Purification and Characterization of Kumamolysin, a Novel Thermostable Pepstatin-insensitive Carboxyl Proteinase from Bacillus novosp. MN-32*

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We have found a novel type of thermostable, pepstatin-insensitive carboxyl proteinase in the culture filtrate of Bacillus novosp. MN-32. The carboxyl proteinase, which was named kumamolysin, was purified about 8,300-fold by column chromatography including DEAE-Sepharose CL-6B, Sephadex G-100, and TSKgel DEAE-5PW. The purified kumamolysin gave a single band corresponding to 41 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular mass of kumamolysin was estimated to be 40 kDa by gel filtration. The isoelectric point of kumamolysin was estimated to be pH 3.5 by isoelectric focusing. Kumamolysin has maximum proteolytic activity at 70 °C and at pH 3.0. Kumamolysin specifically hydrolyzed the Leu<sup>16</sup>-Tyr<sup>16</sup> peptide bond in oxidized insulin B-chain (K<sub>m</sub> = 9.0 × 10<sup>-8</sup> M, K<sub>cat</sub> = 71 s<sup>-1</sup>; at pH 3.0, 30 °C), and additional cleavage at Phe<sup>29</sup>-Tyr<sup>30</sup> was detected at a considerably lower rate. Kumamolysin is insensitive to the known carboxyl proteinase inhibitors pepstatin, diazoacetyl-DL-norleucine methyl ester, and 1,2-epoxy-3-(p-nitrophenoxy)propane. Kumamolysin has no similarity to the thermostable acid protease thermopin from Sulfolobus acidocaldarius (Lin, X.-L., and Tang, J. (1990) J. Biol. Chem. 265, 1490-1495). Thus, the substrate specificity, the inhibitor sensitivity, the molecular mass, and the thermostability all suggest that kumamolysin is a novel type of carboxyl proteinase.

Tracing the historical progress of the investigations on carboxyl proteinases, it is noteworthy that various specific compounds have served as inestimable tools in elucidating the detailed catalytic machinery of carboxyl proteinases, i.e. the development of the active site-directed reagents, diazoacetyl-DL-norleucine methyl ester (1), and 1,2-epoxy-3-(p-nitrophenoxy)propane (2). Furthermore, the discovery of naturally occurring potent and specific inhibitors (pepstatin Ac (3) and pepstatin Isoval (4)) permitted the identification of catalytic residues by detailed biochemical or crystallographic approaches. It is well established that the catalytic sites of most carboxyl proteinases are composed of 2 active aspartic acid residues. Thus these enzymes have now been called aspartic proteinases. Most carboxyl proteinases, irrespective of their origin, are inactivated by diazoacetyl-DL-norleucine methyl ester, 1,2-epoxy-3-(p-nitrophenoxy)propane, and pepstatin. These inhibitors have been utilized as diagnostic agents to deduce the nature of a newly isolated aspartic proteinase, and the inhibitor sensitivity is considered to be a criterion in enzyme nomenclature.

Generally, the individual members of the carboxyl proteinase family share structural and functional similarity obviously different from serine type proteinases that form a strikingly diverse group, such as the trypsin, α-chymotrypsin, and elastase families. The distinctive susceptibility of individual carboxyl proteinase to the inhibitors may be reflected in the different nature of their active sites. The natural occurrence of inhibitor-insensitive carboxyl proteinases was established by the present authors in microbes: first from Scytalidium lignicolium in 1972 (5), and followed by Lentinus edodes (6), Canodermusa lucidum (7), Pseudomonas 101 (8), and Xanthomonas T-22 (9). Consequently, we have proposed that at least two types of carboxyl proteinase may be distinguished: pepstatin-like enzymes, which are inhibited by pepstatin, diazoacetyl-DL-norleucine methyl ester, and 1,2-epoxy-3-(p-nitrophenoxy)propane; Scytalidium-like enzymes, which are not inhibited by one or more of these three compounds (10). The occurrence of thermostable carboxyl proteinase is rare, and the thermostable enzyme, insensitive to these inhibitors, may be an attractive model for the investigation of structure and function in these classes of enzymes. In this paper, we describe the occurrence of a thermostable carboxyl proteinase in Bacillus novosp. MN-32, which is insensitive to pepstatin, diazoacetyl-DL-norleucine methyl ester, and 1,2-epoxy-3-(p-nitrophenoxy)propane. A novel carboxyl proteinase, which we have named kumamolysin, has been isolated and characterized.

