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A CLN2-Related and Thermostable Serine-Carboxyl Proteinase, Kumamolysin: Cloning, Expression, and Identification of Catalytic Serine Residue[1]

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The gene encoding kumamolysin, a thermostable pepstatin-insensitive carboxyl proteinase, was cloned and expressed. (i) Kumamolysin was synthesized as a large precursor consisting of two regions: amino-terminal prepro (188 amino acids) and mature proteins (384 amino acids). (ii) The deduced amino acid sequence of the mature region exhibited high similarity to those of such bacterial pepstatin-insensitive enzymes as *Pseudomonas* carboxyl proteinase (PSCP; EC 3.4.23.37, identity = 37%), *Xanthomonas* carboxyl proteinase (XCP; EC 3.4.23.33, identity = 36%), and human CLN2 gene product (identity = 36%), which is related to a fatal neurodegenerative disease. (iii) The presumed catalytic triad, Glu78, Asp82, Ser278 [three-dimensional structure of PSCP: Wlodawer, A. *et al.* (2001) *Nature Struct. Biol.*, 8, 442–446], was found to be conserved in the amino acid sequence of kumamolysin. (iv) Kumamolysin was inactivated by such aldehyde-type inhibitors as Ac-Ile-Pro-Phe-CHO (K_i = 0.7 ± 0.14 μM). In PSCP, it has been clarified that these inhibitors form a hemiacetal linkage with the catalytic serine residue and inactivate the enzyme. (v) Mutational analysis of the Ser278 residue revealed that the mutant lost both auto-processing activity and proteolytic activity. These results strongly suggest that kumamolysin has a unique catalytic triad consisting of Glu78, Asp82, and Ser278 residues, as previously observed for PSCP.

Key words: catalytic triad, kumamolysin, pepstatin-insensitive carboxyl proteinase, serine-carboxyl proteinase, thermostable proteinase.

Carboxyl proteinases, which have their optimal pH in the acidic region, are classified into two groups on the basis of their sensitivity to inhibitors, pepstatin-sensitive and pepstatin-insensitive (*1–3*). Pepstatin-sensitive carboxyl proteinases, called aspartic proteinases, are inhibited by such affinity labeling reagents as diazoacetyl-DL-norleucine methyl ester (DAN) (*4*) and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (EPNP) (*5*). The consensus catalytic site sequence (-Asp*-Thr-Gly-, Asp*: catalytic residue) is well conserved among the enzymes belonging to this group (*6*).

In 1972, pepstatin-insensitive carboxyl proteinases, A, B, and C, were found in the culture filtrate of *Scytalidium lignicolum* (*7, 8*). Pepstatin (*9*), acetyl-pepstatin (*10*), and DAN did not inhibit these enzymes, and EPNP inhibited only carboxyl proteinase B (*11*). Carboxyl proteinases having similar properties to those of *Scytalidium*-type proteinases have been found to be widely distributed among fungi (*12, 13*) and bacteria (*14–20*).

In 1997, the CLN2 gene encoding a pepstatin-insensitive lysosomal peptidase was found in human brain (*21*). The primary structure of the CLN2 gene product (CLN2 protein) showed high sequence similarity to those of such pepstatin-insensitive carboxyl proteinases from prokaryotes as *Pseudomonas* carboxyl proteinase (PSCP/PCP) (*22*) and *Xanthomonas* carboxyl proteinase (XCP) (*23*). The human CLN2 protein, which was identical to tripeptidyl peptidase I (*24*), is related to a fatal neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis (LINCL). Rawlings and Barrett pointed out in 1999 that PSCP, XCP, and the CLN2 protein might be serine proteinases based on their conserved Gly-Thr-Ser sequence (catalytic motif of serine proteinases) (*25*). However, a conserved histidine, expected for the catalytic triad of serine proteinases, was not observed.

Kumamolysin is the first example of thermostable and pepstatin-insensitive carboxyl proteinase from a thermophile, *Bacillus* novosp. MN-32 (16). It shows its maximum proteolytic activity at 70°C and pH 3.0, and a pair of car-

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boxyl groups is involved in its catalytic function (*17*). Its substrate specificity is notably different from those of PSCP and XCP (*26*). Kumamolysin preferentially hydrolyzes peptides having an Ala or Pro residue in the P_2 position. In contrast, PSCP and XCP preferentially hydrolyze peptides having a Leu or Ala residue in the P_2 position (*27*). This is one of the reasons why kumamolysin is insensitive to tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal), a naturally-occurring inhibitor of PSCP (*28*).

To further our understanding of the structure–function relationship of bacterial pepstatin-insensitive carboxyl proteinases, the kumamolysin gene was cloned, and its expression system in *Escherichia coli* cells was constructed. The results of inhibition studies, mutational analysis, sequence homology, and the three-dimensional structure of PSCP (*29*) strongly suggest that a unique catalytic triad consisting of Glu78, Asp82, and Ser278 residues is essential for catalytic function of kumamolysin.

MATERIALS AND METHODS

Materials—Restriction endonucleases, Klenow enzyme, T4 DNA ligase, deletion kit, calf intestine alkaline phosphatase, and KOD-Dash polymerase were purchased from Nippon Gene (Toyama) or Toyobo (Osaka). [^{32}P-γ]ATP was from Muromachi Chem. (Tokyo). AmpliTaq DNA polymerase Stoffel fragment and Ampli Cycle Sequencing Kit were obtained from Perkin-Elmer (Chiba). Fast Start Taq DNA polymerase was obtained from Roche (Tokyo). Lysozyme, RNase, and AEBSF [4-(2-aminoethyl)benzene sulfonyl fluoride] were obtained from Sigma (St. Louis, MO). Oligonucleotides for cloning and sequencing were either synthesized by the phosphoamidite method using an Applied Biosystems DNA synthesizer model 391, or purchased from Kurabo (Osaka) and Kiko-tech (Osaka). DEAE-Sepharose Fast Flow and Sephadex G-75 were purchased from Amersham Pharmacia Biotech. (Buckinghamshire, UK). Polyvinylidene difluoride (PVDF) membrane was obtained from Bio-Rad Laboratories (Hercules, CA). All other materials were purchased from Wako Pure Chemicals (Osaka).

