Purification and Characterization of a Cold-Active Protease from Psychrotrophic *Serratia marcescens* AP3801

Yasutaka Morita[a], Kenji Kondoh[a], Quamrul Hasan[b], Toshifumi Sakaguchi[a], Yuji Murakami[a], Kenji Yokoyama[a], and Eiichi Tamiya[a,*]

[a]School of Materials Science, Japan Advanced Institute of Science and Technology, Tatsunokuchi, Ishikawa 923-12, Japan, and [b]Research and Development Division, Analytical, Technology and Research Department, Procter & Gamble Far East, Inc., Higashinada-ku, Kobe 658, Japan

ABSTRACT: Protease activity was detected in the culture medium of *Serratia marcescens* AP3801 grown at 10˚C, which was isolated from soil collected from the top of a mountain. The enzyme, designated as CP-58 protease, was purified to homogeneity from the culture broth by ion exchange and gel filtration chromatographies. The molecular mass of the protease was 58 kDa, and its isoelectric point was close to 6.0. Maximal activity toward azocasein was observed at 40˚C and from pH 6.5 to 8.0. The activity was strongly inhibited by 1,10-phenanthroline, suggesting that the enzyme is a metalloprotease. The *N*-terminal amino acid sequence was Ser-Leu-Asn-Gly-Lys-Thr-Asn-Gly-Trp-Asp-Ser-Val-Asn-Asp-Leu-Leu-Asn-Tyr-His-Asn-Arg-Gly-Asn (or Asp)-Gly-Thr-Ile-Asn-Asn-Lys-Pro-Ser-Phe-Asp-Ile-Ala. A search through databases for sequence homology aligned CP-58 protease with metalloprotease. The result of the cleavage pattern of oxidized insulin B-chain suggests that CP-58 protease has a broader specificity than other proteases against the peptide substrate.
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KEY WORDS: Cold-active protease, extracellular protease, metalloprotease, psychrotrophic bacteria, *Serratia marcescens* AP3801.

Extremophiles have adapted to their environments by optimizing their metabolic processes. One class in extremophiles is low-temperature-adapted microorganisms, which can grow at low temperatures, even around 0°C (1–3). Morita (3) defined psychrophiles as those bacteria that have an optimal temperature for growth at about 15°C, a maximal temperature for growth at about 20°C, and a minimal temperature for growth at 0°C or lower. Bacteria capable of growing at low temperature were isolated first by Forster in 1887 from preserved fish (4). Many kinds of cold-active enzymes from cold-adapted microorganisms have been reported (5–8). We have also reported cold-active protease, lipase, and amylase from cold environments (9). Cold-active enzymes have three general characteristics: a curve of activity as a function of temperature that is shifted toward low temperatures, a specific activity k_{cat} or physiological efficiency k_{cat}/K_M that is higher than their mesophilic counterparts at a lower temperature range (<40°C), and a limited thermal stability by their fast denaturation at moderate temperature (10). However, it is still unknown how these enzymes maintain their high catalytic efficiency at low temperature. They might evolve toward a highly flexible structure that is coupled with thermal instability (1,11,12). The information gained from primary sequences of psychrophilic enzymes can be of fundamental importance in further elucidation of psychrophilicity (9,11, 13–15). There are a few reports on nucleotide sequences of cold-active enzymes (13–15).

In this report, we isolated a new psychrotrophic *Serratia marcescens* strain AP3801 from cold environment (soil) and purified and characterized the protease produced by the bacterium.

EXPERIMENTAL PROCEDURES

Bacterial strains. Three hundred forty-seven microorganisms were isolated from soil collected from the top of a mountain (approximately 1,000–2,200 m) in Ishikawa Prefecture. All isolates showed visible growth at 10°C. Strain AP3801, which has high proteolytic activity, was further characterized. To determine the effect of temperature on growth and protease excretion, strain AP3801 was incubated at 4, 10, 15, 20, and 30°C. Growth was monitored by measuring the optical density at 660 nm with a DU 640 spectrophotometer (Beckman, Fullerton, CA). Cell-free supernatant fluids, obtained by centrifugation at 17,000 × *g* and 4°C for 15 min, were assayed for proteolytic activity.

Identification. The characteristics of strain AP3801 were determined with a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan). It was also tested for biochemical characteristics by using API 20 E strips (Bio Merieux S.A., Marcy-l'Etoile, France) at 20°C for 48 h. Further identification was achieved by determination of the 16S rRNA gene.

