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Purification and Characterization of a Fish Scale-Degrading Enzyme from a Newly Identified *Vogesella* sp.

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The objective of the present study is to purify and characterize the fish scale-degrading enzyme from *Vogesella* sp.7307-1, which was newly identified and isolated from fish scales. The enzyme from *Vogesella* sp.7307-1 was assayed with casein and confirmed as a protease. Crude protease was extracted, isolated, and purified 35.7-fold with 19.6% recovery using 20−80% saturation of ammonium sulfate fractionation, Q FF ion exchange chromatography, and Superdex 200 gelfiltration. The molecular weight of the purified enzyme was 119 kDa. The K_m and V_{max} were 0.067 mM and 425.5 U/mg-min, respectively using azo-casein as substrate. The optimum pH of the purified enzyme was 7.5, and the optimum temperature was 50 °C. The enzyme was stable at temperatures below 55 °C and pH range 7.5 to 9.0. The enzyme activity of the purified protease was completely inhibited by EDTA (ethylene diamine teraacetates), indicating the enzyme was a metalloprotease. Hydrolysates from fish scales treated with protease 7307-1 were found having low molecular weight peptides (<1 kDa). The protease 7307-1 is a promising enzyme for preparing smaller peptides from fish scales.

KEYWORDS: Fish scale; metalloprotease; purification; *Vogesella* sp.; collagen peptide

INTRODUCTION

In general, round fish contain about 20−25% edible meat and 75−80% recoverable waste on a live weight basis. The recoverable wastes are predominately viscera, heads, bone, skin, and scale. Some of these wastes were used as fish meal or fertilizer. But, most of them were discarded without valuable utilization. This may result not only in environmental pollution but also in lost value-added byproduct inside the waste. For example, the processing of fish fillets produced numerous skins bearing scales. The skins and scales were discarded before. But, researchers nowadays have paid attention to the recovery of collagen from fish skins and scales (*1*−*4*). The collagen obtained became a useful byproduct and is used widely in pharmaceutical, cosmetic, and biomedical materials and food (*5*).

Main sources of industrial collagen are derived from pig and bovine skin and bones. Since the outbreaks of mad cow disease as well as foot-and-mouth disease in pigs, anxieties regarding the uses of land animal collagen have arisen (*6*). In addition, the use of collagen from the skin and bone of pigs is not allowed in some regions due to religious reasons. This has motivated research efforts toward finding alternative collagen. Thus, the demand for marine collagen has increased year-by-year.

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Marine collagens are commercially produced either from fish skins or scales. Fish skin consists of 6−10% total body weight of round fish, while fish scales contribute to only 3−4%. The recovery of collagen from fish skins is higher than that from fish scales. However, fish skins contain about 3−6% of lipid and fish scale only 0.06% (*7*). Off-flavor caused by lipid oxidation was an important problem during the processing of skin collagen. Fish scale collagen does not suffer such oxidation problems. Nevertheless, both fish skins and scales have type-1 collagen, which is similar to that from human skin (*1*−*4*). The collagen from fish scales is often used in hydrolysate form, i.e., collagen peptide, in practical application probably due to its physical properties, tight structure, and thermal stability (*1*, *2*, *8*). Moreover, collagen peptide was used widely in functional foods, food additives, and cosmetics. There are three ways for preparing collagen peptide from fish scales. (1) Chemical method: treating fish scales by using acidic or alkaline reagents (*6*, *9*). (2) Enzymatic method: using protease to hydrolyze fish scales (*5*, *10*). (3) Microbial fermentation: using microorganisms as a source of enzyme to obtain the collagen peptide (*11*). Among the three methods, the enzymatic method seems to be the best way for preparing collagen peptide from fish scales due to it is efficiency and environmental friendliness. Enzymes used in industries are mostly derived from microorganisms, such as alcalase, protamex, flavourzyme, or protease-N (*12*−*14*). Collagen peptides prepared from fish scales treated with these enzymes had molecular weights of about 2−20 kDa (*15*). However, several researchers had indicated that only

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smaller molecules of peptides possessed physiological functions, such as antioxidative activity (*16*−*18*), antihypertensive activity (*12*, *13*), and ability to proliferate human keratinocytes (*18*).

