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Conversion of Shrimp Shell by Using *Serratia* sp. TKU017 Fermentation for the Production of Enzymes and Antioxidants

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A chitinase (CHT) and a protease (PRO) were purified from the culture supernatant of *Serratia* sp. TKU017, with shrimp shell as the sole carbon/nitrogen source. The molecular masses of CHT and PRO determined by SDS–PAGE were approximately 65 kDa and 53 kDa, respectively. CHT was inhibited by Mn^{2+} and Cu^{2+}, and PRO was inhibited by most tested divalent metals and EDTA. The optimum pH, optimum temperature, pH stability, and thermal stability of CHT and PRO were pH 5, 50°C, pH 5–7, and <50°C, and pH 9, 40°C, pH 5–11, and <40°C, respectively. PRO retained 95% of its protease activity in the presence of 0.5 mM SDS. The result demonstrates that PRO is an SDS-resistant protease and probably has a rigid structure. The 4th-day supernatant showed the strongest antioxidant activity (70%, DPPH scavenging ability) and the highest total phenolic content (196±6.2 μg of gallic acid equiv./ml). Significant associations between the antioxidant potency and the total phenolic content, as well as between the antioxidant potency and free amino groups, were found for the supernatant. With this method, we have shown that shrimp shell wastes can be utilized and it is effective in the production of enzymes and antioxidants, facilitating its potential use in industrial applications and functional foods.

Keywords: Chitinase, protease, antioxidant activity, *Serratia* sp., shrimp shell wastes

Shrimp shell waste is an important source of bioactive molecules. The major components (on a dry weight basis) of shrimp shell waste are protein (48%), chitin (38%), and minerals (14%) [37]. Bioconversion of chitinous materials has been proposed as a waste treatment alternative for the disposal of shellfish wastes [19, 32, 38–40]. However, so far, there are few reports about the bioactive materials from the fermentation of shrimp shell waste. As the shrimp shell waste undergoes rapid putrefaction, it is necessary to preserve the material by adopting environmentally safe techniques, prior to production of enzymes and bioactive components for biotechnological and pharmaceutical applications.

Recent studies on chitin have attaracted interest for converting them to oligosaccharides, because the oligosaccharides are not only water soluble but also possess versatile functional properties such as antitumor activity and antimicrobial activity [19, 30, 38, 39]. Traditionally, chitin oligosaccharides are processed by chemical methods in industries. There are many problems existing in chemical processes, such as a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides, high cost of separation, and also environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost, and reproducibility, chitinase hydrolysis has become more and more popular in recent years [14].

Protease constitutes one of the most important groups of industrial enzymes, accounting for more than 65% of the total industrial enzyme market [1]. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention focussed on exploiting their physiological and biotechnological applications [28]. Proteases are also envisaged as having extensive applications in the development of environmentally friendly technologies, as well as in several bioremediation processes [2, 23, 41, 42]. In addition, proteases have applications in leather processing, food processing, and producing of protein hydrolysates [1]. Recently, the application of protease to the production of certain oligopeptides has received great attention as a viable alternative to the chemical approach [18].

Fermentation techniques can be used for utilization of shrimp shell waste [43, 44]. Literature survey found that

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the shrimp shell wastes are rich sources of phenolic compounds [27]. Phenolic compounds play an important role in antioxidative properties, and phenolic substances were also reported to possess a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory, and vasodilatory actions [5–7]. Besides this, the shrimp shell powder (SSP)-fermented supernatant may also be rich in some compounds with amino groups to enhance its antioxidant properties. It is expected that this bioactive material-rich liquor will have beneficial biological functions owing to the inherent protein and chitin hydrolysis and other bioactive materials production occurring during fermentation. Protein hydrolysates are known to possess strong antioxidative properties [15].

The bacterium *Serratia marcescens* has been widely described as a good chitinase and protease producer [4, 9, 21, 24, 26, 36]. Although *S. marcescens* is well known as a pathogen, these purified enzymes are widely used for medical [46] and industrial [9, 16, 21, 24] applications. In this study, we have recently isolated a novel *Serratia* strain from the soil, producing a chitinase and a protease at the same time and with an unusually broad pH activity and thermostability spectra. The chitinase and protease were also purified, characterized, and compared with chitinases and proteases isolated from other bacterial sources. In addition, this method is effective in the production of antioxidants by *Serratia* sp. TKU017 fermentation. We assessed the phenolic content, free amino groups, and the DPPH free-radical-scavenging activity of the fermented supernatant. The correlation between the antioxidant activity and phenolic content, as well as between the antioxidant activity and free amino groups, was also considered.

Materials and Methods

Materials

The squid pen powder (SPP) and shrimp shell powder (SSP) used in these experiments were prepared as described earlier [43]. Squid pens and shrimp shells were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). For the preparation, the squid pens and shrimp shells were washed thoroughly with tap water and then dried. The dried materials obtained were milled to powders for use as the carbon source for enzymes production. Casein, albumin, gelatin, hemoglobin, fibrin, elastin, keratin azure, myoglobin, azocasein, and azoalbumin were from Sigma Co. DEAE-Sepharose CL-6B, phenyl Sepharose, and Sephacryl S-100 were purchased from GE Healthcare U.K. Ltd (Little Chalfont, Buckinghamshire, England). All other reagents used were of the highest grade available.

