Purification and Some Properties of a Thermostable Metal Proteinase Produced by *Thermomicrobium* sp. KN-22 Strain

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A extreme thermophile that produces a heat-stable proteinase was isolated from hot-spring water and classified as *Thermomicrobium* sp. KN-22 (growth temperature, 50–83°C; and optimum growth temperature, 70°C). The proteinase was purified from the culture broth of this strain by fractionation with ammonium sulfate, chromatography on columns of DEAE-cellulose and CM-Sepharose CL-6B, and HPLC on TSKgel CM-5PW. The purified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis and a single peak after HPLC (yield 8.8%). The enzyme had maximum activity at pH 8.5 and at 75°C and it was stable up to 60°C. The molecular weight of the enzyme was 35,000 by SDS-PAGE. Since the enzymatic activity was completely inhibited by EDTA, *o*-phenanthroline, and phosphoramidon, it appears that the enzyme is a metal proteinase.

Generally, enzymes are unstable to heat, and the purification and storage of an enzyme should be done at low temperature. Since enzymes produced by thermophiles are more thermostable than those produced by mesophiles, enzymes from thermophiles are stable at room temperature for long periods of time. Therefore, enzymes produced by thermophiles have been developed as reagents for clinical analyses. With respect to proteinases produced by extreme thermophiles, only a few enzymes, namely, caldolysin,¹⁾ aqualysin,²⁾ and archaeolysin,³⁾ have been reported. We have been searching for novel thermostable proteinases, and we have already reported a thermostable acid proteinase⁴⁾ produced by a moderate thermophile.

Now we report some taxonomic properties of an extreme thermophile, and the purification and some properties of a proteinase produced by this microorganism are described.

Materials and Methods

Reagents. DEAE-cellulose was obtained from Wako Pure

Chemical Industries, Ltd. CM-Sepharose CL-6B was purchased from Pharmacia. TSKgel CM-5PW was from Tosoh Co. Casein (Hammarsten) was from Merck. Polypeptone and yeast extract were obtained from Wako Pure Chemical Industries, Ltd. Agar (HM-8) was from Ina Food Industries Co. All other chemicals were of analytical grade and obtained from commercial sources.

Isolation of microorganisms. The compositions of the media used for isolation of strains are shown in Table I. Samples of water and mud from hot springs were cultured in rich medium at above 75°C (medium A or B in Table I). Then microorganisms were cultured on an agar medium with casein (medium C in Table I) at temperatures above

Table I. MEDIA FOR SCREENING

A	Glucose	1.0 g/l	C	Casein Na	5.0 g/l
	Polypeptone	1.0		Soluble starch	10.0
	Yeast extract	1.0		Yeast extract	1.0
	Hot-spring water	1.0 l		KH ₂ PO ₄	1.0
				MgSO ₄ ·7H ₂ O	0.5
B	Glucose	1.0 g/l		CaCl ₂	1.0
	Yeast extract	1.0		FeSO ₄ ·6H ₂ O	10.0 mg
	MgSO ₄ ·7H ₂ O	1.0		ZnSO ₄ ·7H ₂ O	1.0
	KH ₂ PO ₄	1.0		CuSO ₄ ·7H ₂ O	1.0
	Hot-spring water	1.0 l		MnSO ₄ ·4H ₂ O	1.0
				Agar (HM-8)	20.0 g
				pH 7.0	

75°C. Colonies with halos as a result of caseinolytic activity were selected as proteinase-producing microorganisms.

Identification of microorganisms. Tests of assimilation of glucose and starch were done by the method of Iizuka and Komagata.⁵⁾ Gram staining was done by a modification of the method of Hucker.⁶⁾ The composition of the cell wall was analyzed by the method of Komagata.⁷⁾ Identification was made by reference to descriptions in Bergey's Manual of Determinative Bacteriology.⁸⁾

Cultivation. *Thermomicrobium* sp. KN-22 strain, after isolation, was cultured in medium with the following constituents: soluble starch, 20 g; polypeptone, 20 g; yeast extract, 2 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.5 g; $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$, 10 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 mg; and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1 mg in 1000 ml of tap water, pH 7.0. Medium for stock cultures included 2% HM-8 agar in the above medium. Strain KN-22 was inoculated from a stock culture on an agar slant and cultured at 70°C for 20 hr. A loopful of cells grown on the agar slant was inoculated into a test tube (diameter 20 mm) which contained 8 ml of medium and was cultured at 70°C for 3–5 hr on a reciprocal shaker (120 strokes per min; seed culture). For production of the proteinase, a 2-liter miniature fermentor (MB type; Iwashita Bio-Science Co., Ltd.) containing 1 l of the medium was used with an air flow of 1.4 l/min and agitation at 700 rpm. A sample of seed culture, equivalent to 5% of the volume of the culture, was inoculated for production of the proteinase. After 14 hr of cultivation, as maximum proteolytic activity was obtained (5.0 unit/ml), the culture broth was used as a source for the enzyme.