Bacterial Strains, Plasmids, and Media—Bacillus novosp. MN-32 was used as a DNA donor. *E. coli* JM109 (*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*, *relA1*, *supE44*, Δ(*lac-proAB*), F′ [*traD36*, *proA*$^+$*B*$^+$, *lacIq*, *lac*ΔM15] and *E. coli* DH5α [*deo R*, *end A1*, *gyr A96*, *hsd R17*(rk$^-$, mk$^+$), *rec A1*, *rel A1*, *sup E44*, *thi-1*, (*lac ZYA-arg F*)U169, ø*80lac*ZΔM15, F$^-$, l$^-$] were used as hosts. Plasmids pUC18 and pUC19 were used for cloning and sequencing. Plasmid pKK223-3 was used for expression (*30*). MN medium (0.75% Polypepton S, 0.75% Polypepton Y, 0.1% yeast extract, 0.3% sodium chloride, 0.05% $MgSO_4 \cdot H_2O$, 0.01% $CaCl_2 \cdot H_2O$, 0.001% $ZnSO_4 \cdot H_2O$, 0.001% $MnCl_2 \cdot H_2O$, 0.001% $FeSO_4 \cdot H_2O$, pH 3.5) was used for the cultivation of *Bacillus* novosp. MN-32. *E. coli* strains were grown in Luria-Bertani broth (LB-broth, 1% Polypepton, 0.5% yeast extract, and 1% sodium chloride, pH 7.0) or super broth (1.2% Polypepton, 2.4% yeast extract, 0.5% glycerol, 1.25% K_2HPO_4, and 0.38% KH_2PO_4, pH 7.0).

Preparation of Anti-Kumamolysin Antibody—An antiserum was prepared by using an authentic kumamolysin. Two milliliters of the emulsified antigen (0.5 to 2 mg/ml) in complete Freund's adjuvant was injected into the hind leg muscles of rabbit. Booster immunization was repeated at two weeks interval after initial sensitization. An anti-kumamolysin antibody was partially purified from the high-titer serum by DEAE-cellulose chromatography. The antibody was stored at –80°C until use.

Determination of Partial Amino Acid Sequence—Kumamolysin (2 mg) was digested with trypsin (12 μg) in 100 mM potassium phosphate buffer, pH 7.8 for 24 h at 37°C. For cyanogen bromide (CNBr) cleavage, the enzyme (1 mg) was incubated in 70% formic acid solution containing 0.1% CNBr under N_2 gas in the dark for 15 h at room temperature. Peptide mixtures obtained by enzymatic or CNBr cleavages were separated by reverse-phase high pressure liquid chromatography. Amino acid sequences of the amino-terminal region of kumamolysin and of peptides derived from trypsin or CNBr cleavages were determined by the Edman degradation method (*31*).

Preparation of DNAs and Transformation—Bacillus novosp. MN-32 chromosomal DNA was prepared by the method of Saito-Miura (*32*). Plasmids were isolated by alkaline lysis followed by polyethylene glycol 6,000 precipitation. Competent cells for transformation were prepared by the method of Hanahan with a slight modification (*33*).

DNA Manipulation—The general procedures for DNA manipulation were based on those described in Sambrook *et al.* (*34*). PCR and sequencing were carried out according to the protocols recommended by the respective manufacturers.

Cloning of the Kumamolysin Gene—Two probes, K4K5 (5′-TAICAIGCIGGICCIGGITGGGAICCIGA-3′) and R3 (5′-CCACGACAT(G/C)ACGGA(G/C)GGGAACAACGACAT-3′), were labeled with [^{32}P-γ]ATP for hybridization. *Bacillus* novosp. MN-32 chromosomal DNAs digested with various restriction enzymes were separated on a 0.7% agarose gel and transferred to a nitrocellulose filter. The filter was pre-hybridized for 2 h at 60°C in a solution comprised of 0.75 M NaCl, 75 mM sodium citrate, 50 mM sodium phosphate, 1× Denhardt's solution, 0.1% SDS, and 0.1 mg/ml salmon testis DNA. Hybridization was carried out in 10 ml of the same solution containing a radioactive probe for 16 h at 60°C. After incubation, the filter was washed twice with 20 ml of a solution comprised of 0.3 M NaCl, 30 mM sodium citrate, and 0.1% SDS for 15 min and twice with 20 ml of the same solution for 1 h at 60°C. After drying, the filter was wrapped and exposed to an X-ray film with an intensifying screen at –80°C.

DNA Sequencing—Nucleotide sequence was determined by the chain termination method with AmpliTaq DNA polymerase Stoffel fragment by using an Applied Biosystems DNA sequencer model 373S. The reaction mixture was loaded on a 5.25% denatured polyacrylamide gel. Nucleotide sequence was analyzed using the DNASIS software programs (Hitachi) for prediction of an amino acid sequence.

Construction of Kumamolysin Expression Plasmids—pK2 was digested with *Sph*I and the resultant 5.6-kbp fragment was self-ligated. The kumamolysin gene including N-Prepro region was located downstream from the *lac* promoter. The resultant plasmid, pK2S1, was transformed into *E. coli* JM109. To construct a superior kumamolysin expression plasmid, a 1.9-kbp fragment of pK2 was amplified. Four sense primers containing an *Eco*RI site, 5′-TTTGAATTCATGCCCATCCCGACTAG-3′ (S1), 5′-TTTGAATTCATGGAGTGGAAGGCGAT-3′ (S2), 5′-TTTGAATTCATGAGCG-

ACATGGAGAA-3′ (S3), and 5′-TTTGAATTCATGGAGAA-GCCTTGGAA-3′ (S4); and an antisense primer containing a *Hin*dIII site, 5′-TTTAAGCTTGGCCAAGTTGTGCTCTC-3′ (A1), were used. PCR (S1 and A1, S2 and A1, and S4 and A1) was performed for 25 cycles of denaturation (96°C, 60 s), annealing (65°C, 90 s), and extension (72°C, 90 s) using pK2 as a template DNA. For PCR using primers S3 and A1, annealing and extension were carried out at 60°C for 90 s and at 67°C for 90 s, respectively. The amplified fragments were digested with *Eco*RI and *Hin*dIII, and each resultant fragment was cloned into the same site of pKK223-3. The resultant plasmids, pS1-A1, pS2-A1, pS3-A1, and pS4-A1 were transformed into *E. coli* JM109 cells.

Construction of Mutant Plasmid, pS278A—A 300-bp fragment of pS3-A1 was amplified with a sense primer SAL, 5′-CACGTCGACTTTCCCGCGGCG-3′, and an antisense primer, 5′-GGCGGCCGTCCCGCCGATGA-3′. A 460-bp fragment of pS3-A1 was amplified with a sense primer, 5′-ATCGGCGGGACGGCCGCCGT-3′, and an antisense primer A1 containing a *Hin*dIII site, 5′-TTTAAGCTTGGCCA-AGTTGTGCTCTC-3′. PCR was performed for 25 cycles of denaturation (96°C for 60 s), annealing (60°C for 90 s), and extension (67°C for 90 s) using pS3-A1 as a template DNA. These fragments were combined and subjected to the second-step PCR using the SAL and A1 primers under the conditions described above. A 760-bp fragment was amplified with a sense primer SAL and an antisense primer A1. The resultant fragment was digested with *Sal*I and *Hin*dIII, and the fragment was inserted into the *Sal*I-*Hin*dIII site of pS3-A1. The mutant plasmid designated as pS278A was transformed into *E. coli* JM109 cells.

