



## Protease Activity of a 90-kDa Protein Isolated from Scallop Shells

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### Abstract

We have previously reported the free radical scavenging activity of a protein with a molecular weight of 90 kDa (90-kDa protein) isolated from the scallop shell. In this study, we found that the 90-kDa protein also shows protease activity. The protein was most active at an alkali pH and at 60°C, and its activity was inhibited by serine protease inhibitors, phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate. Its activity was maintained at approximately 90% of the initial activity, even in the presence of denaturants such as 1% sodium dodecyl sulfate (SDS) and 6 M urea. Substrate specificity analysis performed using synthetic peptides showed that the 90-kDa protein cleaves preferentially at Lys-X and Arg-X bonds. A portion of Phe-X bond was also cleaved by the 90-kDa protein. When casein was treated with the 90-kDa protein, it was digested at the Arg-X, Lys-X, and Phe-X bonds. The 90-kDa protein may be useful for proteome analysis because it retains its activity even in the presence of 1% SDS. To the best of our knowledge, this is the first report of a protease found in scallop shell.

**Keywords:** Alkaline protease, scallop shell, shell matrix protein, thermostable.

### Introduction

Approximately 200,000 t per annum of scallop shells are generated as industrial waste in the Hokkaido region of Japan alone. Approximately 99% of the scallop shell is composed of calcium carbonate, and organic components constitute only 1% of its weight. We have previously shown that the organic components of scallop shells (scallop shell extract) have a variety of biological activities such as free radical scavenging ability, inhibitory ability for the differentiation of 3T3-L1 preadipocytes, and chymotrypsin activity-promoting ability (Liu *et al.*, 2002; Liu and Hasegawa, 2006; Torita *et al.*, 2004; Takahashi *et al.*, 2012).

Previous studies have shown that *Pinctada fucata* shells contain tyrosinase and carbonic anhydrase enzymes (Nagai *et al.*, 2007; Miyamoto *et al.*, 1996). A protein (nacrein) showing carbonic anhydrase activity was identified from the prismatic layer of *Pinctada fucata* shells. Nacrein is thought to be involved in the formation of calcium carbonate crystals, functioning as a carbonic anhydrase that catalyzes  $\text{HCO}_3^-$  formation. Tyrosinase is also

observed in the prismatic layer of *Pinctada fucata* shells. Tyrosinase is secreted from the mantle and transported to the calcified prismatic layer.

Nagai *et al.*, 2007 proposed that the tyrosinase contributes to melanin synthesis, helping in concealment from predators and exclusion of parasites (Nagai *et al.*, 2007). These studies have shown that the mollusc shell contains enzymes that are involved in shell formation.

Many proteases have been isolated from every organisms. Proteases constitute approximately 40% of the total enzyme sales in various industries such as detergent, food, waste management, and pharmaceutical industries (Anwar and Saleemuddin, 1998; Kumar and Takagi, 1999). For industrial applications, many alkaline proteases that are stable for heat, organic solvents, or detergents have been obtained from various microorganisms, particularly *Bacillus species* (Gupta *et al.*, 2002; Johnvesly and Naik, 2001; Rahman *et al.*, 2006; Banik and Prakash, 2004). However, there is still a need for a novel proteases for industrial applications.

In this study, we report the identification and characterization of a protease from scallop shells.

## Materials and Methods

### Materials

Scallops were collected from Funka Bay, Hokkaido. The shells were prepared from the scallop *Patinopecten yessoensis*. Synthetic peptides were purchased from Peptide Institute Inc. (Osaka, Japan).

### Preparation of scallop shell extract

The shells were carefully brushed to remove any adherent materials. The shells were then crushed and ground to a powder as previously described (Liu *et al.*, 2002). For complete decalcification, the powdered shells (approximately 200 g) were dialyzed against 1 L of 5% acetic acid by using a dialysis membrane with a molecular weight cut-off value of 10 kDa. This was followed by exhaustive dialysis against 1 L of deionized water to remove the acetic acid. After dialysis, the decalcified solution was concentrated by lyophilization and the sample was suspended in deionized water. The water-soluble fraction was used as the scallop shell extract in the subsequent experiments.