Measurement of proteolytic activity. Proteolytic activity was measured by the method of Hagihara *et al.*⁹⁾ One unit of enzymatic activity was defined as the amount of enzyme that caused an increase in absorbance at 660 nm of 1.0 in 10 min. In the experiments on optimum pH and pH stability of the enzyme, the following buffer solutions containing 5 mM CaCl_2 were used: 0.1 M citrate–NaOH buffer (pH 3–5), 0.1 M maleate–Tris–NaOH buffer (pH 6–7), 0.1 M Tris–HCl buffer (pH 8–9), and 0.1 M glycine–NaOH buffer (pH 10–12). The enzymatic reaction was done at various pHs for 10 min at 70°C. In the experiments on pH stability, the solution of enzyme was kept at 25°C for 24 hr at various pHs and residual activity was measured at pH 8.0.

Measurement of protein. Protein was measured from the absorbance at 280 nm

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli.¹⁰⁾

Amino acid composition. The sample (total $A_{280} = 0.11$) was dissolved in 3 ml of distilled water and the solution

was evaporated to dryness. The dry matter was hydrolyzed with constant boiling HCl in a sealed tube at 110°C for 48 hr *in vacuo*. The resultant mixture was evaporated to dryness, and distilled water was added to the residue and the mixture again evaporated to dryness. This operation was done five times. The resultant amino acids were analyzed by an automatic amino acid analyzer (Hitachi model L-8500).

Hydrolysis of oxidized insulin B chain by proteinase from strain KN-22. The reaction mixture contained 150 μg of oxidized insulin B chain and 14.5 ng of the proteinase in 325 μl of 50 mM Tris–HCl buffer (pH 8.0). The reaction was done at 37°C for 40 min and analyses were done by HPLC on a column of Nucleosil C_{18} (4 mm i.d. $\times$ 150 mm). The peptides were eluted with a linear gradient, 0.05% trifluoroacetic acid to 40% acetonitrile/0.05% trifluoroacetic acid, for 45 min at a flow rate of 0.8 ml/min.

Separation of hydrolysate of oxidized insulin B chain. The reaction mixture contained 4 mg of oxidized insulin B chain and 0.4 μg of the enzyme in 2 ml of 50 mM Tris–HCl buffer (pH 8.0). The reaction was done at 37°C for 1 hr and the hydrolysis products were isolated as pure samples by HPLC on a column of Nucleosil C_{18} (8 mm i.d. $\times$ 150 mm). The elution of the peptides was the same as above mentioned except for the flow rate (3 ml/min). The isolated samples were lyophilized and used for analysis of amino acid composition.

Results

Isolation of the microorganism

As mentioned in Materials and Methods, strain KN-22 was isolated. The bacteria were rod-shaped (length, $\sim 7 \mu\text{m}$; width, $\sim 2 \mu\text{m}$); they grew under aerobic conditions and produced yellow pigment. The optimum temperature and pH for growth of this microorganism were 70°C (growth temperature, 50–83°C) and 7.0 (range of pH values, 6.0–9.0), respectively.

Identification of the microorganism

Strain KN-22 could not assimilate glucose but could assimilate starch. Gram staining was negative. Catalase and oxidase tests were both positive. This strain was non-motile. *Meso*-diaminopimelic acid, alanine, and glutamic acid were detected as cell wall constituents, but no reducing sugars were detected in the cell wall. According to the taxonomic criteria in

Bergey's Manual of Determinative Bacteriology,⁸⁾ only two genera, *Thermus* and *Thermomicrobium*, are described as Gram-negative aerobes with an optimum growth temperature of 70–75°C. However, *Thermus* can assimilate glucose, while strain KN-22 could not. Strain KN-22 was allocated to the genus *Thermomicrobium* from its physiological properties, so it was concluded that strain KN-22 should be identified as a member of this genus.

Purification of the enzyme

The entire purification was done at room temperature. The culture broth was centrifuged at $7,000 \times g$ for 15 min. The supernatant from the culture broth (10.4 l) was adjusted to pH 8.0, and then it was concentrated about 3-fold at 40°C under reduced pressure. After concentration of the supernatant, the pH of the solution was again adjusted to 8.0, and then the clear supernatant was brought to 80% saturation by addition of solid ammonium sulfate in an ice bath. After standing for 2 days, the solution was centrifuged at $7,000 \times g$ for 5 min; the precipitate was collected and dissolved with a small amount of 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM CaCl_2 . The solution was dialyzed against the same buffer. The insoluble precipitate was removed by centrifugation, and the supernatant was used as the solution of crude enzyme.