Expression of Recombinant Enzyme in E. coli Cells—E. coli JM109 cells harboring the expression plasmid (pK2S1 and its derived plasmids) were cultured at 27°C in 100 ml of super broth containing 50 μg/ml of ampicillin until optical density at 660 nm reached 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and the cultivation was continued for an additional 5 h. Harvested cells were washed twice with 20 mM phosphate buffer, pH 6.5 and resuspended in the same buffer. The suspension (1g wet cells per 10 ml) was disrupted with an Ultrasonic model Astrason XL (output 1 kHz, 2 min at intervals of 1 min, 10 times at 4°C). The resultant suspension was centrifuged at 20,000 ×*g* for 20 min at 4°C. The supernatant was diluted 6-fold with 50 mM sodium acetate, pH 4.7, then incubated at 25°C for 5 h. Western hybridization of the expressed proteins was carried out according to the method of Towbin *et al.* (*35*), by using rabbit anti-kumamolysin antibody and alkaline phosphate-conjugated rabbit IgG antibody.

Processing of the S278A Mutant Precursor by Authentic Kumamolysin—Cell-free extracts of *E. coli* JM109 cells harboring pS3-A1 and pS278A were prepared by the method described above. The supernatants were diluted 6-fold with 50 mM sodium acetate buffer, pH 4.8, then incubated at 25°C for 1 h with or without authentic kumamolysin. The final concentrations of kumamolysin were 1.4 and 0.14 μg/ml, respectively. After incubation, these samples were placed on an ice bath and their proteinase activities were measured at 60°C for 20 min using casein as a substrate. A 10-μl portion of each sample was mixed with 10 μl of 2 M Tris-HCl, pH 9.5 and 5 μl of SDS loading buffer (250 mM Tris-HCl, pH 6.8, 5 mM EDTA, 5% 2-mercaptoethanol, 5% SDS, 0.125% bromophenol blue, and 50% glycerol). The mixtures were loaded onto a 12.5% SDS=polyacrylamide gel after heat denaturation.

Purification of the Enzyme—E. coli JM109 cells harboring pK2S1 or pS3-A1 was used for the production of recombinant kumamolysin. Each strain was aerobically cultured in super broth containing 50 μg/ml of ampicillin at 27°C. When the optical density at 660 nm reached 4.0 (pK2S1) or 2.0 (pS3-A1), IPTG was added to a final concentration of 1 mM, and then the cultivation was continued for an additional 5 h. Harvested cells were washed twice with 20 mM phosphate buffer, pH 6.5 and resuspended in 0.1 g wet cells per milliliter of the same buffer. The cells (pK2S1: 114 g; pS3-A1: 3 g) were disrupted with a Ultrasonic model Astrason XL (pK2S1: output 1 kHz, 2 min at intervals of 1 min, 10 times; pS3-A1: output 1 kHz, 3 s at intervals of 30 s, 80 times) at 4°C. For activation of kumamolysin precursor, each supernatant was diluted 5 to 6-fold with 50 mM sodium acetate, pH 4.8 (buffer A), then incubated at 25°C for 5 h (pK2S1) or 3 h (pS3-A1). For purification of recombinant kumamolysin derived from the pK2S1, a half portion of the activated sample was loaded onto a column of DEAE-Sepharose Fast Flow (ϕ3.2 × 12 cm) equilibrated with 50 mM acetate buffer, pH 5.5 (buffer B). The adsorbed enzyme was eluted with a linear gradient in buffer B from 0 to 0.8 M NaCl. The concentrated enzyme was loaded onto a column of Sephadex G-75 (ϕ2.5 × 43 cm) equilibrated with buffer B. The enzyme was eluted with buffer B at a flow rate of 18 ml/h. Active fractions were loaded onto a column of MonoQ 5/5 (ϕ5.0 × 50 mm) equilibrated with 50 mM acetate buffer, pH 4.7. The enzyme was eluted with 0 to 0.4 M NaCl linear gradient at a flow rate of 60 ml/h. Active fractions were pooled and stored at –20°C until use.

For purification of recombinant kumamolysin derived from the pS3-A1, the activated sample was fractionated by 80% ammonium sulfate saturation. After dialysis, recombinant enzyme was purified by chromatographies on DEAE-Sepharose Fast Flow (ϕ3.2 × 17 cm) and Sephadex G-75 (ϕ1.6 × 50 cm), as described above. Active enzymes were stored at –20°C until use.

Protein Concentration—Protein concentration was calculated from the absorbance at 280 nm by use of the factor $E^{1\%}_{1cm}$ = 12.6 (*17*).

Homogeneity—Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*36*), using a 12.5% polyacrylamide gel in 0.375 M Tris-HCl buffer, pH 8.8, containing 7.3% acrylamide, 0.2% *N,N′*-methylenebisacrylamide, and 0.1% SDS. Electrophoresis was carried out at a constant current of 20 mA at room temperature. The gel was stained with Coomassie brilliant blue R-250 in order to detect protein bands.

Assay for Proteinase Activity and Tripeptidyl Peptidase Activity—Proteinase activity was determined by the folin method with a slight modification using casein as a substrate (*17*). One unit of enzyme was defined as the amount that liberates 1 μg of tyrosine per ml of reaction mixture. Tripeptidyl peptidase activity was assayed as described by Ezaki *et al.* with a slight modification (*37*). The reaction mixture comprised 0.1 μM Ala-Ala-Phe-7-amido-4-methylcoumarin (Ala-Ala-Phe-MCA), 0.1 M sodium acetate buffer, pH 4.0, and enzyme (kumamolysin, 0.42 μg; CLN2, 0.5 μg), in a final volume of 0.2 ml. After incubation for 20 min at

60°C (kumamolysin) or 37°C (CLN2), the reaction was terminated by adding 0.8 ml of 0.1 M TCA containing 30 mM sodium acetate, pH 4.4. One unit of the activity was defined as the enzyme amount that gives fluorescence intensity (excitation, 380 nm; emission, 460 nm).

Inhibition Studies—Ac-Ile-Ala-Phe-CHO (*N*-acetyl-isoleucyl-alanyl-phenylalaninal, *38*), Ac-Ile-Pro-Phe-CHO (*N*-acetyl-isoleucyl-prolyl-phenylalaninal, *38*), tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal, *28*), iodo-tyrostatin, (*N*-isovaleryl-phenylalanyl(4-I)-leucyl-tyrosinal, *38*), and pseudo-tyrostatin (*N*-isovaleryl-tyrosyl-tyrosinal, *38*) were synthesized in our laboratory. The reaction mixture comprised of kumamolysin and each inhibitor was incubated at 60°C, 37°C, or 22.4°C for 10 min. The remaining activities were assayed. K_i values of each inhibitor for kumamolysin were calculated from Lineweaver-Burk plots.