EXPERIMENTAL PROCEDURES

RESULTS

Production and Purification of Kumamolysin—Kumamolysin was an extracellular enzyme produced by a bacterium MN-32 strain at late stationary growth phase. From the taxonomic characterization, MN-32 was considered to be a new member of the genus Bacillus.

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1 Portions of the paper ("Experimental Procedures," part of "Results," Tables I and II, and Figs. 1–5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Kumamolysin was purified from the culture supernatant of *B. novosp.* MN-32 by the procedure described in the Miniprint Section. Typical elution profiles on preparative HPLC\(^2\) using a TSKgel DEAE-5PW column are shown in Fig. 1. The results of the purification procedure are summarized in Table I. The enzyme was purified 8300-fold with 14% recovery; 22 mg of lyophilized enzyme preparation was obtained from 120 liters of culture supernatant. The purified kumamolysin showed a single protein band on polyacrylamide gel electrophoresis (Fig. 2).

**Some Characteristics of Kumamolysin.—**The effect of pH on the proteolytic activity and the stability of kumamolysin was investigated. The optimum pH for the proteolytic activity of kumamolysin on casein is pH 3.0 (incubation temperature: 70 °C). Below pH 1.0 and above pH 6.0, no activity was observed (Fig. 3A). As shown in Fig. 3B, kumamolysin was completely stable in the pH range 2.0-4.0 at 50 °C for 24 h, and in the pH range 1-10 at 5 °C for 3 days. The effect of temperature on the proteolytic activity and the stability of kumamolysin was examined. As shown in Fig. 4A, the optimum temperature was 70 °C (incubation pH was 3.0). Kumamolysin was stable below 70 °C in a 10-min incubation, retaining 60% of the original activity at 80 °C for 10 min, but lost its proteolytic activity after 10 min at 90 °C (Fig. 4B).

The molecular mass of kumamolysin, estimated by SDS-polyacrylamide gel electrophoresis under both native or reducing conditions, was 41 kDa (Fig. 5A). Based on gel filtration by Sephadex G-100, the molecular mass of the enzyme was estimated to be 40 kDa (Fig. 5A).

The isoelectric point of the enzyme was determined by isoelectric focusing using carrier ampholine (pH 3-10). A single peak of proteinase activity and absorbance at 280 nm was detected at pH 3.5 (data not shown).

The UV absorption spectrum of a kumamolysin solution (50 mM sodium acetate buffer, pH 4.0) showed an absorption minimum at 250 nm, maximum at 276 nm, and shoulders at around 282 and 290 nm. The specific absorption coefficient, \(A_{280}^{\text{nm}}\), 280 nm, the absorbance at 280 nm of a 1% solution of kumamolysin in a 1-cm optical path length, was 12.6.

The amino acid composition of kumamolysin is shown in Table II. Kumamolysin is not a glycoprotein as judged by the periodate-Schiff reagent or the phenol-sulfuric acid reagent. The absence of free thiol groups in kumamolysin, assayed with Ellman reagent, and the presence of 4 mol of histidine/molecule indicated the presence of two disulfide bridges/mol of kumamolysin.

**Susceptibility of Kumamolysin to Various Proteinase Inhibitors.—**The effect of various proteinase inhibitors on the proteolytic activity of kumamolysin was determined. Kumamolysin was not inhibited by the tested inhibitors, including carboxyl proteinase inhibitors pepstatin, DANA, and EPNP; serine proteinase inhibitors PMSF, SSI, MAPl (inhibitor of \(\alpha\)-chymotrypsin type and/or thiol proteinase), leupeptin (trypsin and/or thiol proteinase), and chymostatin (\(\alpha\)-chymotrypsin and/or thiol proteinase); thiol proteinase inhibitors IAA, thiolstatin, and PCMB; and metallo proteinase inhibitors EDTA, p-phenanthroline, and tolpenepin.