### Electrophoresis

After column chromatography, SDS sample buffer containing 2% SDS, 20 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol, 10% glycerol, and bromophenol blue was added to each fraction. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli (1970). Native-PAGE was performed without denaturing agents (SDS). After SDS- or native-PAGE, the gel was stained with Coomassie Brilliant Blue (CBB)-R250 or Stains-all (Green *et al.*, 1973). For determination of a molecular weight, molecular weight marker consisting of myosin, 212 kDa;  $\alpha$ -glucosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; maltose-binding protein 2, 42 kDa (Takara, Shiga, Japan) was used.

### Zymography

SDS-PAGE gel containing 2 mg/ml gelatin was used for zymography (Khan *et al.*, 2000). After electrophoresis of scallop shell extract and the 90-kDa protein, a gel was soaked in 2.5% Triton X-100 solution for 60 min, incubated in 50 mM Tris-HCl (pH 7.5) at 37°C for 12 h, and stained with CBB-R250. Areas of gelatin digestion were visualized as non-staining areas in the gel.

### Purification of the 90-kDa Protein

The scallop shell extract was separated using a Sephacryl S-300 gel filtration column equilibrated with deionized water. The fractions with protease

activity were pooled and loaded onto a DEAE-cellulose ion-exchange column equilibrated with 20 mM Tris-HCl (pH 7.5). The adsorbed proteins were eluted using a linear concentration gradient of 0.6 M NaCl and 20 mM Tris-HCl (pH 7.5). The peak fractions with protease activity were pooled and dialyzed and then lyophilized. The peak fraction was separated by native-PAGE and a band with a molecular weight of 90 kDa was excised. The 90-kDa protein was electro-eluted using Electro-Eluter (Bio RAD, CA, U.S.A) and employed in the following experiments.

### Measurement of Protease Activity

Protease activity of each fraction was measured after S-300 gel filtration and DEAE-cellulose column chromatography as follows: 20  $\mu$ l of each fraction was added to 5  $\mu$ l of 10 mM Suc-Ala-Ala-Pro-Phe-pnitroanilide (*p*NA) (chymotrypsin substrate) in 50 mM Hepes-NaOH (pH 7.5) at 37°C (Delmar *et al.*, 1979). After 2 h of incubation, absorbance at 405 nm was measured.

Substrate specificity was measured using several peptidyl-*p*NAs and peptidyl-4-methyl-coumaryl-7-amides (peptidyl-MCAs). Protease activity was measured in a solution containing 10 mM peptidyl-MCA or 10 mM peptidyl-*p*NA, and 50 mM Hepes-NaOH (pH 7.5) at 37°C. The 90-kDa protein (0.026 mg/ml) was added to the substrate solution, and the fluorescence at 460 nm (excitation at 380 nm) for the assay using peptidyl-MCA or the absorbance at 405 nm for the assay using peptidyl-*p*NA was recorded for 30 min.

The effect of pH on protease activity was measured in the range of 2.5–10.5 by using a chymotrypsin substrate at 37°C. The buffer systems used were as follows: 50 mM Gly-HCl for pH 2.5, phosphate buffer for pH 6 and pH 8, and Gly-NaOH buffer for pH 10.5. After adding 90-kDa protein to a final concentration of 0.026 mg/mL, the absorbance at 405 nm was measured for 30 min.

The effect of temperature on protease activity was measured in a solution containing 10 mM chymotrypsin substrate, 50 mM Hepes-NaOH (pH 7.5), and the 90-kDa protein (0.026 mg/mL) at various temperatures for 30 min. To examine the effect of temperature on the stability of the 90-kDa protein, the residual activities were measured after the 90-kDa protein was incubated at various temperatures (30–80°C) for 6, 12 and 24 h.