Growth conditions. The growth medium contained, per liter, the following: 1 g glucose, 5 g yeast extract, 0.5 g casein sodium, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ and 27 g $Na_2CO_3 \cdot 10H_2O$. The pH was adjusted to 10.2 by adding

*To whom correspondence should be addressed at School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-12, Japan. E-mail: tamiya@jaist.ac.jp.

$Na_2CO_3 \cdot 10H_2O$. The medium was sterilized by autoclaving. The bacteria were cultured in 100-mL Erlenmeyer flasks that contained 25 mL culture medium. The incubation was done at 10°C with shaking at 140 rpm in a controlled shaker, NR-80 (Taitec, Saitama, Japan). After 5 d of incubation, 80 mL of the culture medium was transferred to a 5-L jar fermentor, TFA-90 (Tiyoda Manufacturing Co., Ltd., Nagano, Japan), that contained 3.5 L of medium. The incubation was done at 10°C with agitation at 150 rpm. After 5 d of incubation, the culture was treated to remove the cells.

Enzyme purification. After cultivation, cells were removed by centrifugation at 7,000 × *g* for 30 min at 4°C. The protease was precipitated from cell-free supernatant with ammonium sulfate (80% saturation) at 4°C, and the precipitate was dissolved in 20 mM Tris/HCl (pH 8.0) buffer. The precipitate was applied to Sephacryl S-100 (Pharmacia Biotech, Bromma, Sweden) packing in an XK 50/100 column (5 by 100 cm; 2 L), equilibrated with 20 mM Tris/HCl that contained 0.15 M NaCl (pH 8.0). The eluted proteins were monitored by measuring A_{280} of the fractions with a UV-1 monitor (Pharmacia Biotech). Fractions that exhibited proteolytic activity were assayed for proteolytic activity with the azocasein hydrolysis test. Proteolytically active fractions were pooled and concentrated with ammonium sulfate (80% saturation) at 4°C. After concentration, the concentrate was dialyzed against 3 L of 20 mM Tris/HCl (pH 8.0) for 12 h at 4°C. The dialysate was subjected to a Hiload 16/10 Q Sepharose HP column (Pharmacia Biotech), equilibrated with 20 mM Tris/HCl (pH 8.0) and 20 mM Tris/HCl that contained 1 M NaCl (pH 8.0). The active fraction was eluted at a NaCl concentration of 20 mM Tris/HCl (pH 8.0) by using a NaCl linear gradient from 0 to 1 M. All column chromatography was done at 4°C with a fast protein liquid chromatography system (Pharmacia Biotech).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing. Purity and molecular mass of the isolated enzyme were confirmed by SDS–PAGE (16) in a Phast system (Pharmacia Biotech). PhastGel Monogenous 7.5 (7.5% acrylamide gel) (Pharmacia Biotech) and PhastGel SDS buffer strips (Pharmacia Biotech) were used. Isoelectric point determination was carried out with PhastGel IEF 3-9 in the same Phast system by following the manufacturer's instructions. The isoelectric point calibration kit and calibration kits for molecular weight determination from Pharmacia Biotech were used. Protein bands were stained with Coomassie brilliant blue by following the manufacturer's instructions.

Protein assay and proteolytic activity. Protein concentration was determined by the method of Bradford (17) with a Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA) and bovine serum albumin as the standard. The proteolytic activity was determined by a modification of the method of Margesin and Schinner (18,19) with azocasein. The enzyme solution (50 μL) was incubated with 300 μL of 1% azocasein in 100 mM glycine-NaOH buffer, pH 10.5, at 20°C for 20 min. The reaction was stopped by adding 1 mL of 6% trichloroacetic acid. After 15 min, the mixture was centrifuged at 15,000 × *g* for 15 min, and the absorbance of the supernatant was measured at 340 nm. The activity was represented in relative azocasein digestion units (ACU). One unit (ACU) of proteolytic activity was defined as the amount of enzyme required to cause an increase of 0.001 A_{340} unit/min under the described conditions.

