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ORIGINAL PAPER

Conversion and degradation of shellfish wastes by *Serratia* sp. TKU016 fermentation for the production of enzymes and bioactive materials

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Abstract A chitosanase and a protease were purified from the culture supernatant of *Serratia* sp. TKU016 with shrimp shell as the sole carbon/nitrogen source. The molecular masses of the chitosanase and protease determined by SDS–PAGE were approximately 65 and 53 kDa, respectively. The chitosanase was inhibited completely by Mn^{2+}, but the protease was enhanced by all of tested divalent metals. The optimum pH, optimum temperature, pH stability, and thermal stability of the chitosanase and protease were (pH 7, 50°C, pH 6–7, <50°C) and (pH 8–10, 40°C, pH 5–10, <50°C), respectively. SDS (2 mM) had stimulatory effect on TKU016 protease activity. The result demonstrates that TKU016 protease is SDS-resistant protease and probably has a rigid structure. Besides, TKU016 culture supernatant (2% SPP) incubated for 2 days has the highest antioxidant activity, the DPPH scavenging ability was about 76%. With this method, we have shown that shrimp shell wastes can be utilized and it's effective in the production of enzymes, antioxidants, peptide and reducing sugar, facilitating its potential use in biological applications and functional foods.

Keywords Chitosanase · Protease · Antioxidant activity · *Serratia* sp. · Shrimp shell wastes

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Introduction

Shrimp shell waste is an important source of bioactive molecules. The major components (on dry weight basis) of shrimp shell waste are protein (48%), chitin (38%), and minerals (14%) (Wang et al. 2006). Bioconversion of chitinous materials has been proposed as a waste treatment alternative for the disposal of shellfish wastes (Synowiecki and Al-Khateeb 2000; Wang et al. 2006, 2008a, 2009; Liang et al. 2007). 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 adopting the environmentally safe techniques, prior to production of enzymes and bioactive components for biotechnological and pharmaceutical applications.

Most chitosanases are found in microorganisms and a few are found in plants (Lee et al. 2006; Su et al. 2006). However, to date, there was no paper

reported about the production of chitosanase by *Serratia* spp. except *S. ureilytica* TKU011 (Wang et al. 2008b). Almost all of the chitosanase-producing strains will use soluble chitosan or chitosan as a major carbon source. However, preparation of chitin/chitosan involves demineralization and deproteinization of shellfish waste with the use of strong acids or bases (Wang et al. 2006; Liang et al. 2007). The utilization of shellfish waste not only solves environmental problems but also decreases the production cost of microbial chitosanases. The production of inexpensive chitosanase is an important element in the process.

Protease constitutes one of the most important groups of industrial enzymes, accounting for more than 65% of the total industrial enzyme market (Banik and Prakash 2004). 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 (Shikha and Darmwal 2007). Proteases are also envisaged as having extensive applications in the development of environmentally friendly technologies, as well as in several bioremediation processes (Bhaskar et al. 2007; Roberts et al. 2007). In addition, proteases have applications in leather processing, food processing and producing of protein hydrolysates (Banik and Prakash 2004). Recently, the application of protease to the production of certain oligopeptides has received great attention as a viable alternative to chemical approach (Lee et al. 1993).

Fermentation technique can be used for utilization of shrimp shell waste (Wang et al. 2008b). Protein hydrolysates are known to possess strong antioxidative properties (Kim and Mendis 2006). The SSP fermented supernatant may 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 due to the inherent protein and chitin hydrolysis and other bioactive materials production occurring during fermentation.

In this study, we have recently isolated a novel *Serratia* strain from the soil producing a chitosanase and a protease at the same time and with unusually broad pH activity and thermostability spectra. The chitosanase and protease were also purified, characterized, and compared with chitosanase and protease isolated from other bacterial sources. In addition, this method by using another cheap carbon/nitrogen source of squid pen powder is effective in the production of antioxidants, peptide and reducing sugar by *Serratia* sp. TKU016 fermentation.

Materials and methods

Materials

The squid pen powder (SPP) and shrimp shell powder (SSP) used in these experiments were prepared as described earlier (Wang et al. 2008b). Squid pens and shrimp shells were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). For the preparation of the squid pens and shrimp shells were washed thoroughly with tap water and then dried. The dried materials obtained were milled to powders for using 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 and Sephacryl S-100 were purchased from GE Healthcare UK Ltd (Little Chalfont, Buckinghamshire, England). All other reagents used were of the highest grade available.

Isolation and screening of chitosanase/protease producing strains

Microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 0.5% 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 30°C for 2 days. Those organisms obtained from the screening were subcultured in liquid media (containing 0.5% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) in shaking flasks at 30°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 chitosanase/protease activity using the procedure described below. The strain TKU016 that showed the highest chitosanase/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 volume of the resultant media were aerobically cultured at 30°C for 1–5 days on a rotary shaker (150 rpm, Yih Der LM-570R) in 250 ml Erlenmeyer flask. After centrifugation (12,000*g*, 4°C, for 20 min), the supernatants were collected for measurement of chitosanase and protease activity.

Production of chitosanase and protease

For the production of chitosanase and protease, *Serratia* sp. TKU016 was grown in 100 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 1% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7). One milliliter 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 30°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 (1,020 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 column (5 × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). As shown in Fig. 1, one chitosanase was washed from the column with the same buffer and one protease was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions of the two protein peaks showing high chitosanase/protease activity 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).

Sephacryl S-100 chromatography

The resultant enzyme solution (chitosanase and protease) 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 chitosanase and protease activity were respectively obtained, combined, and lyophilized.

