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Degradation of chitin and production of bioactive materials by bioconversion of squid pens

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ABSTRACT

Serratia marcescens TKU011, a protease- and chitosanase-producing bacterium, the optimized condition for protease and chitosanase production was found after the media were heated at 121 °C for 120 min and the culture was shaken at 25 °C for 5 days in 100 mL of medium containing 1% squid pen powder (SPP) (w/v), 0.1% K_2HPO_4, and 0.05% $MgSO_4$. An extracellular metalloprotease with novel properties of solvent stable, and alkaline was purified from the culture supernatant of *S. marcescens* TKU011 with squid pen wastes as the sole carbon/nitrogen source. The enzyme was a monomeric protease with a molecular mass of 48–50 kDa by SDS–PAGE and gel filtration chromatography. The optimum pH, optimum temperature, pH stability, and thermal stability of TKU011 protease were 8, 50 °C, pH 5–11, and <40 °C, respectively. Besides protease and chitosanase, with this method, deproteinization of squid pen for β-chitin, the production of peptide and reducing sugar may be useful for biological applications.

1. Introduction

Protease constitutes one of the most important groups of industrial enzymes, accounting for more than 65% of the total industrial enzyme market (Banik & 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 & 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, Sudeepa, Rashmi, & Tamil Selvi, 2007; Roberts et al., 2007; Wang, Hsu, Liang, Yen, & Wang, 2008; Wang, Wang, & Huang, 2008). A considerable attention has been given to the enzymes that are stable in the presence of detergent and solvents and their biotechnological potentials. Reports on few enzymes that are naturally stable and also exhibiting high activities in the presence of solvent and detergent have got significant importance in the present era (Ustariz, Laca, Garcia, & Diaz, 2004).

Bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes. However, both SCS and SP contain chitin, protein, and inorganic compounds such as calcium carbonate. Conventionally, preparation of chitin from such shellfish chitin wastes involves deproteinization and demineralization with strong bases and acids. However, the use of these chemicals may cause a partial deacetylation of the chitin and hydrolysis of the polymer, resulting in final inconsistent physiological properties (Gagne & Simpson, 1993; Oh, Shih, Tzeng, & Wang, 2000). The chemical treatments also create waste disposal problems, because neutralization and detoxification of the discharged wastewater are necessary. Furthermore, the value of the deproteinization liquid is diminished because of the presence of sodium hydroxide. To overcome the defects of chemical treatments, alternative methods on use of microorganisms or proteolytic enzymes for the deproteinization of shellfish chitin wastes have been cited (Gagne & Simpson, 1993; Oh et al., 2000; Wang, Kao, et al., 2006; Yang, Shih, Tzeng, & Wang, 2000). To further enhance the utilization of the chitin/protein-containing squid pen wastes, we have recently investigated the bioconversion of shellfish chitin wastes for the production of chitooligomers and peptide for antioxidative and anticarcinogenic properties (Wang, Lin, Yen, Liao, & Chen, 2006; Liang, Chen, Yen, & Wang, 2007; Wang, Lin, et al., 2008). In this study, we attempted to optimize the culture conditions of *Serratia marcescens* TKU011 for maximal protease and chitosanase productivity by using cheap carbon/nitrogen source of squid pen powder and deproteinization of squid pen for β-chitin, the production of peptide and reducing sugar. In addition, the protease from *S. marcescens* TKU011 was also purified, characterized, and compared with other bacterial proteases.

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2. Materials and methods

2.1. Materials

The squid pen powder (SPP) used in these experiments was prepared as described earlier (Wang, Wang, et al., 2008). Squid pens were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). In the preparation of the squid pens was washed thoroughly with tap water and then dried. The dried materials obtained were milled to powders for using as the carbon/nitrogen source for protease and chitosanase production. Casein, albumin, gelatin, hemoglobin, fibrin, elastin, 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.