Influence of pH and temperature. The optimal pH of the protease activity was determined at 30°C in a buffer mixture that consisted of 100 mM each of glycine-NaCl and Good's buffers [2-morpholinoethanesulfonic acid monohydrate, 3-morpholinopropanesulfonic acid (MOPS), *N*-tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS), *N*-cyclohexyl-2-aminoethanesulfonic acid, and *N*-cyclohexyl-3-aminopropanesulfonic acid], with pH values ranging from 5.5 to 13.0. Optimal temperature was determined by incubation of the purified enzyme in Tris/HCl buffer (pH 8.0) for 20 min at each temperature and measuring the proteolytic activity as just described. To study stability as a function of pH, the enzyme was incubated in the foregoing solution for 1 h at 20°C. For thermostability, the enzyme was incubated at 10 to 60°C, and the residual proteolytic activity was measured at various time intervals (0–60 min).

Substrate specificity. Proteolytic activity was determined by a modification of the method of Folin and Ciocalteu (20). The enzyme solution (20 μL) and 2 mg of the substrate (casein, collagen, gelatin, hemoglobin, bovine serum albumin, keratin from human hair, keratin from bovine hoofs, or elastin) were mixed with 130 μL of 100 mM glycine-NaOH buffer (pH 10.5) and incubated for 60 min at 30°C with shaking for insoluble substrates. The reactions were stopped by adding 150 μL trichloroacetic acid solution (0.11 M trichloroacetic acid, 0.22 M CH_3COONa, 0.33 M CH_3COOH). The mixture was further incubated at room temperature for 30 min and centrifuged by 15,000 × *g* for 15 min. The supernatant (100 μL), 500 μL of 0.5 M Na_2CO_3 solution, and 100 μL of twofold-diluted Folin-Ciocalteu reagent were mixed and allowed to react for 60 min at room temperature. The absorbance was measured at 660 nm. Activity values were expressed as relative activity against casein.

Effect of protease inhibitors. The following protease inhibitors were used: pepstatin, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline, and EDTA. The mixture (50 μL) of enzyme and inhibitor in 100 mM glycine-NaOH (pH 10.5) was incubated for 60 min at 20°C. The residual activity was measured as described earlier.

Hydrolysis of the oxidized insulin B-chain. For complete hydrolysis, 1.09 mg oxidized insulin B-chain was mixed with 58 μg protease (800 μL) and incubated for 36 h at 25°C. After the reaction, the protease was removed from the reaction mixture by ultrafiltration. Peptides were separated on TSKgel ODS-80 Ts column (4.6 mm × 150 mm; Tosoh, Tokyo, Japan) in high-performance liquid chromatograph (HPLC) equipped with a model CCPM-II multi pump, a model UV8020 ultraviolet (UV) detector, and a model SC-8020 system controller (Tosoh). The peptides were eluted at room temperature with a

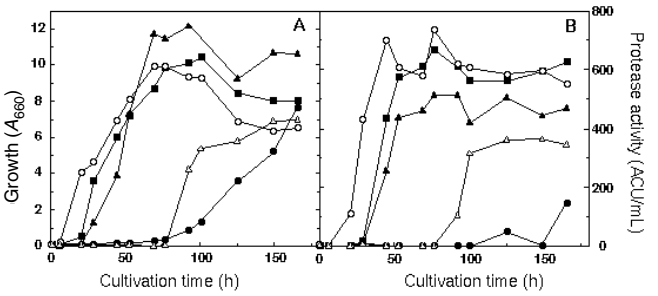

FIG. 1. Growth and secreted proteolytic activity of the strain *Serratia marcescens* AP3801 cultivated at various temperatures. ● 4°C; ▲ 10°C; ■ 15°C; ○ 20°C; △ 30°C. Abbreviation: *A*CU, azocasein digestion units.

FIG. 2. Sodium dodecylsulfate–polyacrylamide gel electrophoresis CP-58 protease. Standard proteins (lane 1): phosphorylase b (94.0 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). The purified protease was applied in lane 2.

120-min linear gradient from 0.05% (vol/vol) trifluoroacetic acid (TFA) to 0.05% TFA, dissolved in 30% (vol/vol) acetonitrile. The eluted peptide fractions were collected under UV at an absorbance of 220 nm. The *N*-terminal amino acids of the purified peptides were determined by Edman sequencing on a pulsed liquid-phase protein sequencer (Model 476A; Applied Biosystems, Foster, CA), equipped with an on-line data analysis system (Model 610A; Applied Biosystems).