Isolation and Screening of Chitinase/Protease-producing Strains

Microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 1% SSP, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 1.5% agar powder (pH 7). The plates were incubated at 25°C for 2 days. Those organisms obtained from the screening were subcultured in liquid media (containing 1% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) in shaking flasks at 25°C and 150 rpm. After incubation for 2 days, the culture broth was centrifuged (4°C and 8,200 ×*g* for 20 min; Kubota 5922) and the supernatants were collected for measurement of chitinase/protease activity using the procedure described below. The strain TKU017 that showed the highest chitinase/protease activity was isolated, maintained on nutrient agar, and used throughout the study.

Enzyme Production and Purification

In the investigation of the culture condition, growth was carried out in a basal medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7), and supplemented with 0.5–2% (w/v) of various carbon sources to be investigated. The carbon sources investigated included SSP or SPP. Various volumes of the resultant media were aerobically cultured at 25°C for 1–5 days on a rotary shaker (150 rpm; Yih Der LM-570R) in 250-ml Erlenmeyer flasks. After centrifugation (12,000 ×*g*, 4°C, for 20 min), the supernatants were collected for measurement of chitinase and protease activities. Maximum chitinase and protease activities were obtained when *Serratia* sp. TKU017 was grown aerobically in 100 ml of the optimum medium (2.0% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) in a 250-ml Erlenmeyer flask at 25°C and pH 7. To reflect the growth of the culture for 12–120 h in this medium by the OD 660 nm measurement, the residual SSP was allowed to settle out.

Production of chitinase and protease. For the production of chitinase and protease, *Serratia* sp. TKU017 was grown in 100 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 2% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7). One ml of the seed culture was transferred into 100 ml of the same medium and was grown in an orbital shaking incubator for 3 days at 25°C and at pH 7 (the pH was 7.5 after autoclaving). After incubation, the culture broth was centrifuged (4°C and 12,000 ×*g* for 20 min), and the supernatant was used for further purification by chromatography.

DEAE-Sepharose CL-6B chromatography. To the culture supernatant (1,070 ml), ammonium sulfate was added (608 g/l). The resultant mixture was kept at 4°C overnight and the precipitate formed was collected by centrifugation at 4°C for 20 min at 12,000 ×*g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), and was dialyzed against the buffer. The resultant dialyzate was loaded onto a DEAE-Sepharose CL-6B

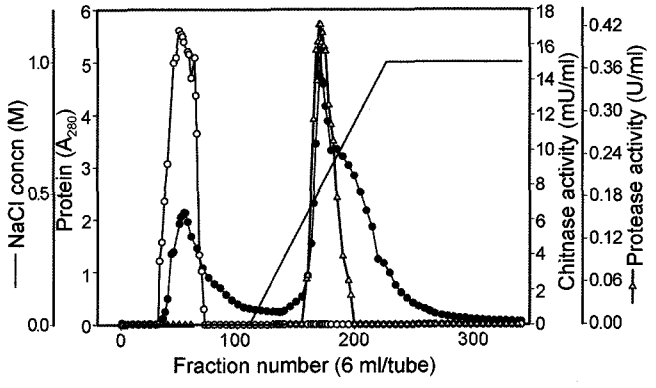

Fig. 1. Elution profile of TKU017 chitinase and protease on DEAE-Sepharose CL-6B: (●) absorbance at 280 nm; (○) chitinase activity (mU/ml); (△) protease activity (U/ml).

column (5×30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). As shown in Fig. 1, one chitinase (CHT) was washed from the column with the same buffer and one protease (PRO) was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions of the two protein peaks showing high chitinase/protease activities were respectively combined and concentrated by ammonium sulfate precipitation. The resultant precipitates were collected by centrifugation and dissolved in 5 ml of 50 mM sodium phosphate buffer (pH 7).

Phenyl Sepharose chromatography. The obtained enzyme solutions (CHT and PRO) were then respectively chromatographed on a column of phenyl Sepharose (1.3×20 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7) containing 1 M $(NH_4)_2SO_4$. CHT was washed from the column with the same buffer and PRO was eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$ in the same buffer. As shown in Fig. 2A and 2B, the CHT and PRO fractions were collected respectively and the enzyme activity was measured. Fractions with confirmed enzyme activity were pooled, dialyzed overnight at 4°C against 50 mM sodium phosphate buffer (pH 7), and used as a purified preparation.

Sephacryl S-100 chromatography. The resultant enzyme solution (CHT and PRO) was loaded respectively onto a Sephacryl S-100 gel filtration column (2.5×120 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7), and was then eluted with the same buffer. Two peaks exhibiting chitinase and protease activities were respectively obtained, combined, and lyophilized.