The solution of crude enzyme was put on a column of DEAE-cellulose (2.5 cm i.d. $\times$ 40 cm) which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM CaCl_2 . The enzyme was not adsorbed. Then the flow-through fraction was dialyzed against

10 mM Tris-HCl buffer (pH 7.5) containing 5 mM CaCl_2 and put on a column of CM-Sephadex CL-6B (5 cm i.d. $\times$ 15 cm) which had been equilibrated with the same buffer. After the column had been washed with the same buffer, adsorbed materials were eluted by a linear gradient of NaCl from 0.0 to 0.5 M in the same buffer. The fraction with the enzyme activity was eluted by 0.2 M NaCl. The active fraction was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM CaCl_2 , and the enzyme was fractionated by preparative HPLC on a column of TSKgel CM-5PW (16 mm i.e. $\times$ 10 cm). The active fraction was eluted by 0.2 M NaCl in good agreement with the peak of protein. Active fractions were pooled as purified enzyme. Approximately 3890-fold purification was achieved with an overall yield of 8.8% from the crude enzyme. The details of the purification are summarized in Table II.

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis.

Electrophoresis was done on a 12% gel at pH 8.8. The protein (about 30 μg) was stained with Coomassie Brilliant Blue R-250. Marker proteins are included for comparison of mobilities. Numerals in parentheses show molecular weights of marker proteins.

Table II. SUMMARY OF PURIFICATION OF PROTEINASE FROM STRAIN KN-22

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture filtrate	10,400	48,000	390,000	0.12	100
$(\text{NH}_4)_2\text{SO}_4$ ppt.	327	38,000	65,700	0.56	79
DEAE-Cellulose	2,115	16,100	306	52.6	34
CM-Sephadex CL-6B	315	10,800	48	225	22
TSKgel CM-5PW	72	4,200	9	467	8.8

Criteria of purity and molecular weight

The homogeneity of the purified enzyme was examined by SDS-PAGE and HPLC. SDS-PAGE was done, and protein was stained with Coomassie Brilliant Blue R-250. As shown in Fig. 1, the purified enzyme gave a single band after electrophoresis, and a single peak after HPLC. The molecular weight of the purified

enzyme was 35,000 by SDS-PAGE and 10,300 by chromatography on TSKgel G2000SW.

Amino acid composition

The amino acid composition of the purified proteinase from KN-22 is shown in Table III. Aspartic acid (including asparagine) and glycine residues were abundant in the enzyme molecule.

Table III. AMINO ACID COMPOSITION OF THE PURIFIED PROTEINASE

Amino acid	Nearest integer ^a
Asp	45
Thr	18
Ser	27
Glu	21
Pro	6
Gly	45
Ala	30
Cys	ND ^b
Val	21
Met	3
Ile	12
Leu	15
Tyr	30
Phe	12
Lys	9
His	6
Arg	12
Trp	ND ^b

^a The nearest integer was calculated on the assumption that the molecular weight was 35,000.

^b ND, not determined.

Effects of pH and temperature on the activity and stability of the proteinase from KN-22 strain

The optimum pH for enzymatic activity was 8–9, and the enzyme was stable from pH 6.0 up to pH 11.0 (data not shown). As shown in Fig. 2, the optimum temperature of the enzyme was 75°C (A), and the enzymatic activity was stable up to 60°C (B). However, the thermostability of the purified enzyme was lower than that of the crude enzyme, as discussed below.

Effects of some reagents on the enzymatic activity

Various reagents such as ethylenediaminetetraacetic acid (EDTA), phosphoramidon, *o*-phenanthroline, phenylmethylsulfonyl fluoride, *Streptomyces* subtilisin inhibitor, and antipain in 1.9 ml of solution were incubated at 70°C for 10 min, and then 0.1 ml of the solution of enzyme was added and the mixture in-

Fig. 2. Effects of Temperature on the Activity and Stability of the Purified Enzyme.

A: Optimum temperature, B: thermostability.

The enzymatic reaction was done at various temperatures for 10 min (pH 8.0) (graph A). The solution of enzyme was heated at various temperatures for 10 min (pH 8.0) and residual activity was measured at pH 8.0 for 10 min (70°C) (graph B).

●, Purified enzyme; ○, crude enzyme (graph B).

Fig. 3. Cleavage Sites for Hydrolysis of Oxidized Insulin B Chain by the Purified Enzyme.

