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Molecular Cloning of the Gene Encoding *Vibrio* Metalloproteinase Vimelysin and Isolation of a Mutant with High Stability in Organic Solvents

Toshihiro Takahashi[1,2], Kenneth K.-S. Ng[3], Hiroshi Oyama[1] and Kohei Oda[1,*]

[1]*Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585;* [2]*Department of Bio Science, Bio College Kyoto, Kyuken-cho, Kamigyo-ku, Kyoto 602-0851; and* [3]*Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4, Canada*

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Vimelysin is a unique metalloproteinase from *Vibrio* sp. T1800 exhibiting high activity at low temperature and high stability in organic solvents such as ethanol. A 1,821 bp open reading frame of the vimelysin gene encoded 607 amino acid residues consisting of an N-terminal pro-region, a mature enzyme, and a C-terminal pro-region. The mature enzyme region showed 80%, 57% and 35% sequence identity with the mature forms of vibriolysin from *V. vulnificus*, pseudolysin from *Pseudomonas aeruginosa*, and thermolysin from *Bacillus thermoproteolyticus*, respectively. The catalytic residues and zinc-binding motifs of metalloproteinases are well conserved in vimelysin. The vimelysin gene was expressed in *E. coli* JM109 cells and the recombinant enzyme was purified as a 38-kDa mature form from cell-free extracts. The purified recombinant enzyme is indistinguishable from the enzyme purified directly from *Vibrio*. To obtain mutants exhibiting higher stability in organic solvents, random mutations were introduced by error-prone PCR and 600 transformants were screened. The N123D mutant exhibits two times higher stability in organic solvents than the wild-type enzyme. A plausible mechanism for the stability of the N123D mutant in organic solvents was discussed based on homology models of vimelysin and the N123D mutant.

Key words: alcohol resistance, metalloproteinase, pseudolysin, random mutagenesis, vimelysin.

Microbial proteinases have many unique characteristics. For instance, *Achromobactor* I proteinase (*1*, *2*) and *Staphylococcus aureus* V8 proteinase (*3*, *4*) are unique in their strict substrate specificities. Thermolysin from *Bacillus thermoproteolyticus* (*5*) exhibits high thermal stability and proteinase from alkalophilic *Bacillus* (*6*) exhibits maximum activity under extremely alkaline conditions.

In order to utilize it as an additive in detergents or for peptide synthesis, we succeeded in isolating *Vibrio* sp. T1800 from marine bacteria, which produce a proteinase exhibiting high stability in organic solvents such as ethanol (*7*, *8*). The purified metalloproteinase, named vimelysin, has a molecular mass of 38 kDa and exhibits higher stability in organic solvents than thermolysin, a representative metalloproteinase from microorganisms. Vimelysin showed 40% activity compared to that of a control in the presence of 10% ethanol, whereas thermolysin showed only 5% activity under the same conditions (*7*, *8*). As for substrate specificity, vimelysin preferred Phe at the P1′ position, while thermolysin preferred Leu at this position (*8–10*). In addition, the optimum temperature of vimelysin was unique. The optimum temperature was 15°C with furylacryloyl-glycyl-leucine amide (FAGLA) as the substrate. The amino-terminal twenty amino acid sequence of vimelysin was similar to those of vibriolysin and pseudolysin, but different from that of thermolysin (*7*, *11–14*).

Vibriolysin is synthesized as a 66-kDa precursor protein consisting of an N-terminal pro-region, a mature enzyme, and a C-terminal pro-region. The N-terminal and C-terminal pro-regions of vibriolysin are cleaved through an autocatalytic mechanism (*15*, *16*). The C-terminal region of vibriolysin is involved in adhesion to protein substrates and the erythrocyte membrane (*16*). Pseudolysin is synthesized as a 54-kDa precursor, which is autocatalytically processed to the mature enzyme (33 kDa) (*12*, *17*).

It is well known that the addition of an organic solvent to the reaction mixture increases the yield of a product because of a shift in the thermodynamic equilibrium in favor of peptide synthesis. Thus, increasing the resistance of proteinases to organic solvents will greatly facilitate the development of novel bio-catalysts.

Thermolysin is used as a bio-catalyst for the synthesis of aspartame precursors in organic solvents (*18*, *19*). It has been reported that a single amino acid substitution in the enzyme enhances its thermostability, but improvement of its stability in organic solvents has not yet been investigated (*20–22*). It has also been reported that pseudolysin shows high stability in organic solvents, whereas for vibriolysin such stability has not been reported at all (*23*). Thus, the factors responsible for the stability of the enzyme in organic solvents are particularly interesting and important for their applications.

*To whom correspondence should be addressed. E-mail: bika@ipc.kit.ac.jp

To understand the molecular mechanism of the organic solvent–resistance of vimelysin, the following were carried out: (i) cloning of the vimelysin gene and determination of its nucleotide sequence; (ii) expression, purification and characterization of the recombinant vimelysin; and (iii) random mutagenesis to obtain mutants with more superior organic solvent–resistance than that of vimelysin.

EXPERIMENTAL PROCEDURES

Materials—Authentic vimelysin was purified from the culture filtrate of *Vibrio* sp. T1800. Restriction endonuclease, *TaKaRa Taq* DNA polymerase, *Pyrobest* DNA polymerase, a DNA Ligation Kit ver. 2, and oligonucleotide primers were purchased from Takara Bio (Shiga, Japan). A BigDye Terminator Cycle Sequencing Kit (ver. 1.1) was purchased from Applied Biosystems (California, USA). An AlkPhos Direct System, DEAE-Sepharose CL-6B, and a HIC-Phenyl column were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Furylacryloyl-Gly-Leu-NH_2 (FAGLA) and MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 [MOCAc-PLGL-A_2pr(Dnp)-AR-NH_2] were from Bachem (Bubendorf, Switzerland) and the Peptide Institute (Osaka, Japan), respectively. A Diversify^{TM} PCR Random Mutagenesis Kit was purchased from Clontech (California, USA). Bacterial protein extraction reagent B-PER II was from PIERCE (Illinois, USA). Goat anti–rabbit IgG antibody alkaline phosphatase conjugate was from Zymed Laboratories (San Francisco, USA). The polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad Laboratories (California, USA). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan). Rabbit anti-vimelysin antibodies were prepared using authentic vimelysin as an antigen.