The optimum pH (as shown in Fig. 3A) of the proteolytic activity of kumamolysin indicated that the enzyme may be classified as belonging to the carboxyl proteinase group. The insensitivity of kumamolysin to the inhibitors specific for carboxyl proteinases was examined in detail. In the case of pepstatin, typical carboxyl proteinases (such as porcine pepsin) were completely inhibited by pepstatin in an equimolar dose response mode (enzyme/inhibitor = 1:1, molar ratio). As shown in Fig. 6A, kumamolysin was not inhibited by pepstatin at a large molar excess (approximately 1300-fold molar excess). In the case of DANA, treatment of kumamolysin with a 210-fold molar excess of DANA did not affect the activity of kumamolysin (Fig. 6B). And as shown in Fig. 6C, EPNP did not inhibit kumamolysin. In the positive control experiments, inhibition of porcine pepsin by these active-site-directed inhibitors was confirmed (shown as closed circles in Fig. 6, A-C).

**Substrate Specificity of Kumamolysin.—**The cleavage site of oxidized insulin B-chain by kumamolysin was determined. Reversed-phase HPLC analysis of the final reaction products from the oxidized insulin B-chain following kumamolysin hydrolysis indicated the formation of only four products. Under the optimum reaction conditions (70 °C, pH 3.0), the hydrolysis rate was so fast that the reaction process could not be followed. Even at lower incubation temperature (37 °C), the substrate was completely hydrolyzed within 20 s at a mass ratio of substrate/enzyme of 20:1. Accordingly, the time course of the reaction was followed under suboptimum conditions: incubation temperature, 20 °C; substrate/enzyme ratio 100,000:1 (by mass ratio); pH 3.0. As shown in Fig. 7A, two peptides, designated 2 and 3, were predominant during the initial incubation time. After prolonged incubation times, product 3 was gradually hydrolyzed to yield products 1 and 4 (Fig. 7B). After the disappearance of the oxidized insulin B-chain peak, no additional hydrolysis products were detected in further incubations.

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\(^2\) The abbreviations used are: HPLC, high-performance liquid chromatography; DAN, diazoacetyl-L-lysine methyl ester; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; PMSF, phenylmethylsulfonyl fluoride; IAA, iodoacetic acid; PCMB, p-chloromercuribenzoate; FAB-MS, fast atom bombardment-mass spectrometry; PAD, pyridine-2-azo-p-dimethylaniline; Nph, p-nitrophenylalaine; Sta, (S,S,S)-4-amino-3-hydroxy-6-methylheptanoic acid; SSL, Streptomyces subtilisin inhibitor; MAPl, microbial alkaline proteinase inhibitor.
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Oxidized insulin B-chain

**Fig. 7.** Time course of oxidized insulin B-chain hydrolysis by kumamolysin. A. 1 ml of oxidized insulin B-chain (0.5 mg) in 0.1 M HCl-KCl buffer, pH 3.0, was incubated with kumamolysin (5 ng) at 20 °C. At adequate time intervals, a aliquot (10 μl) of the reaction mixture was analyzed by HPLC. B, the reaction conditions were same as A, except for enzyme concentration (kumamolysin = 50 ng). Details of the chromatography are under “Experimental Procedures.” The HPLC columns were Nucleosil 5C18 (A) and TSKgel ODS-120T (B).

These peptides were isolated by preparative HPLC, and their structures were deduced by amino acid analysis, NH₂-terminal amino acid identification, and FAB-MS. As shown in Table III, the corresponding sequence in the original substrate was elucidated as follows; product 1 (residues 26 to 30), product 2 (1–15), product 3 (16–30), product 4 (16–25). From these results, the main cleavage site was deduced to be Leu₁⁵-Tyr₂⁶, and Phe₁⁰-Tyr₂⁶ bond was very slowly attacked. The scissile bond in the substrate was shown to be unique, compared to various carboxyl proteinases hitherto reported.

The predominant cleavage at the Leu₁⁵-Tyr₂⁶ peptide bond was studied kinetically. The hydrolysis rate was estimated by measuring peptides 2 and 3 by reversed-phase HPLC (external standard method). From the Lineweaver-Burk plot, $K_m$ was $9.0 \times 10^{-5}$ M, and $K_{cat}$ was 71 s⁻¹, at 30 °C, pH 3.0.

**Fig. 8.** pH-dependence of $K_{cat}/K_m$ for kumamolysin-catalyzed hydrolysis of oxidized insulin B-chain at 30 °C. Experimental details are under “Experimental Procedures.”