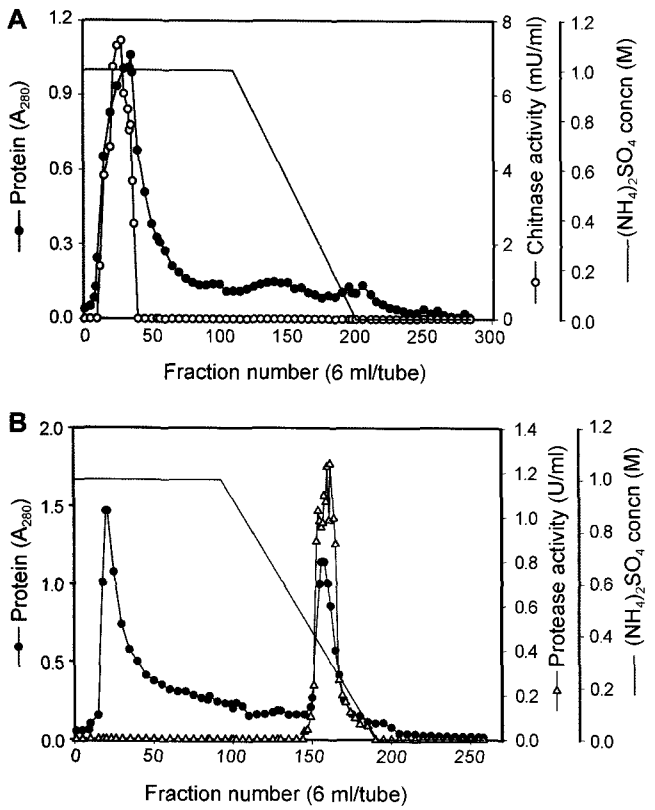

Fig. 2. Elution profile of TKU017 chitinase and protease on phenyl Sepharose 6 Fast Flow.
A. Chitinase fractions; **B.** protease fractions. (●) Absorbance at 280 nm; (○) chitinase activity (mU/ml); (△) protease activity (U/ml).

Protein Determination

Protein content was determined by the method of Bradford [3] using Bio-Rad dye reagent concentrate and bovine serum albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.

Measurement of Enzyme Activity

Colloidal chitin (1.3% in 50 mM phosphate buffer) was used as the substrate for the measurement of chitinase activity. The mixture of enzyme solution (0.5 ml) and substrate (1 ml) was incubated at 37°C for 30 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita [12] with *N*-acetylglucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmole of reducing sugars per minute.

For measuring protease activity, a diluted enzyme solution (0.2 ml) was mixed with 1.25 ml of 1.25% casein in pH 7 phosphate buffer and incubated for 30 min at 37°C. The reaction was terminated by adding 5 ml of 0.19 M trichloroacetic acid (TCA). The reaction mixture was centrifuged and the soluble peptide in the supernatant fraction was measured by the method of Todd [35] with tyrosine as the reference compound. One unit of protease activity was defined as the amount of enzyme required to release 1 μmole of tyrosine per minute.

Polyacrylamide Gel Electrophoresis and Zymograms

The molecular masses of the purified chitinase and protease were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [17]. The standard proteins (Geneaid, Taiwan) used for calibration were phosphorylase b (molecular mass, 97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.3 kDa). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercaptoethanol. The gel of PRO was stained with Coomassie Brilliant Blue R-250 in methanol–acetic acid–water [5:1:5 (v/v)], and was decolorized in 7% acetic acid. The protein band of CHT on the gel after electrophoresis was visualized by silver staining. The molecular masses of CHT and PRO in the native form were also determined by a gel filtration method. The sample and standard proteins were applied to a Sephacryl S-100 column (2.5×120 cm) equilibrated with 50 mM phosphate buffer (pH 7). Bovine serum albumin (molecular mass, 67 kDa), *Bacillus* sp. α-amylase (50 kDa), and hen egg white lysozyme (14 kDa) were used as molecular mass markers.

Gelatin zymography for proteolytic activity was performed in polyacrylamide slab gels containing SDS and gelatin (0.1%) as a copolymerized substrate, as described by Heussen and Dowdle [11] with some modifications. After electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 1 h at 25°C to remove the SDS and were then incubated in 50 mM phosphate buffer (pH 7). After 30 min of incubation at 37°C, the gels were stained in a solution of 0.5% (w/v) amido black 10B.

Effects of pH and Temperature on the Enzyme Activities

The optimum pHs of CHT and PRO were studied by assaying the samples at different pH values. The pH stabilities of CHT and PRO were determined by measuring the residual activity at pH 7, as described above, after the sample had been dialyzed against a 50 mM

buffer solution of various pH values (pH 3–11) in seamless cellulose tubing (Sankyo). The buffer systems used were glycine·HCl (50 mM, pH 3), acetate (50 mM, pH 4–5), phosphate (50 mM, pH 6–8), and Na_2CO_3–$NaHCO_3$ (50 mM, pH 9–11). To determine the optimum temperatures for CHT and PRO, the activity values of the samples were measured at various temperatures (25–90°C). The thermal stabilities of CHT and PRO were studied by incubating the samples at various temperatures for 30 min. The residual activity was measured as described above.

Effects of Various Chemicals

The effects of various chemicals on the enzyme activity were investigated by preincubating the enzyme with chemicals in 50 mM phosphate buffer solution (pH 7) for 10 min at 37°C, followed by measuring the residual chitinase and protease activities.

Effects of Various Surfactants

The enzyme solutions (250 μl) were incubated, in the absence or presence of 0.25 ml of surfactant solutions, at 25°C for 30 min. The residual activities were estimated by the assay procedure described above.

Scavenging Ability on 1,1-Diphenyl-2-Picrylhydrazyl Radicals

TKU017 culture supernatant (150 μl) was mixed with 37.5 μl of methanolic solution containing 0.75 mM DPPH (Sigma) radicals. The mixture was shaken vigorously and was left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank [29]. The scavenging ability was calculated as follows: Scavenging ability (%)=[(ΔA_{517} of control–ΔA_{517} of sample)/ΔA_{517} of control]×100.