Bacterial Strains, Plasmids, and Media—*Vibrio* sp. T1800 was used as a DNA donor. *E. coli* JM109 [*e14-*(*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_k^-, m_k^+) *supE44 relA1*(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*Z M15*)] was used as a host for a plasmid. Plasmid pUC18 was used for cloning and sequencing. Plasmid pKK223-3 was used for expression. Nealson's medium (0.6% glycerol, 0.5% peptone, 0.3% yeast extract and 50 mM Tris-HCl, pH 7.8, containing 0.3 M NaCl, 8 mM KCl, 50 mM $MgSO_4$ and 10 mM $CaCl_2$) was used for the cultivation of *Vibrio* sp. T1800. *E. coli* cells were grown in Luria-Bertani broth (LB-broth; 1% polypepton, 0.5% yeast extract and 1% NaCl, pH 7.4) containing 100 μg/ml ampicillin.

Preparation of Genomic DNA—Genomic DNA was prepared from *Vibrio* sp. T1800 according to the protocol described by Oda *et al*. (*24*).

DNA Manipulation—The general procedures used for DNA manipulation were those described by Sambrook *et al*. (*25*).

DNA Sequencing—Nucleotide sequences were determined with an Applied Biosystems DNA sequencer model ABI 3100-Avant Genetic Analyzer (Applied Biosystems, California, USA) by the chain termination method with the BigDye Terminator Cycle Sequencing Kit (ver. 1.1). The nucleotide sequence was analyzed using the DNASIS software (Hitachi, Tokyo, Japan) for prediction of an amino acid sequence.

Cloning of the Vimelysin Gene—A sense primer, 5′-AARACNACNMGNTAYGARTAYGG-3′ (VLN-F1), and an antisense primer, 5′-TGNACRTTNARNCCRTCRTA-3′ (VLN-R1), were designed based on the partial amino acid sequence of vimelysin. Degenerate PCR was performed for 35 cycles of denaturation (95°C, 30 s), annealing (40°C, 30 s), and extension (72°C, 30 s) using *TaKaRa Taq* DNA polymerase and *Vibrio* sp. T1800 genomic DNA as a template. The resultant fragment was sequenced to confirm that it was indeed the amplified vimelysin gene. The fragment was labeled with alkaline phosphatase using the Alkphos Direct kit. *Vibrio* sp. T1800 genomic DNA was digested with various restriction enzymes. Each restriction digest was separated on a 0.7% agarose gel and then transferred to a nitrocellulose filter. A 5.5-kbp *Hin*dIII-digested fragment hybridized with the labeled fragment described above. The fragment was extracted and purified from an agarose gel, and then cloned into *E. coli* JM109 cells using pUC18. The plasmid was designated as pUC18-VLN. Based on the restriction map of pUC18-VLN, the inserted fragment was divided and subcloned into *E. coli* JM109 cells using pUC18. All divided fragments were sequenced for both strands.

Construction of a Vimelysin Expression Plasmid—A sense primer, 5′-TGCGGAATTCATGAACCAACAACGTCAACTAAGC-3′ (VLN-EF1: the underlined sequence shows the position of an *Eco*RI site), and an antisense primer, 5′-TGCGAAGCTTGACTCGCTCTAACTCAATCTAA-3′ (VLN-ERH: the underlined sequence shows the position of a *Hin*dIII site), were used for PCR. PCR was performed for 25 cycles of denaturation (95°C, 10 s), annealing (55°C, 20 s), and extension (72°C, 60 s) using *Pyrobest* DNA polymerase and pUC18-VLN as a template. A 1.9-kbp amplified fragment was digested with *Eco*RI and *Hin*dIII, and then inserted in the *Eco*RI–*Hin*dIII sites of pKK223-3. The resultant plasmid, designated as pKK223-3-VLN, was transformed into *E. coli* JM109 cells.

Expression and Purification of the Recombinant Vimelysin—*E. coli* JM109 cells harboring pKK223-3-VLN were aerobically cultured in LB-broth containing 1% (w/v) glucose and 100 μg/ml ampicillin at 20°C for 36 h with shaking (140 rpm). Harvested cells were washed twice with Buffer A (50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$) and the resuspended at 0.1 g of wet cells per ml in the same buffer. The cells were disrupted with an Ultrasonic model Astrason XL [output 20% (110 W), 5 s at intervals of 30 s, 60 times] at 4°C. The resultant suspension was centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was incubated at 50°C for 20 min and then centrifuged at 20,000 × *g* for 30 min at 4°C. The resultant supernatant was fractionated with ammonium sulfate (0–60% saturation) and then dialyzed against Buffer A. The dialysate was loaded on a column of DEAE-Sepharose CL-6B (column size: ϕ10 mm × 170 mm) equilibrated with Buffer A. The column was washed with the same buffer and then the enzyme was eluted with a linear gradient of 0 to 0.3 M NaCl in Buffer A. The active fractions were pooled and then ammonium sulfate was added to a final concentration of 1 M. The solution was loaded onto a HIC-Phenyl column (column size: ϕ16 mm × 25 mm) equilibrated with Buffer A containing 1 M ammonium sulfate. After the column had been washed with the same buffer, the enzyme was eluted with a linear gradient of 0.2 to 0 M ammonium sulfate in Buffer A. The active fractions were collected and then stored at –80°C until use.

Protein Concentration—Protein concentrations were estimated by the method of Lowry *et al.* (*26*) using bovine serum albumin as a standard.

SDS-PAGE and Western Blot Analysis—Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*27*), using 12.5% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250. Western blot analysis was carried out according to the method of Towbin *et al.* (*28*), using a rabbit anti-vimelysin antibody and an alkaline phosphatase–conjugated rabbit IgG antibody.

Assay Methods for Enzyme Activities—Proteinase activity was measured by the folin method with a slight modification using casein as a substrate (*29*). One unit of the enzyme was defined as the amount that liberates 1 μg of tyrosine per ml of reaction mixture. Peptide hydrolytic activity was measured according to the method described by Oda *et al.* (*7*).

Kinetic Assays—(1) Chromogenic peptide substrate. Kinetic analysis was performed at 25°C in 0.1 M MES buffer, pH 6.5, using FAGLA as a substrate. Cleavage of the substrate between Gly and Leu was monitored spectrophotometrically by following the decrease in absorbance at 345 nm using a BECKMAN DU-7000. The initial rate was measured with eight concentrations of the substrate, and then kinetic constants K_m and V_{max} were calculated from Lineweaver-Burk plots. The k_{cat} value was derived from $V_{max} = k_{cat}\ [E]_0$, where $[E]_0$ is the enzyme concentration.