2.2. Microorganism and culture conditions

TKU011 was isolated from the soil in Taiwan and maintained on nutrient agar plates at 30 °C as reported previously (Wang, Peng, Liang, & Liu, 2008). TKU011 was identified as *S. marcescens* by the Bioresource Collection and Research Center (Shin-Chu, Taiwan) (Wang, Peng, et al., 2008). In our preliminary experiments, we found 100 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$, pH 7) containing 1% SPP was better for the production of protease and chitosanase by strain TKU011 at 25 °C. However, some materials might be extracted or the structure of SP might be changed in an autoclave (121 °C for 15 min). Thus, various time (15–180 min) of the resultant medium in an autoclave was investigated. *S. marcescens* TKU011 was inoculated in a 100 mL medium in a 250-mL Erlenmeyer flask, aerobically cultured at 25 °C for 1–7 days on a rotary shaker (150 rpm). After centrifugation (12,000*g*, 4 °C, for 20 min), the supernatants were collected for measurement of enzymes activity.

2.3. Purification of the protease

For the production of protease, *S. marcescens* TKU011 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SPP, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7). One milliliter of the seed culture was transferred into 100 mL of the same medium heated at 121 °C for 120 min and grown in an orbital shaking incubator (150 rpm) for 5 days at 25 °C and pH 7 (the pH after being autoclaved was 7.5). 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.

2.3.1. DEAE–Sepharose CL-6B chromatography

To the culture supernatant (900 mL), ammonium sulfate was added (608g/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 dialyzed against the buffer. The resultant dialysate (25 mL) was loaded onto a DEAE–Sepharose CL-6B column (5 × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). The unadsorbed materials were washed from the column with the same buffer, and the protease was fractionated with linear gradient 0–1 M NaCl in 50 mM phosphate buffer (Fig. 1). The fractions with high protease activity were combined and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 5 mL of 50 mM sodium phosphate buffer (pH 7).

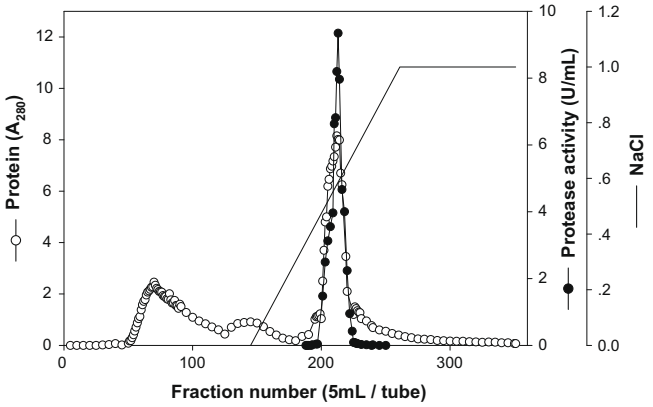

Fig. 1. Elution profile of TKU011 protease on DEAE–Sepharose CL-6B: (○) absorbance at 280 nm; (●) protease activity (U/mL).

2.3.2. Sephacryl S-100 chromatography

The resultant enzyme solution was loaded onto a Sephacryl S-100 gel filtration column (2.5 × 120 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7), then eluted with the same buffer. One peak exhibiting protease activity was obtained, combined, and used as a purified preparation.

2.4. Protein determination

Protein content was determined by the method of Bradford (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.

2.5. Measurement of enzymes activity

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 (Wang, Hsu, et al., 2008). One unit of protease activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per min.

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 min.

2.6. Polyacrylamide gel electrophoresis and zymograms

The molecular mass of the purified protease was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970). The standard proteins used for calibration were phosphorylase b (molecular mass, 97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactabumin (14.4 kDa). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercatpethanol. The gels were stained with Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (5:1:5, v/v), and decolorized

in 7% acetic acid. The molecular mass of the TKU011 protease in the native form was 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. The activity band was observed as a clear zone depleted of gelatin in the gel against the blue background. The demonstration of a protease activity band was also done after electrophoresis in native PAGE gel by the method of Davis (1964) in a 7.5% (w/v) polyacrylamide gel containing gelatin (0.1%) with Tris/glycine buffer, pH 8.3. After electrophoresis, the gels were incubated in 50 mM phosphate buffer (pH 7) at 37 °C and then stained with amido black 10B. A clear zone was also observed in the gel.