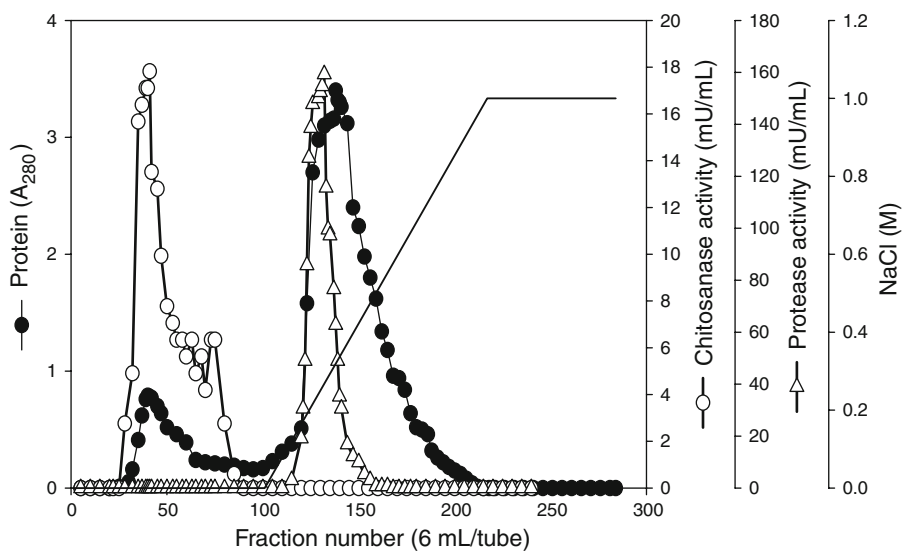

Fig. 1 Elution profile of TKU016 chitosanase and protease on DEAE-Sepharose CL-6B: (*filled circle*) absorbance at 280 nm; (*opened circle*) chitosanase activity (mU/ml); (*opened square*) protease activity (mU/ml)

Protein determination

Protein content was determined by the method of Bradford (1976) 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

Chitosanase activity of the enzyme was measured by incubating 0.2 ml of the enzyme solution with 1 ml of 0.3% (w/v) water soluble chitosan in 50 mM phosphate buffer, pH 7 at 37°C for 30 min. The reaction was stopped by heating it at 100°C for 15 min. The amount of reducing sugar produced was measured by the method of Imoto and Yagishita (1971) with glucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol 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 with tyrosine as the reference compound (Todd 1949). One unit of protease activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per minute.

Polyacrylamide gel electrophoresis and zymograms

The molecular masses of the purified chitosanase and protease were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970). The standard proteins (Geneaid, Taiwan) used for calibration were phosphorylase b (molecular weight, 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 TKU016 protease 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 TKU016 chitosanase on the gel after electrophoresis was visualized by silver staining. The molecular masses of chitosanase and protease 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 co-polymerized substrate, as described by Heussen and Dowdle (1980) with some modifications. After electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 1 h at 25°C to remove 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.

Effect of pH and temperature on the enzyme activities

The optimum pH of chitosanase and protease were studied by assaying the samples at different pH values. The pH stability of chitosanase and protease 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 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 temperature for chitosanase and protease, the activity values of the samples were measured at various temperatures (25–90°C). The thermal stability of chitosanase and protease were studied by incubating the samples at various temperatures for 30 min. The residual activity was measured as described above.

Effect of various chemicals

The effect 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 chitosanase and protease activities.

Effect 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.

Thin layer chromatography analysis

The hydrolysis products of chitosan oligosaccharides and chitin oligosaccharides produced by TKU016 crude enzyme were analyzed by Silica Gel thin layer chromatography (TLC) using 5:4:3 (v/v/v) *n*-butanol/methanol/16% aqueous ammonia as the mobile phase (Kadokura et al. 2007). 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), followed by heating.

Scavenging ability on 1,1-dipheny l-2-picrylhydrazyl radicals

TKU016 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 (Shimada et al. 1992). The scavenging ability was calculated as follows: Scavenging ability (%) = [(ΔA_{517} of control-ΔA_{517} of sample)/ΔA_{517} of control] × 100.

Results and discussion

Identification of the strain TKU016

TKU016 is a gram-negative and non-spore-forming bacillus, 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, TKU016 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%. On the basis of glucose metabolic patterns, TKU016 conforms to the description of the *Serratia* species. From these data, we conclude that TKU016 belongs to the *Serratia* species. Further identification by the Bioresource Collection and Research Center (Shin-Chu, Taiwan), found that TKU016 belongs to a new species strain.

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 0.5% SSP was better for the production of chitosanase and protease by strain TKU016 at 30°C for 3 days. To study the effect of SSP concentration on the production of chitosanase and protease, we found that 1% (w/v) of SSP was more suitable for the production of chitosanase and protease than the concentrations of 0.5, 1.5, and 2% (data not shown). To investigate the effect 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 (1%, w/v) of SSP or SPP, respectively. It was found that SSP (22 mU/ml and 160 mU/ml, respectively) was more suitable as an inducer for the production of chitosanase and protease than SPP (8 mU/ml and 90 mU/ml, respectively). Therefore, the shrimp shell was chosen to investigate by the following experiment. To study the time course of cultivation, 100 ml of the media (1% SSP contained basal medium, pH 7) was used, and the relationship between incubation time (1–5 days), chitosanase and protease activity were investigated. The maximum activities of chitosanase and protease were both found at the third day and then decreased gradually (Fig. 2).