(2) Fluorescent substrate. —Kinetic analysis was performed at 25°C in 50 mM MES buffer, pH 6.5, containing 10 mM $CaCl_2$ and 0.005% Triton X-100, using MOCAc-PLGL-A_2pr(Dnp)-AR-NH_2 as a substrate. Cleavage of the substrate between Gly and Leu was monitored fluorometrically by following the increase in fluorescence intensity at 450 nm with excitation at 360 nm using a fluorescence plate reader MTP-100F (CORONA). The initial rate was measured with eight concentrations of the substrate, and then kinetic constants K_m, V_{max} and k_{cat} were calculated from Lineweaver-Burk plots.

CD Spectra—CD spectra were measured in the far-UV region using a JASCO model J-720 spectropolarimeter at 25°C with 0.19 mg/ml of protein in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$.

Amino-Terminal Amino Acid Sequence—The purified enzyme was separated by SDS-PAGE and then transferred to a PVDF membrane. Proteins were stained with Coomassie Brilliant Blue R-250 on the membrane. The N-terminal five amino acid residues of the stained protein material were sequenced with a Shimadzu model PPSQ-23 gas-phase sequencer (Shimadzu Biotech, Kyoto, Japan).

Mass Spectra—The purified enzyme was desalted and then subjected to MALDI-TOF analysis. Mass analysis of the enzyme was performed on an AXIMA-CFR mass spectrometer (Shimadzu Biotech, Kyoto, Japan).

Error-Prone PCR—Error-prone PCR was carried out using the Diversify™ PCR Random Mutagenesis Kit, and a sense primer, 5′-AGCGCCCTTTTTACTTTATTGAT-3′ (VSF3), and an antisense primer (VLN-ERH). Plasmid pKK223-3-VLN was used as a template. PCR was carried out according to the protocol recommended by the respective manufacturers. The amplified fragments were digested with *Dra*III and *Hin*dIII. These digested fragments were inserted into the *Dra*III–*Hin*dIII sites of pKK223-3-VLN. The resultant plasmids were transformed into *E. coli* JM109 cells. Each transformant was cultured with shaking (220 rpm) in LB-broth containing 1% (w/v) glucose and 100 μg/ml ampicillin at 20°C for 48 h. Harvested cells were resuspended in B-PERII and then centrifuged. Each cell lysate was incubated at 37°C for 5 h in the presence of 50% ethanol. After evaporating ethanol with a centrifugal concentrator, the remaining proteinase activity of each sample was measured by means of the casein plate assay (37°C, 16 h).

Purification of Vimelysin N123D—The transformant having the vimelysin N123D gene was grown at 20°C for 36 h in LB broth containing 1% (w/v) glucose and 100 μg/ml ampicillin. Vimelysin N123D was purified by the same procedures as those used for wild-type vimelysin. The active enzyme was stored at –80°C until use.

Stabilities of Wild-Type Vimelysin and Vimelysin N123D in Organic Solvents—Each purified enzyme (0.2 mg/ml) was mixed with an equal volume of ethanol or 2-propanol. After incubation at 37°C for 1 or 3 h, the remaining activity of each sample was measured by the standard assay method.

Modeling—Three-dimensional homology models of vimelysin were built using the SWISS-MODEL program (http://swissmodel.expasy.org//SWISS-MODEL.html) and MODELLER (version 8.1) (*30*) based on the three-dimensional structures of pseudolysin [PDB: 1EZM (*31*) and 1U4G] and thermolysin [PDB: 4TMN (*32*)]. The predicted hydrogen bonds and electrostatic interactions in both wild-type vimelysin (PDB: 2A4I) (*33*) and vimelysin N123D were evaluated using the programs Swiss-PdbViewer (*34–36*) and PyMol (*37*).

RESULTS

Cloning of the Vimelysin Gene and Its Nucleotide Sequence—A 3.6-kbp *Eco*RI–*Pst*I fragment including the vimelysin gene was isolated from *Vibrio* sp. T1800 chromosomal DNA. An open reading frame of the vimelysin gene comprised 1,821 bp and encoded 607 amino acid residues (Fig. 1). Putative promoter sequences, the –35 and –10 regions, and the putative ribosome binding site were located upstream from the initiation codon (Fig. 1). The putative transcription terminator was located downstream from the termination codon (Fig. 1). By comparing the amino-terminal amino acid sequence of the authentic enzyme with that deduced from the nucleotide sequence of the vimelysin gene, the amino terminal residue of the mature enzyme was identified as Ala-196 of the deduced amino acid sequence of the open reading frame (Fig. 1). The molecular mass of the mature enzyme was estimated to be 33,801.3 on mass spectrometric analysis. These results suggest that the mature enzyme corresponds to a polypeptide composed of residues 196 to 506 (molecular mass calculated from the deduced amino acid sequence, 33,799.1). The vimelysin precursor was shown to be composed of the N-terminal pro-region (residues 1 to 195), the mature enzyme (residues 196 to 506), and the C-terminal pro-region (residues 507 to 607).

Sequence homology searches using the BLAST server revealed that the amino acid sequence of the mature enzyme showed 80% identity with vibriolysin (UniProt accession No. Q8GR94) from *Vibrio vulnificus*, 57%