Thin-Layer Chromatography Analysis

The antioxidant materials produced from TKU017 culture supernatant were analyzed by silica gel thin-layer chromatography (TLC). TLC analysis was performed on silica gel using CH_2Cl_2–MeOH in various proportions as the mobile phase. Silica gel TLC plates (0.25 mm) were obtained from E. Merck. Compounds were detected by spraying a *p*-anisaldehyde–ethanol–sulfuric acid mixture followed by heating at 100°C for 5 min. Another system was analyzed by using 5:4:3 (v/v/v) *n*-butanol–methanol–16% aqueous ammonia as the mobile phase [14]. After developing the TLC plates, the compounds were visualized by spraying with an aqueous solution of 2.4% (w/v) phosphomolybdic acid, 5% (v/v) H_2SO_4, and 1.5% (v/v) H_3PO_4 (phosphomolybdic acid reagent) or ethanol containing 0.5% (w/v) ninhydrin (ninhidrin reagent) or the DPPH solution described above, followed by heating.

Determination of Total Phenolic Content

The total phenolics of the culture supernatants were determined [13] and expressed as micrograms of gallic acid equivalents per milliliter of culture supernatant. Two ml of deionized water and 1 ml of Folin–Ciocalteu's phenol reagent were added to 0.3 ml of each sample. Five ml of 20% aqueous sodium carbonate solution (w/v) was added and mixed well, and then the mixture was allowed to stand at ambient temperature for 20 min. Absorbance of the developed dark blue-purple color was measured by a spectrophotometer at 735 nm. The content of total phenolics in each sample was determined using a standard curve prepared with gallic acid at varied concentrations (0, 50, 100, 200, 400, 600, and 800 mg/ml).

Results and Discussion

Identification of the Strain TKU017

TKU017 is a Gram-negative and nonspore-forming bacterium, with catalase and oxidase, which grows in both aerobic and anaerobic environments. According to the result of a 16S rDNA partial base sequence and the API identification system, TKU017 is most close to *Serratia* sp., such as *Serratia marcescens* subsp. *marcescens, S. marcescens* subsp. *sakuensis*, and *S. ureilytica*, and the similarity of 16S rDNA partial base sequence was more than 98%. The identification of strain TKU017 was carried out by the Bioresource Collection and Research Center (Shin-Chu, Taiwan).

Culture Conditions and Enzyme Production

In our previous experiments (data not shown), we found that 100 ml of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing 1% SSP was better for the production of chitinase and protease by strain TKU017 at 25°C for 3 days. To study the effect of SSP concentration on the production of chitinase and protease, we found that 2% (w/v) of SSP was more suitable for the production of chitinase and protease than the concentrations of 0.5%, 1%, and 1.5% (data not shown). To investigate the effects of carbon and nitrogen sources on the production of these enzymes, growth was carried out in 100 ml of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing additional carbon/nitrogen sources [2% (w/v)] of SSP or SPP, respectively. It was found that SSP (12 mU/ml and 0.18 U/ml, respectively) was more suitable as an inducer for the production of chitinase and protease than SPP (5 mU/ml and 0.14 U/ml, respectively). Therefore, the shrimp shell was chosen to be investigated by the following experiment. To study the time course of cultivation, 100 ml of the medium (2% SSP-containing basal medium, pH 7) was used, and the relationships between incubation time (12–120 h), and chitinase and protease activities were investigated. As shown in Fig. 3, both the cell concentration and enzyme activity reached the maximum after 3 days of fermentation and decreased gradually thereafter. This result indicates that production of chitinase and protease is cell growth dependent.

Isolation and Purification

The purification of the TKU017 chitinase and protease from the culture supernatant (1,070 ml) is described in the Materials and Methods section. First, the supernatant was submitted to ion-exchange chromatography (Fig. 1) showing two protein peaks, the unadsorbed fractions that display chitinase activity and the adsorbed fractions that display protease activity. The unadsorbed chitinase fractions and the adsorbed protease fractions were collected and combined, followed by hydrophobic interaction chromatography,

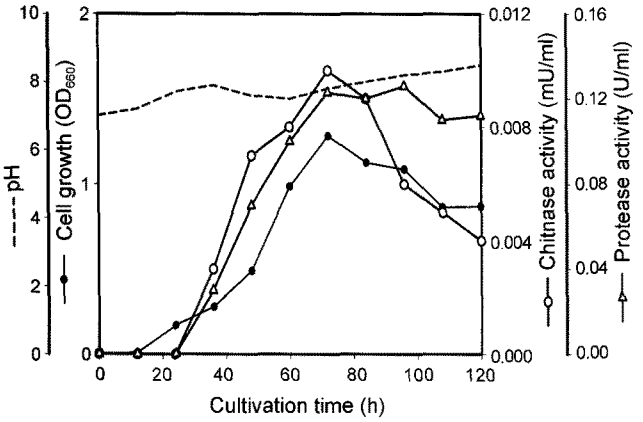

Fig. 3. Time courses of cell growth, and chitinase and protease production in a culture of *Serratia* sp. TKU017 on shrimp-shell-containing media: (●) cell growth; (○) chitinase activity (U/ml); (△) protease activity (U/ml); (---) pH.

