Purification and Characterization of Protease Q: A Detergent- and Urea-Stable Serine Endopeptidase from *Bacillus pumilus*

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An extracellular endopeptidase from a strain of *Bacillus pumilus* displaying high stability in 10% (w/v) sodium dodecyl sulfate (SDS) and 8 M urea has been purified and characterized. The enzyme, named protease Q, is a basic protein (pI = 9.35) composed of 309 amino acids with a molecular weight of 31 100. It is stable at 25 °C in the pH range 5−12 and most active at pH 10.5. Like other bacterial subtilisins, protease Q contains no cysteine and cystine residues. Protease Q possessed neither esterase nor amidase activity on synthetic substrates with arginine at position 1 and substrates of trypsin-like proteases. However, the enzyme showed strong activity on substrates with phenylalanine at position 1, substrates for chymotrypsin-like proteases. It also exhibited keratinase-like activity on keratin in 8 M urea. Inhibition studies indicated that protease Q was not sensitive to either trypsin-specific inhibitors, such as tosyllysine chloromethyl ketone (TLCK) and soybean trypsin inhibitor (SBTI), or chymotrypsin-specific inhibitors, such as tosylamido-2-phenylethyl chloromethyl ketone (TPCK). However, it was irreversibly inhibited by serine protease inhibitors such as PMSF. Although the N-terminal sequence of protease Q exhibited a high degree of homology with those of subtilisin Carlsberg and subtilisin BPN′, it did not exactly match with any of the known enzymes in the protein data bank. The specificity of protease Q on the oxidized insulin B-chain was distinctly different from those of subtilisin Carlsberg and proteinase K. One of the distinguishing properties of protease Q is that it is remarkably more stable than subtilisin Carlsberg in 5% SDS at 50 °C. These data suggest that protease Q is a new member of the subtilisin family of endopeptidases.

Keywords: *Bacillus pumilus; endopeptidase; detergent-stable protease; alkaline protease; urea-stable protease*

INTRODUCTION

Serine proteases are widespread in nature. They are found in viruses, bacteria, and eukaryotes, and they include exopeptidases, endopeptidases, oligopeptidases, and omega peptidases (Rawlings and Barrett, 1993). *Bacillus* and other Gram-positive bacteria have historically been used as sources of industrial enzymes, especially proteases (Priest, 1977). Subtilisin Carlsberg, an alkaline serine endopeptidase that is widely used in commercial detergent formulations, is the most economically significant enzyme produced by *Bacillus*. In this paper we report on the purification and characterization of an extremely sodium dodecyl sulfate (SDS)- and urea-stable extracellular serine endopeptidase isolated from a soil microorganism which has been identified as *Bacillus pumilus* (Han and Damodaran, 1997a,b). This protease appears to be a new member of the subtilisin family of serine endopeptidases not previously reported in the literature. The enzyme has been tentatively named as "protease Q". Previously, we have shown that protease Q exhibits remarkable stability in concentrated SDS and urea solutions (Han and Damodaran, 1997b). In this paper, we describe the physicochemical characteristics of protease Q and the major differences between protease Q and proteinase K and subtilisin Carlsberg.

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MATERIALS AND METHODS

Peptone, yeast extract, and other media components were obtained from Difco (Detroit, MI). Casein, synthetic oligopeptides, subtilisin Carlsberg, proteinase K, and all other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Protein molecular weight markers (low molecular weight) were purchased from Promega, (Madison, WI). All other chemicals and reagents used in this study were of analytical grade.

Microorganism and Growth Conditions. The organism, *Bacillus pumilus*, selected from a single colony on the agar plate, was cultured at 37 °C in 250 mL conical flasks using a fermentation medium containing 1.8% (w/v) peptone, 0.2% K_2HPO_4, 0.1% $MgSO_4$, 0.3% yeast extract, and 0.02% antifoam reagent. The initial pH of the medium was 7.06. The flasks were shaken in an environmental shaker (Lab-Line Instruments Co., Melrose Park, IL) at 120 rpm. Growth rate was monitored by measuring turbidity of the medium at 610 nm using uncultivated medium as the blank. Fermentation was carried out for 120 h.

Purification of Protease Q. All isolation and purification steps were carried out at 5 °C if not otherwise mentioned. The fermentation broth was centrifuged at 12100*g* for 15 min, and the clear supernatant (1000 mL) was mixed directly with DEAE-Sephadex A-50 which had been previously equilibrated with 20 mM Tris-HCl buffer (pH 8.3) containing 1.0 mM $CaCl_2$ (buffer A). The suspension was filtered and the filtrate was freeze-dried. The freeze-dried sample was dissolved in buffer A and fractionated on a Sephacryl-100 gel permeation column (2.5 × 100 cm) using buffer A as the eluent. The fractions with alkaline protease activity were collected and further purified on a Blue-Sepharose column (1.2 × 25 cm). The column was washed with buffer A containing 1.0 M NaCl, and

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bound proteins were then eluted with a NaCl gradient (from 1.0 to 0 M) in buffer A. A 173-fold purification was achieved after the Blue-Sepharose column chromatography. Further purification was carried out by preparative isoelectric focusing (IEF) using a Rotofor preparative IEF cell (Bio-Rad, Hercules, CA). Fractions from IEF were collected and passed through a Sephacryl-100 gel permeation column (1.6 × 95 cm). The column was eluted with buffer A, and fractions with proteolytic activity were collected. The highly purified enzyme was stored at −80 °C until used.

Proteolytic Activity Assay. Proteolytic activity on casein was determined according to the method described by Walter (1984). The casein concentration was typically 0.5%. The enzyme−substrate mixture was incubated at 37 °C for 20 min and stopped by the addition of an aliquot of 12% (w/v) trichloroacetic acid to a final TCA concentration of 3%. The samples were then incubated in ice for 10 min, and the absorbance of TCA soluble materials was determined at 280 nm after centrifugation at 14000*g* for 10 min. One casein unit (CU) was defined as the amount of enzyme which increased the absorbance at 280 nm of TCA-soluble materials by 0.001 unit/min.