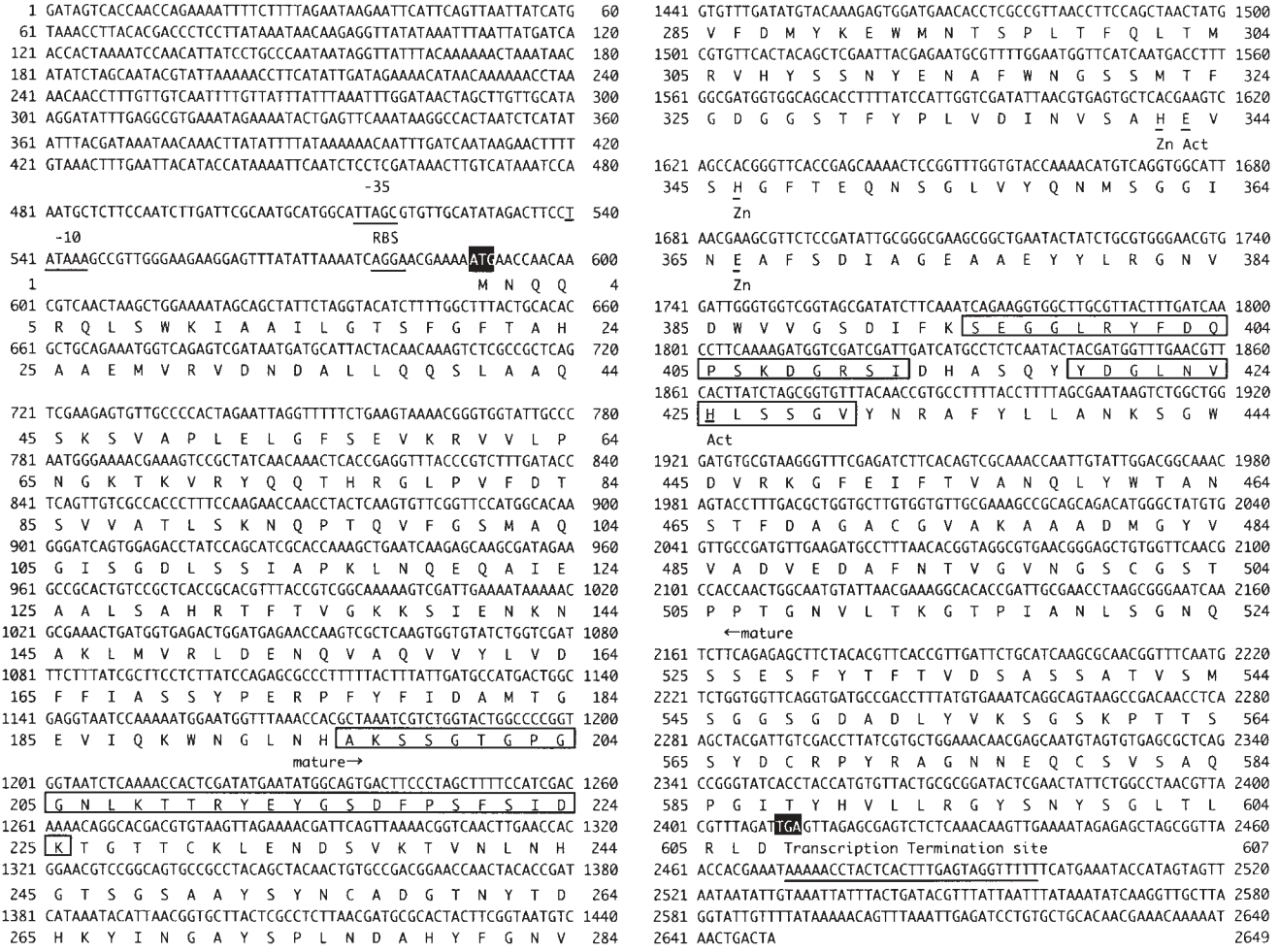


Fig. 1. **Nucleotide sequence and deduced amino acid sequence of the vimelysin precursor.** The numbering of amino acid residues starts at the amino terminus, Met, of the precursor protein. The amino acid sequences determined by protein sequencing are boxed. The putative promoter sequences, –35 and –10 regions, putative ribosome-binding site, and putative transcription terminator are underlined. Act and Zn indicate catalytic residues and Zn-binding motifs, respectively.

identity with pseudolysin (UniProt accession No. P14756) from *Pseudomonas aeruginosa*, and 35% identity with thermolysin (UniProt accession No. P00800) from *Bacillus thermoproteolyticus* (Fig. 2). The catalytic residues (Glu-148 and His-230) and zinc-binding motifs (His-147, His-151 and Glu-171), characteristics of the metalloproteinase family, are well conserved in vimelysin (Fig. 2).

Expression of the Vimelysin Gene in E. coli *Cells and Purification of the Enzyme—E. coli* JM109 cells harboring pKK223-3-VLN were cultivated at 20°C for 36 h. Two point nine mg of the recombinant vimelysin per 1 g of wet cell paste was produced (Fig. 3A). When the transformant was cultivated at 25°C or 30°C, the recombinant enzyme was expressed as a precursor form and most of the expressed vimelysin accumulated in an insoluble form. In addition, soluble precursor proteins did not show any activity after incubation at 37°C for 3 h. Expression of the vimelysin gene at lower temperature may be important for normal folding of the precursor proteins. The recombinant enzyme was purified from cell-free extracts by heat treatment, ammonium sulfate fractionation, DEAE-Sepharose, and HIC-Phenyl. The purified enzyme gave a single band on SDS-PAGE (Fig. 3B). Thirty-two mg of the recombinant enzyme was obtained from 24 g of wet cell paste, the overall yield being 40%.

Enzymatic and Physical Properties of the Recombinant Enzyme—The recombinant enzyme showed maximal activity at 15°C and pH 6.5 with FAGLA as a substrate. In contrast, maximal activity was observed at 50°C and pH 8.0 with casein as a substrate. The enzyme showed 40% of the control activity with FAGLA as a substrate in the presence of 10% ethanol (25°C and pH 6.5). These enzymatic properties were identical to those of the authentic enzyme. The far-UV CD spectra for the recombinant enzyme and the authentic enzyme were also nearly identical (Fig. 4). Finally, the amino-terminal amino acid sequence of the recombinant enzyme was determined to be AKSSG, which is identical to that of the authentic enzyme. Thus, the recombinant enzyme is indistinguishable from the authentic enzyme with respect to enzymological and physical properties.