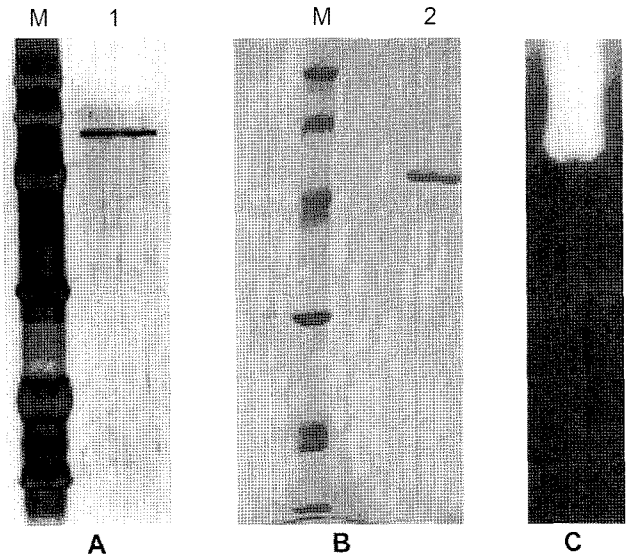

Fig. 4. SDS-PAGE analysis of the purified chitinase (**A**) and protease (**B**) produced by strain TKU017.
Lanes: M, molecular mass markers (97.4, 66.2, 45, 29, 20.1, 14.4 kDa); 1, CHT; 2, PRO. **C**. Zymogram of PRO in SDS-PAGE. Further details are described in Materials and Methods.

respectively. As shown in Fig. 2A and 2B, the chitinase and protease were purified respectively by hydrophobic interaction chromatography. As shown in Table 1, the purification steps were combined to give an overall purification of about 61.3-fold (CHT) and 30.5-fold (PRO). The overall activity yields of the purified enzymes were 2.0% (CHT) and 1.4% (PRO). The molecular masses of CHT and PRO were determined by gel filtration, which gave a peak at 66 kDa for CHT and at 50 kDa for PRO. The purified CHT and PRO were further both confirmed to be homogeneous by SDS-PAGE (Fig. 4A and 4B). The molecular masses of CHT and PRO were calculated to be 65 kDa and 53 kDa by SDS-PAGE, respectively. Their molecular masses remained unchanged with β-mercaptoethanol treatment, suggesting that the proteins were monomeric and lacked intermolecular disulfide bonds.

The molecular mass of PRO (53 kDa) was obviously smaller than most of the other *Serratia* proteases, such as *S. marcescens* serine protease (66.5 kDa) [24], *S. marcescens* metalloprotease (61 kDa) [34], and *S. rubidaea* metalloprotease CP-1 (97 kDa) [26]. The *Serratia* proteases that had the similar molecular mass as *Serratia* sp. TKU017 protease included the metalloprotease of *S. marcescens* (50 kDa) [36], *S. marcescens* ATCC 25419 (53.5 kDa) [24], *S. marcescens* (50.9 kDa) [26], and *S. ureilytica* TKU013 (P1, P2) (50 kDa, 50 kDa) [40]. With regard to CHT, the molecular mass of CHT (65 kDa) was greater than other reported *Serratia* sp. chitinases, such as *Serratia* sp. KCK (57 kDa) [16], *S. marcescens* NK1 (57 kDa) [21], *S. marcescens* BJL200 (55.5 kDa) [4], *S. marcescens* QMB1466 (58 kDa) [9], *S. marcescens* 2170 (50 and 47 kDa) [31], *S.*

Table 1. Purification of chitinase and protease from *Serratia* sp. TKU017.

Step	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification fold	Yield(%)
		CHT/PRO	CHT/PRO	CHT/PRO	CHT/PRO
Culture supernatant	20,356	15.2/473.3	0.7/23.3	1.0/1.0	100.0/100.0
$(NH_4)_2SO_4$ ppt	2,125	4.2/72.1	2.0/33.9	2.9/1.5	27.6/15.2
DEAE-Sepharose					
CHT[a]	203	2.1	10.3	14.7	13.8
PRO[b]	638	43.4	68.0	2.9	9.2
Phenyl Sepharose					
CHT	41	0.7	17.1	24.4	4.6
PRO	85	33.1	389.4	16.7	7.0
Sephacryl S-100					
CHT	7	0.3	42.9	61.3	2.0
PRO	9	6.4	711.1	30.5	1.4

[a]: Chitinase; [b]: protease.

plymuthica HRO-C48 (60.5 kDa) [8], *Serratia* sp. (35.5–58.2 kDa) [25], and *S. ureilytica* TKU013 (C1) (60 kDa) [40]. Most chitinases of *Serratia* spp. had a molecular mass in the range of 35.5–60 kDa.

Effects of pH and Temperature

The pH activity profile of CHT and PRO showed maximum values at pH 5 and pH 9, respectively. CHT and PRO were stable at pH 5–7 and pH 5–11, respectively. The optimum temperature for CHT and PRO was 50°C and 40°C, respectively. CHT maintained its initial activity from 25 to 50°C, and PRO maintained its initial activity from 25 to 40°C. However, CHT was inactivated at 70°C, and PRO was completely inactivated at 60°C (Fig. 5).

The pH optimum of PRO (pH 9) was different from that of *S. marcescens* ATCC 25419 metalloprotease (pH 8.5) [24], and *S. rubidaea* metalloprotease CP-2 (pH 8) [26]. Compared with other *Serratia* proteases, such as the metalloprotease of *S. marcescens* NRRLB-23112 (pH 6–10) [26] and two metalloproteases (CP-1, CP-2) of *S. rubidaea* (pH 6–11, pH 5–9) [26], the pH stability of PRO exhibited a rather broad pH activity range (pH 5–11). The optimum temperature of PRO (40°C) was slightly lower than that of *S. marcescens* ATCC 25419 metalloprotease (45°C) [24], *S. marcescens* metalloprotease (42°C) [26], and *S. marcescens* ATCC 25419 serine protease (48°C) [24]. The activity of PRO maintained about more than 95% of its initial activity from 25 to 40°C. It was completely inactivated at greater than 60°C. The property of thermal instability was similar to the metalloprotease of *S. marcescens* NRRLB-23112 [26], the serine protease of *S. marcescens* ATCC25419 [24], the metalloproteases (CP-1, CP-2) of *S. rubidaea* ATCC 25419 [26], and the metalloproteases (P1, P2) of *S. ureilytica* TKU013 [40].