In this study, we describe an enzyme having the ability to produce low molecular weight peptides (< 1 kDa) from fish scales. This enzyme is an extracellular protease derived from a newly identified bacterial strain and is a promising enzyme for future applications.

MATERIALS AND METHODS

Fish Scales. Fish scales were collected from a fishery processing plant that produced tilapia fillets at Pingtung in Southern Taiwan. Fish scales used in microorganism screening were fresh ones which were stored in cold conditions and sent to the laboratory immediately. Fish scales for routine use in culture medium were washed and dried at 100 °C for 2 h. The dried fish scales were then milled into powder and stored at room temperature until use (*19*).

Microorganism and Growth Conditions. The bacterium used in this study was isolated and purified from a fresh fish scale. In purification of the bacterium, the fish-scale enrichment culture containing 200 mL of sterile distilled water and 0.5% fresh fish scale was incubated with shaking at 150 rev min^{-1}. After 24 h of culture, the enrichment broth was diluted with sterile distilled water and spread onto a casein medium. A single colony was picked from the casein medium after several streaking and picking series to verify a pure culture. A bacterial strain was isolated and identified as a new strain, *Vogesella* sp. 7307-1 (abbreviated as 7307-1) according to its 16S rDNA sequence. The strain 7307-1 was found to have the ability not only to produce protease but also to completely degrade intact fish scales. Routine preparation of crude protease was obtained from the centrifuged growth medium of 7037-1 incubation. The compositions of growth medium were as follows: 0.016% tryptone, 0.027% yeast extract, 0.005% sodium chloride, 0.0005% trace element stock solution, and 0.5% fish scale powder. The trace element stock solution contained per liter: 2.86 g of H_3BO_3, 2.03 g of $MnSO_4 \cdot 4H_2O$, 0.22 g of $ZnSO_4 \cdot 7H_2O$, 0.08 g of $CuSO_4 \cdot 5H_2O$, 0.14 g of $Na_2MoO_4 \cdot 2H_2O$, and 0.1 g of $FeCl_3$. The pH of medium was adjusted to 7.5 and sterilized by an autoclave. The bacterium was cultivated in test tubes (20 by 1.5 cm), each of which contained 10 mL of culture medium. After one day of incubation, 10 mL of the culture medium was transferred to a 3-L flask containing 1.0 L of medium. After 4 days of incubation, the medium was collected for enzyme purification. All incubations were done at 37 °C with shaking at 150 rpm in a shaker (Yieder Co., Taiwan).

16S rDNA Sequencing and Phylogenetic Analysis. Amplification and sequence analysis of the 16S rRNA gene was done according to the method of Chen el al (*20*). Universal Primers FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RD1 (5′-CAGGCCTAACACATGCAAGTC-3′) were used for amplification of bacterial 16S rRNA genes by PCR. These primers correspond to nucleotide positions 8−27 and 1524−1540 of the *Escherichia coli* 16S rRNA gene, respectively, and can be used for amplifying the nearly full-length 16S rRNA gene. The sequence was compared with data available in GenBank and Ribosomal Database Project II. The multiple-sequence alignment including strain 7307-1 and its closest relatives was performed using the BioEdit software (*21*). The phylogenetic reconstruction was inferred by using the neighbor-joining method (*22*). A bootstrap analysis (confidence values estimated from 1000 replications of each sequence) was performed using the CLUSTAL w 1.7 program (*23*). A phylogenetic tree was drawn using the TREEVIEW program (*24*). Sequence identities were calculated using the BioEdit software (*21*).