2.7. Mass spectrometry and protein identification

Bands of interest on SDS–PAGE gel were excised and in-gel digested with trypsin. The identification of TKU011 protease was determined by using liquid chromatography–tandem mass spectrometry (LC–MS/MS) by the Mission Biotech, Taiwan. Fragment spectra were searched against the bacteria subset of NCBInr database of GenBank. Database searches were carried out using the MASCOT search engine.

2.8. N-terminal amino acid sequence analysis

The purified protease was subjected to electrophoresis on 12.5% SDS–PAGE and then transferred to a PVDF membrane. The region containing the protease band on the PVDF was excised and the protein N-terminal amino acid sequence was determined by the Edman degradation method (Academia Sinica, Taiwan).

2.9. The isolation and measuring of remaining squid pens

The culture broth was centrifuged for 20 min at 12,000*g*, the solid mass obtained was washed with distilled water, followed by the removal of cells and the dissolved materials by filtration, and the process was repeated twice. Then, after freeze drying, the dry weight of remaining squid pens was calculated.

2.10. Deproteinization of squid pens by alkali

SPP was mixed with 2 N NaOH solutions at a ratio of 3:8 (w/v). The mixture was allowed to react at 100 °C for 30 min. The ratio of chitinous materials to solvent, 3:40 (w/v), was the same as the deproteinization method for the preparation of crustacean chitin (Wang, Kao, et al., 2006). After centrifugation, the solid residues were washed with de-ionized water until became a neutral pH, and then dried at 65 °C in an oven. The dried residues were used for the analysis of deproteinization. The resultant supernatant was also used for analysis of protein concentration by the method of Bradford as described above.

3. Results and discussions

3.1. Culture conditions and enzymes production

TKU011 was isolated from the soil in Taiwan and maintained on nutrient agar plates at 30 °C as reported previously (Wang, Peng, et al., 2008). TKU011 was identified as *S. marcescens* by the Bioresource Collection and Research Center (Shin-Chu, Taiwan) (Wang, Peng, et al., 2008). The biological utilization of squid pen was less investigated and reported. The squid pen is rich in protein and chitin. The carbon/nitrogen sources with both protein and chitin were more suitable as an inducer for protease and chitosanase production by microorganism than carbon/nitrogen sources with only protein but no chitin (Wang, Kao, et al., 2006; Wang & Yeh, 2006). The protein/chitin/mineral salts composition of SP is 61%/38%/1% obtained in our previous experiments (Wang, Kao, et al., 2006). Therefore, the squid pen biowaste was used to produce protease and chitosanase by TKU011 in the current study. In our preliminary experiments, we found 100 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing 1% SPP was better for the production of protease and chitosanase by strain TKU011 at 25 °C. However, some materials might be extracted or the structure of SP might be changed in an autoclave (121 °C for 15 min). Thus, various time of the medium in an autoclave was investigated. To study the time (15–60 min) in an autoclave, 100 mL of the media (1% SPP contained basal medium, pH 7) was used, and the relationship between incubation time (0–7 days), protease and chitosanase activity were investigated. As shown in Fig. 2, the protease and chitosanase activity increased with an increase in heating time. These parameters were found to be the best