Activity on synthetic oligopeptides was determined using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF) as substrate as described by DelMar et al. (1979). One unit of activity is expressed as the amount of enzyme which can liberate 1.0 mM *p*-nitroaniline/min. The concentration of *p*-nitroaniline was determined using a molar absorption coefficient of 8480 at 410 nm (DelMar et al., 1979). The trypsin-like enzyme activity was measured by the method of Arnon (1970) using (α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) as substrate.

Esterase activity on benzoyl-L-arginine ethyl ester (BAEE) substrate was determined according to the method described by Rick (1965). Esterase activity on *N*-benzoyl-L-tyrosine ethyl ester (BTEE) was determined using the method of Walsh (1970).

pH Stability. The universal buffer (5 mM) described by Terell and Stenhagen (1938) was used to vary the pH (the activity of protease Q was not significantly affected by ionic strength below 0.5). For the determination of pH stability, the enzyme solution (0.1 mL, 90 casein units) was mixed with 0.9 mL of universal buffer at various pH and incubated for 120 min at 25 °C. The residual activity of the enzyme was determined by mixing 0.25 mL of enzyme solution with 0.75 mL of 0.5% casein in 0.2 M Tris-HCl buffer (pH 8.7) and incubating for 20 min at 37 °C. The substrate-to-enzyme ratio was 166 mg/CU. For the determination of optimal reaction pH, the activity of the enzyme at various pH was determined using casein as substrate without preincubating the enzyme at the indicated pH for 120 min. The other reaction conditions were similar to that of pH stability studies as described above.

Thermal Stability. The enzyme solution in 0.1 M Tris-HCl buffer, pH 7.5, containing 1.0 mM $CaCl_2$ was incubated at a chosen temperature. An aliquot of the enzyme solution (20 μL, 18 CU) was withdrawn at various time intervals and immediately mixed with 280 μL of 0.5% casein in 0.1 M Tris-HCl buffer, pH 8.7. The reaction was carried out at 25 °C for 10 min and stopped by the addition of 100 mL of 12% trichloro acetic acid.

The optimal temperature of the enzyme was determined by using casein as the substrate and incubating the enzyme−substrate mixture at various temperatures for 10 min. The other experimental conditions and procedures were similar to those for thermal stability determination.

Protease Inhibitors. The effect of various protease inhibitors on protease Q were studied as follows. An aliquot of the enzyme solution (0.1 mL, 90 CU) was mixed with 0.4 mL of a stock solution of inhibitor in 0.1 M Tris-HCl buffer, pH 8.3. The enzyme−inhibitor mixture was incubated at 25 °C for 30 min followed by the addition of 0.25 mL of a stock solution of casein substrate to a final concentration of 0.5%. The reaction mixture was then incubated for 20 min at 37 °C. The activity was determined as described above and expressed as the percentage of the activity of the enzyme under identical conditions in the absence of inhibitors.

Proteolysis in the Presence of Urea and SDS. The effect of sodium dodecyl sulfate on the activity of protease Q was studied by using RNase-I and γ-globulins as substrates. The enzyme was first preincubated for 10 min at room temperature in 50 mM Tris-HCl buffer, pH 8.3, containing SDS. To this solution was added an aliquot of a stock solution of either RNase-I or γ-globulins in the Tris-HCl buffer to a final enzyme-to-substrate ratio of 5 CU/mg of substrate protein. The reaction mixture was incubated for 30 min at 37 °C in the case of RNase-I and for 45 min at 37 °C in the case of γ-globulins. The reaction was stopped by heating the solution for 5 min in a boiling water bath. The extent of hydrolysis of the proteins was analyzed by using Tricine-SDS-PAGE.

The effect of urea on the activity of protease Q was studied using keratin as substrate. The enzyme was first preincubated for 10 min at room temperature in 50 mM Tris-HCl buffer, pH 8.3, containing 8 M urea. To this was added a stock solution of keratin in the Tris-HCl−urea buffer to a final enzyme-to-substrate ratio of 5 CU/mg substrate protein. The reaction mixture was incubated for 45 min at 37 °C and stopped by heating the solution for 5 min in a boiling water bath. The extent of hydrolysis of the proteins was analyzed by using Tricine-SDS-PAGE.

Stability in SDS. The kinetic stability of protease Q, subtilisin Carlsberg, and proteinase K in 5% SDS solution was studied as follows. The enzyme solutions in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM $CaCl_2$ and 5% (w/v) SDS were incubated at 25 and 50 °C. An aliquot (20 mL) of the solution was withdrawn at various time intervals, and the remaining activity was determined using casein as substrate (see above). The final SDS concentration in the proteolysis reaction mixture was 0.25% (w/v) for the study at 50 °C and was about 0.07% (w/v) for the study at 25 °C. The proteolysis reaction was carried out for 10 min at 37 °C.

Liquid-Phase Isoelectric Focusing. The isoelectric point (pI) of protease Q was determined by using a preparative isoelectric focusing cell (Bio-Rad). Isoelectric focusing was performed using 2.0% (v/v) Bio-Lyte ampholyte (pH 3−10 for the first run and pH 8−10 for all subsequent runs) in a total volume of 60 mL. Focusing of the enzyme was performed at 1 °C for 3.5 h at 15 W constant power supply. Twenty fractions were collected, and their pH value, protein concentration, and proteolytic activity were determined. Fractions with proteolytic activity were pooled, and the ampholyte was removed by passing through a Sephacryl-100 gel filtration column.

Electrophoresis. A discontinuous Tricine-SDS-PAGE as described by Schagger and von Jagow (1987) was used. The slab gel was made of a 4% stacking gel and a 16.5% separating gel. The Tricine-SDS-PAGE gel was prerun with the running buffer containing 0.001% (w/v) thioglycolic acid for 10 min at 10 mA constant current before the samples were loaded. After running, the gel was fixed overnight and stained with silver nitrate, as described by See and Jackowski (1989).