A reaction mixture (50 μl) containing 100 mM sodium formate buffer (pH 3.5), 10 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride), and 0.1 μM kumamolysin was incubated at 60°C for 1 h. The remaining activity was measured by the casein-folin method.

RESULTS

Partial Amino Acid Sequence of Kumamolysin—The amino-terminal amino acid sequence of kumamolysin was determined to be A-A-P-T-A-Y-T-P-L-D-V-A-Q-(X)-Y. Amino acid sequences of seven peptides derived from trypsin or CNBr digestion were also determined. Based on these partial amino acid sequences of kumamolysin, two oligonucleotide probes (K4K5 and R3) were designed and synthesized for further experiments.

Cloning of Kumamolysin Gene—The location of the kumamolysin gene was determined by Southern blot analyses of the restriction enzyme-digested chromosomal DNA. A 5.8-kbp *Pst*I fragment hybridized with both probes, K4K5 and R3. The fragment had been extracted from agarose gel

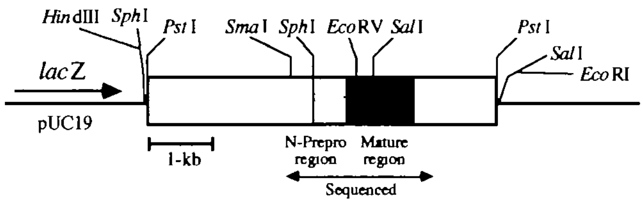

Fig. 1. **Partial restriction map of the 5.8 kb chromosome DNA fragment inserted into *Pst*I site of pUC19.** The restriction map of pK2 is shown. N-Prepro and mature regions are indicated by shaded and closed boxes, respectively.

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       CCCGGGCGGGAGATTCCTGCTGCTCGAATGGGACAAGCGTCCCATGGAGATGGGGCCACCGGTCGAGGAGCGTTTGTCGATCCACGCGTGTGAGGAGGCGC  101
     TTCGCACGGCCGGTTTTGAGATCCTTTATCGCATTTTTCCGAACGACGTCCAATACGGAATTGTGGCCGAGCGCCCAGGCCGCCCGTCCACGCCGTGAGCACCCG  206
     GCTGCATGTAGCACAGCAGGGGAGTCGCAGGCTCCGCGCAGCCGACCGCAGATGCCTAAATCTTGCGTAAAATCCACAACTTTTCCTCCAGCCGTGTTATACTGA  311
                         SphI
     GGAGAGCGAAGCCTGAAGCCCTGCATGCCCATCCCGACTAGGAGAATGGAGTGGAAGGCGATGAGCGACATGGAGAAGCCTTGGAAGGAAGAGGAGAAGCGCGAG  416
-200                       M  P  I  P  T  R  R  M  E  W  K  A  M  S  D  M  E  K  P  W  K  E  E  E  K  R  E
     GTCCTCGCGGGACACGCTCGCAGGCAGGCGCCGCAGGCTGTCGATAAGGGACCGGTGACCGGGGACCAGCGGATTTCCGTTACGGTCGTGCTGCGCCGCCAACGA  521
-173 V  L  A  G  H  A  R  R  Q  A  P  Q  A  V  D  K  G  P  V  T  G  D  Q  R  I  S  V  T  V  V  L  R  R  Q  R
     GGCGATGAACTCGAGGCGCATGTGGAACGCCAGGCCGCGCTCGCACCTCACGCGCGAGTGCATCTGGAGCGAGAAGCGTTTGCCGCTTCGCACGGCGCTTCGCTC  626
-138 G  D  E  L  E  A  H  V  E  R  Q  A  A  L  A  P  H  A  R  V  H  L  E  R  E  A  F  A  A  S  H  G  A  S  L
     GACGACTTTGCGGAGATTCGAAAGTTCGCGGAAGCGCACGGGCTCACGCTCGATCGCGCCCACGTGGCTGCGGGCACCGCGGTGCTGAGCGGCCCGGTGGACGCC  731
-103 D  D  F  A  E  I  R  K  F  A  E  A  H  G  L  T  L  D  R  A  H  V  A  A  G  T  A  V  L  S  G  P  V  D  A
     GTTAACCAAGCGTTTGGGGTCGAGTTGCGCCATTTCGATCATCCAGACGGATCCTATCGAAGCTACGTCGGCGACGTGCGTGTGCCGGCTTCCATCGCGCCTCTG  836
-68  V  N  Q  A  F  G  V  E  L  R  H  F  D  H  P  D  G  S  Y  R  S  Y  V  G  D  V  R  V  P  A  S  I  A  P  L
     ATTGAAGCGGTGTTTGGCCTGGACACGCGCCCGGTGGCGCGGCCCCACTTTCGGCTGCGGAGGCGCGCCGAGGGCGAGTTTGAGGCGAGATCGCAGTCCGCGGCG  941
-33  I  E  A  V  F  G  L  D  T  R  P  V  A  R  P  H  F  R  L  R  R  R  A  E  G  E  F  E  A  R  S  Q  S [A  A
     CCGACTGCGTACACGCCGCTCGACGTCGCGCAGGCGTACCAATTTCCCGAGGGGCTCGACGGACAGGGACAGTGCATCGCCATCATCGAATTGGGCGGCGGCTAC 1046
3   [P  T  A  Y  T  P  L  D  V  A  Q  A  Y] Q  F  P  E  G  L  D  G  Q  G  Q  C  I  A  I  I  E  L  G  G  G  Y
     GACGAGACATCTCTCGCGCAGTATTTCGCATCGCTTGGCGTGTCCGCGCCGCAGGTGGTGAGCGTCTCGGTGGACGGCGCCACCAATCAGCCCACGGGCGATCCG 1151
38   D  E  T  S  L  A  Q  Y  F  A  S  L  G  V  S  A  P  Q  V  V  S  V  S  V  D  G  A  T  N  Q  P  T  G  D  P
     AATGGCCCGGACGGCGAGGTCGAGCTCGATATCGAAGTGGCGGGAGCGCTCGCCCCGGGCGCCAAGATTGCCGTGTATTTCGCGCCGAACACGGACGCCGGCTTC 1256
73   N  G  P  D  G  E  V  E  L  D  I  E  V  A  G  A  L  A  P  G  A [K  I  A  V  Y  F  A  P  N  T  D  A  G  F]
     CTGAACGCCATCACGACCGCCGTTCACGATCCCACCCACAAGCCGTCCATCGTGTCCATCAGCTGGGGTGGCCCTGAGGACAGTTGGGCGCCGGCTTCCATCGCG 1361
108 [L  N  A  I  T  T  A  V  H  D  P  T  H  K][P  S  I  V  S  I  S  W  G  G  P] E  D  S  W  A  P  A  S  I  A
     GCGATGAACCGCGCGTTTCTGGACGCGGCGGCGCTGGGGGTGACGGTCCTCGCGGCGGCGGGCGACAGCGGATCCACGGACGGCGAGCAGGACGGCCTGTACCAC 1466
143  A [M  N  R  A  F  L  D  A  A  A  L  G  V  T  V  L  A  A  A  G  D  S  G  S  T  D  G  E  Q  D  G  L  Y] H
     GTCGACTTTCCCGCGGCGTCGCCGTACGTGCTGGCCTGCGGCGGTACGCGGCTTGTGGCGAGCGCGGGCCGCATCGAGCGAGAGACCGTGTGGAACGACGGCCCG 1571
178  V  D  F  P  A  A  S  P  Y  V  L  A  C  G  G  T [R  L  V  A  S  A  G  R  I  E  R  E  T  V  W  N  D  G  P]
     GATGGAGGATCGACGGGCGGCGGCGTGAGCCGCATCTTTCCGCTGCCCTCGTGGCAGGAGCGCGCGAACGTGCCTCCTTCGGCGAATCCGGGCGCTGGCAGCGGC 1676
213 [D  G] G  S  T  G  G  G  V  S  R [I  F  P  L  P  S  W  Q  E  R  A  N  V] P  P  S  A  N  P  G  A  G  S  G
     CGCGGCGTGCCGGATGTGGCTGGCAATGCCGATCCGGCCACGGGGTACGAGGTCGTGATCGACGGCGAGACTACGGTCATCGGCGGGACGAGCGCCGTGGCGCCG 1781
248  R  G  V  P  D  V  A  G  N  A  D  P  A  T  G  Y  E  V  V  I  D  G  E  T  T  V  I  G  G  T  S  A  V  A  P
     CTTTTCGCCGCGCTGGTGGCCCGCATCAACCAGAAGCTCGGCAAGCCAGTCGGCTATTTGAACCCGACACTCTACCAGTTGCCTCCGGAGGTGTTTCACGACATC 1886
283  L  F  A  A  L  V  A  R  I  N  Q [K  L  G  K  P  V  G  Y  L  N  P  T  L  Y  Q  L  P  P  E  V  F  H  D  I]
     ACCGAGGGCAACAACGACATCGCGAACCGGGCGAGGATCTATCAGGCGGGGCCGGGATGGGATCCGTGCACGGGGCTCGGGAGCCCCATTGGGATCCGATTCGCT 1991
318 [T  E  G  N  N  D  I  A  N  R  A  R  I  Y  Q  A  G  P  G  W  D  P  C  T  G  L  G  S  P  I  G  I  R] F  A
     TCAGGCGCTGCTGCCGAGCGCTTCACAGGCCCAGCCGTAACGCGCGACCTCGCTTCAGGCAAGGAATACAATTGCGAAAGTTTGCAAGCGATGCTATAGTAGCGC 2096
353  S  G  A  A  A  E  R  F  T  G  P  A  V  T  R  D  L  A  S  G  K  E  Y  N  C  E  S  L  Q  A  M  L  *  *
     CACGTAGAGATGGCGTCGGCACATGGCGACGCCCGGACGAACATCCGGGCGCCGCTTGTGTGTATCAGGGCCTGTTGCCCATCGCGGCGCCAGCCTTGATTCTGC 2201
     GAAGGAGGAGAGCACAACTTGGCCTTCACCGCCTCAATCGGATTTCCCCGCATGGGGCCTCGCCGCGAGCTCAAGCGGATGGTGGAACAGTTTTGGCAGGGCAAA 2306
     ATTGGGGAGGACGAGCTCACGTCCCGTGCCGCGGAACTGCGAAAACTGCGCTGGCAGGTGCAAAAGGACCGCGGCGTCAAGTGGATCGTCGCGAACGACTTTTCC 2411
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Fig. 2. **Nucleotide sequence and the deduced amino acid sequence of kumamolysin precursor.** The nucleotide sequence is numbered from *Sma*I site of the 5′ terminus of pK2. Numbering of amino acid residues was started at the amino-terminus, Ala, of the mature enzyme. Amino acid sequences determined by protein sequencing are boxed. Predicted Shine-Dalgarno sequence and putative transcription terminator is indicated by an underline and a dashed line, respectively. The termination codon is indicated by an asterisk.