Further Evidence for Identification of Kumamolysin as a Carboxyl Proteinase—To clarify the assignment of kumamolysin to the carboxyl proteinase group, the following two independent experiments were performed. First, the pH dependence of kumamolysin-catalyzed cleavage of oxidized insulin B-chain (hydrolysis at Leu₁⁵-Tyr₂⁶ bond) was investigated kinetically. As shown in Fig. 8, the pH profile of the ratio $K_{cat}/K_m$ of kumamolysin is bell-shaped, which has been taken to indicate the presence of two catalytically important prototropic groups, having pK values of 1.97 and 3.47. Second, to investigate the participation of carboxyl groups in the catalytic site of kumamolysin, binding of zinc(II)-PAD, an active-site probe for carboxyl proteinases, was studied spectrophotometrically. The zinc(II)-PAD complex had an absorption maximum at around 530 nm (PAD: $2.91 \times 10^{-5}$ M; ZnSO₄: $2.00 \times 10^{-3}$ M, in 50 mM sodium acetate buffer, pH 5.0). An addition of kumamolysin ($4.65 \times 10^{-5}$ M) to the

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**Table III**

| Peptide | 1 | 2 | 3 | 4 |
| --- | --- | --- | --- | --- |
| Amino acid analysis | | | | |
| Asx | 1.0(1) | | | |
| Thr | 0.7(1) | 0.9(1) | | |
| Ser | 0.8(1) | 2.0(2) | 1.0(1) | 1.0(1) |
| Gly | 1.4(1) | 1.0(1) | 0.6(1) | | |
| Ala | 1.0(1) | 2.0(2) | 2.1(2) | 2.1(2) |
| Cysteine acid | 0.6(1) | 0.6(1) | 0.7(1) | | |
| Val | 1.9(2) | 1.1(1) | 1.0(1) | | |
| Leu | 3.1(3) | 1.2(1) | 0.7(1) | | |
| Tyr | 1.0(1) | 1.8(2) | 0.7(1) | | |
| Phe | 1.0(1) | 2.1(2) | 1.8(2) | | |
| Lys | 1.0(1) | | | 1.0(1) |
| His | 1.9(2) | | | |
| Arg | | | | |

| NH₂-terminal analysis | ND | Phe | Tyr | ND |
| --- | --- | --- | --- | --- |

| FAB-MS [MH]+ | | | |
| --- | --- | --- | |
| Observed | 579.53 | 1715.22 | 1799.28 | 1238.90 |
| Theoretical | 579.51 | 1715.19 | 1799.20 | 1238.87 |

| Matching sequence in the substrate | Tyr₂⁵, Phe₁⁰, Ty₂⁶, Tyr₂⁶, Phe₁⁰ |
| --- | --- | --- | |
| Ala₁⁰⁰, Leu₁⁰⁰, Ala₁⁰⁰, Phe₁⁰⁰ |

*Analysis was performed on a 24-h hydrolyzate, using constant boiling HCl at 110 °C.

*ND, not determined.*
Aspergillus saitoi (conditions: pH 4.5, 25 °C, 30 min) for 15 min (28). At the time when we found kumamolsyn in 1988, kumamolsyn was the only thermostable carboxyl proteinase reported. However, recently, there was a report of an extremely thermostable carboxyl proteinase from a thermophilic archaeabacterium (29).

Substrate Specificity—Generally, carboxyl proteinases show a broad substrate specificity, which has been characterized by a preference for aromatic or bulky amino acid residues at the cleavage site (P₁-P₂₋). Hydrolysis by kumamolsyn of the oxidized insulin B-chain was restricted to only two peptide bonds, Leu₁⁴-Tyr₁⁶ and Phe₂₅-Tyr₂₆. The hydrolysis of the former peptide bond was exceptionally fast (Kcat = 71 s⁻¹, at suboptimum temperature (30 °C)). This specificity is one of the unique characteristics of kumamolsyn not yet reported in the microbial carboxyl proteinase group.