Enzyme Purification. All steps were conducted at 4 °C. Three liters of a 4-day culture of *Vogesella* sp. 7307-1 were centrifuged at 20,000*g* at 4 °C for 30 min, and the supernatant was filtered through a filter paper (Toyo No. 1) to obtain a crude enzyme, which was then fractionated by ammonium sulfate precipitation at 20 to 80% saturation and centrifuged at 20,000*g* for 20 min. The pellet was dissolved in a minimal volume of Tris buffer (25 mM, pH 7.5) and dialyzed against the same buffer. The dialysate was applied to a Q FF (Amersham Pharmacia, Uppsala, Sweden) column (1.6 × 10 cm), and the enzyme was eluted with Tris buffer (25 mM, pH 7.5) or the same buffer containing 0.5 M NaCl at a flow rate of 0.5 mL/min. The Q FF column separation fractions having enzyme activity were collected and concentrated using ultrafiltration (MW cutoff 10 kD). The active fractions were pooled onto a Superdex 200 (Amersham Pharmacia, Uppsala, Sweden) column (1.0 × 30 cm) eluted with 0.05 M potassium phosphate (pH 7.5) at a flow rate of 0.5 mL/min using a Fast Protein Liquid Chromatrophy system (FPLC, Amersham Pharmacia, Uppsala, Sweden). The calibration kits (gelfiltration standard, Amersham Pharmacia, Uppsala, Sweden) for molecular weight contain catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), and ovalbumin (43 kDa). The purity of the purified enzyme was determined with 7.5% native polyacrylamide gel electrophoresis (PAGE) using a Bio-Rad mini-PAGE instrument (Hercules, Ca, USA) and silver stain for protein detection (*25*).

Assay of Enzyme Activity. The enzyme extract was incubated with fish scales at 50 °C for 2 h. The enzyme activity was determined by measuring the release of peptides or amino acids from fish scales. Fish scale powder (50 mg) was diluted with 4 mL of 0.05 M Tris buffer (pH 7.5). The reaction was initiated by adding 1 mL of enzyme extract and stopped by adding 5% TCA after 2 h of incubation at 50 °C. The reaction mixture was then filtered with filter paper (Toyo No.5) after setting at room temperature for 30 min. The peptides or amino acids released were assayed with the orthophtaldialdehyde (OPA) method (*26*) using a spectrophotometer (Amersham Pharmacia U-2000) at 340 nm. One unit(U) of enzyme activity was defined as the increased of absorbance at 340 nm by 0.01 per mL enzyme for 2 h.

Collagen Peptide Prepared from Protease. Collagen peptides were prepared from protein substrates treated with protease 7307-1 and alcalase (provided by Novo Nordisk, USA). Protein substrates include scales from tilapia, milk fish, and skins from tilapia, cobia, porcine, lamb, chicken, and duck. The reaction mixture containing 1 g of protein substrates and 100 mL of protease 7307-1 and alcalase (3000 U) in buffer solutions of designated pH was incubated at 50 °C for 2 h with constant agitation at 150 rpm. The designated pH values of protease 7307-1 and alcalase were 7.5 and 8.0, respectively. Peptides or amino acids formed were assayed with either the OPA method or Fast Protein Liquid Chromatrophy (FPLC). The FPLC system was equipped with a superdex peptide column (1.0 × 30 cm, Amersham Pharmacia, Uppsala, Sweden) at a flow rate of 0.5 mL/min and monitored at 215 nm. The Superdex peptide column showed excellent efficiency on separation of the peptides within MW 0.1 to 7 kDa as described by the supplier. The calibration standards for molecular weight contain ribonuclease (13.7 kDa), insulin (5.7 kDa), vitamin B12 (1.3 kDa), glutathione (307 Da), and tyrosine (181 Da).

RESULTS AND DISCUSSION

Bacterial Strain Identification. The bacterial strain, *Vogesella* sp. 7307-1 (abbreviated as 7307-1), was isolated from a fresh fish scale. Strain 7307-1 was able to grow at temperatures ranging from 25 to 50 °C and pH values between 6.0 and 9.0. The gram stain reaction was negative. Nearly the full length (1200 bp) of the 16S rRNA gene sequence of strain 7307-1 was obtained. Comparison of the 16S rRNA gene sequence of strain 7307-1 with available 16S rRNA gene sequences in public databases (GenBank and Ribosomal Database Project II) indicated that strain 7307-1 belonged to the family Neisseriaceae of the class Betaproteobacteria. In the phylogenetic tree based on the 16S rRNA gene sequence, strain 7307-1 formed a distinct subline within the genus *Vogesella*. Calculations of sequence similarity indicated that strain 7307-1 was closely related to *Vogesella perlucida* strain DS-28^T (99.5% similarity) and *Vogesella indigofera* strain ATCC 19706^T (97.3% similarity). Lower sequence similarities (< 94.1%) were found with representative members of all other genera listed in **Figure 1**. The strain, *Vogesella* sp. 7307-1, was found having the ability to degrade casein or gelatin, while *Vogesella perlucida* strain DS-28^T did not have that ability. Moreover, so far there are four *Vogesella* species in this genus including *V. indigofera* (*27*), *V. lacus* (*28*), V. *mureinivorans* (*29*), and *V. perlucida* (*30*). No pathogenic bacterial strain had been reported in this genus.