Amino-terminal sequence. The *N*-terminal amino acid sequence was determined by Edman degradation on an automated pulsed liquid-phase protein sequencer (Procise series Model 494; Applied Biosystems). The sequence data were compared with GenBank, Protein Data Bank (PDB), SwissProt, and Protein Identification Resource (PIR) databases.

RESULTS AND DISCUSSION

Strain characterization. In total, 347 strains were isolated and tested for excretion of protease. The best cold-active protease producer, strain AP3801, isolated from the top of a mountain (approximately 1,000 m), was selected for further characterization. This bacterium is gram-negative and aerobic. It is rod-shaped and has a size of approximately 1.0×1.7 μm, based on observations with a transmission electron microscope. The results of biochemical characterization with API 20 E strips suggested that strain AP3801 belongs to *S. marcescens.* 16S rRNA data analysis (not shown) also confirmed that it belongs to *S. marcescens*. Therefore, this bacterium was named as *S. marcescens* AP3801 in this study. Figure 1 (A and B) shows that *S. marcescens* AP3801 grew best at 10°C (92.5 h). Furthermore, slow growth was observed at 0°C. However, the highest amount of protease activity was present at 20°C. Protease activity was negligible at 30°C. These properties might be related to the bacterium's ability to survive in a cold environment. We propose classifying the strain AP3801 as a psychrotroph.

Enzyme purification. A typical purification method for protease from *S. marcescens* AP3801 is summarized in Table 1. The overall yield was only 1.8%. The reason why specific activity decreased after ammonium sulfate precipitation may be that metal ions that are part of the CP-58 protease were taken off by sulfide ions, which may make the CP-58 protease inactive. Generally, cold-active protease is labile (10). CP-58 protease was mixed with other protease in the medium, because other protease activity was detected after ion exchange chromatography on a Hiload 16/10 Q sepharose HP column (data not shown). The enzyme was homogeneous, as judged by SDS-PAGE. The purified protease, subjected to SDS-PAGE, gave only one protein band with a molecular mass of 58 kDa, as shown in Figure 2. The molecular mass of the protease was also determined by gel filtration to be 58 kDa (data not shown). This result means that CP-58 protease is monomeric. We named this protease CP-58 protease. The pI value of CP-58 protease was close to 6.0.

Effect of pH. The pH characteristics of CP-58 protease were determined with azocasein as substrate. The six buffers covered the range between 5.5 and 13.0. High enzyme activity was observed between pH 6.5 and 8.0 (in 100 mM MOPS and TAPS buffer), as shown in Figure 3A. About 40% of the

TABLE 1
Summary of the Purification of CP-58 Protease[a]

Step	Volume (mL)	Total protein (mg)	Total activity (ACU)	Specific activity (ACU/mg)	Yield (%)	Purification (fold)
Culture supernatant	3390	111	657200	5920	100	1
Ammonium sulfate precipitation	65	47.1	73360	1560	11	0.26
Sephacryl S-100	390	41.7	38390	920	5.8	0.16
Dialysis	56	5.2	23190	4460	3.5	0.75
Q Sepharose	58	4.2	11910	2840	1.8	0.48

[a]ACU, azocasein digestion units. Sephacryl S-100 and Q Sepharose (Pharmacia Biotech, Bromma, Sweden).

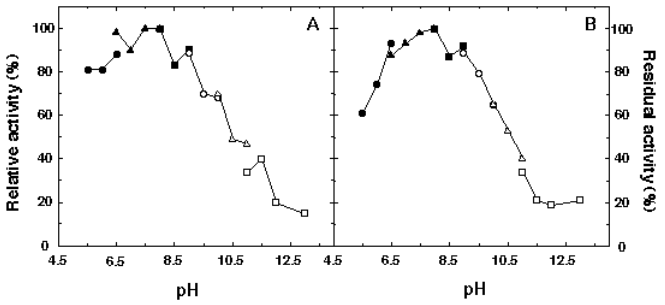

FIG. 3. (A) Effect of pH on enzymatic activity. Caseinolytic activity was measured in the following buffers (100 mM): ● 2-morpholinoethane sulfonic acid monohydrate (pH 5.5–6.5), ▲ 3-morpholinopropanesulfonic acid (pH 6.5–8.0), ■ *N*-tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid (pH 8.0–9.0), ○ *N*-cyclohexyl-2-aminoethanesulfonic acid (pH 9.0–10.0), △ *N*-cyclohexyl-3-aminopropanesulfonic acid (pH 10.0–11.0), □ Glycine-NaCl/NaOH (pH 11.0–13.0). (B) Effect of pH on stability of CP-70 protease. After CP-58 protease was preincubated at 20°C for 60 min in the same buffer, residual activity was measured at 30°C with azocasein as substrate. The original enzymatic activity before preincubation at various pH values was taken as 100%.

protease activity was still detectable at pH 5.5 and 11.5.