The pH optimum of CHT (pH 5) was similar to the other *Serratia* sp. chitinases, such as that of *S. marcescens* NK1 (pH 6.2) [21], *S. marcescens* BJL200 (pH 5–6) [4], *S. ureilytica* TKU013 (pH 6) [40], and *S. plymuthica* HRO-C48 (pH 5.4 and 6.6) [8]. The optimum temperature of CHT (50°C) was similar to the other *Serratia* sp. chitinase, such as that of *S. marcescens* NK1 (47°C) [21], *S. marcescens* BJL200 (50–60°C) [4], *S. marcescens* 2170 (60°C) [31], *S. plymuthica* HRO-C48 (55°C) [8], *Serratia* sp. (55°C) [25], and *S. ureilytica* TKU013 (50°C) [40].

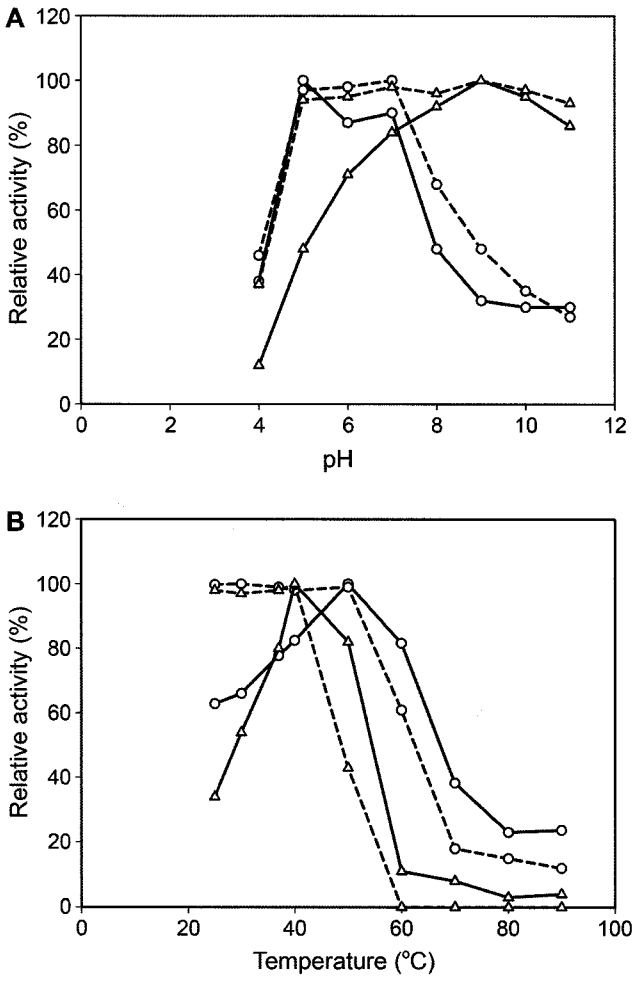

Fig. 5. Effects of pH (**A**) and temperature (**B**) on the activity (solid line) and stability (dashed line) of CHT and PRO. (○) CHT; (△) PRO.

Substrate Specificity

The activities of PRO toward various substrates are summarized in Table 2. The enzyme showed especially high activity toward casein but no activities toward fibrin and elastin. The activities of CHT upon chitin, chitosan, and other polysaccharides were investigated. The effect of the degree of deacetylation (DD) [33] of chitosan on enzyme activity was studied using chitosan of varying DD as the substrate. As shown in Table 3, CHT was specific for colloidal chitin, but had almost no activity toward

Table 2. Substrate specificity of PRO.

Substrates	Relative activity (%)	
	Method A[a]	Method B[b]
Casein	100	
Albumin	38	
Fibrin	0	
Elastin	0	
Hemoglobin	15	
Gelatin	60	
Myoglobin	3	
Azocasein		100
Azoalbumin		90

[a]The activities of these substrates were measured by the method of Todd as described in Materials and Methods.
[b]The activities of these substrates were determined by measuring the absorbance at 440 nm as described in the protocol of Sigma Co.

Table 3. Substrate specificity of CHT

Substrate	Relative activity (%)
Colloidal chitin	100
Chitin (α-type)	30
Chitin (β-type)	90
Chitosan (98% DD)	0
Chitosan (85% DD)	48
Chitosan (60% DD)	82
CMC	0
Glycol chitosan	0

chitosan with higher deacetylation. These results indicate that the physical form of the substrate affects the rate of hydrolysis.

Effects of Various Chemicals

To further characterize CHT and PRO, we next examined the effects of some known enzyme inhibitors and divalent metals on their activities. The results are summarized in Table 4. Cu^{2+} and Mn^{2+} inhibited almost completely the chitinase activity of CHT. Compared with other *Serratia* spp. chitinases, the chitinase of *S. plymuthica* HRO-C48 [8] and *S. ureilytica* TKU013 [40] were also inhibited by Cu^{2+}. However, the protease activity of PRO was inhibited slightly by most tested divalent metals. An inhibitor of serine protease [phenylmethanesulfonyl fluoride (PMSF)] had no significant effect on the enzyme activities of CHT and PRO. EDTA, a chelator of divalent cations, was an inhibitor of PRO, suggesting the metal ions were essential for the catalytic action of the enzyme.