N-Terminal Sequence and Amino Acid Composition. N-terminal sequence and amino acid composition of the enzyme were determined according to the microsequencing method. The enzyme was transblotted from the Tricine-SDS-PAGE gel on to a poly(vinylidene difluoride) (PVDF) membrane as described by Matsudaira (1993). Transblotting was performed in a Trans-Blot SD semi-dry transfer cell (BIO-RAD, CA) at 12 V constant voltage for 12 h. The PVDF membrane with transblotted proteins was stained with Coomassie Blue-R 250 for 5 min and destained using the SDS-PAGE destaining solution for 5 min. The membrane was incubated with deionized water for 1 h before drying. The transblotted enzyme band in the dried PVDF membrane was used for N-terminal sequencing and amino acid composition determination. Analysis of the N-terminal sequence of the intact enzyme was performed using an automatic amino acid sequencer; the amino acid composition was determined from an acid hydrolysate of the sample using a Beckman amino acid analyzer. Both N-terminal sequencing and amino acid analysis were performed at the Protein/Nucleic Acid Shared Facility (The Medical College of Wisconsin, Milwaukee, WI).

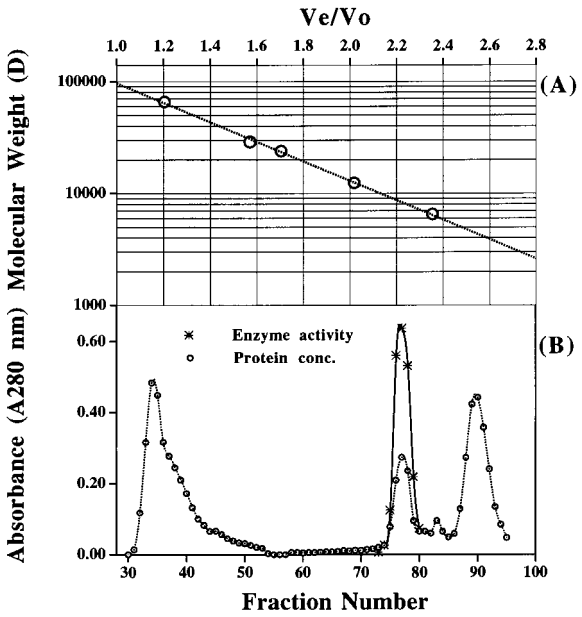

Figure 1. Size exclusion chromatography of protease Q on a Sephacryl-100 column. The column size was 2.5 × 100 cm. (A) Elution of molecular weight marker proteins. (B) Elution profile of protease Q preparation obtained after DEAE-Sephadex A-50 ionexchange chromatography. ○, absorbance at 280 nm; ∗, proteolytic activity.

Determination of Proteolytic Specificity of Protease Q. The specificity of protease Q was determined using insulin B-chain as a substrate. The oxidized B-chain of insulin, purchased from Sigma Co. (St. Louis, MO), was dissolved in 20 mM Tris-HCl buffer, pH 8.7, and passed through a Sephadex G-25 gel permeation column to remove sodium in the sample. The protein was eluted with 5% acetic acid, and the tubes containing the sample were pooled and freeze-dried. The freeze-dried insulin B-chain was dissolved in 50 mM Tris-HCl buffer (pH 8.7) and was mixed with protease Q to a final insulin B-chain concentration of 0.2% (w/v) and to keep an enzyme-to-substrate ratio of 1:980. The reaction mixture was incubated at 25 °C for different time intervals, and the reaction was stopped by mixing one volume of 4% acetic acid with two volumes of the reaction solution to bring the pH value of the reaction mixture to 3.6. The hydrolyzed protein samples were stored at −80 °C until further analysis by a matrix-assisted laser desorption/ionization (MALDI) mass spectrometer.

RESULTS AND DISCUSSION

Purification of Protease Q. Protease Q was purified to 173-fold from the centrifuged fermentation broth by first treating with DEAE-Sephadex A-50 ion exchange gel, followed by chromatography on a Sephacryl-100 gel permeation column and a Blue agarose column. Protease Q did not bind to DEAE-Sephadex A-50 at the pH of the fermentation broth (∼9.0). The gel permeation chromatography step was used immediately after treatment with the ion-exchange gel because protease Q exhibited an abnormal mobility in the gel permeation matrix (either Sephadex G-75 or Sephacryl-100) even when the column was eluted with a buffer containing 0.5 M NaCl. The enzyme moved on this column as a 10 000 protein, probably because of nonspecific interactions with the gel matrix. As a consequence, all contaminant proteins with molecular weights greater than 20 000 could be easily removed after this gel permeation chromatography step (Figure 1). It has been reported that proteinase K also exhibited abnormal mobility on gel permeation columns, which led to its molecular weight being originally reported as 18 500 ± 500 (Ebeling et al., 1974). However, its true molecular weight was later determined to be 28 930 from its amino acid sequence (Jany et al., 1986).

Protease Q was further purified to homogeneity by liquid-phase preparative isoelectric focusing (IEF), followed by a Sephacryl-100 gel permeation column. Protease Q collected from the gel permeation column showed a single band on a silver-stained Tricine-SDS-PAGE gel (data not shown). The enzyme preparation was treated with 1 mM PMSF prior to electrophoresis in order to inhibit possible autolysis during electrophoresis.

Table 1. Amino Acid Composition of Protease Q, Subtilisin Carlsberg, and Protease K[a]

	no. of residues		
amino acid	Q	C	K
Asx	46		
Asp		9	14
Asn		19	17
Thr	15	19	21
Ser	30	32	36
Glx	17		
Glu		5	5
Gln		7	7
Pro	17	9	9
Gly	35	35	34
Ala	40	41	33
Val	37	31	19
Met	4	5	5
Ile	12	10	10
Leu	17	16	13
Tyr	10	13	16
Phe	5	4	6
His	5	5	4
Lys	11	9	8
Arg	7	4	12
Cys	0	0	5
Trp		1	2
total	309	274	277
nonpolar	132	117	97
polar and charged	177	145	180

[a] Data from DeLange and Smith (1968) and Jany et al. (1986) for subtilisin Carlsberg and proteinase K, respectively. Abbreviations refer to the following: Q, protease Q; C, subtilisin Carlsberg; K, proteinase K.