Effect of temperature. The optimal temperature for the reaction was 40°C in Tris/HCl buffer, pH 8.0, as shown in Figure 4A. In general, the optimal temperature of protease from mesophilic bacteria is around 60°C (21–23). Thus, a shift of at least 20°C toward lower temperature was observed for CP-58 protease. CP-58 protease had the characteristics of a typical psychrotrophic enzyme. Similar properties, including optimal activity at low temperature, were observed with other reported proteases from psychrophiles and psychrotrophs. For example, an extracellular proteinase of psychrophilic *Escherichia freundii* exhibited the optimal temperature of 25°C at pH 10.0 and of 40°C at pH 8.0 (24); a psychrotroph, *Pseudomonas fluorescens* 114, produced a protease with an optimal temperature of 35°C (25); an Antarctic yeast, *Candida humicola*, produced a protease with an optimal temperature of 37°C (26); and psychrophilic *Vibrio* sp. strain 5709 produced a protease with optimal temperature at 40°C (27). The thermal stability of CP-58 was assessed in 100 mM glycine-NaCl buffer pH 10.5 after heating several times at various temperatures (10–60°C). The enzyme was stable up to 30°C, as shown in Figure 4B. CP-58 protease lost 33% activity after 60 min at 40°C. Complete loss of activity was observed after heating at 60°C for 30 min. However, mesophilic subtilisin Carlsberg lost its activity at higher than 60°C (data not shown). Thus, CP-58 protease is thermolabile, compared with its mesophilic counterparts. The thermolability of the enzyme is assumed to be caused by its loose structure, which is easily denatured by the change of an external parameter, such as temperature.

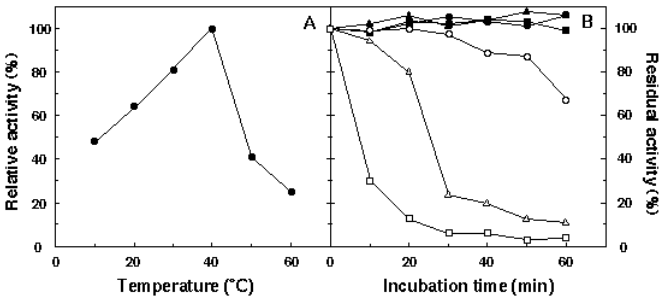

FIG. 4. (A) Effect of temperature on enzymatic activity. Relative enzyme activity was determined at pH 10.5 and different temperatures by the standard activity assay. Caseinolytic activity was measured in the following buffers: ● 100 mM Tris/HCl buffer (pH 8.0). (B) Effect of temperature on enzyme stability. CP-58 protease was preincubated for each time at various temperatures in 100 mM glycine-NaOH buffer (pH 10.5). Heating of the enzyme was stopped by cooling in an ice bath. Aliquots were used for measurement of residual activity at 20°C. The enzymatic activity of unheated enzyme was taken as 100%. Enzyme was preincubated at the following temperatures: ● 10°C, ▲ 20°C, ■ 30°C, ○ 40°C, △ 50°C, □ 60°C.

To be cold-active, an enzyme must fulfill the requirement of having a curve of activity as a function of temperature that is shifted toward low temperatures and limited thermal stability by its fast denaturation at moderate temperature (10). Because we found these properties in CP-58 protease, we concluded that it is a cold-active enzyme.

Effect of protease inhibitor. The enzyme was treated with various inhibitors for 60 min at 20°C in 100 mM glycine-NaOH pH 10.5. 1,10-Phenanthroline inhibited the enzyme activity by 15 (1 mM) and 7% (10 mM), as shown in Table 2. The result suggests that CP-58 protease can be classified as a metalloprotease. This result was also supported by the homology of the *N*-terminal amino acid sequence through a database search because a significant match with metalloproteases was found. Inhibitions by EDTA were 51 (1 mM) and 40% (10 mM). CP-58 protease was unaffected by pepstatin, E-64, and PMSF.