Inhibitor Sensitivity—The proteolytic activity of kumamolsyn was not inhibited by all the tested protease inhibitors, including serine, metallo, thiol, and carboxyl proteinase inhibitors. This insensitivity to the inhibitors was quite unique, when compared to the hitherto reported carboxyl proteinases, the exceptions being the carboxyl proteinase from mesophilic microorganisms, L. edodes (26), S. lignicolum A enzyme (10), and G. lucidum (7), which were also not inhibited by pepstatin, DAN, and EPNP, as reported by us previously. Accordingly, kumamolsyn is the fourth case of the natural occurrence of an unique inhibitor-insensitive carboxyl proteinase.

As described in the introductory statement, we propose that carboxyl proteinases may be classified into two groups. Group I: this is the well known typical proteinases (now classified as aspartyl proteinases); e.g., pepsin, cathepsin D, and carboxyl proteinases of various microorganisms. These are inhibited by specific inhibitors of carboxyl proteinases, such as pepstatin, EPNP, and DAN. Group II: this group of enzymes is insensitive to these inhibitors, as described above. It has been well established that the active site of pepsin is composed of 2 aspartic acid residues (Asp³⁰ and Asp³⁵). Their carboxyl side chains are sufficiently close to each other that they may share a proton (oxygen-oxygen distance are assumed to be 2.9 Å). The former aspartic residue is susceptible to esterification by DAN in the presence of copper; the latter residue is reactive to EPNP. Irrespective of origin, these two catalytic residues are retained in group I enzymes in a close geometric arrangement. The insensitivity of kumamolsyn to the two active-site-directed reagents, DAN and EPNP, suggests striking differences in the active site. The participation of the carboxyl groups in the catalytic action of kumamolsyn was deduced from the following experimental results.

The pH dependence of the kinetic constant for kumamolsyn was examined by using oxidized insulin B-chain as substrate (cleavage at Leu¹⁸-Tyr¹⁹ bond). The pH profile of the Kcat/Km ratio (as shown in Fig. 8) indicated two ionizing group with pK values of 1.97 and 3.47. These values were comparable to that of S. lignicolum acid protease A (pK₁ = 1.53; pK₂ = 3.77) (30), which is a member of group II enzyme. Klote et al. (31) pointed out from the change in the visible absorption spectrum that the azo dye PAD which could not be bound to pepstatin by itself, could be bound in the presence of certain transition metal ions such as zinc(II). Based on these results, we (32) showed that all the tested acid (carboxyl) proteinases also exhibited the same spectral changes as pepsin at pH 5. However, lysozyme (hen egg white), amylases (bacterial a-amylase and fungal glucoamylase), and neutral or alkaline proteinases such as a-chymotrypsin or subtilisin did not show any such a spectral change at pH 5. The spectral change of zinc(II)-PAD caused by carboxyl proteinases is very similar to that by oxalic acid. It is reasonable to consider that the spectral change of zinc(II)-PAD on reaction with oxalic acid is due to the formation of the mixed complex, as shown in Scheme I, in which the two carboxyl moieties of oxalic acid are located in a close geometric arrangement. The distance between the two oxygen atoms of the carboxyl moiety, which could form a ternary complex with the zinc(II)-PAD reagent, was estimated to be 3 Å (33). This value is comparable to the distance of two active carboxyl groups (Asp³⁰ and Asp³⁵) in pepsin, assumed from the crystallographic investigations. The spectral change of zinc(II)-PAD, caused by the addition of carboxyl proteinases (such as pepsin), is the same as that observed with oxalic acid. An identical spectral change was observed for all the tested carboxyl proteinases, even in the case of group II carboxyl proteinases. It is quite reasonable to consider that the zinc(II)-PAD reagent as an excellent and specific active site probe of carboxyl proteinases, suggesting the presence of two adjacent catalytic carboxylate groups in...
the active site (33) in a similar geometric arrangement as aspartic acid. As described under "Results," the presence of two adjacent carboxyl groups in kumamolysin was deduced from the zinc(II)-PAD experiment. The difference in absorbance due to the zinc(II)-PAD-kumamolysin ternary complex formation was 0.032 at 530 nm (kumamolysin, 4.65 \times 10^{-5} M). This value was considered to be significant, compared to the following data (34): 0.058 for pepsin (2.46 \times 10^{-5} M); 0.043 for carboxyl proteinase A2 from S. lignicolum (2.17 \times 10^{-5} M); 0.136 for carboxyl proteinase B from S. lignicolum (2.13 \times 10^{-5} M), the latter two enzymes are members of group II. The value of the absorbance increase was characteristic of the individual enzymes, which may depend on differences in the active site of each enzyme. The absorbance increase with kumamolysin was not observed at 37 °C (data not shown). When the temperature was raised to 50 °C, the absorbance increase was observed. As described under "Results," the optimum temperature for kumamolysin was 70 °C, using casein as substrate. The absorbance increase with kumamolysin (ΔA₃₉₅ = 0.034; E = 4.65 \times 10^{-5} M), observed at suboptimum temperature (50 °C), may be rather small compared to other carboxyl proteinases.

