latter. These proteases in eucaryotes are implied to be involved in propheromone or prohormone processing. In this regard, it is very interesting that procaryotic protease VII has properties similar to those of eucaryotic enzymes. It may be a prototype of such eucaryotic proteases. Protease VII may function in the processing or maturation of some specific membrane or periplasmic proteins. Its role may be the protection of cells against toxic factors in the environment. The penetration of toxins or nutrients into cells may be catalyzed by this enzyme. An *E. coli* mutant lacking protease VII activity has been isolated recently (K. Sugimura, Biochem. Biophys. Res. Commun., in press), and the physiological role of this enzyme will be studied by using this mutant.

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