Substrate specificities of native proteins. Among proteins tested, casein was the most suitable substrate for CP-58 protease. The protease was ineffective in hydrolyzing other substrates, including scleroproteins. The rates of hydrolysis of

TABLE 2
Effect of Protease Inhibitors on the Activity of CP-58 Protease

Inhibitor[a]	Target protease	Conc. (mM)	Residual activity (%)
None			100
		0.01	93
Pepstatin	Aspartic		
		0.1	65
		0.01	105
E-64	Cysteine		
		0.1	87
		1	100
PMSF	Serine		
		10	113
	Metallo-/	1	15
1,10-Phenanthroline	metal-activated	10	7
	Metallo-/	1	51
EDTA	metal-activated	10	40

[a]Abbreviations: E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane; PMSF, phenylmethane-sulfonyl fluoride.

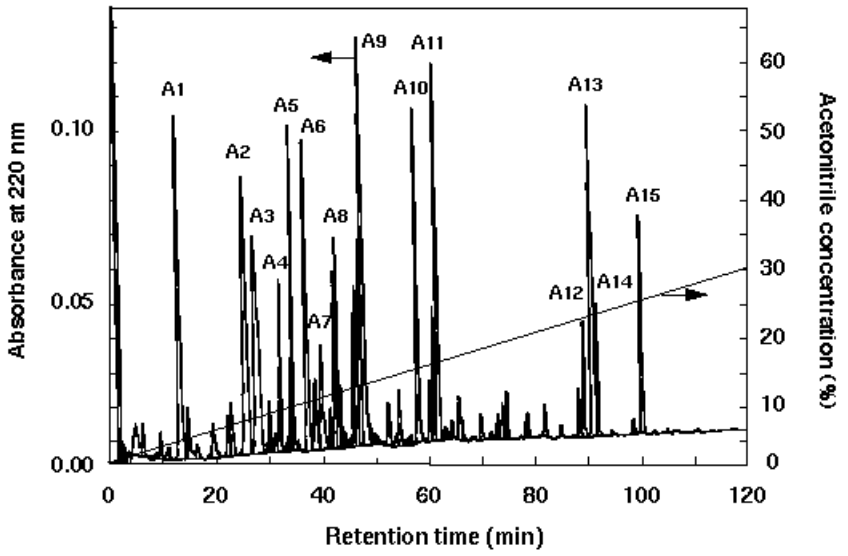

FIG. 5. Chromatogram of the hydrolysate of oxidized insulin B-chain. Oxidized insulin B-chain (1.09 mg) was hydrolyzed by 58 μg protease in 800 μL of the same buffer for 36 h at 25°C. Peptides were separated by high-performance liquid chromatography on a column of TSKgel ODS-80 Ts (4.6 mm × 150 mm; Tosoh, Tokyo, Japan). The peptides were eluted at room temperature with a 120-min linear gradient from 0.05% (vol/vol) trifluoroacetic acid (TFA) to 0.05% TFA, dissolved in 30% (vol/vol) acetonitrile. Hydrolysis by CP-58 protease for 36 h at 25°C.

gelatin, hemoglobin, albumin, human hair keratin, bovine hoof keratin, collagen, and elastin were 84, 5, 8, 14, 4, 3, and 3%, respectively (casein 100%).

Specificity of cleavage by CP-58 protease of the oxidized insulin B-chain. The oxidized insulin B-chain was hydrolyzed at 25°C for 36 h with 58 μg protease, and the hydrolysate was fractionated by HPLC, as shown in Figure 5. A number of peaks were eluted, of which 15 major peaks (A1–A15) were analyzed. Figure 6 shows that the principal sites of cleavage of the substrate by CP-58 protease were the carbonyl bonds of Asn3, His10, Tyr16, Arg22, and Tyr26. CP-58 also acted on the carbonyl bonds of Val2, Val12, Glu13, Ala14, Leu15,