Purification of the Enzyme. The strain 7307-1 almost completely degraded intact fish scales within 3 days of incubation at 37 °C under 150 rev min^{-1} of shaking (data in isolation of microorganisms),

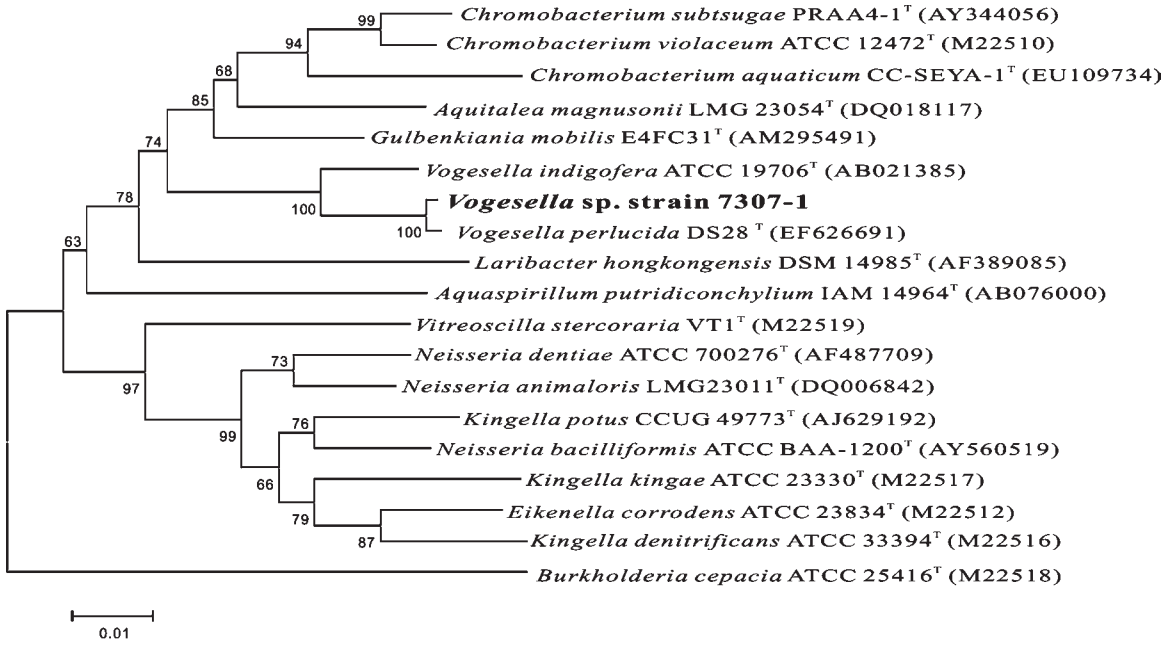

Figure 1. Phylogenetic tree of *Vogesella* sp. strain 7307-1 and related taxa in class Betaproteobacteria based on a distance matrix analysis of the 16S rDNA sequence. The neighbor-joining method was employed with the Kimura two-parameter model. Numbers at the nodes are bootstrap percentages based on both neighbor-joining tree-making algorithms. The sequence of *Burkholderia cepacia* ATCC 25416^T was used as an outgroup. The bar represents 0.01 substitutions per nucleotide position.