Isolation and purification

The purification of the TKU016 chitosanase and protease from the culture supernatant (1,020 ml) is described in the "Materials and methods". First, the supernatant was submitted to ion exchange

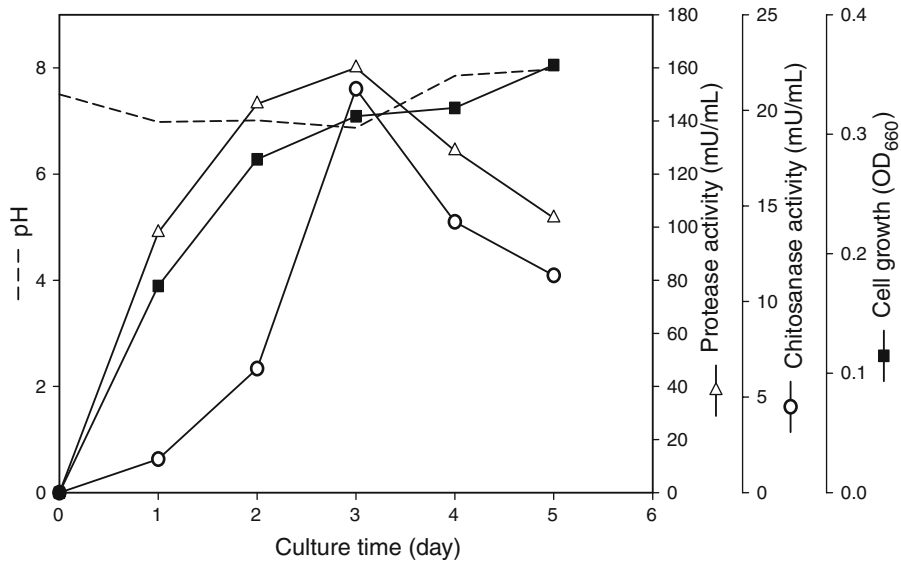

Fig. 2 Time courses of chitosanase and protease production in a culture of *Serratia* sp. TKU016 on shrimp shell containing media: (*opened circle*) chitosanase activity (mU/ml); (*opened square*) protease activity (mU/ml); (*solid line*) pH; (*filled square*) OD_{660}

chromatography (Fig. 1) showing two protein peaks, the unadsorbed fractions that display chitosanase activity and the adsorbed fractions that display protease activity. The unadsorbed chitosanase fractions and the adsorbed protease fractions were collected and combined followed by gel filtration chromatography, respectively. The chitosanase and protease were purified respectively. As shown in Table 1, the purification steps were combined to give an overall purification of about 21.7-fold (chitosanase) and 21.6-fold (protease). The overall activity yields of the purified enzymes were 5.7 (chitosanase) and 9.8% (protease). The molecular masses of chitosanase and protease were determined by gel filtration, which gave a peak at 63 kDa for chitosanase and at 50 kDa for protease. The purified chitosanase and protease were further both confirmed to be homogeneous by SDS–PAGE (Fig. 3a, b). The molecular masses of chitosanase and protease were calculated to be 65 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 TKU016 protease (53 kDa) was obviously smaller than most of the other *Serratia* proteases, such as, *S. marcescens* serine protease (66.5 kDa) (Romero et al. 2001), *S. marcescens*

Table 1 Purification of chitosanase and protease from *Serratia* sp. TKU016

Step	Total protein (mg)	Total activity (mU) chitosanase/ protease	Specific activity (mU/mg) chitosanase/ protease	Purification fold chitosanase/ protease	Yield(%) chitosanase/ protease
Culture supernatant	12,210	16,230/74,152	1.3/6.1	1.0/1.0	100.0/100.0
$(NH_4)_2SO_4$ ppt	1,420	3,830/46,258	2.7/32.6	2.1/5.3	23.6/62.4
DEAE-sepharose					
Chitosanase	210	1,240	5.9	4.5	7.6
Protease	138	14,450	104.7	17.2	19.5
Sephacryl S-100					
Chitosanase	33	930	28.2	21.7	5.7
Protease	55	7,250	131.8	21.6	9.8

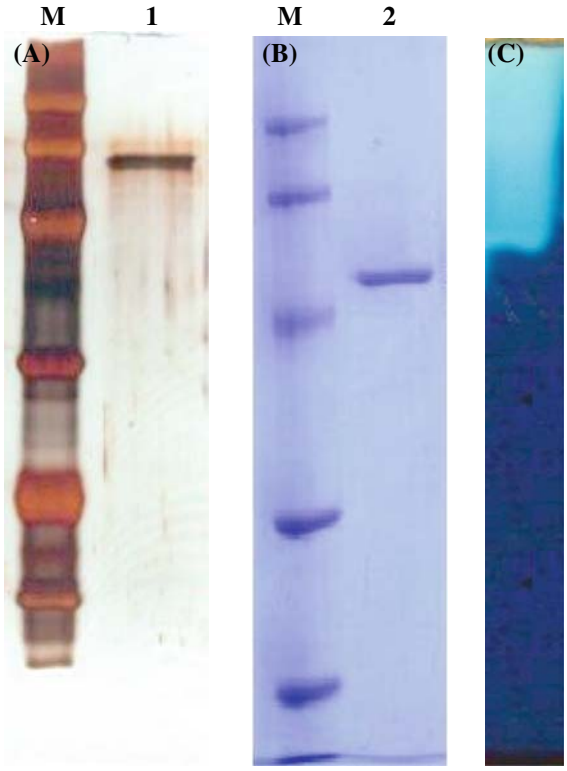

Fig. 3 SDS–PAGE analysis of the purified chitosanase (**a**) and protease (**b**) produced by strain TKU016. *Lanes*: *M*, molecular markers (97.4, 66.2, 45, 29, 20.1, 14.4 kDa); *1*, chitosanase; *2*, protease. (**c**) Zymograms of TKU016 protease in SDS–PAGE. Further details are described in "Materials and methods"