In the above-mentioned zinc(II)-PAD experiment, the possibility of participation by cysteine and/or histidine residues in the active site of kumamolysin may be anticipated. (About cysteine: in the presence of Hg²⁺ ion, PAD was used as a titrant for sulfhydryl groups in protein (35).) Kumamolysin contains 4 cysteine residues, as shown in Table II. These residues were deduced to form two disulfide bridges in kumamolysin, estimated by Ellman reagent titration in the reductive or nonreductive condition. The activity of kumamolysin was not inhibited by thiol proteinase inhibitors such as PCMB, IAA, and thiolstatin. Accordingly, the participation of cysteine residues in the spectral change of the zinc(II)-PAD reagent was considered to be improbable. (About histidine: from the two independent experimental results, 1) activity-pH profile (optimum pH of kumamolysin was pH 3.0); 2) pH-Kcat/Km profile (pKcat = 1.57; pKm = 3.47), the allocation of histidine residue to the active site considered to be improbable.)

Addition of the substrate (oxidized insulin B-chain) to the zinc(II)-PAD-kumamolysin complex revealed thatbound zinc(II)-PAD was liberated from the ternary complex. Accordingly, it is reasonable to assume that the location of the two reactive carboxyl groups in kumamolysin are at the active site. We also reported the presence of two carboxyl groups in the active site of S. lignicolum carboxyl proteinase (A and B enzyme) by the same spectrophotometric method, using angiotensin I as the substrate (34) and the pH dependence of the ratio Kcat/Km (30). Together with these results, this assumption was confirmed from another line of evidence. A naturally occurring carboxyl proteinase inhibitor, pepstatin, which is a transition-state analog inhibitor, had no effect in liberating the dye probe from the zinc(II)-PAD-kumamolysin complex. Pepstatin showed no inhibitory effect on kumamolysin, and interaction with or binding to kumamolysin was not observed by a difference spectrophotometric experiment (data not shown). Inability of pepstatin to liberate the dye probe from the active site of kumamolysin was considered to be consistent with the lack of inhibitory effect. Finally, we concluded that kumamolysin is apparently related to the carboxyl proteinase group II.

One of the unique characteristics of kumamolysin is its substrate specificity. In general, irrespective of enzyme origin and inhibitor susceptibility, the substrate specificity of carboxyl proteinases was shown to be relatively broad compared to serine type proteinases. For example, hydrolysis of oxidized insulin B-chain by porcine pepsin occurs at large at His¹⁶, Leu¹⁷, Val¹², Gln¹³, Ala¹⁴, Leu¹⁵, Tyr¹⁴-Leu¹⁷, and Phe²⁴-Phe²⁵ (see Ref. 36). The cleavage sites of various carboxyl proteinases, determined with the same substrate, was shown to be in two main regions which were located around the sequence Ala¹⁴-Leu¹⁵-Yr¹⁴-Leu¹⁷ and Phe²⁴-Phe²⁵, where amino acids with large hydrophobic side chains were clustered. However, additional cleavage sites were characteristic of individual enzymes, as most carboxyl proteinases showed multiple attack sites, usually more than five, in the oxidized insulin B-chain. Nevertheless, as shown in Fig. 7 and Table III, the cleavage sites of kumamolysin were restricted to only two peptide bonds, Leu¹⁴-Yr¹⁴ and Phe²⁴-Phe²⁵. This unique substrate specificity closely resembled those of the aspartic proteinase from pig intestinal mucosa (37). However, they apparently differ in their susceptibility to pepstatin, DAN, and EPNP; i.e. pig intestinal mucosa aspartic proteinase was inhibited by these inhibitors. The absence of kinetic investigations on the hydrolysis reaction of various aspartic proteinases using oxidized insulin B-chain prevents a direct comparison with that of kumamolysin. However, the rate constant of kumamolysincatalyzed hydrolysis of the Leu¹⁴-Yr¹⁴ bond was remarkably high (Kcat = 71 s⁻¹), even though the reaction was done at suboptimum temperature (50 °C). Detailed experiments on the substrate specificity of kumamolysin are now in progress. The kinetic constant of newly synthesized peptides containing the sequence around the scissile bond in the oxidized insulin B-chain, was evaluated: H-Val-Glu-Ala-Leu-Yr-Leu-OH was an equally favorable substrate (hydrolysis point: Leu-Yr-), but deletion of the NH₂-terminal valine residue (corresponding to P₁) lowered the rate constant to one-hundredth of that for the parent peptide, and further deletion of the NH₂-terminal glutamic acid residue (corresponding to P₂) resulted in no hydrolysis (results not shown). These preliminary experiments indicate the active-site clef of kumamolysin is a rather expanded one.