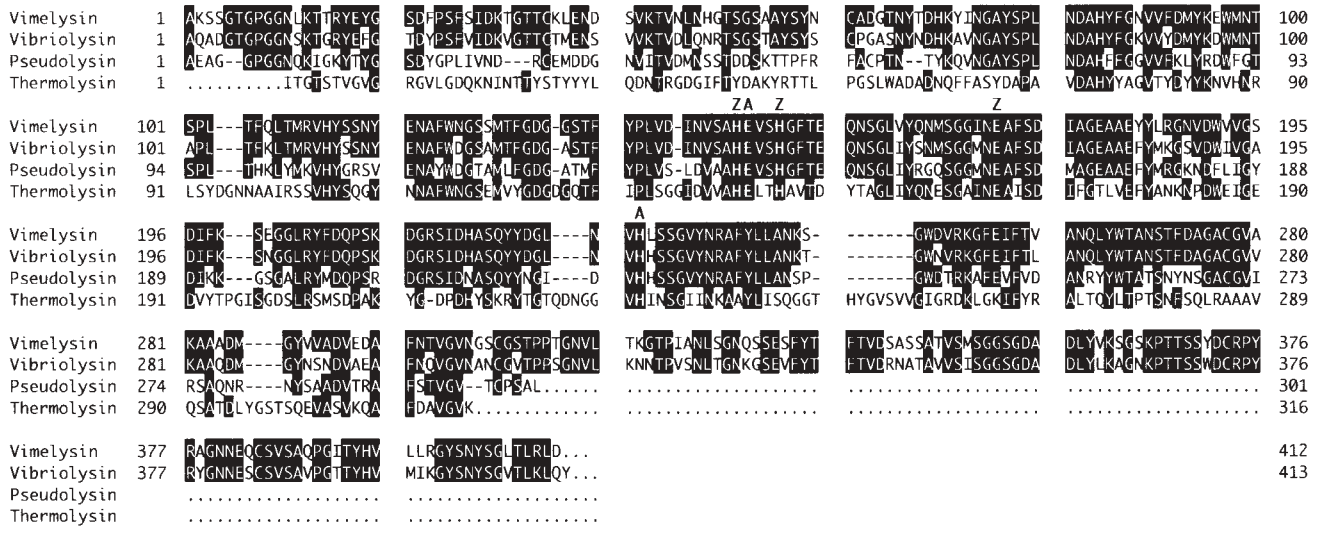


Fig. 2. **Sequence alignment of vimelysin and related proteinases**. The enzymes in this alignment are vibriolysin, pseudolysin and thermolysin. The amino acid sequences are numbered from the amino-terminal amino acid residue of the mature enzyme. Vimelysin and vibriolysin contain a C-terminal pro-region (vimelysin: residues 312–412). A and Z indicate catalytic residues and Zn-binding motifs, respectively.

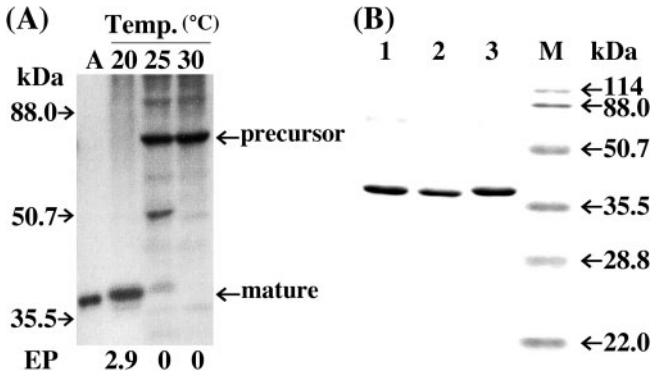


Fig. 3. **Expression of the vimelysin gene in *E. coli* JM109 cells and purification of the enzyme.** (A) Western blot analysis of the vimelysin expressed in *E. coli* JM109 cells: *E. coli* JM109 cells harboring pKK223-3-VLN were cultured at 20, 25, and 30°C for 36 h. A cell-free extract of each sample was electrophoresed in a 12.5% SDS–polyacrylamide gel and analyzed by Western blotting. The enzyme activities were measured using casein as a substrate. EP indicates the amount of enzyme (mg) per 1 g of wet cell paste. Authentic vimelysin (0.5 μg) is shown in A. (B) SDS-PAGE of wild-type vimelysin and vimelysin N123D: Each sample was loaded onto a 12.5% gel and the protein bands were stained with Coomassie Brilliant Blue R-250. Lane 1, wild-type vimelysin; lane 2, authentic vimelysin; lane 3, vimelysin N123D; lane M, prestained molecular mass standards, phosphorylase B (114 kDa), bovine serum albumin (88.0 kDa), ovalbumin (50.7 kDa), carbonic anhydrase (35.5 kDa), soybean trypsin inhibitor (28.8 kDa), and lysozyme (22.0 kDa).

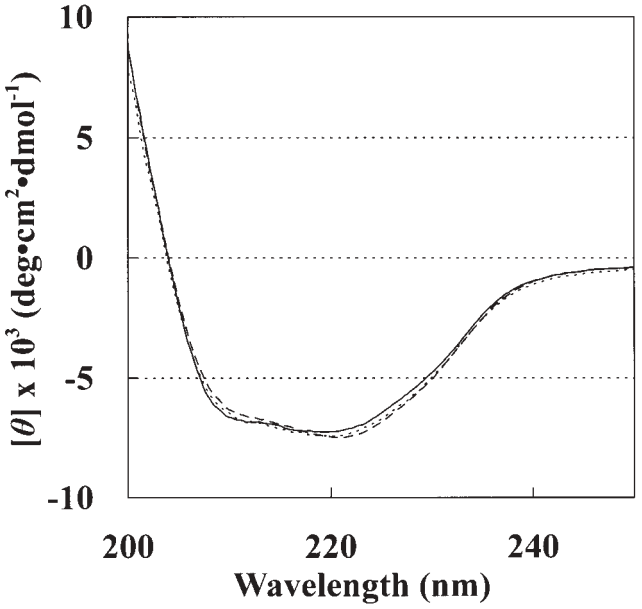
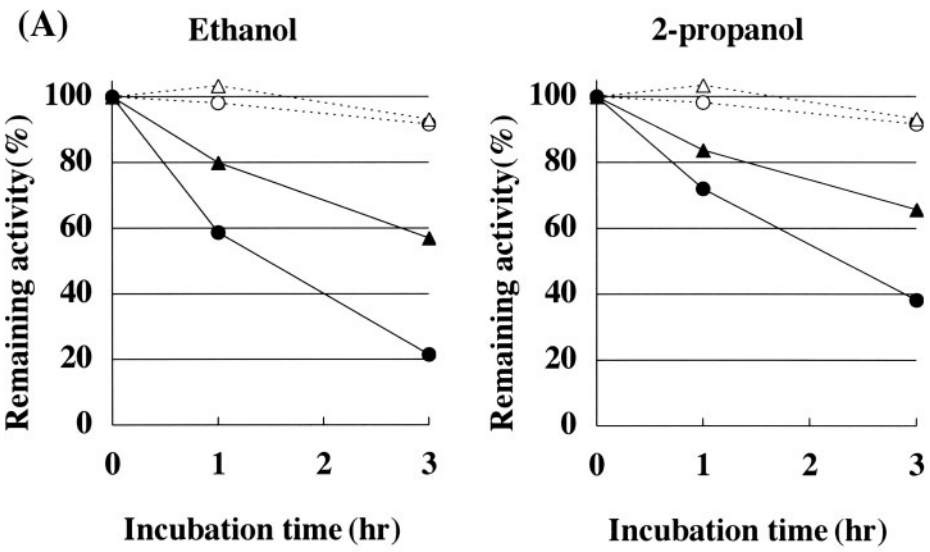


Fig. 4. **CD spectra of authentic vimelysin (solid line), wild-type vimelysin (dotted line), and vimelysin N123D (dashed line).** CD spectra were measured on a JASCO J-720A spectropolarimeter at 25°C.