Table 1. Purification of Protease from *Vogesella* sp. 7307-1

stage	total activity (U[a])	total protein(mg)	specific activity (U/mg protein)	purification fold	yield (%)
crude enzyme	21600	501.1	43.1	1.0	100
20−80% $(NH_4)_2SO_4$	19030	142.4	133.6	3.1	88.1
Q FF (Q1)	15228	15.9	956.8	22.2	70.5
Sephadex 200 (S2)	4234	2.7	1538.7	35.7	19.6

[a] One unit (U) of enzyme activity was defined as the absorbance at 340 nm increased by 0.01 per mL of enzyme for 2 h.

indicative of its secretion of extracellular enzymes. The bacterium 7307-1 was cultivated for 4 days in a medium enriched with fish scales (as described in Materials and Methods). The crude extracellular enzyme was obtained by centrifuging the media at 20,000*g*. The crude enzyme was assayed with casein to confirm it as a protease (data not shown). The crude protease of 7307-1 was further purified by ammonium sulfate fractionation, Q FF ion exchange column chromatography, and gel filtration on Superdex 200 (**Table 1**). The fraction precipitated with ammonium sulfate between 20 and 80% resulted in a 3.1-fold purification with 88.1% recovery (**Table 1**). Almost 71.5% protein was removed after ammonium sulfate fractionation. Only 12% of enzyme activity was lost in this step. The dialysate of the 20−80% saturation of ammonium sulfate was applied to a Q FF column and eluted with Tris buffer or Tris buffer containing with 0.5 M NaCl (**Figure 2**). Most of the enzyme activity was found in the fractions between 40 and 90 mL eluted with Tris buffer without NaCl. Only 15% of enzyme activity was found in the fraction between 125 and 240 mL, which was eluted with Tris buffer containing 0.5 M NaCl (**Figure 2**). The recovery after Q FF ion exchange was 70.5%, and purification of enzyme activity was 22.2-fold (**Table 1**). The active fraction (Q1) from Q FF column separation was pooled onto a Superdex 200 column, and the enzyme activity was eluted between 20 and 28 mL (**Figure 3**) with 19.6% recovery and 35.7-fold purification (**Table 1**). The active fraction (S2) from Superdex 200 gel filtration was collected, dialyzed, and concentrated. The purity was examined by native polyacrylamide gel electrophoresis (PAGE). The electropherogram showed only one band (**Figure 4**), which was also

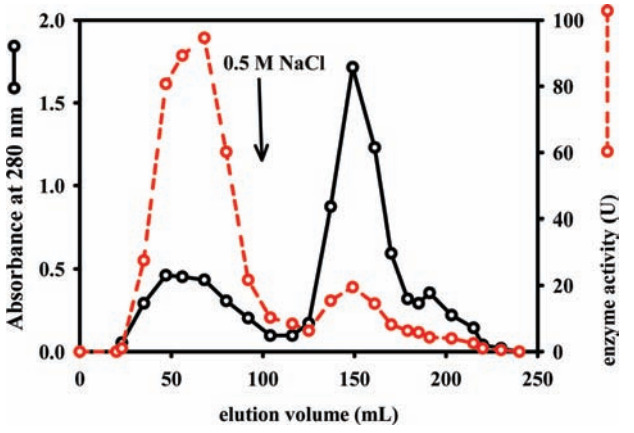

Figure 2. Anion exchange chromatography of the enzyme from *Vogesella* sp. 7307-1 on a Q FF column (1.6 × 10 cm) pre-equilibrated with 25 mM Tris buffer (pH 7.5) at a flow rate of 0.5 mL/min. The enzyme was eluted with 25 mM tris buffer (pH 7.5) and the same buffer containing 0.5 M NaCl. Fractions of 3 mL were collected and assayed for protein content and protease activity.

performed with activity staining by casein to confirm as protease (data not shown). The molecular weight of the native protease was 119 kD as shown by Superdex 200 gel filtration. When S2 was examined with SDS−PAGE, three bands were found in the electropherogram indicating that at least three subunits (85, 70, and 64 kD) existed in the enzyme structure (**Figure 5**).