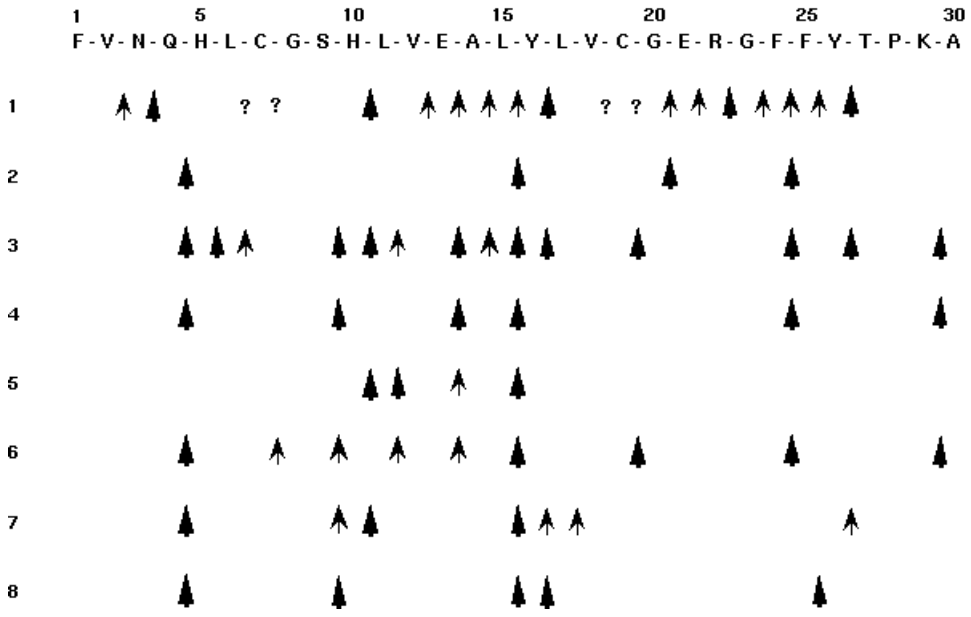

FIG. 6. Cleavage patterns of protease on oxidized insulin B-chain. Thick arrows indicate major cleavage sites; thin arrows, minor sites. 1, CP-58 protease; 2, subtilisin S41; 3, alkaline M protease; 4, alkaline no. 221; 5, alkaline AH101; 6, alkaline elastase YaB; 7, subtilisin Carlsberg; 8, subtilisin BPN'.

Gly20, Glu21, Gly23, Phe24, and Phe25 to a lesser extent. Either Leu6 or Cys7 and Val18 or Cys19 was the site of cleavage, though the incapability of the sequencer to detect cysteine residues made it impossible to pinpoint the site. For comparison, the complete cleavage sites on the B-chain by seven other microbial proteases (11,28–33) are listed in Figure 6. Cleavage specificity of CP-58 protease for the oxidized insulin B-chain was different from and broader than that of any other reported proteases. Schechter and Berger (34) have demonstrated that the rate of hydrolysis of a given bond in a peptide chain is influenced by a sequence of up to seven amino acid residues around the bond to be split. The variation in the rates of hydrolysis of the different bonds in the B-chain by the CP-58 protease might therefore in part be explained by the existence of favorable or unfavorable amino acid sequences.

This enzyme showed no significant preference for the bond with the specific amino acid located in the P_1 site, as shown in Figure 6. The cleavage result of the oxidized insulin B-chain shows that CP-58 protease has less strict amino acid specificity. However, three of the four major cleavage sites were a dissociated amino acid, and one of the four major cleavage sites was a hydrophilic amino acid located in a P_1 site. Hydrophobic amino acids are not located in the P_1 site. This fact may indicate that CP-58 protease does not prefer the bond with a hydrophobic amino acid located in the P_1 site. However, eight of the 11 minor cleavage sites were hydrophobic amino acids, and three of the 11 cleavage sites were dissociated amino acids located in P_1 sites. No hydrophilic amino acid was located in the P_1 site. In comparison with some other proteases, CP-58 protease showed a quite different specificity toward the oxidized insulin B-chain. The structure around the catalytic site of CP-58 may be different from those of general proteases, which may also explain its cold-active properties.

Amino-terminal sequences. The *N*-terminal amino acid sequence of CP-58 protease, determined by Edman sequencing, was Ser-Leu-Asn-Gly-Lys-Thr-Asn-Gly-Trp-Asp-Ser-Val-Asn-Asp-Leu-Leu-Asn-Tyr-His-Asn-Arg-Gly-Asn (or Asp)-Gly-Thr-Ile-Asn-Asn-Lys-Pro-Ser-Phe-Asp-Ile-Ala. A search through the GenBank, PDB, SwissProt, and PIR databases for sequence homology yielded a significant match with metalloproteases. The identities between CP-58 protease and matched metalloproteases were 50–61%.

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