The effects of metal ions on protease activity were measured in a solution containing 10 mM chymotrypsin substrate, 50 mM Hepes-NaOH (pH 7.5), and the 90-kDa protein (0.026 mg/mL) in the presence of various metal ions at 37°C for 30 min.

The effects of protease inhibitors were studied using phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), EDTA, and iodoacetamide. Protease activity was measured in a

solution containing 10 mM Suc-Ala-Ala-Pro-Phe-pNA, the 90-kDa protein (0.026 mg/ml), 50 mM Hepes-NaOH (pH 7.5) and 1 mM protease inhibitor at 37°C for 30 min.

The effects of some surfactants (e.g., Triton X100, Tween 20, and saponin) or denaturants (SDS and urea) were measured in a solution containing 10 mM chymotrypsin substrate, the 90-kDa protein (0.026 mg/ml), and 50 mM Hepes-NaOH (pH 7.0) in the presence of 1% surfactant, 1% SDS, or 6 M urea at 37°C for 30 min. The stability of the 90-kDa protein in the presence of SDS was examined by measuring the residual activity in a solution containing 10 mM chymotrypsin substrate, the 90-kDa protein (0.026 mg/ml), and 50 mM Hepes-NaOH (pH 7.5) after incubation with 0.1%, 0.5%, or 1% SDS at 37°C for 1, 2, 3, 6, 12, or 24 h.

One unit of protease activity was defined as the release of 1 nmol of pNA or MCA per min at 37°C.

Each experiment was performed at least twice and usually three times for confirming the result.

### Digestion of Milk Casein with the 90-kDa Protein

Milk casein (0.33 mg/ml) was digested in a solution containing 10 mM Tris-HCl (pH 10.5), 6 M urea, and 0.026 mg/ml 90-kDa protein at 37°C for 12 h. The digested fragments were concentrated with ZipTip C18, and subsequently spotted onto the MALDI sample plate with a solution of  $\alpha$ -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid (TFA). Mass spectra were acquired on an Ultraflex tandem time-of-flight (TOF/TOF) instrument (Bruker Daltonics, MA, U.S.A.) in the positive reflection mode. The TOF/TOF spectra of casein fragments was measured, data were processed by flex analysis and BioTools™ software (Bruker Daltonics), and each fragment was assigned using the MASCOT search engine (Perkins *et al.*, 1999).

Digested casein fragments from casein were loaded on a C18 reverse-phase column equilibrated with 0.1% TFA and eluted using a concentration gradient (0–50%) of acetonitrile. Three fragments were isolated, and N-terminal amino acid sequence analysis were performed using an Applied Biosystems 491 protein sequencer (Perkin-Elmer, MA, U.S.A.). The determined amino acid sequences were assigned by BLAST analysis.