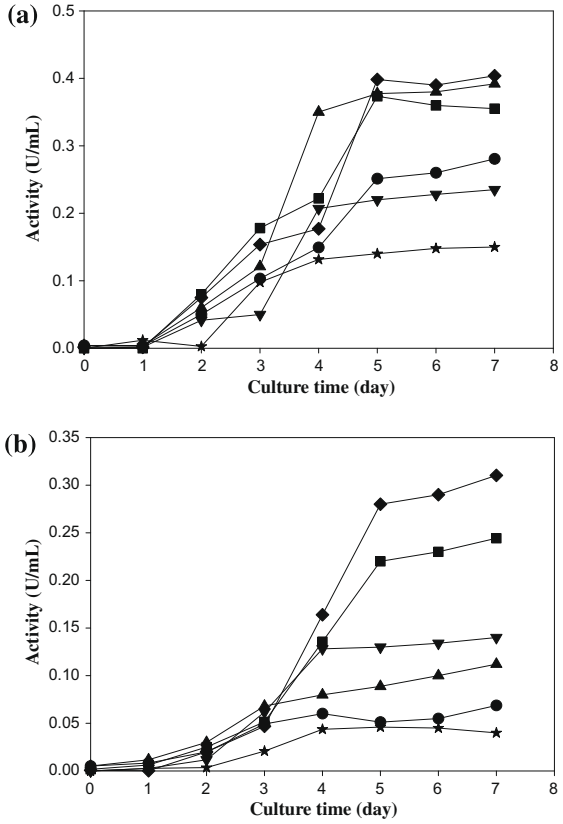

Fig. 2. Time courses of enzyme production in a culture of *S. marcescens* TKU011 on squid pen containing media for various heating time (121 °C for 15–180 min): (★) 15 min; (●) 30 min; (▼) 45 min; (▲) 60 min; (♦) 120 min; (■) 180 min; (a) protease; (b) chitosanase.

for 60 min heating after 4 days of cultivation in a flask culture at 25 °C and 150 rpm (Fig. 2). Whether the enzymes activity increased continuously with an increase in heating time or not. To study further the effect of the time in an autoclave for enzymes activity, prolonged heating time (to 180 min) of the medium in an autoclave, and the relationship between incubation time (0–7 days), protease and chitosanase activity were investigated. As shown in Fig. 2, the enzyme production was affected certainly by heating time of SPP medium. The results shown that the optimal conditions of enzyme production was the medium heating 121 °C for 120 min after 5 days of cultivation in a flask culture at 25 °C and 150 rpm (Fig. 2). It is assumed that the heating treatment (121 °C for 120 min) provides a sufficient enzyme production effect to utilize SP by TKU011.

3.2. Isolation and purification of the protease

TKU011 chitosanase had been purified, characterized, and reported previously (Wang, Peng, et al., 2008). In this study, the purification of the TKU011 protease from the culture supernatant (900 mL) was described under Section 2. As shown in Table 1, the purification steps were combined to give an overall purification of about 22-fold for protease. The overall activity yield of the purified protease was 33%, with specific protease activities of 0.324 U/mg. The final amount of TKU011 protease obtained was 210 mg. The purified enzyme migrated as a single band in SDS–PAGE and apparent molecular mass of the protease was about 50 kDa (Fig. 3a), whereas it was estimated as approximately 48 kDa by Sephacryl S-100 gel filtration. Zymogram activity staining also revealed one clear zone of proteolytic activity against the blue background in both native and SDS–PAGE (Fig. 3b).

The molecular mass of TKU011 protease (50 kDa) was obviously different from most of the other *Serratia* proteases, such as, *S. marcescens* serine protease (66.5 kDa) (Romero, Garcia, Salas, Diaz, & Quiros, 2001), *S. marcescens* metalloprotease (61 kDa) (Tao, Long, Liu, Tao, & Liu, 2006), and *S. rubidaea* metalloproteases (CP-1, CP-2) (97 kDa, 45 kDa) (Salamone & Wodzinski, 1997). The *Serratia* proteases that had the similar molecular weight as *S. marcescens* TKU011 protease included the metalloprotease of *S. marcescens* ATCC 25419 (53.5 kDa) (Romero et al., 2001) and *S. marcescens* (50.9 kDa) (Salamone & Wodzinski, 1997).