Molecular Weight and Amino Acid Composition. According to its amino acid composition, protease Q contains 309 amino acid residues with a molecular weight of 31 100 Da (Table 1). The amino acid composition of subtilisin Carlsberg also is given in Table 1 for comparison. The molecular weight of protease Q is higher than that of subtilisins, such as subtilisin Carlsberg (274 amino acid residues) and subtilisin BPN′ (275 amino acid residues) (Wong, 1996).

The amino acid composition of protease Q (Table 1) shows that it is devoid of cysteine and cystine residues. Since all trypsin-related enzymes contain such residues (Jany et al., 1986), protease Q is apparently not related to the trypsin family. On the other hand, bacterial subtilisins generally contain no cysteine/cystine residues, with the exception of the subtilisin from *Bacillus smithii* (EMBL: L24202). Therefore, protease Q may belong to the subtilisin family of serine proteases. The most significant feature of the amino acid composition of protease Q is its Asx content (Asp plus Asn residues), as compared with other subtilisins. Protease Q contains 46 amino acid residues of Asx, corresponding to 14.8 mol %, whereas subtilisin BPN′ (produced by *Bacillus amyl*

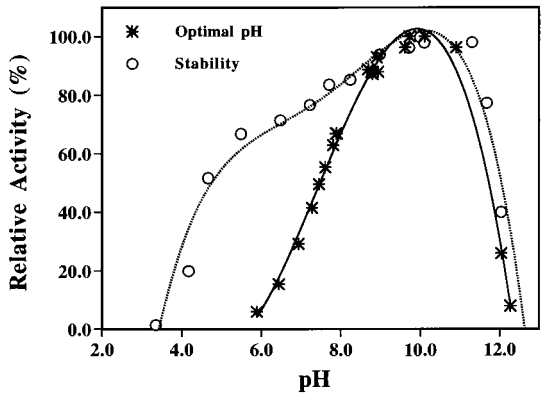

Figure 2. pH stability (○) and optimal reaction pH (∗) of protease Q. Data are the mean of duplicate measurements. The relative activity is expressed as the percent of the activity at the optimum pH.

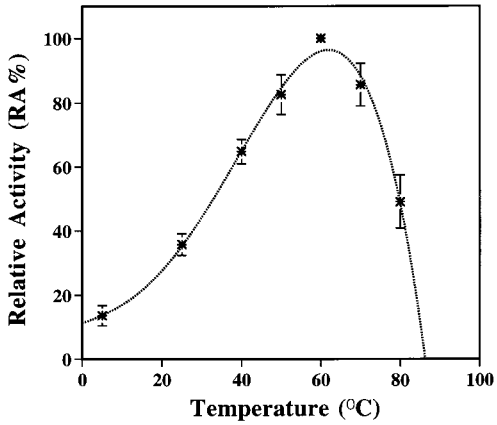

Figure 3. Temperature versus activity of protease Q. Data are the mean of triplicate measurements, and the bars denote standard error. The relative activity is expressed as the percent of the maximum activity in the activity−temperature profile.

oliquifaciens) and subtilisin Carlsberg (produced by *Bacillus licheniformis*) contain only 10.2 and 9.45 mol %, respectively. Since the Lys and Arg contents of all these three enzymes are very similar and the pI of all these enzymes is in the range of 9.3−9.4, we infer that the excess amount of Asx in protease Q must be related to a high Asn content.

General Physicochemical Properties of Protease Q. Results of preparative isoelectric focusing of the enzyme indicated that protease Q is a basic protein with a pI value of 9.35. Protease Q was stable over a broad range of pH values from 5 to 12 and was active over the pH range 7.5−11.5 (Figure 2). The optimal reaction pH of protease Q was in the range of 10.0−10.5 for the hydrolysis of casein at 37 °C. No proteolytic activity of the enzyme was detected at pH values lower than 6.0, although the enzyme was relatively stable at pH 4.0. The fact that the enzyme was active only above pH 6.0 (Figure 2) may imply involvement of a histidine residue ($pK = 5.6-7.0$) at the active site, which is a well-known characteristic of serine proteases (Rawlings and Barrett, 1993).

The optimal reaction temperature of protease Q was about 60 °C for a 10 min hydrolysis of casein (Figure 3). However, at longer reaction time periods, the optimum temperature was around 50 °C, which is similar to that of other alkaline proteases from *Bacillus* species (Outtrup and Boyce, 1990). The most significant property of protease Q was that even though it contained no disulfide bond, it was reasonably stable at or below 50 °C (Figure 4A**)**. Above 50 °C, the stability decreased with an increase of temperature. It was inactivated within 6 min at 80 °C. Table 2 shows the half-life of protease Q at various temperatures. At 25 °C the half-life was about 2822 h, and even at 70 °C it survived for over 20 min. The Arrhenius plot of the thermal stability data showed a linear relationship over the temperature range 37−80 °C (Figure 4B). The activation energy (E_a) for thermal inactivation of the enzyme, calculated from the slope of the Arrhenius plot (Figure 4B), was about 45.8 kcal/mol.