Engineering of Vimelysin by Random Mutagenesis—To improve the stability of vimelysin in organic solvents, random mutations were introduced into the vimelysin gene. Six hundred transformants were obtained by random mutagenesis using error-prone PCR. A cell-free extract of each transformant was incubated at 37°C for 5 h in the presence of 50% ethanol and then the remaining proteinase activity in each extract was measured. One mutant enzyme exhibited higher residual activity than that of a control. On comparing the nucleotide sequence of the mutant enzyme with that of the wild-type enzyme, it was revealed that Asn (AAT) at position 123 of the mature enzyme was replaced with Asp (GAT). The mutant enzyme was named vimelysin N123D.

Cultivation and purification of vimelysin N123D were carried out using the same procedures as for the wild-type enzyme. The purified vimelysin N123D gave a single band on SDS-PAGE (Fig. 3B). The molecular mass of



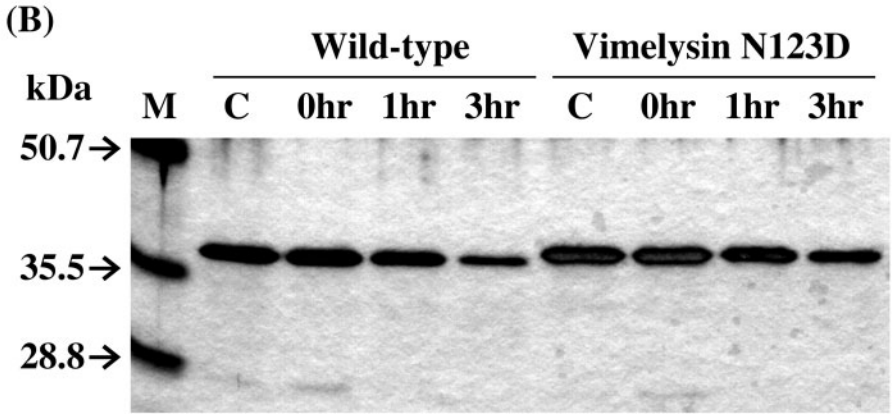


Fig. 5. **Effects of organic solvents on the stabilities of wild-type vimelysin and vimelysin N123D.** (A) Stability in organic solvents: Each enzyme was mixed with an equal volume of ethanol or 2-propanol. After incubation at 37°C for 1 or 3 h, the remaining activity was measured using MOCAc-PLGL-A_2pr(Dnp)-AR-NH_2 as a substrate. The control reaction was carried out using water instead of an organic solvent. Organic solvents: solid circles, wild-type; solid triangles, N123D; water: open circles, wild-type; open triangels, N123D. (B) Auto-degradation of wild-type vimelysin and vimelysin N123D: Each sample was mixed with an equal volume of ethanol and then incubated at 37°C for 1 or 3 h. Samples were electrophoresed in a 12.5% SDS–polyacrylamide gel and the proteins were visualized by silver staining. Lane M, prestained molecular mass standards, ovalbumin (50.7 kDa), carbonic anhydrase (35.5 kDa), soybean trypsin inhibitor (28.8 kDa); lane C, control enzymes.

vimelysin N123D was determined to be 33,903.6 by mass spectrometric analysis, which was larger by 102 than that of the wild-type enzyme. The amino-terminal amino acid sequence of vimelysin N123D was determined to be AKSSG, which is identical to that of the wild-type enzyme. The CD spectrum of vimelysin N123D was identical to that of the authentic enzyme (Fig. 4). These results suggest that vimelysin N123D corresponds to the polypeptide composed of residues 196 to 507 (Fig. 1). The C-terminal processing site of vimelysin N123D appears to differ slightly from that of the wild-type enzyme, which suggests that C-terminal processing in vimelysin may exhibit some heterogeneity.

Stability of Vimelysin N123D in Organic Solvents—The stability of vimelysin N123D in organic solvents was evaluated (Fig. 5A). After treatment with 50% ethanol at 37°C for 3 h, the wild-type vimelysin exhibited a level of activity that was only 22% of the level exhibited by a control sample. In contrast, vimelysin N123D showed 57% of the control level under the same conditions. After treatment with 50% 2-propanol at 37°C for 3 h, the wild-type enzyme showed 38% of the control level, while vimelysin N123D showed 66% of the control level under the same conditions. These results indicate that vimelysin N123D showed about two times higher stability in organic solvents than the wild-type enzyme. To confirm the higher stability of the N123D mutant, each enzyme was incubated at 37°C and pH 6.5 for 3 h in the presence of 50% ethanol, and then each enzyme was subjected to SDS-PAGE. As shown in Fig. 5B, vimelysin N123D exhibited lower auto-degradation activity than the wild-type enzyme.

Enzymatic Properties of Vimelysin N123D—The specific activity (unit/mg protein) of vimelysin N123D with casein as a substrate was 82% of that of the wild-type enzyme. The catalytic constants for synthetic substrates are summarized in Table 1. The k_{cat}/K_m value of vimelysin N123D for FAGLA was 55% of that of the wild-type enzyme, while the k_{cat}/K_m value of vimelysin N123D for a fluorescent substrate was 66% of that of the wild-type enzyme.

Modeling—Homology models of vimelysin and the N123D mutant were constructed based on the three-dimensional structures of pseudolysin [PDB: 1EZM (*31*) and 1U4G] and thermolysin [PDB: 4TMN (*32*)]. Similar models were obtained using both Swiss-Model and Modeller (v. 8.1). These models showed that Asn-123 of vimelysin is located near the S2 subsite, which is a central region of the enzyme that is likely very important for substrate-binding, catalysis and protein stability. In particular,

Table 1. **Catalytic constants of wild-type vimelysin and vimelysin N123D.**

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1}) $\times 10^5$
(A)			
Wild-type	3.13	137	0.44
N123D	2.39	58	0.24
(B)			
Wild-type	12.6×10^{-3}	0.89	0.71
N123D	15.7×10^{-3}	0.73	0.47

(A) Substrate, FAGLA; reaction mixture, 0.1M MES (pH 6.5) at 25°C. (B) Substrate, MOCAc-PLGL-A_2pr(Dnp)-AR-NH_2; reaction mixture, 50 mM MES (pH 6.5), 10 mM $CaCl_2$, 0.005% Triton X-100 at 25°C.

the Phe121-Trp-Asn-Gly124 sequence flanking residue Asn-123 is highly conserved in metalloproteinases, with aromatic residues playing key structural roles by bracing parts of the active-site cleft, and the asparagine or sometimes the aspartate residue associating with the following glycine residue to form a stable turn. One of the side-chain oxygen atoms of the Asn or Asp residue stabilizes the following turn by accepting hydrogen bonds from main-chain amide groups in the turn (Fig. 6).