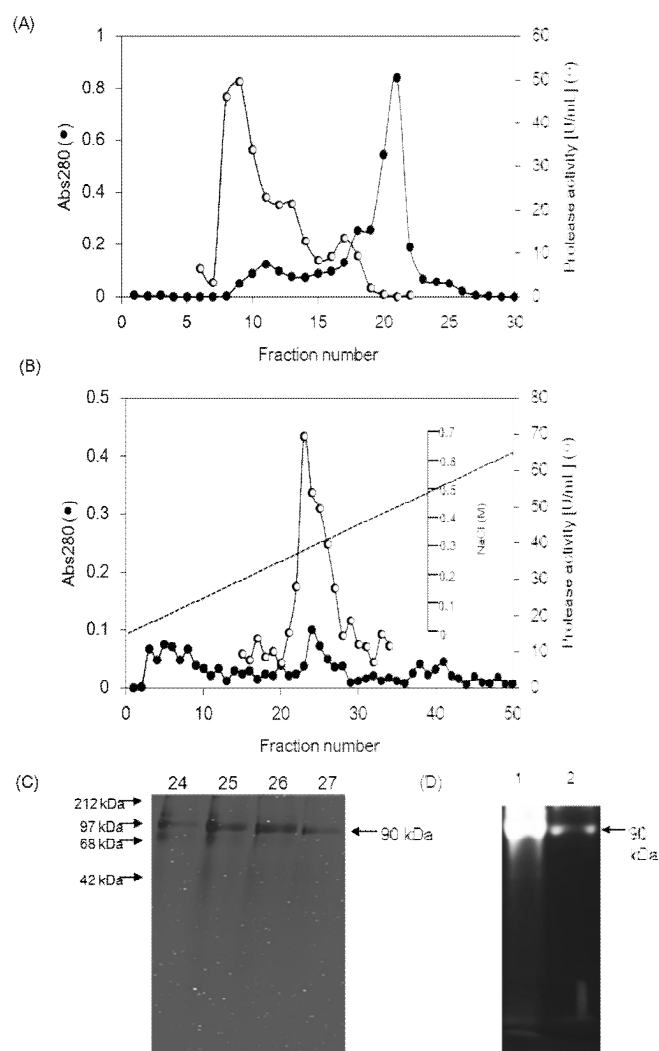
### Results

We previously showed that scallop shell extract could promote chymotrypsin activity (Liu *et al.*, 2002). In the process of searching for chymotrypsin activity-promoting substances, we found that the scallop shell extract shows protease activity that cleaves a chymotrypsin substrate. To detect the protease in scallop shells, the scallop shell extract was subjected to S-300 gel filtration column

chromatography (Figure 1A). Subsequently, the protease activity of each fraction was examined, and it was found in 2 fractions (fractions 9–13 and 16–18). The high molecular weight fractions (fraction 9–13) showing higher enzyme activity were pooled and subjected to DEAE-cellulose column chromatography for protease isolation. The bound proteins were eluted using a linear concentration gradient of NaCl (0–0.6 M) in 20 mM Tris-HCl (pH 7.5) (Figure 1B). The protease activity was observed in a single peak, which was eluted around the 0.3 M NaCl fraction. SDS-PAGE analysis of this peak did not show any bands by CBB staining, but showed a main band with a molecular weight of approximately 90 kDa by Stains-all staining (Figure 1C). To purify the protease further, the fractions after DEAE-cellulose were separated by native-PAGE. The band with a molecular weight of approximately 90 kDa (90-kDa protein) was excised and the protein was recovered from the gel by electrophoretic elution. A summary of the protease purification is given in Table 1. The protease was purified 17-fold with a yield of 9.3% from the scallop shell extract. Protease activity of isolated 90-kDa protein was also confirmed by zymography (Figure 1D). Interestingly, the 90-kDa protein had the same molecular weight as a free radical-scavenging protein that we previously isolated (Mitsubishi *et al.*, 2013). In fact, the 90-kDa protein showed scavenging activity for superoxide anion radicals produced by xanthine and xanthine oxidase (data not shown). The reduction of xanthine oxidase activity was not observed during measurement. These results show that the 90-kDa protein was identical to a free radical scavenging protein in scallop shells.

The effect of temperature on protease activity was examined by measuring the activity at different temperatures by using a chymotrypsin substrate (Figure 2A). The 90-kDa protein was active over a broad temperature range of 10–80°C, and the optimal temperature for enzyme activity was found to be 60°C. The activity declined at higher temperatures, but even at 80°C, approximately 80% of the maximum activity was retained. The thermal stability was determined by incubating the 90-kDa protein at different temperatures, followed by measuring the residual activity (Figure 2B). The 90-kDa protein retained more than 70% of its activity after exposure to 30°C for 24 h. The activity declined to approximately 27% upon exposure to 60°C for 24 h, but approximately 60% of the activity remained even after treatment at 50°C for 24 h, suggesting that the 90-kDa protein is a heat-stable protease.