Table 4. Effects of various chemicals on enzyme activity.

Chemicals	Concentration	Relative activity (%)	
		CHT	PRO
None	0	100	100
PMSF	5 mM	75	86
EDTA	5 (10) mM	83	54 (2)
Mg^{2+}	5 mM	90	91
Cu^{2+}	5 (10) mM	8 (4)	78 (66)
Fe^{2+}	5 mM	67	71
Ca^{2+}	5 mM	100	72
Zn^{2+}	5 mM	92	73
Mn^{2+}	5 (10) mM	28 (0)	70
Ba^{2+}	5 mM	93	85
SDS	0.5 (2) mM	23 (0)	95 (73)
Tween 20	0.5 (2)%	102 (99)	99 (94)
Tween 40	0.5 (2)%	102 (103)	100 (95)
Triton X-100	0.5 (2)%	101 (90)	98 (82)

Purified enzymes were preincubated with the various reagents at 25°C for 30 min and residual enzyme activities were determined as described in the text. One hundred percent was assigned to the activity in the absence of reagents.
Anionic surfactant: SDS.
Nonionic surfactants: Tween 20, Tween 40, Triton X-100.

Effects of Various Surfactants

Enzymes are usually inactivated by the addition of surfactants to the reaction solution. The effects of different surfactants on the stabilities of CHT and PRO were also studied. CHT and PRO were incubated with surfactants at 25°C for 30 min and the remaining enzymatic activity was determined under normal assay conditions. The enzyme activity of the sample without any surfactants (control) was taken as 100%. It was found that in the presence of 2% nonionic surfactants Tween 20, Tween 40, or Triton X-100, CHT and PRO retained more than 82% of their original activity. In the presence of 0.5 mM SDS (anionic surfactant), the activity of CHT was inhibited, but the activity of PRO was not significantly effected (Table 4). These differences between both enzymes might be related to the dissimilarity of the ratio of their hydrophobic and hydrophilic amino acids.

The above results show that PRO is resistant to SDS. The result was confirmed using the in-gel protease assay. Fig. 4C shows that SDS and β-mercaptoethanol had no effects on the protease activity of PRO. These results suggested that the disulfide bond in the protein molecule is not associated with its proteolytic activity. Besides this, in the in-gel protease assay (Fig. 4C), PRO exhibited marked gel retardation, migrating much less than in plain SDS-PAGE (Fig. 4B). The results may reflect the fact that PRO is SDS-resistant and can bind to the gelatin molecules incorporated in the SDS gel used for the in-gel assay. Indeed, after the gel was further incubated at 37°C, no matter with or without Triton X-100 treatment, the gelatin to which the protease binds degraded. This is consistent with the findings that a clear lane was found in detecting SDS-resistant protease by in-gel protease assay. These results suggest that PRO has a rigid structure and is therefore SDS-resistant. The stability of the protease from *Serratia* sp. TKU017 may make it useful for industrial applications.

Scavenging Ability on 1,1-Diphenyl-2-Picrylhydrazyl Radicals

It has been reported that chitin, chitosan, and peptides have antioxidative [10, 20, 22, 40, 45] and anticarcinogenic [19, 39] properties. To increase the utilization of these chitin/protein-containing shrimp shell wastes, we incubated *Serratia* sp. TKU017 for 1–5 days under the optimal culture conditions described above (0.5–2% SSP, 25°C) and analyzed the antioxidant activity of the culture supernatant. The antioxidant activity assayed was the DPPH scavenging ability. It was found that TKU017 culture supernatant (2% SSP) incubated for 4 days had the highest antioxidant activity, where the DPPH scavenging ability of the TKU017 culture supernatant was about 70% (Fig. 6). In further investigations on the effects of different carbon/nitrogen (SPP) on the production of antioxidant materials by TKU017, the antioxidant

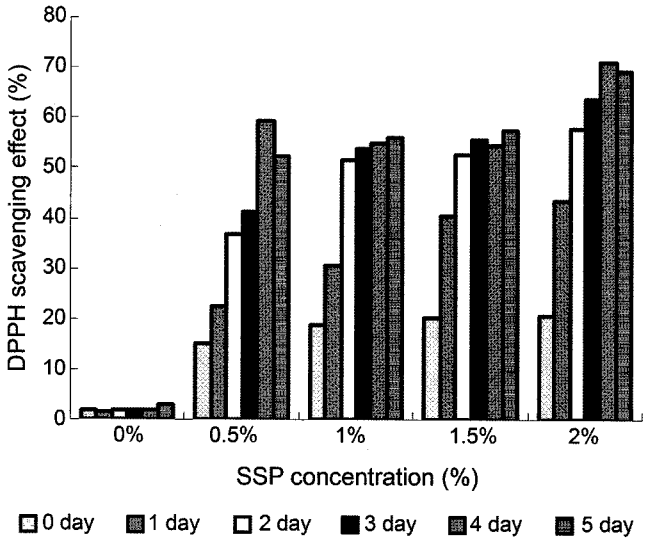

Fig. 6. DPPH scavenging effects of the culture supernatants at various concentrations of SSP by using *Serratia* sp. TKU017 fermentation.

activity had no significant increase (data not shown). To analyze the antioxidant activity of the culture medium at day 0, we heated these marine wastes in an autoclave (121°C for 15 min), and the antioxidant activities were 15–20% and 20–30% in the SSP and SPP supernatants, respectively. However, as shown in Fig. 6, it was found that the antioxidant activities increased after fermentation by TKU017. SSP was the suitable carbon/nitrogen source for antioxidant materials production by strain TKU017. It is assumed that even though the treatment (121°C for 15 min) degrades the marine waste and produces some of the antioxidant materials, most of the antioxidant materials are produced by strain TKU017.