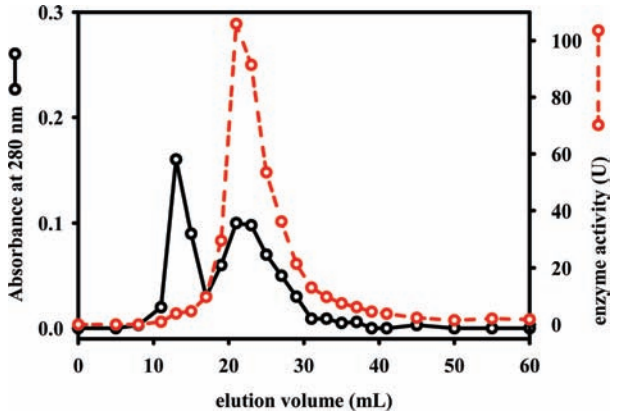

Figure 3. Gel filtration chromatography of the enzymes from *Vogesella* sp. 7307-1 on a Superdex 200 column (1 × 30 cm) equilibrated with 25 mM phosphate buffer containing 0.15 M NaCl (pH 7.5) at a flow rate of 0.5 mL/min. The enzyme were eluted with the same buffer. Fractions of 1 mL were collected and assayed for protein content and protease activity.

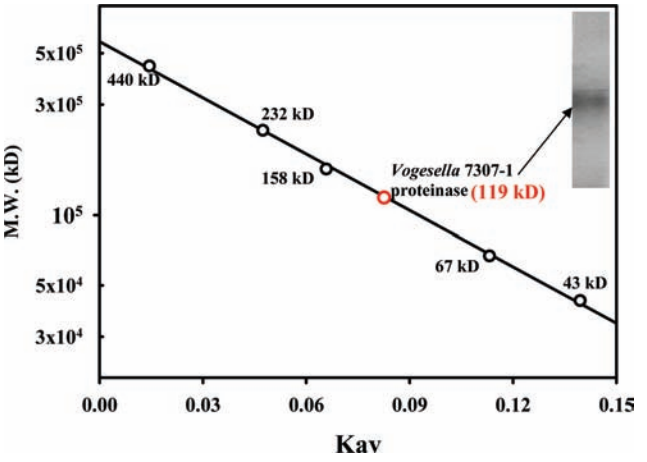

Figure 4. Estimation of the molecular weight of protease 7307-1 on Superdex 200 gel filtration.

Properties of Protease. The optimal pH of protease 7307-1 was 7.5 (**Figure 6**). At pH 9.0, 54% activity remained, and at pH 5.0, only 20% activity remained. The optimal temperature of purified protease 7307-1 was 50 °C (**Figure 7**). An abrupt decline in activity occurred above 60 °C, whereas 70% of the maximal activity remained at 35 °C. However, almost no loss in enzyme activity was found when protease 7307-1 was incubated at 50 °C for 48 h (**Figure 8**). In the practical application of commercial proteases such as alcalase, protamex, bromelin, and papain, the reaction temperature is usually 50−60 °C. Protease 7307-1 seems to be very suitable for commercial applications. The effect of inhibitors on the activity of protease 7307-1 was examined by measuring its residual activity after incubation at room temperature for 30 min in the presence of 1 mM concentration of the following inhibitors: phenylmethanesulfonyl fluoride (PMSF), glutathione (GSH), and ethylene diamine tetraacetic acid (EDTA). The activity of the protease 7307-1 was inhibited completely by EDTA, whereas inhibitors of serine and thiol proteases (PMSF and GSH) had no significant effect (**Table 2**). These results suggested that protease 7307-1 belonged to the class of metalloprotease (*31*). On the basis of a Lineweaver−Burk plot, the K_m of the purified protease 7307-1 was 0.067 mM, and the V_{max} was 425.5 U/mg-min using azocasein as the substrate (**Table 3**).

Collagen Peptide Prepared from Protease 7307-1. The commercial products of collagen peptide were usually prepared from the