The effect of pH on the protease activity was investigated at 37°C (Figure 3). Protease activity was present over a wide pH range, between pH 2.5 and pH 10.5. The highest activity was observed at pH 10.5, at which the activity was approximately 3-fold more than that at pH 6.0. This result shows that the protease is an alkaline protease, which has optimal activity at an alkaline pH.



**Figure 1.** The 90k-Da protein was isolated by S-300 gel filtration column chromatography (A) and DEAE-cellulose ion exchange chromatography (B). SDS-PAGE of the indicated fraction number after DEAE-cellulose ion exchange column chromatography. The gel was stained with Stains-all (C). Zymograph of isolated 90k-Da protein. Lane 1, scallop extract, 90k-Da protein (D).

**Table 1.** Summary of purification of protease from shell extract

Step	Protein (mg)	Specific activity (U/mg protein)	Activity (U)	Purification (fold)	Recovery (%)
Scallop shell extract	19.78	7.85	155.3	1	100
S-300 gel filtration	1.29	82	105.8	10.4	97.8
DEAE-cellulose	0.77	102.7	79.1	13.1	51
Native-PAGE	0.11	131.1	14.4	16.8	9.3

The effects of the denaturants, SDS and urea, on the protease activity were examined. Approximately 90% of the enzyme activity was maintained in the presence of 1.0% or 6M urea (Figure 4A and 4B). The protease retained the activity of more than 70% in the presence of ionic (1% saponin) and non-ionic (1% Tween 20 and 1% Triton X-100) surfactants (Figure 4C). The stability of the 90-kDa protein in the

presence of SDS was examined (Figure 4D). Approximately 50% of the activity remained, even after incubation for 24 h in the presence of 0.5% SDS, suggesting that the 90-kDa protein is resistant to SDS.

The effects of various inhibitors on the protease activity were examined (Table 2). Protease was inhibited by DFP and PMSF, but EDTA and iodoacetamide did not inhibit protease activity. These

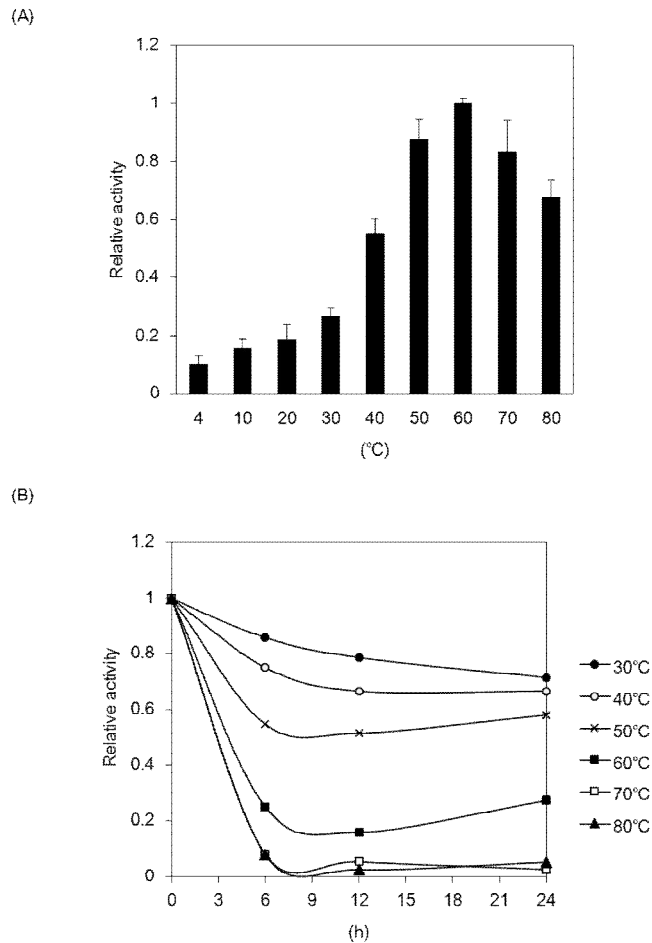


Figure 2. Effect of temperature on protease activity (A) and (B).