and cloned into *E. coli* DH5α cells by using pUC19. After the restriction sites of the fragment had been determined, each small fragment was sequenced in both strands (Fig. 1). Within the DNA sequence, four ATG start codons at positions 336, 357, 372, and 381, were found (Fig. 2). The translational initiation site of kumamolysin was deduced to be ATG at position 372, since a Shine-Dalgarno sequence (GGAG) similar to the putative *E. coli* ribosome-binding site (*39*) was present 10 nucleotides upstream from the initiation site. A palindromic sequence was located downstream from the stop codon TAG at position 2088. These data revealed that the open reading frame of the kumamolysin gene was composed of 1,716 base pairs and encoded 572 amino acid residues with a molecular mass of 59,866 Da. By comparing the amino-terminal amino acid sequence of authentic enzyme with that deduced from the kumamolysin gene, it was revealed that kumamolysin was synthesized as a precursor comprised of N-Prepro (188 amino acids) and mature proteins (384 amino acids), as observed in PSCP (*22*) and XCP (*23*).

Purification and Some Properties of the Recombinant Enzyme—We constructed an expression vector for the kumamolysin gene, pK2S1. *E. coli* cells harboring pK2S1 produced an immunoreactive protein (K2S1). The expression of K2S1 was induced by the addition of IPTG. From 114 g (wet weight) of the cell paste, 5.4 mg of the purified recombinant kumamolysin having a specific activity of 6,600 units/mg was obtained (Table I). The purified enzyme showed a single protein band on a SDS-PAGE (Fig. 3). After incubation at 70°C and pH 4.0 for 10 min, the recombinant enzyme retained 70% of its original activity. The amino-terminal amino acid sequence of recombinant kumamolysin was determined to be A-A-P-T-A-. Other sequences, S-A-A-P- and S-Q-S-A-A-P- were also detected. These se-

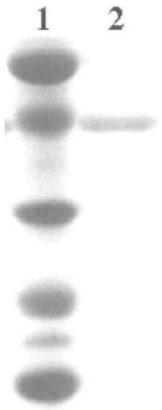

Fig. 3. **SDS–polyacrylamide gel electrophoresis of purified recombinant kumamolysin.** Samples were loaded onto a 12.5% SDS–polyacrylamide gel after denaturation with SDS and 2-mercaptoethanol. After electrophoresis, proteins were stained with Commassie Brilliant Blue R-250. Lane 1, molecular weight standards, bovine serum albumin (62 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). Lane 2, purified enzyme (3 μg).