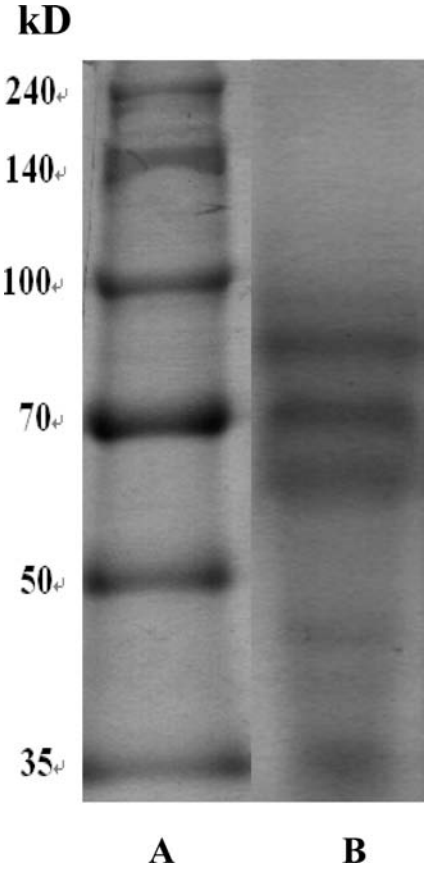

Figure 5. SDS−polyacrylamide gel electrophoresis of the purified protease 7307-1. (**A**) Molecular weight marker and (**B**) protease from Superdex 200 gel filtration.

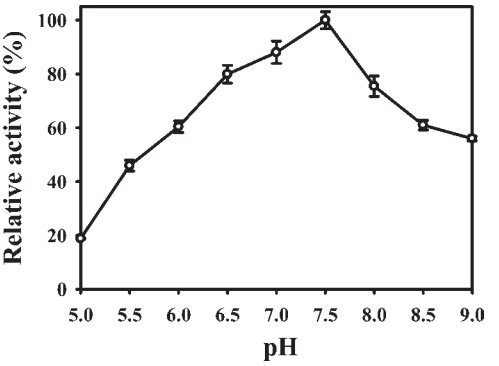

Figure 6. pH profile on the protease activity from *Vogesella* 7307-1. Fish scale was used as the substrate and reacted at 50 °C for 2 h. The buffer systems included an acetate buffer ranging in pH from 5.0 to 5.5, phosphate buffer ranging in pH from 6.0 to 7.0, Tris buffer at pH 7.5 to 8.0, and borate buffer ranging in pH from 8.5 to 9.0.

collagen of porcine skin as well as fish skin or fish scales. Various sources of collagen including scales from tilapia and milk fish, and skins from tilapia, cobia, porcine, lamb, chicken, and duck were assayed for their availability for preparing collagen peptide by using protease 7307-1. Reactivity of the enzyme toward these collagens was measured with the amount of released peptides or amino acids using the OPA method. Data obtained showed that protease 7307-1 exhibited the highest reactivity toward tilapia scales, followed by milk fish scale, lamb skin, cobia skin, tilapia skin, porcine skin, chicken skin, and duck skin (**Table 4**). Tilapia scale is a suitable substrate for producing collagen peptide using

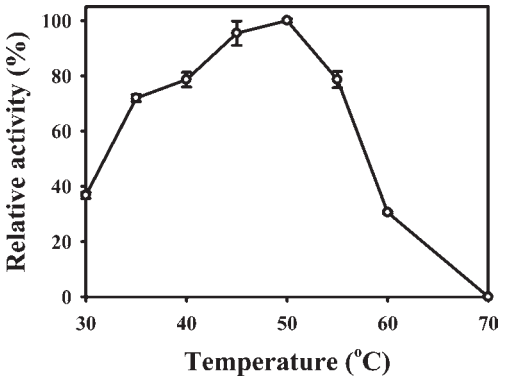

Figure 7. Temperature profile on the protease activity from *Vogesella* sp. 7307-1 in 25 mM Tris buffer (pH 7.5) using fish scale as the substrate.

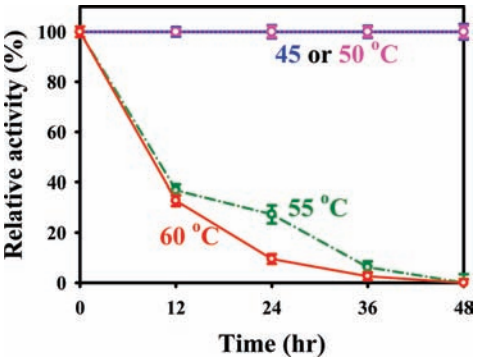

Figure 8. Thermal stability of protease 7307-1 at different temperatures for 48 h.

Table 2. Effect of Protease Inhibitors on the Enzyme Activity of Protease 7307-1

inhibitor	specificity of inhibitor	concentration (mM)	relative activity (%)
none			100.0 ± 2.03
PMSF[a]	serine protease	0.1	102.2 ± 3.53
		1.0	100 ± 3.19
GSH[b]	thiol protease	0.1	94.0 ± 1.50
		1.0	90.5 ± 1.53
EDTA[c]	metalloprotease	0.1	18.7 ± 2.9
		1.0	0 ± 3.2

[a] Phenylmethanesulfonyl fluoride. [b] Glutathione. [c] Ethylenediaminetetraacetic acid.