metalloprotease (61 kDa) (Tao et al. 2006), and *S. rubidaea* metalloproteases CP-1 (97 kDa) (Salamone and Wodzinski 1997). The *Serratia* proteases that had the similar molecular mass as *Serratia* sp. TKU016 protease included the metalloprotease of *S. marcescens* (50 kDa) (Ulrich 1994), *S. marcescens* ATCC 25419 (53.5 kDa) (Romero et al. 2001), *S. marcescens* (50.9 kDa) (Salamone and Wodzinski 1997), and *S. ureilytica* TKU013 (P1, P2) (50 kDa, 50 kDa) (Wang et al. 2009). With regard to TKU016 chitosanase, the molecular mass of TKU016 chitosanase (65 kDa) is greater than other reported *Serratia* sp. chitinases/chitosanases, such as, *Serratia* sp. KCK (57 kDa) (Kim et al. 2007), *S. marcescens* NK1 (57 kDa) (Nawani and Kapadnis 2001), *S. marcescens* BJL200 (55.5 kDa) (Brurberg et al. 1996), *S. marcescens* QMB1466 (58 kDa) (Green et al. 2005), *S. marcescens* 2170 (50 and 47 kDa) (Suzuki et al. 2002), *S. plymuthica* HRO-C48 (60.5 kDa) (Frankowski et al. 2001), *Serratia* sp. (35.5–58.2 kDa) (Sakurai et al. 1995), and *S. ureilytica* TKU013 (C1) (60 kDa) (Wang et al. 2009). Most chitinases/chitosanases of *Serratia* spp. had molecular mass in the range of 35.5–60 kDa.

Effect of pH and temperature

The pH activity profile of TKU016 chitosanase and protease showed maximum values at pH 7 and pH 8–10, respectively. The chitosanase and protease were stable at pH 6–7 and pH 5–10, respectively. The optimum temperature for the chitosanase and protease were 50 and 40°C, respectively. The chitosanase and protease both maintained their initial activity at <50°C. However, The chitosanase was inactivated completely at 80°C, and the protease was inactivated at 60°C (Fig. 4).

The pH optimum of TKU016 chitosanase (pH 7) was higher than the other *Serratia* sp. chitinase/

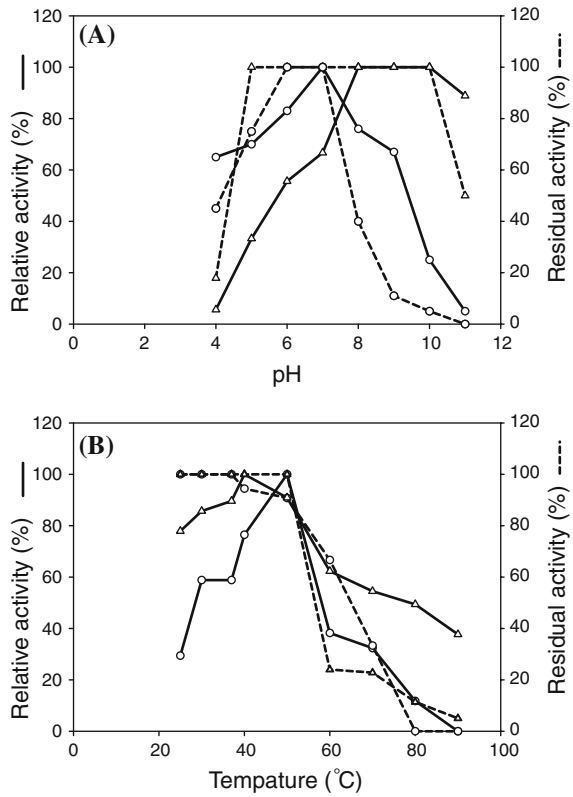

Fig. 4 Effect of pH (**a**) and temperature (**b**) on the activity (*solid line*) and stability (*dashed line*) of the purified chitosanase and protease. (*opened circle*) chitosanase; (*opened square*) protease

chitosanase, such as, 6.2 of *S. marcescens* NK1 (Nawani and Kapadnis 2001), 5–6 of *S. marcescens* BJL200 (Brurberg et al. 1996), 6 of *S. ureilytica* TKU013 (Wang et al. 2009), 5.4 and 6.6 of *S. plymuthica* HRO-C48 (Frankowski et al. 2001). The optimum temperature of TKU016 chitosanase (50°C) was similar to the other *Serratia* sp. chitinase/chitosanase, such as, *S. marcescens* NK1 (47°C) (Nawani and Kapadnis 2001), *S. marcescens* BJL200 (50–60°C) (Brurberg et al. 1996), *S. marcescens* 2170 (60°C) (Suzuki et al. 2002), *S. plymuthica* HRO-C48 (55°C) (Frankowski et al. 2001), *Serratia* sp. (55°C) (Sakurai et al. 1995), and *S. ureilytica* TKU013 (50°C) (Wang et al. 2009).