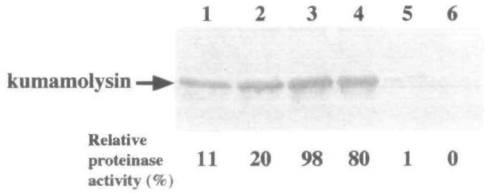

Fig. 4. **Western blot analysis of N-Prepro deletion mutants expressed in *E. coli* cells.** *E. coli* cell extracts after acidic activation were electrophoresed in a 12.5% SDS–polyacrylamide gel and analyzed by Western blotting using rabbit anti-kumamolysin antiserum. Proteinase activities of each cell-free extract were measured using casein as a substrate. Relative activity was estimated based on the proteolytic activity per wet cell weight. Lane 1, cell-free extract of pS1-A1–expressed cells; lane 2, cell-free extract of pS2-A1–expressed cells; lane 3, cell-free extract of pS3-A1–expressed cells; lane 4, cell-free extract of pS4-A1–expressed cells; lane 5, cell-free extract of pK2S1-expressed cells; lane 6, cell-free extract of pKK223-3-expressed cells.

TABLE I. **Summary of the purification of recombinant kumamolysin.**

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Activation	79,400	4,900	16	1	100
DEAE-Sepharose	61,500	270	230	14	76
Sephadex G-75	48,800	27	1,800	110	62
MonoQ	35,600	5.4	6,600	410	45

E. coli cells harboring pK2S1 were used for the production of recombinant kumamolysin.

TABLE II. **Inhibition constants of aldehyde-type inhibitors for kumamolysin.**

Inhibitor				K_i (μM)*		
P_4	P_3	P_2	P_1	60°C	37°C	22.4°C
Ac	Ile	Ala	Phe-CHO	> 2	2.2 ± 0.30	0.9 ± 0.14
Ac	Ile	Pro	Phe-CHO	> 2	1.5 ± 0.11	0.7 ± 0.14
Isoval (iodo-tyrostatin)	Phe(4-I)	Leu	Tyr-CHO	> 2	1.9 ± 0.12	1.2 ± 0.24
	Isoval (pseudo-tyrostatin)	Tyr	Tyr-CHO	> 2	> 8	17 ± 3.0
Isoval (tyrostatin)	Tyr	Leu	Tyr-CHO	> 2	> 8	2.8 ± 0.5

*Kumamolysin and inhibitors were incubated for 10 min at pH 3.5 before adding substrate. Reactions were performed at 60°C, 37°C, and 22.4°C. Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu was used as a substrate (K_m = 69 ± 11 μM at 60°C, K_m = 24 ± 3 μM at 37°C, and K_m = 17 ± 2 μM at 22.4°C).

quences correspond to the amino acid residues from –1 to 3 and –3 to 2 of the kumamolysin precursor, respectively. The specific activity of kumamolysin for Ala-Ala-Phe-MCA was 0.2% of that of the CLN2 protein. Ala-Ala-Phe-chloromethylketone, an inhibitor of the CLN2 protein, showed no effect on the proteolytic activity of kumamolysin (data not shown).

Construction of High Expression System—To improve the expression system using pK2S1, we focused our attention on Met residues at the amino-terminal of the kumamolysin precursor. Their N-Prepro truncation mutants were cloned into an expression vector, pKK223-3. *E. coli* cells harboring pS1-A1 (sequence number: 336 to 2088), pS2-A1 (357 to 2088), pS3-A1 (372 to 2088), or pS4-A1 (381 to 2088) produced a 43 kDa immunoreactive protein (S1-A1, S2-A1, S3-A1, or S4-A1) against the anti-kumamolysin antibody (Fig. 4). *E. coli* cells harboring pS3-A1 showed the highest expression level among them, which was two orders of magnitude higher than that of *E. coli* cells harboring pK2S1. When *E. coli* cells harboring pS3-A1 were used for enzyme production, 25 mg of the purified enzyme (specific activity: 6,300 units/mg) was obtained from 2.4 liter of culture medium.

Inhibition Studies—As reported previously, kumamolysin is insensitive to tyrostatin (*N*-isovaleryl-tyrosyl-**leucyl**-tyrosinal), a specific inhibitor of PSCP and XCP (*28*). The reason for this insensitivity is that kumamolysin has a unique preference for the P_2 position of a substrate, preferentially hydrolyzing peptides having an Ala or Pro residue at this position, while PSCP and XCP prefer peptides having hydrophobic and bulky amino acid residues such as Leu at the P_2 position. Based on this substrate specificity of kumamolysin, two aldehyde inhibitors were synthesized: Ac-Ile-Ala-Phe-CHO and Ac-Ile-Pro-Phe-CHO, which have an Ala or Pro residue at the P_2 position (*38*). Their inhibitory activities against kumamolysin are summarized in Table II, along with those of tyrostatin derivatives. These aldehyde-type inhibitors showed temperature-dependent inhibition: inhibitory activity was detected at 22.4 and 37°C but not at 60°C. Ac-Ile-Pro-Phe-CHO, Ac-Ile-Ala-Phe-CHO, and iodo-tyrostatin showed inhibition in the μM range for kumamolysin at 22.4°C. Tyrostatin and pseudo-tyrostatin

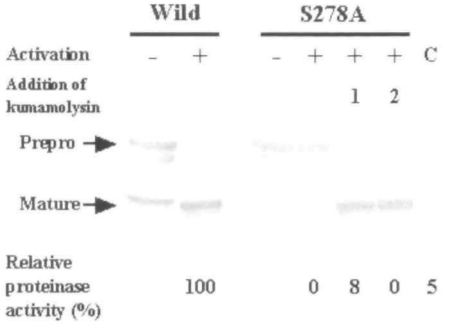

Fig. 5. **Western blot analysis of the S278A mutant expressed in *E. coli* cells.** Cell-free extracts of pS3-A1–expressed and pS278A-expressed cells were incubated for 1 h at 25°C and pH 4.8 with or without authentic kumamolysin. These samples were electrophoresed in a 10% SDS–polyacrylamide gel and analyzed by Western blotting using rabbit anti-kumamolysin antiserum. Proteinase activity was measured using casein as a substrate. Acidic activation: before (–), after (+); kumamolysin concentration: 1.4 μg/ml (1), 0.14 μg/ml (2); authentic kumamolysin: 1.4 μg/ml (C).