3.3. Effect of pH and temperature

The effect of pH on the catalytic activity was studied by using casein as a substrate under the standard assay conditions. The pH activity profile of the protease was with maximum values at pH 8 (Fig. 4a). The pH optimum of the protease was similar to 8.5 of *S. marcescens* ATCC 25419 metalloprotease (Romero et al., 2001), 8 of *S. rubidaea protease* metalloprotease CP-2 (Salamone & Wodzinski, 1997).

The pH stability profile of the protease activity was determined by the measurement of the residual activity at pH 7 after incubation at various pH values at 37 °C for 60 min. The protease activity was stable at pH 5–11 (Fig. 4a). The TKU011 protease had broader pH stability range (pH 5–11) than the metalloprotease of *S. marcescens* NRRLB-23112 (pH 6–10) (Salamone & Wodzinski, 1997) and two metalloproteases (CP-1, CP-2) of *S. rubidaea* metalloproteases (pH 6–11, pH 5–9) (Salamone & Wodzinski, 1997).

Table 1
Purification of protease from *Serratia marcescens* TKU011.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture sup.	14,378	209	0.015	1	100
$(NH_4)_2SO_4$ ppt	1964	181	0.092	6	87
DEAE–Sepharose	695	130	0.187	12	62
Sephacryl S-100	210	68	0.324	22	33

Serratia marcescens TKU011 was grown in 50 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 2% shrimp shell powder, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ in a rotary shaker (150 rpm) for 2 days at 25 °C.

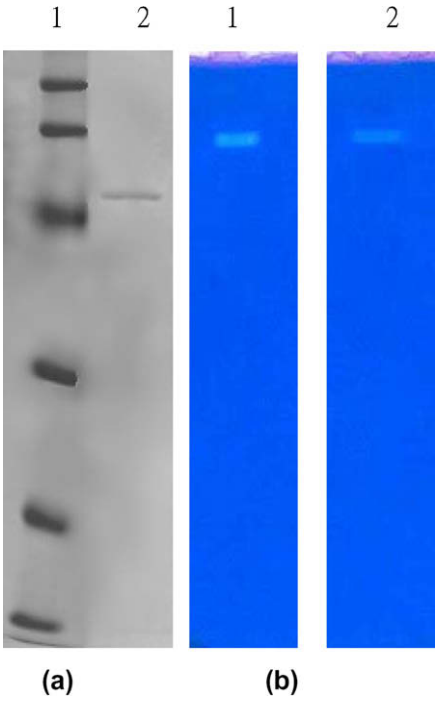

Fig. 3. (a) SDS–PAGE analysis of the purified protease produced by strain TKU011. Lanes: 1, molecular markers (97.4, 66.2, 45, 29, 20.1, 14.4 kDa); 2, Sephacryl S-100 purified protease. (b) Zymograms of the purified protease in SDS–PAGE (1) and native PAGE (2). Further details are described in Section 2.

The effect of temperature on the activity of protease was studied with casein as a substrate. The temperature profile of protease activity is presented on Fig. 4b. The optimum temperature for TKU011 protease was 50 °C, which was slightly higher than *S. marcescens* ATCC 25419 metalloprotease (45 °C) (Romero et al., 2001) and *S. marcescens* metalloprotease (42 °C) (Salamone & Wodzinski, 1997), and similar to *S. marcescens* ATCC 25419 serine protease (48 °C) (Romero et al., 2001). To examine the heat stability of TKU011 protease, the enzyme solution in 50 mM phosphate buffer (pH 7) was allowed to stand for 60 min at various temperatures, and then the residual activity was measured. TKU011 protease maintained about more than 95% of its initial activity from 25 to 40 °C but was rapidly inactivated, retaining only 10% of its initial activity at 50 °C and was completely inactivated at greater than 60 °C. The property of thermal instability was similar to the metalloprotease of *S. marcescens* NRRLB-23112 (Salamone & Wodzinski, 1997), the serine protease of *S. marcescens* ATCC25419 (Romero et al., 2001), and the metalloproteases (CP-1, CP-2) of *S. rubidaea* ATCC 25419 (Salamone & Wodzinski, 1997).