The pH optimum of TKU016 protease (pH 8–10) was similar to 8.5 of *S. marcescens* ATCC 25419 metalloprotease (Romero et al. 2001), 8 of *S. rubidaea* metalloprotease CP-2 (Salamone and Wodzinski 1997). Compared with other Serratia proteases, such as, the metalloprotease of *S. marcescens* NRRLB-23112 (pH 6–10) (Salamone and Wodzinski 1997) and two metalloproteases (CP-1, CP-2) of *S. rubidaea* metalloproteases (pH 6–11, pH 5–9) (Salamone and Wodzinski 1997), the pH stability of TKU016 protease also exhibited a rather broad pH activity range (pH 5–10). The optimum temperature of TKU016 protease (40°C) was slightly lower than *S. marcescens* ATCC 25419 metalloprotease (45°C) (Romero et al. 2001), *S. marcescens* metalloprotease (42°C) (Salamone and Wodzinski 1997), and *S. marcescens* ATCC 25419 serine protease (48°C) (Romero et al. 2001). The activity of TKU016 protease maintained its initial activity from 25 to 50°C. But it was inactivated at greater than 60°C. The property of thermal instability was similar to the metalloprotease of *S. marcescens* NRRLB-23112 (Salamone and Wodzinski 1997), the serine protease of *S. marcescens* ATCC25419 (Romero et al. 2001), the metalloproteases (CP-1, CP-2) of *S. rubidaea* ATCC 25419 (Salamone and Wodzinski 1997), and the metalloproteases (P1, P2) of *S. ureilytica* TKU013 (Wang et al. 2009).

Substrate specificity

The activities of TKU016 protease 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 TKU016 chitosanase upon chitin and chitosan were investigated. The effect of the degree of deacetylation (DD) (Tan et al. 1998) of chitosan on enzyme activity was studied using chitosan of varying DD as the substrate. As shown in Table 3, TKU016 chitosanase showed activities toward chitosan and chitin. The most susceptible chitosan and chitin were 95% deacetylation and β form, respectively. Preparation 60–85% deacetylated chitosan and α form chitin were less susceptible to the enzyme action. These results indicate that the physical form of the substrate affect the rate of hydrolysis.

In Table 3, TKU016 chitosanase have high activities against chitosan (95% DD) and chitin (β-type). This enzyme may be also chitinase. The hydrolysis products of chitosan oligosaccharides and chitin oligosaccharides produced by TKU016 crude enzyme were analyzed using TLC. The TLC mobility results indicate that (GlcNAc) and $(GlcNAc)_2$ are the

Table 2 Substrate specificity of TKU016 protease

Substrates	Relative activity (%)	
	Method A[a]	Method B[b]
Casein	100	
Albumin	87	
Fibrin	0	
Elastin	0	
Hemoglobin	85	
Gelatin	60	
Myoglobin	5	
Azocasein		100
Azoalbumin		56

[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 TKU016 chitosanase

Substrate	Relative activity (%)
Chitosan (95% DD)	100
Chitosan (85% DD)	67
Chitosan (73% DD)	44
Chitosan (60% DD)	33
Glycol chitosan	23
Chitin (α-type)	44
Chitin (β-type)	100

oligosaccharides produced by the hydrolysis of TKU016 crude enzyme on β-chitin (Fig. 5b-2). The products did not react with ninhydrin (Fig. 5c-2), indicating that TKU016 crude enzyme hydrolyaed β-chitin to (GlcNAc) and $(GlcNAc)_2$. For the hydrolysis of TKU016 crude enzyme on water-soluble chitosan, phosphomolybdic acid reagent was used to visualize the oligosaccharide, TLC analysis of the reaction mixture showed two products (Fig. 5b-1), which were also visualized by ninhydrin reagent (Fig. 5c-1). These results suggest that the hydrolysates of water-soluble chitosan are a mixture of oligosaccharides possessing free amino groups. Although the mobility of the two compounds corresponded to that of chitin oligosaccharides [(GlcNAc) and $(GlcNAc)_2$], their structures need to be further confirmed in the future. However, for the hydrolysis of TKU016 crude enzyme on α-chitin, $(GlcNAc)_2$ was visualized by spraying with phosphomolybdic acid reagent (Fig. 5b-3), but no compounds were visualized by spraying with ninhydrin reagent (Fig. 5c-3). The TLC mobility results indicate that $(GlcNAc)_2$ is the oligosaccharide produced by the hydrolysis of TKU016 crude enzyme on α-chitin (Fig. 5b-3). Therefore, the specific activities of TKU016 crude enzyme towards powdered β-chitin and water-soluble chitosan were higher than towards powdered α-chitin. TKU016 chitosanase was found to exhibit chitinase activity. In our previous experiments (data not shown), we found that TKU016 crude enzyme hydrolyzed water-soluble chitosan more easily than β-chitin. Some *Serratia* sp. chitinases were also found to exhibit chitosanase activity (Nawani and Kapadnis 2001; Green et al. 2005; Kim et al. 2007).