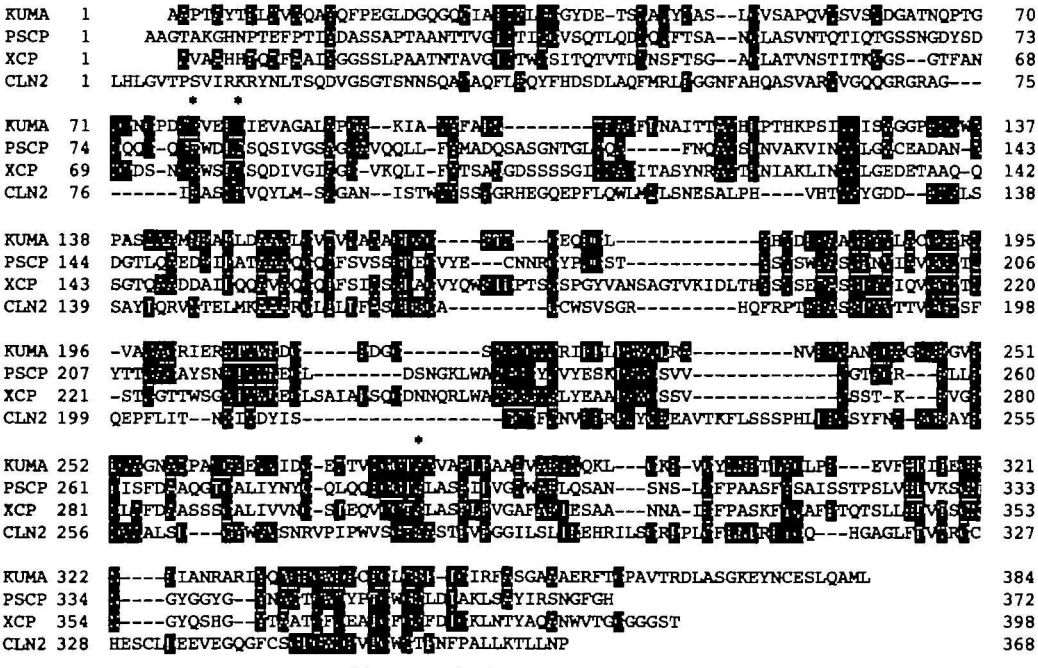

Fig. 6. **Comparison of the primary structure of kumamolysin with those of related carboxyl proteinases.** The amino acid sequence was numbered from the amino-terminal residue of mature enzymes. The sequences are: KUMA, kumamolysin (this work); PSCP, *Pseudomonas* serine-carboxyl proteinase; XCP, *Xanthomonas* carboxyl proteinase; CLN2, human CLN2 protein. Heavy shading indicates identical amino acid residues. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The asterisks indicate the catalytic residues identified by a three-dimensional structure of PSCP (*29*).

did not show inhibitory activities at 37°C. In addition, kumamolysin was not inhibited at all by AEBSF, a typical serine proteinase inhibitor, even at 10 mM concentration of inhibitor.

Mutational Analysis of Ser278 Residue—The Gly-Thr-Ser sequence, a typical catalytic motif of serine proteinases, is found in PSCP, XCP, and the CLN2 protein (*25*). The corresponding serine residue in kumamolysin is Ser278. To clarify the involvement of the Ser278 residue in the catalytic function, mutational analysis was carried out. As shown in Fig. 5, *E. coli* JM109 cells harboring pS3-A1 and pS278A produced immunoreactive proteins (wild-type and S278A) against the anti-kumamolysin antibody. *E coli* JM109 harboring pS3-A1 produced a 64 kDa precursor protein and the partially processed protein before acidic activation. These proteins were autocatalytically converted to a 43 kDa active form after incubation under acidic conditions. In contrast, *E. coli* JM109 cells harboring pS278A produced only the 64 kDa precursor protein, and this was not processed to an active protein after incubation under acidic conditions. The precursor protein of the S278A mutant was converted to a mature form by addition of catalytic amounts of authentic kumamolysin, but the mature protein lacked proteolytic activity.

DISCUSSION

Kumamolysin is the first thermostable and pepstatin-insensitive carboxyl proteinase isolated from prokaryotic cells. To clarify the structure-function relationship of kumamolysin, we have cloned the gene from *Bacillus* novosp. MN-32 and determined its nucleotide sequence.

Kumamolysin was synthesized as a large precursor consisting of two regions: N-Prepro (188 amino acids) and mature proteins (384 amino acids). The N-Prepro region might have a chaperone-like activity that causes the mature protein to fold correctly, as observed in PSCP (K. Oda *et al.*, unpublished data). The mature region of kumamolysin showed no significant sequence similarity to any pepstatin-sensitive carboxyl proteinases (aspartic proteinases) reported so far or to those of the thermophile enzyme, thermopsin (*40, 41*), and the fungal pepstatin-insensitive carboxyl proteinases (*42, 43*). However, it showed significant sequence similarities to PSCP (*22*), XCP (*23*), and the CLN2 protein (*21*) (Fig. 6). The identity was 37% for PSCP, 36% for XCP, and 36% for the CLN2 protein.

E. coli cells harboring pK2S1 and pS3-A1 produced about 1.2 and 25 mg of recombinant kumamolysin per liter of culture medium, respectively. These amounts were about 7 and 150 times higher than that of authentic kumamolysin produced in *Bacillus* novosp. MN-32 (*17*). In pS3-A1, the length of the N-Prepro part was adjusted to get a higher expression level. Specific activity of recombinant kumamolysin (6,600 units/mg) was also 2.6 times higher than that of authentic enzyme (2,500 units/mg) (*17*). One reason for such differences might be the simple purification of recombinant kumamolysin, by which self-degradation was prevented. Enzymatic properties of recombinant kumamolysin were similar to those of the authentic enzyme, despite a slight difference in the amino-terminal amino acid sequence. During destruction of *E. coli* cells and/or acidic activation, the precursor protein might be cleaved by *E. coli* proteinases and/or kumamolysin.

We demonstrated previously that kumamolysin preferentially hydrolyzed such peptides as Lys-Pro-Ile-Pro-Phe-Nph-Arg-Leu and Lys-Pro-Ile-Ala-Phe-Nph-Arg-Leu, having a Pro or Ala residue at the P_2 position (*26*). The CLN2 protein could not cleave such octa-peptides, but it cleaved shorter peptides such as P_3-P_2-P_1-P_1'-P_2'-P_3' effectively (B.M. Dunn and K. Oda, unpublished data). In this study, we confirmed that the recombinant kumamolysin showed very weak tripeptidyl peptidase activity. The specific activity of kumamolysin for Ala-Ala-Phe-MCA was 0.2% of that of the CLN2 protein. In addition, Ala-Ala-Phe-chloromethylketone, an inhibitor of the CLN2 protein, did not inhibit kumamolysin. Thus, it was strongly suggested that the subsite structure of kumamolysin, especially the S_2 and S_3 subsites, was very different from those of PSCP (*27*) and the CLN2 protein (*37*).