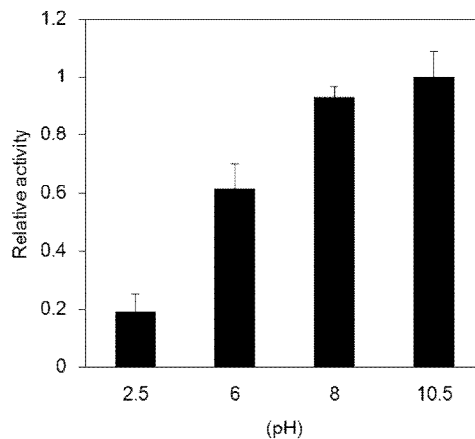
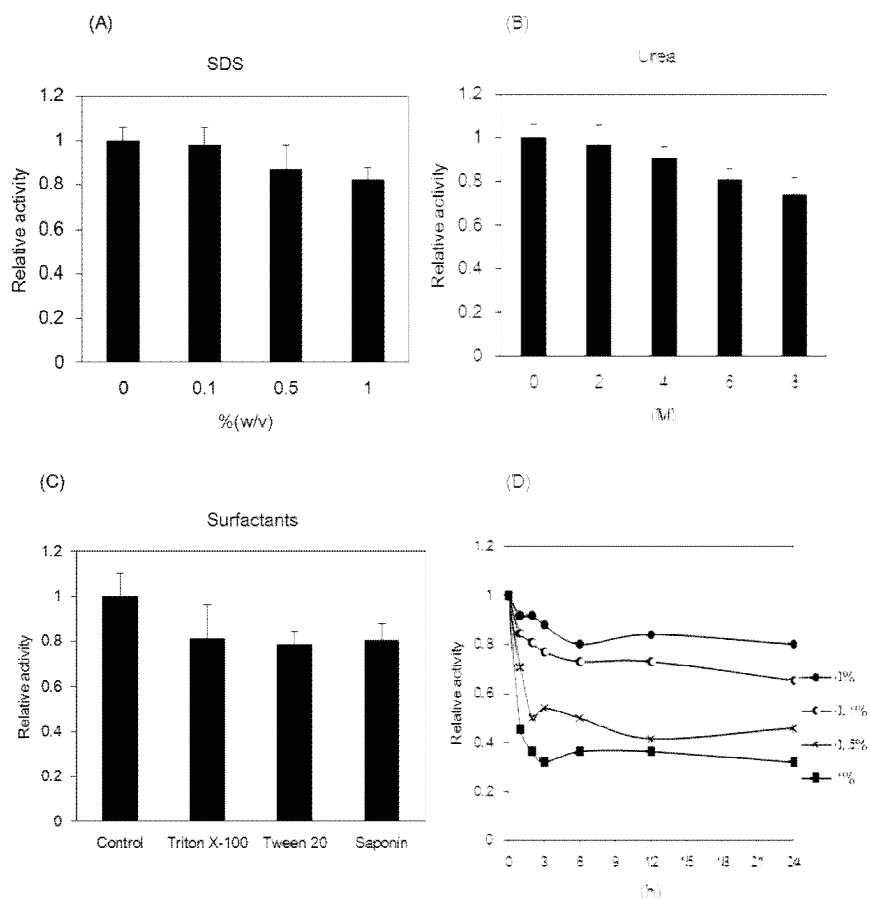


Figure 3. Effect of pH on protease activity.

results indicated that the 90-kDa protein is a serine protease. Among metal ions,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Fe}^{3+}$  ions inhibited the activity to 27%, 62%, and 49%, respectively, but  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  did not affect the enzyme activity (Table 1).  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Mn}^{2+}$  tended to promote enzyme activity.