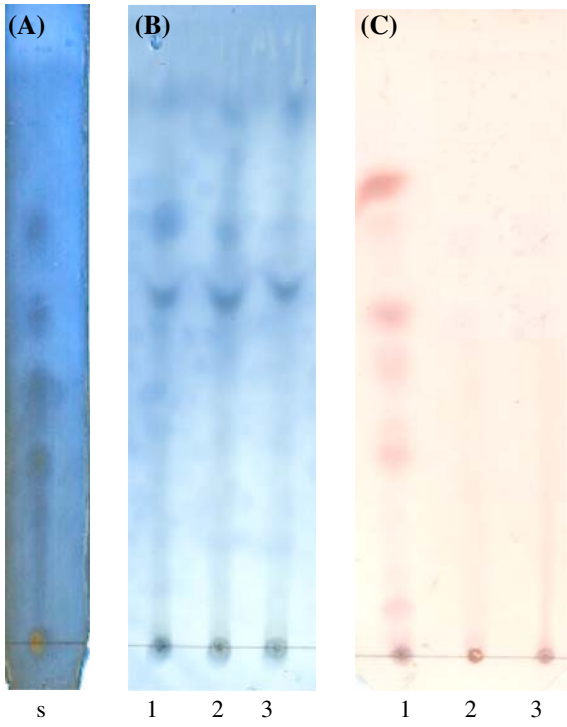

Fig. 5 TLC analysis of oligosaccharides produced by TKU016 crude enzyme. The method of hydrolysis was followed the measurement of chitosanases activity using water-soluble chitosan, β-chitin, and α-chitin, respectively as the substrate. After developing the TLC plates, the oligosaccharides were visualized using the following reagents: **a**, **b** phosphomolybdic acid reagent, **c** ninhydrin reagent. *Lane s N*-acetylchitooligosaccharide standards, *lane 1* water-soluble chitosan, *lane 2* β-chitin, *lane 3* α-chitin

Effects of various chemicals

To further characterize TKU016 chitosanase and protease, we next examined the effect of some known enzyme inhibitors and divalent metals on their activities. The results are summarized in Table 4. Cu^{2+} and Zn^{2+} inhibited slightly the chitosanase

Table 4 Effects of various chemicals on enzyme activity

Chemicals	Concentration	Relative activity (%)	
		Chitosanase	Protease
None	0	100	100
PMSF	5 mM	98	91
EDTA	5 mM	57	127
Mg^{2+}	5 mM	100	146
Cu^{2+}	5 mM	85	109
Fe^{2+}	5 mM	98	127
Ca^{2+}	5 mM	100	136
Zn^{2+}	5 mM	75	118
Mn^{2+}	5 mM	0	182
SDS	0.5 (2) mM	90 (51)	105 (145)
Tween 20	0.5 (2)%	95 (92)	105 (155)
Tween 40	0.5 (2)%	93 (92)	191 (318)
Triton X-100	0.5 (2)%	94 (94)	109 (127)

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 absence of reagents

Anionic surfactant: SDS; Nonionic surfactants: Tween 20, Tween 40, Triton X-100

activity, but Mn^{2+} inhibited completely the chitosanase activity. Compared with other *Serratia* spp. chitinase/chitosanase, the chitinase of *S. plymuthica* HRO-C48 (Frankowski et al. 2001) and *S. ureilytica* TKU013 (Wang et al. 2009) were also inhibited by Cu^{2+}. However, all of tested divalent metals resulted in enhancement in TKU016 protease activity. Inhibitor of serine protease (phenylmethanesulfonyl fluoride [PMSF]) had no significant effect on the enzyme activity of TKU016 chitosanase and protease. EDTA, a chelator of divalent cations, was a inhibitor for TKU016 chitosanase, suggesting the metal ions were essential for the catalytic action of the enzyme.

Effect of various surfactants

Enzymes are usually inactivated by the addition of surfactants to the reaction solution. The effect of different surfactants on the stability of TKU016 chitosanase and protease were also studied. The chitosanase and protease 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 of Tween 20, Tween 40, or Triton X-100, the activities of the chitosanase retained more than 92% of its original activity. At the presence of 2 mM SDS (anionic surfactant), the activity of TKU016 chitosanase was inhibited, but the protease was activated (Table 4). These differences between both enzymes might be related to the dissimilarity of the ratio of their hydrophobic and hydrophilic amino acids. Surfactants such as SDS, Triton X-100, Tween 20 and Tween 40 had stimulatory effect on TKU016 protease activity which may be due to change in the conformation of enzyme thus increasing the substrate accessibility. The protease activity was highly stimulated (about threefold higher) in presence of Tween 40 (2%).

The above results show that TKU016 protease is resistant to SDS. The result was confirmed using the in-gel protease assay. Figure 3c showed that SDS and β-mercaptoethanol had no effect on TKU016 protease activity. These results suggested that the disulfide bond in the protein molecule is not associated with its proteolytic activity. Besides, in the in-gel protease assay (Fig. 3c), TKU016 protease exhibited marked gel retardation, migrating much less than in plain SDS–PAGE (Fig. 3b). The results may reflect the fact that TKU016 protease 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 TKU016 protease has rigid structure and is therefore SDS-resistant. The stability of the protease from *Serratia* sp. TKU016 may make it useful for industrial applications.

Degradation of shellfish wastes and production of antioxidants

It has been reported that chitin, chitosan and peptide have antioxidative (Pinero Estrada et al. 2001; Lin and Chou 2004; Xing et al. 2005; He et al. 2006; Wang et al. 2009) and anticarcinogenic (Liang et al. 2007; Wang et al. 2008a) properties. To increase the utilization of these chitin/protein-containing shellfish wastes, we incubated *Serratia* sp. TKU016 for 1–4 days under the optimal culture conditions described above (0.5–2% SSP and SPP, 30°C) and analyzed the antioxidant activities of the culture supernatants. The antioxidant activity assayed was the DPPH scavenging ability. As shown in Fig. 6, it was found that TKU016 culture supernatant (2% SPP) incubated for 2 days has the highest antioxidant activity, the DPPH scavenging ability of TKU016 culture supernatant was about 76% (Fig. 6b). To analyze the antioxidant activity of the culture medium at the 0 day, we heated these marine wastes in an autoclave (121°C for 15 min), the antioxidant activities were 15–20 and 20–30% in the shrimp shell powder and squid pen powder supernatants, respectively. As shown in Fig. 6, it was found that the antioxidant activities increased after fermentation by TKU016. The antioxidant activities showed approximately 50 (Fig. 6b) and 30% (Fig. 6a) increases in the SPP and SSP supernatants, respectively. It was also found that the relative weight of the recovered squid pen decreased with an increase in cultivation time (data not shown). It is possible that the structure of SP might be changed in an autoclave to make degradation more easily by TKU016. Besides, in the SPP culture supernatant, the