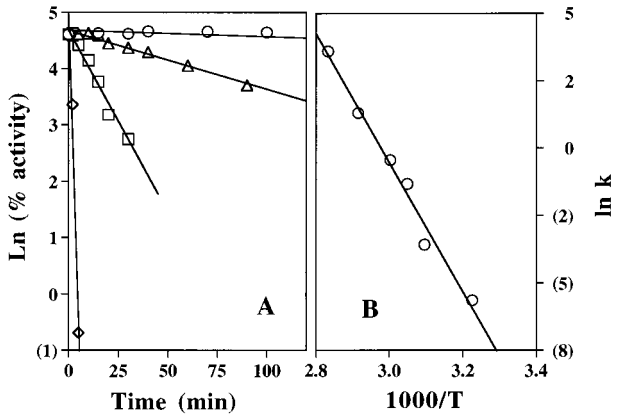

Figure 4. Thermal stability of protease Q. (A) First-order plot of activity (left) of the enzyme as a function of time at 50 (○), 60 (△), 70 (□), and 80 °C (◊). The data are the mean of duplicate measurements. (B) Arrhenius plot of the data. *k* is the first-order rate constant of inactivation of the enzyme.

Table 2. Half-Life of Protease Q at Various Temperatures

temp (°C)	$t_{1/2}$ (h)	temp (°C)	$t_{1/2}$ (h)	temp (°C)	$t_{1/2}$ (h)
25	2822.0	55	3.1	70	0.2
37	725.0	60	1.3	80	0.02
50	30.0				

Protease Q exhibited both esterase and amidase activities on oligopeptides with Tyr or Phe at position P_1, such as *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF), which are specific substrates for chymotrypsin-like proteases (DelMar et al., 1979; Walsh, 1970). However, the activity on AAPF does not necessarily mean that protease Q is a chymotrypsin-like enzyme. Most of the microbial members of the subtilisin family have specificity somewhat similar to that of chymotrypsin (Rawlings and Barrett, 1993). In addition, protease Q showed neither esterase nor amidase activity on synthetic substrates with P_1 = Arg, such as benzoyl-L-arginine ethyl ester (BAEE) and α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), which are substrates for trypsin-like proteases (Rick, 1965). The catalytic specificity of protease Q indicates that it is not a trypsin-like protease.

The effects of various protease inhibitors on the activity of protease Q are summarized in Table 3. The proteolytic activity of protease Q was not influenced by pepstatin which is an aspartyl protease inhibitor (Smith et al., 1968). Protease Q was not inhibited by inhibitors of trypsin-like enzymes, such as tosyllysine chloromethyl ketone and soy bean trypsin inhibitor (SBTI), suggesting further that it is not a trypsin-like protease. The enzyme was also not sensitive to leupeptin, which

Table 3. Influence of Inhibitors on Proteolytic Activity[a]

inhibitors	concn	rel activity (%)
pepstatin	(0.1 mM)	123.9 ± 4.36
leupeptin	(1 mM)	97.2 ± 3.60
EDTA	(5 mM)	79.1 ± 3.86
NEM	(5 mM)	173.0 ± 6.12
ME	(0.1 M)	109.0 ± 0.77
	(0.5 M)	94.2 ± 2.90
	(1.0 M)	89.0 ± 3.20
SBTI	(0.1 mg/mL)	112.3 ± 8.23
TLCK	(0.4 mg/mL)	136.5 ± 6.71
TPCK	(2 mM)	97.4 ± 4.72
PMSF	(1 mM)	10.9 ± 2.13 (30 min incubation)
		0.8 ± 0.37 (90 min incubation)

[a] The concentrations of inhibitors shown were final concentrations in the reaction system. 100% activity refers to activity in the absence of inhibitors under identical experimental conditions. Data are the mean of triplicate determinations + standard error. The abbreviations refer to the following: ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; TLCK, tosyllysine chloromethyl ketone; TPCK, L-1-tosylamido-2-phenyl)ethyl chloromethyl ketone; EDTA, ethylenediamine tetraacetic acid.

is an inhibitor of trypsin-like serine proteases and also most cysteine proteases (Umezawa, 1976).

Although protease Q showed strong amidase activity on AAPF, it was not sensitive to tosylamido-2-phenylethyl chloromethyl ketone, which is an inhibitor of chymotrypsin-like enzymes (Shoellman and Shaw, 1963), suggesting that it is not a chymotrypsin-like enzyme. However, like all other serine proteases, protease Q irreversibly lost its proteolytic activity in the presence of 1.0 mM phenylmethanesulfonyl fluoride (PMSF) (89% of its activity was lost after 30 min incubation and 99% after 90 min), indicating involvement of serine in the catalytic activity. In the presence of ethylenediaminetetraacetic acid (EDTA), which is an inhibitor of metalloproteases, the activity of protease Q decreased to 79% of its original value. It did not however completely inhibit the activity of the enzyme, indicating that protease Q is not a metalloprotease.

Proteolytic Activity in SDS and Urea. Parts A and B of Figure 5 show hydrolysis of γ-globulin and RNase-I, respectively, by protease Q both in the presence and absence of 2% SDS. In 2% SDS, intact RNase-I was completely hydrolyzed within 30 min by protease Q, whereas in the absence of 2% SDS some amount of intact RNase-I was still present after 30 min reaction. RNase-I treated with protease Q in the presence of 2% SDS lost its activity immediately upon the addition of protease Q (data not shown). The γ-globulins were hydrolyzed more in the presence of 2% SDS than in its absence. This is probably because the native γ-globulins were more resistant to proteolysis than the denatured γ-globulins in SDS. In should be pointed out that in all these experiments protease Q was preincubated for 10 min at room temperature in 2% SDS before the addition of the substrate. Thus, the data also indicate that protease Q was very stable and active in 2% SDS.

Human keratin is a protein soluble only in 8 M urea. After 30 min incubation with protease Q in the presence of 8 M urea, the intact keratin was completely hydrolyzed to low molecular weight polypeptides (Figure 5C, lane 2). This demonstrates that protease Q was not denatured by 8 M urea. Further studies on its stability in the presence of urea revealed that even in 10 M urea, a saturated concentration of urea at room temperature, protease Q retained much of its activity.