Substrate specificity of the protease was examined at 37°C and pH 7.5 by using several peptidyl-*p*NA and peptidyl-MCAs as substrates

(Table 3). The 90-kDa protein preferentially cleaved the trypsin substrate Boc-Leu-Ser-Thr-Arg-*p*NA, Glt-Gly-Arg-MCA, and Z-His-Glu-Lys-MCA. Similarly, the 90-kDa protein efficiently cleaved the  $\alpha$ -chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-*p*NA, but Bz-Tyr-*p*NA, Suc-Leu-Leu-Val-Tyr-MCA, Suc-Ile-Ile-Trp-MCA and Suc-Ala-Ala-Pro-Phe-MCA, also  $\alpha$ -chymotrypsin substrate, were not cleaved. These results suggest that the 90-kDa protein



**Figure 4.** Effect of SDS (A), urea (B) and surfactants (C) on protease activity. Stability of the 90-kDa protein in the presence of SDS (D)

**Table 2.** Effect of inhibitors and metal ions on protease activity

Control	1.00
DEP	0.1
PMSF	0.24
EDTA	0.82
Iodacetamide	0.85
CaCl <sub>2</sub>	1.16
MgCl <sub>2</sub>	1.14
NiSO <sub>4</sub>	1.00
MnCl <sub>2</sub>	1.11
CoSO <sub>4</sub>	1.04
ZnSO <sub>4</sub>	0.67
CuSO <sub>4</sub>	0.27
Fe <sub>2</sub> Cl <sub>3</sub>	0.49

preferentially cleaves Arg-X and Lys-X bonds and partially cleaves Phe-X. To confirm the substrate specificity of the protease, milk casein was digested with the 90-kDa protein at 37°C for 12 h in the presence of 6 M urea. The digested fragments were separated by reverse-phase C18 column chromatography, and the N-terminal amino acid sequences of 3 isolated fragments were determined. The amino acid sequences of digested fragments were also assigned by MALDI-TOF/TOF, as described in Materials and Methods. Assignments of the digested

fragments showed that the 90-kDa protein digested casein at the C-termini of Lys, Arg, and Phe residues (Figure 5).

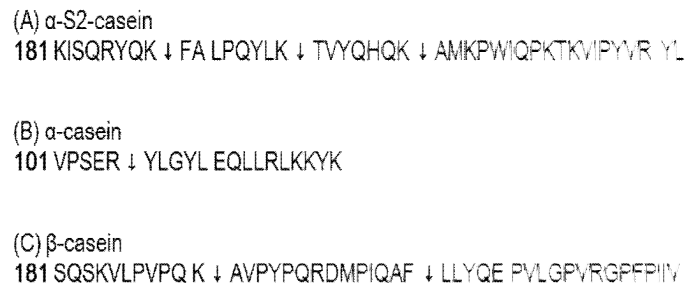
## Discussion

Alkaline proteases are defined as proteases that are active in a neutral to alkaline pH range (Gupta *et al.*, 2002). They are classified as either serine proteases or metalloproteinases. The 90-kDa protein was active at both neutral and alkaline pH, and its activity was inhibited by PMSF and DFP, suggesting that the 90-kDa protein is an alkaline serine protease. Since alkaline serine proteases are stable at alkaline pH, proteases find applications in numerous industries. Because of the utilization of proteases as industrial enzymes, such as in the detergent, food, and pharmaceutical industries, there have been many reports of alkaline proteases that show activity over a wide range of temperatures or in the presence of metal ions, surfactants, or organic solvents (Johnvesly and Naik, 2001; Rahman *et al.*, 2006; Banik and Prakash, 2004). Isolated 90-kDa protein from scallop shells also retains activity over 90% of its activity in the presence of SDS. The 90-kDa protein showed approximately 50% residual activity after incubation in the presence of 0.5% SDS for 24 h. This property