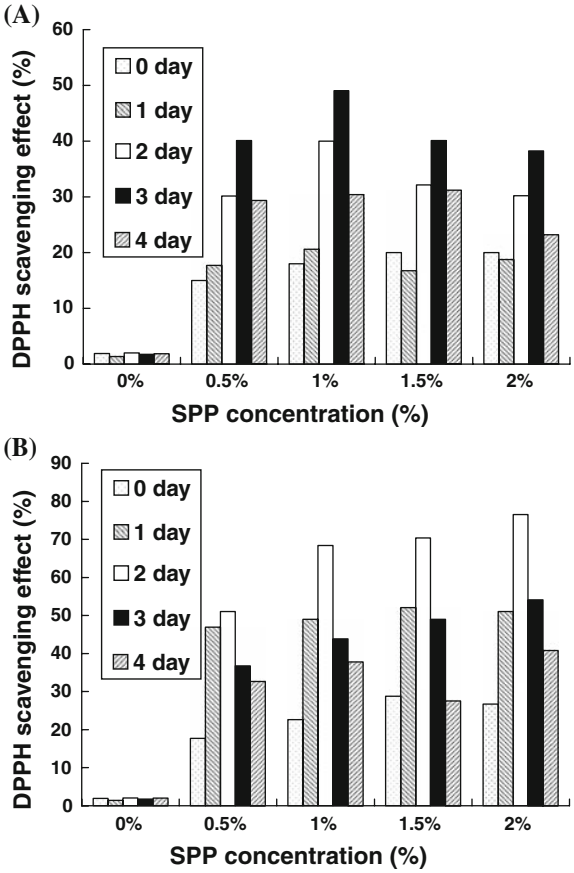

Fig. 6 DPPH scavenging effects of the culture supernatants at various concentrations of SSP (**a**) and SPP (**b**) by using *Serratia* sp. TKU016 fermentation

protein concentration and reducing sugar dramatically increased on the 2nd day (data not shown). Therefore, squid pen powder was the suitable carbon/nitrogen source for antioxidant materials or some bioactive materials production by strain TKU016. It is assumed that even though the treatment (121°C for 15 min) degrades the SPP and produces some of the antioxidant materials, but most of the antioxidant materials are produced by strain TKU016.

Another chitosanase/protease-producing strain (*Serratia marcescens* TKU011) in our laboratory can also use shrimp shell wastes as the sole carbon/nitrogen source (Wang et al. 2008b). The antioxidant activity of the SPP culture supernatant was analyzed and compared with that of strain TKU016. The results showed that the DPPH scavenging ability of the culture supernatant of *S. marcescens* TKU011 and *Serratia* sp. TKU016 was about 22 and 76%, respectively. We investigated the effect 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). Both of the species studied here, *S. marcescens* TKU011 was a chitosanase (had no chitinase activity)/protease-producing strain, while *Serratia* sp. TKU016 was a chitosanase (had chitinase activity)/protease-producing strain. The most obvious difference between the enzymes produced by these two protease-producing strains in *Serratia* species is that strain TKU011 chitosanase does not have chitinase activity and strain TKU016 chitosanase has chitinase activity. The different results of antioxidant productivity might be related to the substrate specificity of these two enzymes.

In conclusion, we have purified and characterized chitosanase and protease from the culture supernatant of *Serratia* sp. TKU016 using shrimp shell as the sole carbon/nitrogen source. In addition, it was also found that the SPP culture supernatant has antioxidant activity as well. With this method, TKU016 chitosanase and protease were produced in the SSP medium at the 3rd day, the antioxidant materials, reducing sugar, and peptide were produced in the SPP medium at the 2nd day. These results may be useful for biological applications in relation to enzyme production and chitin/protein degradation.

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References

Banik RM, Prakash M (2004) Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol Res 159:135–140

Bhaskar N, Sudeepa ES, Rashmi HN, Tamil Selvi A (2007) Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. Biores Technol 98:2758–2764

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brurberg MB, Nes IF, Eijsink VGH (1996) Comparative studies of chitinases A and B from *Serratia marcescens*. Microbiology 142:1581–1589

Frankowski J, Lorito M, Scala F, Schmid R, Berg G, Bahl H (2001) Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. Arch Microbiol 176:421–426

Green AT, Healy MG, Healy A (2005) Production of chitinolytic enzymes by *Serratia marcescens* QMB1466 using various chitinous substrates. J Chem Technol Biotechnol 80:28–34

He H, Chen X, Sun C, Zhang Y, Gao P (2006) Preparation and functional evaluation of oligopeptide-enriched hydrolysate from shrimp (*Acetes chinensis*) treated with crude protease from *Bacillus* sp. SM98011. Biores Technol 97:385–390

Heussen C, Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem 102:196–202

Imoto T, Yagishita K (1971) A simple activity measurement of lysozyme. Agric Biol Chem 35:1154–1156