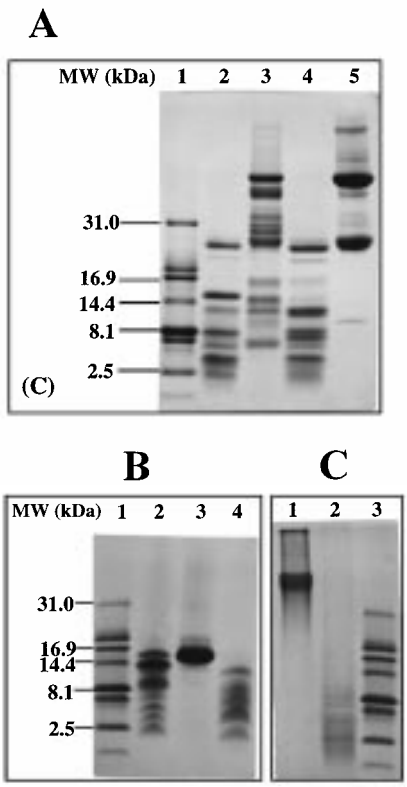

Figure 5. (A) Proteolysis of γ-globulin by protease Q: lane 1, protein markers; lane 2, hydrolysis in buffer containing 1% SDS; lane 3, hydrolysis in 50 mM Tris-HCl buffer; lane 4, hydrolysis in buffer containing 2% SDS; lane 5, γ-globulin control. (B) Tricine-SDS-PAGE profile of RNase-I treated with protease Q: lane 1, molecular weight markers; lane 2, RNase-I treated with protease Q without SDS; lane 3, RNase-I control; lane 4, RNase-I treated with protease Q in the presence of 2% SDS. (C) Proteolysis of keratin by protease Q in the presence of 8 M urea: lane 1, keratin control; lane 2, keratin treated with protease Q in the presence of 8 M urea; lane 3, molecular weight markers.

Although protease Q can hydrolyze keratin, it is not a keratinase, because keratinases cannot utilize oligopeptides such as AAPF as substrates (Nickerson and Durand, 1963), whereas protease Q does. Other than proteinase K, only a few microbial alkaline proteases possess keratinase activity. A not well-characterized protease from the fungus *Trichophyton mentagrophytes* has been reported to possess keratinase activity, but it has a molecular weight of 48 400 with an optimal reaction pH of 7.0 (Yu et al., 1969). Another alkaline protease from *Alkalophilic streptomyces* has also been reported to have keratinase activity, which has a molecular weight of 50 000 and is extremely alkalophilic with an optimal reaction pH higher than pH 12 (Nakanishi and Yamamoto, 1974). Thus, protease Q does not appear to be related to any of the enzymes mentioned above.

N-Terminal Sequence of Protease Q. Figure 6 shows the N-terminal sequence of protease Q. Protein data bank (SwissProt and Brookhaven) search results indicated that no protein with this exact N-terminal sequence has been reported in the literature. Thus, protease Q appears to be a new member of serine endopeptidases. However, the N-terminal sequence of protease Q showed a high degree of homology with that of subtilisin Carlsberg and subtilisin BPN′. A fragment from the C-terminal region of protease Q also showed a high degree of homology with residues 221−250 of

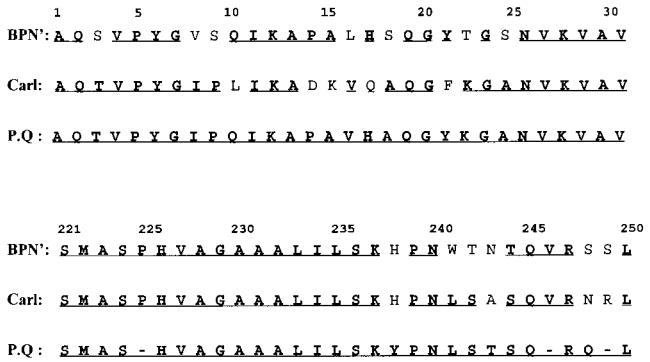

Figure 6. Comparison of N-terminal sequences of subtilisin BPN′, subtilisin Carlsberg, and protease Q.

subtilisin Carlsberg and subtilisin BPN′ (Figure 6). Alignment of the N-terminal sequence of protease Q (from 1 to 30 residues) with those of subtilisin Carlsberg and subtilisin BPN′ showed that the N-terminal sequence of protease Q is a hybrid of the two enzymes. That is, at the locations where protease Q did not match with subtilisin Carlsberg, it matched with subtilisin BPN′. Whether or not this trend continued significantly in other parts of the molecule as well remains to be studied. The C-terminal fragment 221−250 showed differences at positions 238(Tyr), 243(Thr), and 248-(Gln). It should be noted that the residue 221(Ser) is part of the catalytic triad in subtilisin Carlsberg and subtilisin BPN′ (McPhalen and James, 1988). This position is conserved in protease Q as well. However, the total number of amino acid residues in the primary sequences of subtilisin Carlsberg and subtilisin BPN′ is 274 and 275, respectively, whereas it is about 309 in protease Q based on amino acid composition.

Specificity of Protease Q. Figure 7 shows a typical MALDI mass spectra of insulin B-chain hydrolyzed by protease Q. The hydrolysis products at two different reaction conditions (enzyme-to-substrate ratios of 1:490 and 1:980) were almost identical. The fragment with molecular mass 1799.3 Da, which is one of the products of cleavage at the Leu_{15}-Tyr_{16} position was always dominant in all MALDI spectra. This suggests that the bond Leu_{15}-Tyr_{16} is the most preferred site for protease Q. The cleavage at the Leu_{15}-Tyr_{16} site produces two polypeptides with molecular masses of 1799.3 and 1715.2 Da. Similar results have been reported for subtilisin Carlsberg (Johansen et al., 1968). The only cutting site inside the fragment of 1799.3 Da was Tyr_{26}-Thr_{27}, which liberates peptide fragments of molecular masses 1401.8 and 415.5 Da. The peak with a molecular mass of 550.1 Da in Figure 7B is a reference signal.