Kumamolysin is a thermostable enzyme from a thermophilic bacterium (*16*). In the case of the neutral proteinase of *Bacillus stearothermophilus*, its thermostability was improved by the introduction of proline into its primary structure (*44*). The proline content (8.3%) of kumamolysin was 2.6 times higher than that of PSCP (identity with kumamolysin = 37%). It was suggested that the high proline content contributed to the thermostability of kumamolysin.

The molecular mass (43 kDa) of recombinant kumamolysin on SDS-PAGE was clearly different from the predicted M_r of 39,092. The same phenomenon was observed in analysis of scytalidopepsin B (*45*). The percentages of Asp and Glu residues in kumamolysin were 6.3 and 4.7%, respectively. Those in scytalidopepsin B were 9.2 and 6.3%, respectively (*45*). Zirwes *et al.* reported that the negatively charged amino acid residues weakened SDS binding efficiency (*46*). This might be one of the reasons for low electrophoretic mobility.

To identify the catalytic residue, we studied the effect of aldehyde-type inhibitors, which were designed and synthesized based on the chemical structure of tyrostatin and the substrate specificity of kumamolysin (*26, 28*). In PSCP (*29*), such aldehyde-type inhibitors form a hemiacetal linkage with the catalytic serine residue and inactivate the enzyme. This will be further discussed below, in conjunction with the catalytic triad of PSCP. The inhibitory activities of the aldehyde-type compounds were temperature-dependent. The strongest inhibition was observed at 22.4°C (Table II). At higher temperature, the binding ability of the inhibitors for kumamolysin may have been decreased due to rapid dissociation of the hemiacetal product, or the binding affinity may have been too low to provide a productive complex. Kumamolysin was inhibited by Ac-Ile-Pro-Phe-CHO, Ac-Ile-Ala-Phe-CHO, and iodo-tyrostatin with inhibition constants at 22.4°C in the μM range. Tyrostatin is a competitive inhibitor for PSCP with a K_i value of 2.6 nM (*47*). The K_i values of the inhibitors for kumamolysin were three orders of magnitude lower than that of tyrostatin for PSCP. This suggested that the preference of kumamolysin at the P_1 position, as well as the P_2 position, was also different from that of PSCP.

The inhibition studies described above showed that serine residue was crucial for the catalytic function of kumamolysin. Based on the sequence similarity among PSCP, XCP, and the CLN2 protein, the Ser278 residue was selected as the likely catalytic residue of kumamolysin, and its involvement in the catalytic function was confirmed by

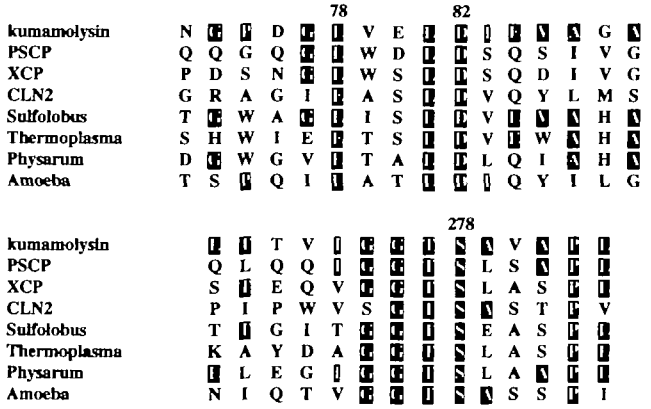

Fig. 7. **Partial sequence alignment for selected members of the family of serine-carboxyl proteinases.** Abbreviations for the enzymes are as follows: CLN2, human tripeptidyl peptidase I (*21*); *Sulfolobus*, protease-related protein in *Sulfolobus solfataricus* (*48*); Thermoplasma, XCP-related protein in *Thermoplasma acidophilum* (*49*); Physarum, cell-specific *php* protein from the slime mold *Physarum polycephalum* (*50*); Amoeba, lysosomal pepstatin-insensitive carboxyl proteinase from *Amoeba proteus* (*51*). Heavy shading indicates identical amino acid residues.

mutational analysis. Precursor protein of the S278A mutant was not converted to mature protein after acidic activation (Fig. 5). By addition of catalytic amounts of authentic kumamolysin, the precursor protein was converted to a 43 kDa mature form, but the processed protein showed no proteolytic activity. These results indicated that the S278A mutant lost both auto-processing activity and proteolytic activity. Thus, it was elucidated that the Ser278 residue was essential for the catalytic function of kumamolysin.

Finally, catalytic residues of kumamolysin will be discussed based on the sequence homology and the structure of PSCP. In 2001, Wlodawer *et al.* reported a three-dimensional structure of PSCP (*29*). The following unique features were elucidated: (i) the unexpected fold of PSCP was a superset of the subtilisin fold, (ii) the catalytic residues were composed of a unique triad of Glu80, Asp84, and Ser287 residues. The catalytic triad of subtilisin is composed of Asp32, His64, and Ser221 residues. The Glu80 residue of PSCP, equivalent to the His64 residue in subtilisin, works as a general base. This is the one of the unique features of PSCP. Thus, PSCP was classified into a novel family (MEROPS S53) of serine-carboxyl proteinases with a catalytic triad consisting of Glu80, Asp84, and Ser287 residues.

PSCP-related proteins were detected in a BLAST search of the SwissPlot database. As shown in Fig. 7, a unique catalytic triad comprising Glu, Asp, and Ser was found to be conserved in kumamolysin and other homologous proteins such as PSCP (*22*), XCP (*23*), the CLN2 protein (*21*), protease-related protein in *Sulfolobus solfataricus* (*48*), XCP-related protein in *Thermoplasma acidophilum* (*49*), cell-specific *php* protein from the slime mold *Physarum polycephalum* (*50*), and lysosomal pepstatin-insensitive carboxyl proteinase from *Amoeba proteus* (*51*).

The results of inhibition studies, mutational analysis, sequence alignment, and the structure of PSCP strongly suggest that kumamolysin has a unique catalytic triad comprising Glu78, Asp82, and Ser278 residues (corresponding to Glu80, Asp84, and Ser287 residues in PSCP).

Three-dimensional structure analyses of kumamolysin with and without inhibitors are currently underway. We hope to obtain more information on the structure-function relationship of kumamolysin from these studies.

We appreciate the assistance of Dr. Ken-ichi Fukuhara (Ajinomoto) in the determination of peptide sequences.

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