Replacement of Asn with Asp changes the amide moiety of the side chain into an oxygen, and changes the neutral side chain into a negatively charged one. As a result of this mutation, the carboxylate group in Asp-123 may form stronger hydrogen bonds with either or both the main chain amide and side chain hydroxyl groups of Ser-126 (Fig. 6). In addition, it is interesting to note that Arg-110, which is surface-exposed and located near this loop, would not be expected to interact with Asn-123 in the wild-type vimelysin, but would be expected to form a salt-bridge with the negatively charged side chain of the N123D mutant (Fig. 6). The occurrence of this salt-bridge interaction would be expected to confer a significant degree of stability on the N123D mutant. The formation of electrostatic interactions such as ion pairs is one of the more commonly observed mechanisms for increasing the thermostability of enzymes (*38*). It is interesting to note that Lys-103 in pseudolysin occupies a similar position to that predicted for Arg-110 in vimelysin, but the shorter Lys side chain does not allow a salt-bridge to form with Asp-116 of pseudolysin, which is equivalent to Asn-123 in vimelysin. Ser-103 in thermolysin, which is equivalent to Arg-110 in vimelysin, does not interact with Asn-116 in thermolysin (Asn-123 in vimelysin), but it does form a hydrogen bond with Gln-119 to possibly stabilize the turn following Asn-116.

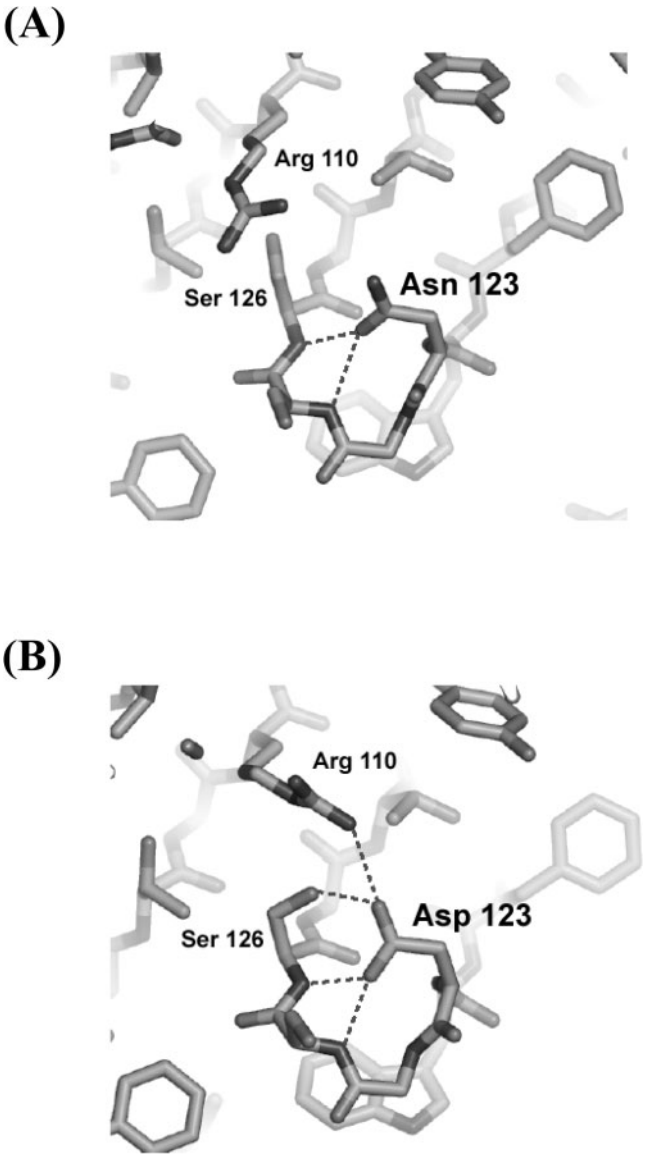


Fig. 6. **Homology models of (A) wild-type vimelysin and (B) the vimelysin N123D mutant.** Homology models were generated with MODELLER v. 8.1, based on the three-dimensional structures of pseudolysin and thermolysin. The region near residue 123 and possible interactions with its side chain are drawn. Carbon, nitrogen and oxygen atoms are colored green, blue and red, respectively. Hydrogen bonds are drawn as dashed red lines. This figure was prepared using PyMol (*32*).

DISCUSSION

The vimelysin gene was cloned into *E. coli* JM109 cells and its nucleotide sequence was determined (Fig. 1). An open reading frame of the vimelysin gene encoded 607 amino acid residues. The sequence starting at Ala-196 was identical to the amino terminal amino acid sequence of authentic vimelysin. The molecular mass of the expressed enzyme in *E. coli* cells was calculated to be 33,801.3 by mass spectrometry, which was approximately equal to the molecular mass of the 311 residues between residue 196 and residue 506 (33,799.1). The amino terminal amino acid sequence, CD spectrum, and several enzymological properties of the recombinant wild-type enzyme were identical to those of the authentic enzyme purified directly from *Vibrio* sp. T1800. These results indicate that the vimelysin precursor is processed on both the N- and C-terminal sides. That is, the N-terminal pro-region (residues 1 to 195) and C-terminal pro-region (residues 507 to 607) are removed post-translationally, leaving the mature enzyme (residues 196 to 506). On comparing the amino terminal amino acid sequence of vimelysin with those of vibriolysin and PA-proteinase, the first 25 amino acid residues of the N-terminal pro-region of vimelysin were presumed to be

a signal peptide (*39*, *40*). This signal peptide may be cleaved off during its passage through the inner membrane by endogenous signal peptidases, as observed for vibriolysin (*15*).

Vibriolysin is synthesized as a 66-kDa precursor protein consisting of an N-terminal pro-region, a mature enzyme, and a C-terminal pro-region, and showed 69% identity with the vimelysin precursor. The N-terminal pro-region of vibriolysin is cleaved autocatalytically in the periplasmic space (*15*), and an intermediate with 45 kDa was detected during the processing. After incubation at 37°C and pH 7.5 for 24 h, the intermediate was autocatalytically converted to a 35-kDa mature enzyme (*16*). For vimelysin, both the authentic and recombinant enzymes were purified as 38-kDa polypeptides in the mature form of the enzyme (Fig. 3B). Therefore, it was thought that the vimelysin precursor was rapidly converted to the mature enzyme through two steps after expression in host cells. Thus, it was assumed that the processing of the precursor protein in the vimelysin molecule occurred autocatalytically.