In Figure 8 and in Table 4 the proteolytic specificity of protease Q is compared with those of subtilisin Carlsberg and proteinase K on oxidized insulin B-chain. All these three proteases could not cut the site at Arg_{22}-Gly_{23}, a typical hydrolysis site for trypsin (Sanger and Tuppy, 1951). Protease Q could not cut the two sites, Ser_9-His_{10} and Leu_{17}-Val_{18}, at which both proteinase K and subtilisin Carlsberg could cut (Figure 8 and Table 4). The 1715.2 Da fragment appears to be first cut at the His_5-Leu_6 site, liberating a 643.7 Da fragment. It should be noted that both proteinase K and subtilisin Carlsberg do not cut at this site (Table 4). Since a fragment corresponding to 1071.5 Da (segments 6−15) does not appear in MALDI, it must have been cut immediately at the Leu_{11}-Val_{12} site, liberating a 677.0 Da fragment (Figure 9). It should be noted that after

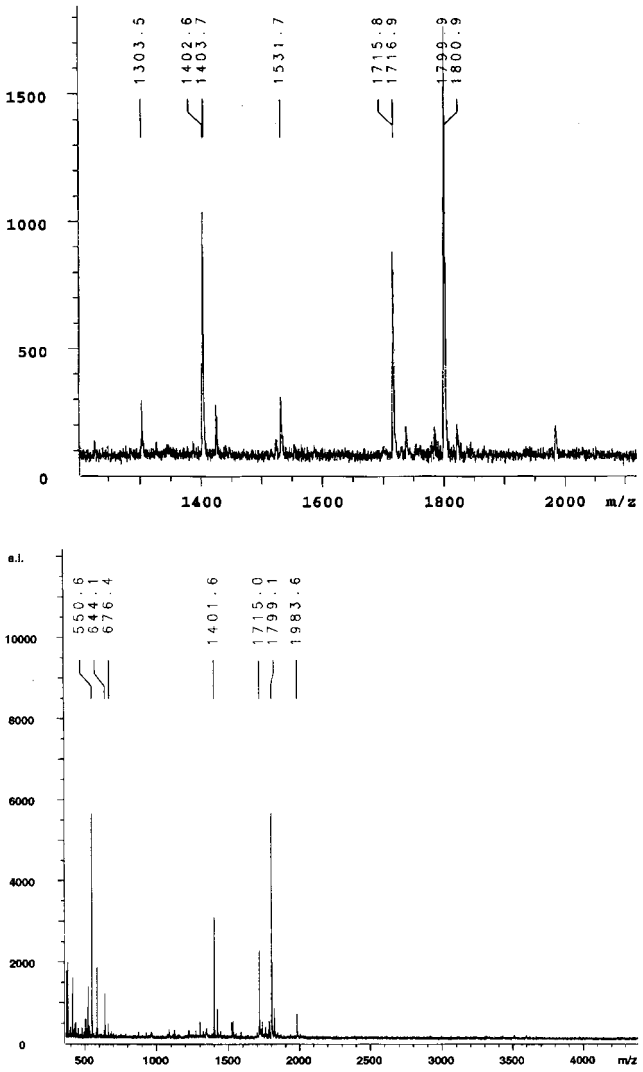

Figure 7. MALDI spectra of hydrolysis products of oxidized insulin B-chain. (A, top) Proteolysis was carried at at 25 °C for 2 h with an enzyme-to-substrate ratio of 1:980 in 50 mM Tris-HCl buffer, pH 8.7. (B, bottom) Proteolysis carried out at 37 °C for 20 min with an enzyme-to-substrate ratio of 1:490 in 50 mM Tris-HCl buffer, pH 8.7.

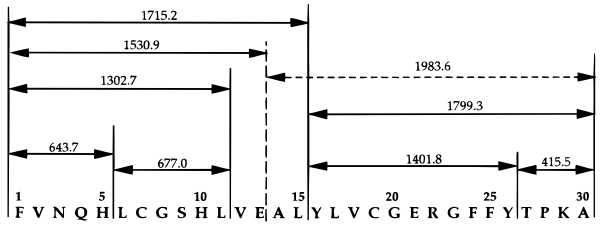

Figure 8. Major (—) and minor (- -) cleavage sites for protease Q in oxidized insulin B-chain.

the initial cut at the Leu_{15}-Tyr_{16}, protease Q does not cut at the Leu_{17}-Val_{18} site of the 1799.3 Da fragment, whereas it could cut a similar site at Leu_{11}-Val_{12} of the 1071.5 Da fragment. This contradiction could be attributed to the difference in the number of amino acid residues present at the N-terminus of the cutting site. In the case of Leu_{11}-Val_{12}, there are six amino acid residues at the N-terminus, which may facilitate binding of the substrate to the enzyme. This may suggest that the activity of protease Q may require a P_{1-3}−S_{1-3} binding mechanism where P_{1-3} refers to three amino acid residues from the cutting site of the substrate and

Table 4. Major (Large Arrows) and Minor (Small Arrows) Cutting Sites for Proteinase K (PK), Subtilisin Carlsberg (SC), and Protease Q (PQ) in the Oxidized Insulin B-Chain