Determination of the Antioxidative Components

It has been reported that shrimp shell waste contains natural antioxidants, mainly phenolic compounds [27]. In an attempt to elucidate the antioxidative components in TKU017-fermented supernatant, the contents of total polyphenols in the supernatants were determined. As shown in Table 5, the 4th day supernatant contained the most abundant amount of total polyphenols (196±6.2 μg of gallic acid equiv./ml), followed by the 5th day supernatant (182±3.5 μg of gallic acid equiv./ml), the 3rd day supernatant (160±5.3 μg of gallic acid equiv./ml), and the 2nd day supernatant (125±4.6 μg of gallic acid equiv./ml). Data analysis revealed that the supernatants with high phenolic contents also showed high DPPH radical-scavenging activity (Table 5). This result suggested that phenolic compounds might be responsible for the activity.

As shown in Table 5, the antioxidant activity of the 4th day supernatant was the highest than the others, so the 4th day supernatant was selected for further study. The

Table 5. Antioxidative assay and contents of total polyphenols of TKU017 culture supernatants.

Cultivation time (day)	DPPH scavenging ability (%)	Total polyphenols[a] (μg of gallic acid equival/ml of culture supernatant)
0	20.8	nd[b]
1	43.5	nd
2	57.9	125±4.6d
3	63.7	160±5.3c
4	70.9	196±6.2a
5	69.4	182±3.5b

[a]All data are expressed as mean±SD from three different experiments (each experiment was conducted in triplicate). Data with different letters are significantly different at $p<0.05$.
[b]nd, not detected.

antioxidant compounds in the 4th day supernatant were analyzed using TLC. The supernatant was developed on the TLC plates, and three compounds were visualized by spraying with anisaldehyde reagent, ninhydrin reagent, phosphomolybdic acid reagent, and DPPH solution (data not shown). These results suggest that the antioxidant compounds in the 4th day supernatant had free amino groups and phenolic compounds. However, it was difficult to use the current data to elucidate the chemical structure of the compound in the supernatant unless it was compared with the respective authentic standards. We will further purify and confirm the structure by NMR, IR, and mass spectral analyses in the future.

Compared with the optimal enzyme production conditions, the difference in culture time was that the optimal enzyme production was on the 3rd day but the production of antioxidant materials was on the 4th day. The increase in antioxidant activity of culture supernatant in the culture time suggested that antioxidant materials were related to hydrolysis of the enzymes. To study the relationship between the antioxidant production and the enzymes (chitinase or protease), we measured the antioxidant activity respectively after the incubation of chitin/protein-containing materials (SSP, SPP, colloidal chitin, and chitin) with the purified enzymes at 37°C for 1 h. As shown in Table 6, the antioxidant activities of the purified chitinase and protease were 12.40% and 6.23%, respectively. With the same concentrations, these hydrolysates showed higher antioxidant activity than only chitinase or protease, especially using SSP as the substrate (Table 6). The result suggested that the chitooligosaccharides and peptides present in the hydrolysates were related to antioxidant activity. However, the antioxidant activity of the 4th day culture supernatant was still the highest (70%). These results suggested that antioxidant components in the culture supernatant might contain the hydrolysates from TKU017 enzymes and other materials fermented by TKU017. The increase in antioxidant activity might also be due to the synergistic effect of the hydrolysates and

Table 6. Antioxidative assay of the various hydrolysates from the purified chitinase and protease.

Sample	DPPH scavenging ability (%)
SSP	12.24
SPP	6.89
Colloidal chitin	4.92
Chitin	4.54
SSP+chitinase	44.48
SPP+chitinase	20.77
Colloidal chitin+chitinase	19.13
Chitin+chitinase	16.50
Chitinase	12.40
SSP+protease	35.74
SPP+protease	16.83
Colloidal chitin+protease	6.74
Chitin+protease	6.05
Protease	6.23

other materials. Another chitosanase/protease-producing strain (*Serratia marcescens* TKU011) in our laboratory can also use shrimp shell wastes as the sole carbon/nitrogen source. The antioxidant activity of the culture supernatant was analyzed and compared with that of strain TKU017. The results showed that the DPPH scavenging ability of the culture supernatant of *S. marcescens* TKU011 and *Serratia* sp. TKU017 was about 22% and 70%, respectively. We investigated the effects of different carbon/nitrogen (shrimp shell powder, crab shell powder, squid pen powder, chitin powder, chitosan powder) on the production of antioxidant materials by TKU011, and found no increase on the antioxidant activity (data not shown). *S. marcescens* TKU011 was a chitosanase/protease-producing strain, whereas *Serratia* sp. TKU017 was a chitinase/protease-producing strain. The most obvious difference between the enzymes produced by these two protease-producing strains in *Serratia* species is that strain TKU011 does not have chitinase activity whereas strain TKU017 dose, which is the difference in the other reported *Serratia* that, have either chitosanase or chitinase. The different results of antioxidant productivity might be related to the substrate specificity of these two enzymes.

In conclusion, we have purified and characterized chitinase and protease from the culture supernatant of *Serratia* sp. TKU017 using shrimp shell as the sole carbon/nitrogen source. In addition, it was found that the culture supernatant has antioxidant activity as well.

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