It has also been reported that the C-terminal pro-region of vibriolysin, which is not essential for its activity, is involved in adhesion to protein substrates and the erythrocyte membrane (*16*). The C-terminal pro-region of vimelysin showed 71% identity with that of vibriolysin (Fig. 2). Pseudolysin and thermolysin have no C-terminal propeptide (Fig. 2). It is likely that the C-terminal pro-region of vimelysin has a function similar to that of vibriolysin.

In order to improve the stability of vimelysin in organic solvents, random mutations were introduced into the vimelysin gene by error-prone PCR. The most solvent-resistant mutant obtained with this procedure was the N123D mutant, which showed higher residual activity (57%) than that of the wild-type enzyme (22%) after treatment with 50% ethanol at 37°C for 3 h. Following treatment with 50% 2-propanol, the remaining activity of vimelysin N123D (66%) was also higher than that of the wild-type enzyme (38%) under the same conditions (Fig. 5A). Thus, the alcohol resistance of vimelysin N123D was about twice that of the wild-type enzyme. The molecular mass of vimelysin N123D determined by mass spectrometry was larger by 102 Da than that of the wild-type enzyme. The nucleotide sequence of vimelysin N123D, excluding the mutation site, was identical to that of the wild-type enzyme. The CD spectrum of vimelysin N123D was also the same as that of the wild-type enzyme (Fig. 4). The discrepancy between the wild-type enzyme and vimelysin N123D is only one amino acid residue on the C-terminal side. Homology models of vimelysin indicate that the C-terminus of the mature enzyme is far from the active site. It seems unlikely that the slight difference in C-terminal processing can significantly affect the alcohol resistance of the N123D mutant. It is more likely that the superior stability of vimelysin N123D in organic solvents can be attributed to other factors.

Ogino *et al.* reported that pseudolysin from *P. aeruginosa* PST-01 showed high activity and stability in organic solvents (*23*), and that two disulfide bonds in pseudolysin were important contributors to the stability of the enzyme (*41*). The mature region of vimelysin is 57% identical to that of pseudolysin. The four Cys residues that form the two disulfide bonds of pseudolysin are well conserved in vimelysin. These observations suggest that at least some of the structural features contributing to the high stability of vimelysin in organic solvents are similar to those of pseudolysin. The stabilities of vimelysin and pseudolysin in organic solvents were higher than that of thermolysin, which is the archetypal metalloproteinase from bacteria (*7*, *23*). In their primary amino acid sequences, Asn-123 in the vimelysin molecule corresponds to Asp-116 in pseudolysin and Asn-116 in thermolysin, respectively. As reported here, the alcohol resistance of vimelysin N123D was significantly higher than that of the wild-type enzyme (Fig. 5A). These results suggest that interactions between the residue at position 123 and other residues of vimelysin may have important effects on alcohol resistance.

It is instructive to compare our observations on vimelysin with ones on mutants of subtilisin E that were generated by sequential random mutagenesis and showed superior stability in organic solvents (*42*). The 10 substitutions of the subtilisin E mutants were clustered on one face of the enzyme, near the active site and substrate-binding pocket, and all were located in loops that connect core secondary-structure elements. It was thought that these variable surface loops may be effective handles for "tuning" of the activity of enzyme (*42*). Homology models of vimelysin indicate that Asn-123 of vimelysin is located near the S2 subsite, and may participate in key hydrogen bonding and ion-pair interactions with core elements of the enzyme, depending on the nature of the side chain at this position.

It is particularly interesting to look at the replacement of Asp-218 by Ser in subtilisin E, which is a mutation that significantly improves the stability of the enzyme in organic solvents (*43*): the subtilisin D218S mutant showed superior stability in 80% DMF and 2-times higher specific activity than the wild-type enzyme, apparently because of optimization of internal hydrogen bonds (*43*). Homology models of vimelysin and the N123D mutant suggest that an altered and possibly stronger hydrogen bond network may be formed by the carboxylate side chain of Asp-123. In addition, an ion pair may form between Asp-123 and Arg-110, which may further stabilize the enzyme against denaturation in organic solvents.

Kinetic constants for wild-type vimelysin and the N123D mutant were also obtained using two standard substrates, FAGLA and a fluorescent substrate (Table 1). The k_{cat}/K_m value of vimelysin N123D was about 60% of that obtained for the wild-type enzyme. The N116D mutant of the thermolysin-like protease from *Bacillus stearothermophilus*, which is equivalent to vimelysin N123D, was 1.7 times more active than the wild-type enzyme (*44*). Apparently, electrostatic changes near the substrate-binding cleft and the active site can affect the catalytic efficiency of related enzymes in opposite ways. As shown in Fig. 5B, vimelysin N123D also showed lower auto-degradation activity than the wild-type enzyme. The increased stability of vimelysin N123D is likely to be partly attributable to the lower level of autoproteolysis, but the increase in the stability of the N123D mutant is clearly too large to be entirely due to a lower degree of activity in this mutant. Further experiments are needed to distinguish between the contributions of reduced enzymatic activity and increased structural stability to the overall resistance of the N123D mutant against inactivation by organic solvents. In comparison,

the subtilisin D218S mutant described above exhibited increases in both activity and stability in organic solvents (*43*). In general, changes in specific activity are not highly correlated with changes in stability, and it is likely that the decrease in specific activity of vimelysin N123D is not directly correlated with increased stability in organic solvents.

The replacement of Asn 123 by Asp in vimelysin clearly increases the stability of the enzyme in organic solvents. The changes in hydrogen bonding and electrostatic interactions accompanying this mutation suggest possible mechanisms for how this mutation may increase enzyme stability. Further mutagenesis studies of this residue, as well as residues that may interact through hydrogen-bonding (Ser-125 and Ser-126) or electrostatic interactions (Arg-110), should help to clarify the roles of these residues in enzyme activity and stability. Crystallographic studies of vimelysin and vimelysin mutants would also be helpful for further clarification of the contributions of different residues towards stabilization of the enzyme in organic solvents.

CONCLUSIONS

The gene encoding *Vibrio* metalloproteinase vimelysin was cloned and sequenced, and then expressed and purified in large amounts. The purified recombinant enzyme was shown to have physical and enzymological properties identical to those of the native enzyme purified directly from *Vibrio* sp. T1800. The vimelysin N123D mutant was generated by error-prone PCR mutagenesis and shown to exhibit superior stability in organic solvents.

The nucleotide sequence and the amino acid sequence data reported in this paper appear in the DDBJ/EMBL/Gene Bank nucleotide sequence databases under accession number AB080995 and in the UniProt databases under accession number Q76LC2, respectively. The homology models of vimelysin have been deposited in the Protein Data Bank (PDB code 2A4I).
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