**Table 3.** Substrate specificity of 90 k-DA protease

Syntetic peptide name	Relative activity
(A)	
Boc-Leu-Ser-Thr-Arg- <i>p</i> NA	1
Bz-DL-Arg- <i>p</i> NA	0.36
Suc-Ala-Ala-Pro-Phe- <i>p</i> NA	0.63
Bz-Tyr- <i>p</i> NA	0.06
Z-Ala-Ala-Leu- <i>p</i> NA	0.08
Pyr-Phe-Leu- <i>p</i> NA	0.11
Gly-Pro- <i>p</i> NA Tos	0.12
Suc-Ala-Ala-Ala- <i>p</i> NA	0.06
(B)	
Glt-Gly-Arg-MCA	1
Z-His-Glu-Lys-MCA	1.64
Suc-Ala-Ala-Pro-Phe-MCA	0.08
Suc-Leu-Leu-Val-Tyr- MCA	0.05
Suc-Ile-Ile-Trp- MCA	0.01

The activity was represented as relative activity against the activity in Boc-Leu-Leu-Ser-Thr-Arg-*p*NA (A) and Glt-Gly-Arg-MCA (B).



**Figure 5.** Determination of cleavage sites of milk casein by the 90-kDa protein. The amino acid sequences of  $\alpha$ -S2 casein (accession no: P02662), and  $\beta$ -casein (accession no: P02666), and cleavage sites of the 90-kDa protein are shown by arrowheads. Amino acid numbers are indicated in the left panel.

of the 90-kDa protein may be characteristic because only a few reports have been published for SDS-stable enzymes (Oberoi *et al.*, 2001; Joo *et al.*, 2003). Stability at high temperature, an alkaline pH, and resistance to several surfactants of the 90-kDa protein may be attractive as industrial enzymes. However, the utilization of the 90-kDa protein as an industrial enzyme may be difficult because the relative activity of the 90-kDa protein is about one-thousandth that of trypsin (data not shown).

While many alkaline proteases show broad substrate specificity (Anwar and Saleemuddin, 1998), the 90-kDa protein has relatively higher substrate specificity. Studies of substrate specificity, using several synthetic peptide substrates, showed that the 90-kDa protein preferentially cleaves Arg-X and Lys-X bonds, and partially cleaves Phe-X bond. Digestion of milk casein by the 90-kDa protein showed that the protein could cleave Lys-X, Phe-X, and Arg-X bonds. The 90-kDa protein did not cleave all of Lys-X and Arg-X bonds in casein. The 90-kDa protein cleaved Suc-Ala-Ala-Pro-Phe-*p*NA, but Suc-Ala-Ala-Pro-Phe-MCA was not cleaved. These results suggest that the digestion may be influenced by the surrounding structure of Lys, Arg, and Phe residues. The substrate specificity of 90-kDa protein may be useful for

proteome analysis because the 90-kDa protein can cleave proteins in the presence of 0.5% SDS, and limited autolysis of the 90-kDa protein does not occur during incubation at 37°C for 24 h (data not shown).

This is the first report that the scallop shell contains a protease. Hjelmeland *et al.* (1983) found that a trypsin-like protease acts as an antibacterial factor in rainbow trout mucus. Aranishi (1999) suggested that cathepsin L- and B-like proteases from the epidermal cell layer of the Japanese eel are involved in an integral mechanism in primary defense. The 90-kDa protein may also act as an antibacterial agent on the scallop shell.

The 90-kDa protein is a glycoprotein (Mitsubishi *et al.*, 2013). Amino acid composition analysis of the isolated 90-kDa protein showed that the 90-kDa protein is rich in Asx (Asp or Asn), Ser, and Gly residues. The amino acid composition is similar to those of soluble shell matrix proteins (Sarashina and Endo, 2001), but not to those of proteases such as trypsin, chymotrypsin, and alkaline proteases. In addition, BLAST analysis of partial amino acid sequences of the 90-kDa protein did not show homology to other proteases. It will be necessary to determine the complete sequence and compare it to sequences of other proteases.

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