Vertical arrows show cleavage sites for hydrolysis. Arrows with solid line indicate first attack sites, and those with broken line indicate minor sites. Arrows written sideways indicate peptides obtained by hydrolysis of oxidized insulin B chain. The values in parentheses are yield (%) of peptides of oxidized insulin B chain obtained after 1 hr of incubation with KN-22 proteinase.

cubated at 70°C for 30 min (pH 8.0). Samples of the mixture (0.5 ml) were used as the solution of enzyme. The enzyme activity was inhibited by specific inhibitors of metal proteinases, EDTA (1.0 mM), phosphoramidon (0.1 mM), and *o*-phenanthroline (1.0 mM) by 84.7, 99.1, and 98%, respectively. Other reagents did not inhibit the enzymatic activity. Therefore, it appears that the purified enzyme was a metal proteinase.

The cleavage sites of hydrolysis for oxidized insulin B chain by proteinase from strain KN-22

The hydrolysis products for oxidized insulin B chain by the enzyme included 7 peptides. The resultants were isolated as pure samples and their amino acid compositions were analyzed. The yields of peptides corresponding to Phe²⁵-Ala³⁰, Phe¹-Gly²³, Phe¹-Phe²⁴, Phe²⁴-Ala³⁰, Phe¹-Tyr¹⁶, Leu¹⁷-Ala³⁰, and Phe¹-His¹⁰ were 27.3, 22.4, 21.6, 20.9, 3.2, 2.5, and 2.2%, respectively. Judging from the course of the hydrolysis and the yields of peptides, the peptide bonds of Gly²³-Phe²⁴ and Phe²⁴-Phe²⁵ were hydrolyzed preferentially and then the peptide bonds of His¹⁰-Leu¹¹ and Tyr¹⁶-Leu¹⁷ were hydrolyzed in succession. Therefore, the cleavage sites of hydrolysis for oxidized insulin B chain by the enzyme were decided as in Fig. 3, including cleavage sites of other thermostable metal proteinases. The

specificity of the enzyme for the substrate is broad, resembling other microbial metal proteinases.

Discussion

With respect to metal proteinase produced by extreme thermophiles, only one example, caldolysin,¹⁾ has been reported. Caldolysin is not inhibited by 10 mM *o*-phenanthroline (zinc-specific chelator) and was inhibited by 1 mM EDTA and 10 mM EGTA (calcium-specific chelators), 40% and 45%, respectively. As to this phenomenon, Cowan and Daniel assumed the following: (a) caldolysin contains no essential zinc ion cofactor or (b) any zinc atom present was inaccessible or too tightly bound to be chelated and (c) calcium was important for the maintenance of molecular integrity. By contrast, the proteinase from KN-22 was completely inhibited by EDTA and *o*-phenanthroline. The molecular weights of the proteinase from KN-22 and caldolysin are 35,000 (SDS-PAGE) and 21,000, respectively. Therefore, the proteinase from KN-22 is different from caldolysin.

Thermolysin, produced by a mesothermophile (*Bacillus thermoproteolyticus*), is completely inhibited by phosphoramidon at a molar ratio of one to one. However, as shown in Fig. 4, 50% inhibition of activity of the proteinase from KN-22 (0.14 nmol) occurred

Fig. 4. Inhibition of the Purified Enzyme by Phosphoramidon.

The enzyme was incubated at 70°C for 30 min with phosphoramidon at pH 8.0 and the residual activity was measured at 70°C. I indicates inhibitor (phosphoramidon), and E indicates the purified enzyme.

with addition of 6.16 nmol phosphoramidon (molar ratio of one to forty-four of the enzyme to the reagent), and the affinity of the enzyme to phosphoramidon was low, as compared with that of thermolysin. Thus, the proteinase from KN-22 is clearly different from thermolysin.

The molecular weight of the proteinase from KN-22 was 35,000 by SDS-PAGE and 10,300 by gel filtration with TSKgel G2000SW. Furthermore, the enzyme was filtrable through an ultrafiltration membrane (UF-10PS; Tosoh Co.; molecular size cut off, 10,000 daltons). These results suggest that the enzyme has a rather complex tertiary structure (*e.g.*, rod shaped) in solution.

Generally, enzymes produced by extreme thermophiles are thermostable, for example, caldolysin is stable to heating at 85°C for 11 hr. By contrast, the thermostability of the proteinase from KN-22 was almost the same as that of proteinases from mesophiles, even

though it was produced by an extreme thermophile, *Thermomicrobium* sp. The temperature at which 50% of the activity was destroyed by heating for 10 min, was about 15°C lower than that for the crude enzyme. Therefore, we followed the thermostability of the proteinase through the purification procedure. After the solution of crude enzyme was put on the column of DEAE-cellulose, its thermostability apparently decreased. Therefore, some material that was adsorbed to DEAE-cellulose may participate in the thermostability of the proteinase. The column of DEAE-cellulose was eluted by a linear gradient of NaCl from 0.0 to 1.0 M. When dialyzed fractions from the column were added to the purified proteinase, the thermostability of the enzyme returned to the original level. Further investigations of this phenomenon are now in progress. The results will be reported elsewhere.

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