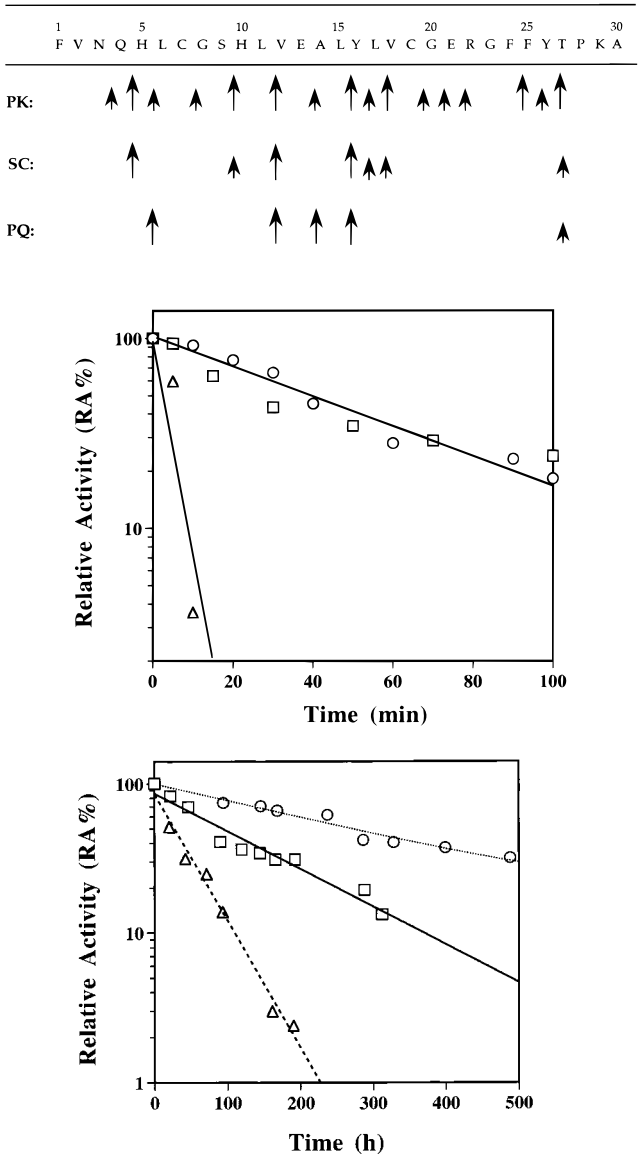

Figure 9. Kinetic stability of protease Q (□), subtilisin Carlsberg (△), and proteinase K (○) in 5% SDS at 50 °C (A, top) and at 25 °C (B, bottom). The relative activity refers to the percentage of enzyme activity at zero time in the absence of SDS.

S_{1-3} refers to complementary amino acid residues at the active site of the enzyme (Morihara and Oka, 1970). In the case of Leu_{17}-Val_{18}, the N-terminus of the fragment 1799.2 Da (peptide 16−30) contains only one amino acid residue (Tyr_{16}). Therefore, the peptide could offer only the P_2 binding site to the enzyme. Since there was no P_3 site available for the substrate to bind with the S_3 site of the enzyme, the cut at the Leu_{17}-Val_{18} could not occur. This again confirms the finding that the binding cleft of subtilases consist of at least six subsites, S_4−S_3−S_2−S_1−S_1'−S_2' (Morihara and Oka, 1970). Recently, it has been reported that the binding interactions involve eight subsites (Gron et al., 1992). Notwithstanding the evidence, the possibility that the Tyr residue at P_2 was not a favorable residue for binding to S_2 of the enzyme cannot be ruled out. Nonetheless, the inability of protease Q to cut at the Leu_{17}-Val_{18} site indicates that the secondary binding between subsites of an enzyme and the binding site in substrates is critical to facilitate proteolysis. The proteolytic specificity of a protease, thus, could not be determined by using synthetic short polypeptides.

The Glu_{13}-Ala_{14} site is a typical cutting site for all cathepsins except for cathepsin G (Keil, 1992). The strong proteolytic specificity of protease Q and proteinase K at Glu_{13}-Ala_{14} site implies that these two enzymes also has cathepsin-like proteolytic specificity. This may explain why proteinase K and protease Q have a strong proteolytic activity on native protein substrates, in which Glu residues are predominantly located on the exterior of proteins. At comparable activity units as judged from their activity on AAPF, subtilisin Carlsberg always exhibits lower proteolytic activity than proteinase K and protease Q on native protein substrates. The most significant difference between proteinase K and other two enzymes is that the former can cut the Phe_{24}-Phe_{25} site while the latter cannot. Proteinase K shows a broad proteolytic specificity compared to that of the other two proteases.

Stability in SDS. The relative stability of protease Q, proteinase K, and subtilisin Carlsberg in SDS at 50 and 25 °C are shown in Figure 9A,B, respectively. In a solution containing 5% SDS at 50 °C, subtilisin Carlsberg lost almost all of its activity within 10 min, whereas both protease Q and proteinase K were inactivated at a much slower rate. Both protease Q and proteinase K retained about 40% of their original activity after 60 min incubation in 5% SDS (Figure 9A). At 25 °C however proteinase K was the most stable in 5% SDS. Although protease Q was not as stable as proteinase K in 5% SDS at 25 °C, it was much more stable than subtilisin Carlsberg (Figure 9B). The data indicate that although protease Q is highly homologous to subtilisin Carlsberg based on their N-terminal sequences, the amino acid substitutions that have occurred in protease Q seem to provide it with a better stability against SDS. Although protease Q and proteinase K are not homologous, they display a similarity in stability against SDS.

The results presented here show that although protease Q and subtilisin Carlsberg belong to subtilisin family of serine endopeptidases and their N-terminal sequences are homologous, there are some distinguishing characteristics between the two proteases. Protease Q is more stable than subtilisin Carlsberg in concentrated SDS and urea solutions. In this respect protease Q is more like proteinase K. Protease Q also exhibits keratinase activity which has not been reported for other subtilisins. The specificity of protease Q on the oxidized B-chain of insulin is also different from those of subtilisin Carlsberg and proteinase K. The stability of protease Q in detergent solutions may have useful applications in several industrial formulations, including detergent formulations, and in molecular biology techniques. Because protease Q is very stable in SDS solutions, it can be used for inactivating RNase during purification of RNA from cell homogenates. In this respect protease Q may have an advantage over proteinase K: Because protease Q contains no cysteine and cystine residues, it is active in the presence of reducing agents, such as β-mercaptoethanol and dithiothrietol, whereas proteinase K is not. Further investigations on the structural bases for its resistance to denature in the presence of concentrated SDS and urea solutions would be helpful in designing detergent-stable enzymes by protein engineering.

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