Kadokura K, Rokutani A, Yamamoto M, Ikegami T, Sugita H, Itoi S, Hakamata W, Oku T, Nishio T (2007) Purification and characterization of *Vibrio parahaemolyticus* extracellular chitinase and chitin oligosaccharide deacetylase involved in the production of heterodisaccharide from chitin. Appl Microbiol Biotechnol 75:357–365

Kim S, Mendis E (2006) Bioactive compounds from marine processing byproducts-A review. Food Res Intern 39: 383–393

Kim HS, Timmis KN, Golyshin PN (2007) Characterization of a chitinolytic enzyme from *Serratia* sp. KCK isolated from kimchi juice. Appl Microbiol Biotechnol 75: 1275–1283

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lee KH, Lee PM, Siaw YS, Morihara K (1993) Kinetics of aspartame precursor synthesis catalysed by *Pseudomonas aeruginosa* elastase. J Chem Technol Biotechnol 56: 375–381

Lee YS, Yoo JS, Chung SY, Lee YC, Cho YS, Choi YL (2006) Cloning, purification, and characterization of chitosanase from *Bacillus* sp. DAU101. Appl Microbiol Biotechnol 73:113–121

Liang TW, Chen YJ, Yen YH, Wang SL (2007) The antitumor activity of the hydrolysates of chitinous materials hydrolyzed by crude enzyme from *Bacillus amyloliquefaciens* V656. Process Biochem 42:527–534

Lin HY, Chou CC (2004) Antioxidant activities of water-soluble disaccharide chitosan derivatives. Food Res Intern 37:883–889

Nawani NN, Kapadnis BP (2001) One-step purification of chitinase from *Serratia marcescens* NK1, a soil isolate. J Appl Microbiol 90:803–808

Pinero Estrada JE, Bermejo Bescoś P, Villar del Fresno AM (2001) Antioxidant activity of different fractions of Spriulina platensis protean extract. IL FARMACO 56:497–500

Roberts DP, McKenna LF, Lakshman DK, Meyer SLF, Kong H, Souza JT, Lydon J, Baker CJ, Buyer JS, Chung S (2007) Suppression of dumping-off of cucumber caused by Pythium ultimum with live cells and extracts of Serratia marcescens N4–5. Soil Biol Biochem 39:2275–2288

Romero FJ, Garcia LA, Salas JA, Diaz M, Quiros LM (2001) Production, purification and partial characterization of two extracellular proteases from *Serratia marcescens* grown in whey. Process Biochem 36:507–515

Sakurai K, Funaguma T, Hara A (1995) Studies on chitinases produced by *Serratia* sp. isolated from soil. Meijodai Nogakuho 31:21–31

Salamone PR, Wodzinski RJ (1997) Production, purification and characterization of a 50-kDa extracellular metalloprotease from *Serratia marcescens*. Appl Microbiol Biotechnol 48:317–324

Shikha AS, Darmwal NS (2007) Improve production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. Biores Technol 98:881–885

Shimada K, Fujikawa K, Yahara K, Nakamura T (1992) Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 40:945–948

Su C, Wang D, Yao L, Yu Z (2006) Purification, characterization, and gene cloning of a chitosanase from *Bacillus* species strain S65. J Agric Food Chem 54: 4208–4214

Suzuki K, Sugawara N, Suzuki M, Uchiyama T, Katouno F, Nikaidou N, Watanabe T (2002) Chitinases A, B, and C1 of *Serratia marcescens* 2170 produced by recombinant *Escherichia coli*: enzymatic properties and synergism on chitin degradation. Biosci Biotech Biochem 66:1075–1083

Synowiecki J, Al-Khateeb NAAQ (2000) The recovery of protein hydrolysate during enzymatic isolation of chitin from shrimp *Crangon crangon* processing discards. Food Chem 68:147–152

Tan SC, Khor E, Tan TK, Wong SM (1998) The degree of deacetylation of chitosan: advocating the first derivative UV-spectrophotometry method of determination. Talanta 45:713–719

Tao K, Long Z, Liu K, Tao Y, Liu S (2006) Purification and properties of a novel insecticidal protein from the locust pathogen *Serratia marcescens* HR-3. Curr Microbiol 52:45–49

Todd EW (1949) Quantitative studies on the total plasmin and trypsin inhibitor of human blood serum. J Exp Med 39:295–308

Ulrich B (1994) Crystal structure of the 50 kDa metallo protease from *Serratia marcescens*. J Mol Biol 242: 244–251

Wang SL, Kao TY, Wang CL, Yen YH, Chern MK, Chen YH (2006) A solvent stable metalloprotease produced by Bacillus sp. TKU004 and its application in the deproteinization of squid pen for β-chitin preparation. Enzyme Microb Technol 39:724–731

Wang SL, Lin HT, Liang TW, Chen YJ, Yen YH, Guo SP (2008a) Reclamation of chitinous materials by bromelain for the preparation of antitumor and antifungal materials. Biores Technol 99:4386–4393

Wang SL, Peng JH, Liang TW, Liu KC (2008b) Purification and characterization of a chitosanase from *Serratia marcescens* TKU011. Carbohydr Res 343:1316–1323

Wang SL, Lin CL, Liang TW, Liu KC, Kuo YH (2009) Conversion of squid pen by *Serratia ureilytica* for the production of enzymes and antioxidants. Biores Technol 100:316–323

Xing R, Yu H, Liu S, Zhang W, Zhang Q, Li Z (2005) Antioxidative activity of differently regioselective chitosan sulfates in vitro. Bioorg Med Chem 13:1387–1392