Even in the case of group I aspartic proteinases, some differences in inhibition rate by pepstatin, acyl-Val-Val-Stat'-Ala-Stat'-OH, are always observed with individual enzymes. This difference may be explained by the energy of interaction, derived from the Stat' residue to the 2 catalytic aspartic acid residues and probable interaction to the binding sites (38). The complete insensitivity of kumamolysin to pepstatin probably also indicates the different nature of the subsites in the active-site cleft of kumamolysin as compared to pepsin. Favorable substrates for various aspartic proteinases (39), such as H-Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu-OH, are extremely poor substrates for kumamolysin (results not shown). Therefore, it is likely that the subsite length and/or the nature of the active-site cleft in kumamolysin are quite different from various other aspartic proteinases.

Recently, a thermostable "acid proteinase," named thermolysin, was reported (29). However, kumamolysin is obviously unrelated to thermolysin based on inhibitor sensitivity, substrate specificity, amino acid composition (as shown in Table II), absence of carbohydrate, and other characteristics. Kumamolysin is the fourth case of the natural occurrence of carboxyl proteinases of a bacterial origin; i.e. kumamolysin, thermolysin (29), and two pepstatin-insensitive carboxyl proteinases from mesophilic bacteria (Xanthomonas sp. and Pseudomonas sp.), the latter two enzymes were reported by previously the present authors (8, 9). These microorganisms are the most ancient organism that produces carboxyl proteinases. Comparative investigations of these bacterial enzymes may serve as a beginning in the understanding of carboxyl
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and/or aspartic proteinase evolution.

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**METHODS**

**Experimental Methods**

**Materials.** Crystalline pancreatic Ac (Avicel—Vol-Sil—Alu Silico-OFF) (1) Matrig (microsomal albumin protein inhibitor), triglutamate (2), and diiodoterm (1) were purified by the method of Murao et al. (4). The following materials were purchased from the sources indicated in parentheses: DEAE-Sephadex A-50, Sephadex G-100, protein samples for molecular weight calibrations, and Pharmacia (Pharmacia LKB Biotechnology, Uppsala, Sweden). D-TEA (pH 7.5) and 1,000- (4°C, 24 h, 70°C, 15 min), and 10,000- (4°C, 90 min) dye-adsorbed proteins were purchased from Pharmacia (UP-70, Uppsala, Sweden). Oxa-ACCEPTANCE. 25. Aoyagi, T., Kunimoto, S., Morishima, H., Takeuchi, T., and Umezawa, H. (1971) *J. Antibiot.* 24, 687-694. 26. Murao, S., Oda, K., Matsuhashi, Y. (1972) *Agric. Biol. Chem.* 36, 1647-1650. 27. Terasita, T., Oda, K., Muro, S., and Murao, S. (1981) *Agric. Biol. Chem.* 45, 167-168. 28. Terasita, T., Oda, K., Kono, M., and Murao, S. (1984) *Agric. Biol. Chem.* 48, 1029-1035. 29. Oda, K., Nakazima, T., Terasita, T., Suzuki, K., and Murao, S. (1987) *Agric. Biol. Chem.* 51, 3073-3080. 30. Murao, S., and Oda, K. (1985) *Agric. Proteinases and Their Inhibitors* (Kostas, V., ed) pp. 379-399. Walter de Gruyter, Berlin. 31. Murao, S., and Sato, T. (1970) *Agric. Biol. Chem.* 34, 1265-1267. 32. Fukuhara, K., Katsura, M., and Murao, S. (1985) *Agric. Biol. Chem.* 49, 895-897. 33. Claus, D., and Berleby, R. C. W. (1986) *Bergey's Manual of Systematic Bacteriology* (volume 2, part A; Mair, N. S., Sharpe, M. E., and Holt, J. G., ed.) Vol. 2, pp. 1105-1139. Williams & Wilkins, Baltimore. 34. Weber, K., Pringle, J. R., and Osborn, M. (1972) *Methods Enzymol.* (Hirs, C. H. W., and Timasheff, S. N., eds, Vol. 29-32, pp. 1077-1107. Academic Press, New York).