Table 3. Kinetic Parameters of Protease 7307-1 Using Azocasein As Substrate

pH	7.5
ionic strength	25 mM
temperature	50 °C
K_m	0.067 mM
V_{max}	425.5 U/mg-min

protease 7307-1. In order to determine the molecular weight of collagen peptides derived from tilapia scales treated with protease 7307-1, peptides formed were further analyzed using a superdex peptide column in an FPLC system monitored at 215 nm (**Figure 9**). Commercial protease of alcalase hydrolyzes fish muscle protein to form a dipeptide (*14*). Fish scales were also treated with alcalase for comparison. Fish scales in 25 mM phosphate buffer (pH 7.5) treated without enzyme (as control) showed no peptide formation (**Figure 9A**). Powdered samples of fish scales reacted with alcalase released peptides mainly of 1−10 kDa (**Figure 9B**). Fish scales treated with protease 7307-1 formed peptides of molecular distribution with

Table 4. Substrate Specificity of Protease 7307-1

substrate	relative enzyme activity (%)
tilapia scale	100.0 ± 2.4
milkfish scale	82.1 ± 3.7
tilapia skin	60.7 ± 2.5
cobia skin	64.3 ± 1.8
porcine skin	53.6 ± 0.6
lamb skin	66.3 ± 2.2
chicken skin	25.1 ± 1.7
duck skin	14.3 ± 0.7

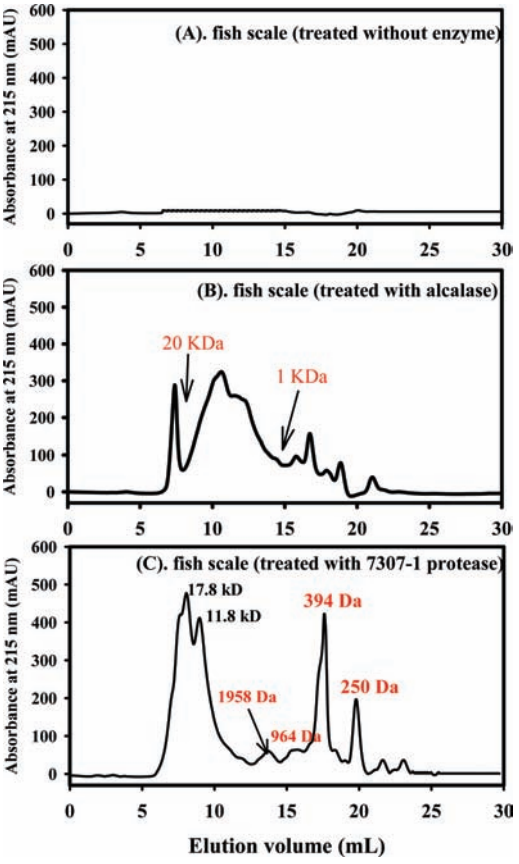

Figure 9. Gelfiltration profile on a Superdex peptide column (1 × 30 cm) for the soluble fraction of tilapia scales in 25 mM phosphate buffer (pH 7.5) treated (**A**) without adding enzyme, (**B**) with alcalase at 50 °C for 2 h, and (**C**) with protease 7307-1 at 50 °C for 2 h.

more of smaller molecular peptides than those from alcalase. Two major peaks of smaller peptides were found in 384 and 250 Da (**Figure 9C**). Four peptides having antihypertensive activity in vivo were isolated from the hydrolysate of sea bream scales. Their amino acid sequences were Gly-Tyr (256 Da), Val-Tyr (298 Da), Gly-Phe (240 Da), and Val-Ile-Tyr (429 Da) (*12*). The peptide peak with a molecular weight of 250 Da in **Figure 9C** was probably a dipeptide; the 394 Da was a tripeptide. The protease 7307-1 seems to be a promising enzyme for preparing smaller peptides from fish scales. The physiological function of the derived peptides needs further study.

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