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The active kumamolysin fraction (230 mg) was desalted by filtration on Sephadex G-25 columns, followed by thorough dialysis against distilled water. Lyophilization of the resulting solution yielded 22 mg of kumamolysin, without loss of activity.

| Steps                  | Total activity (mg) | Total protein (mg) | Specific activity (mg/mg) | Yield (%) | Purification fold |
|------------------------|---------------------|--------------------|---------------------------|-----------|------------------|
| Culture supernatant    | 460000              | 338000             | 0.3                       | 100       | 1                |
| (NH₄)₂SO₄ ppt          | 240000              | 356000             | 0.9                       | 80        | 5                |
| DEAE-Sephadex CL-6B    | 260000              | 260000             | 1.0                       | 51        | 253               |
| Sephadex G-100 fast    | 115000              | 176                 | 65.3                      | 29        | 2180              |
| Sephadex G-100 slow    | 82000               | 90                  | 900                       | 21        | 3600              |
| TS gel DEAE-SF 1st     | 65000               | 79                  | 2240                      | 16        | 1470              |
| TS gel DEAE-SF 2nd     | 52000               | 31                  | 2060                      | 14        | 8330              |

Table II Amino acid composition of kumamolysin, thermolysin, and carboxyl proteinase of Pseudomonas sp.

| Amino acid | Kumamolysin | Thermolysin | Pseudomonas a,b |
|------------|-------------|-------------|----------------|
| Ala        | 40          | 40          | 50             |
| Thr        | 25          | 28          | 34             |
| Ser        | 26          | 27          | 52             |
| Glu        | 34          | 16          | 37             |
| Pro        | 25          | 14          | 14             |
| Gly        | 51          | 79          | 56             |
| Ala        | 35          | 19          | 46             |
| Cys 1/2    | 4           | 4           | 1              |
| Val        | 30          | 20          | 23             |
| Met        | 5           | 4           | 0              |
| Ile        | 19          | 13          | 29             |
| Leu        | 11          | 24          | 18             |
| Phe        | 10          | 9           | 13             |
| Lys        | 5           | 2           | 8              |
| His        | 5           | 0           | 2              |
| Arg        | 12          | 2           | 7              |
| Trp        | 2           | 4           | 10             |
| Total      | 401         | 299         | 417            |

a Data from PVA sequence (see ref. 29). b Data from amino acid analysis (ref. 8).

c Values estimated from amino acid composition.
d Measured as cysteic acid on a 24 hr hydrolysis after performic acid oxidation.
e Heart activated spectrophotometrically.
f Other values are average of 24, 48, and 72 hr hydrolysis.

Fig. 1. Electropherogram of kumamolysin.
Fig. 2. Polyacrylamide disc gel electrophoresis of purified kumamolysin.
Fig. 3. Effect of pH on the activity (A), and stability (B) of kumamolysin.
Fig. 4. Effect of temperature on the activity (A), and stability (B) of kumamolysin.
Fig. 5. Molecular weight determination of kumamolysin.

Electrophoresis was done under the following conditions: (A) under 4 mA constant current for 2 hr electrophoresis, pH 2.3, 7% gel; (B) 3 mA for 3 hr, pH 8.8, 7.5% gel. After electrophoresis, the protein (40 μg protein was applied) was stained with Coomassie Brilliant Blue.