3.4. Substrate specificity

The activity of the purified protease toward various substrates was investigated. The enzyme showed especially high activity toward casein and gelatin but no activities toward elastin, albumin, and myoglobin (data not shown). This result was similar to that of *Lactobacillus paracasei* subsp. *paracasei* protease (Wang, Wang, et al., 2008). Peng, Huang, Zhang, and Zhang (2003) have measured the ratio of fibrinolytic activity and caseinolytic activity (F/C) of

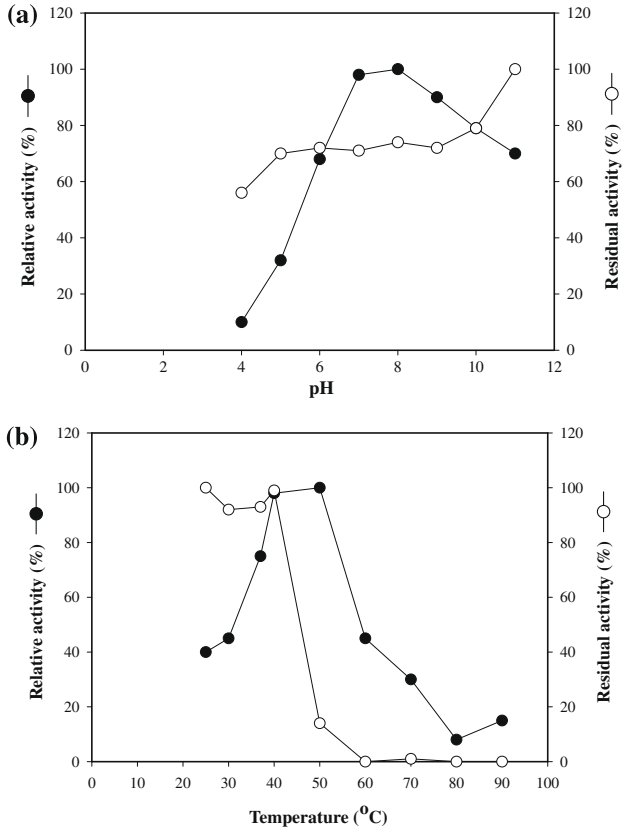

Fig. 4. Effect of pH and temperature on the activity and stability of the purified protease. (a) Effect of pH: (●) optimum pH, (○) pH stability; (b) Effect of temperature: (●) optimum temperature, (○) thermal stability.

various microbial proteases. The F/C (%) of *B. amyloliquefaciens* DC-4 (serine protease), *B. subtilis* (neutral protease), *B. pumilus* (alkaline protease), and *B. licheniformis* (alkaline protease) were 1.92, 0.92, 0.75, and 0.39, respectively (Wang, Wang, et al., 2008). Different to the above microbial proteases, the F/C ratio of TKU011 protease was 0%.

3.5. Effects of various chemicals

To further characterize the *S. marcescens* TKU011 protease, we next examined the effects of some known enzyme inhibitors and divalent metals on their activities. The effects of various chemicals on the enzyme activity were investigated by preincubating the enzyme with chemicals in 50 mM phosphate buffer (pH 7) for 10 min at 37 °C and then measuring the residual protease activity by using casein as a substrate. The results were summarized in Table 2. Mg^{2+} and Ba^{2+} increased protease activity by 109% and 102% of the control, respectively. However, Ca^{2+}, Mn^{2+} and Zn^{2+} inhibited the enzyme activity by 71%, 51% and 67%, respectively. The protease activity was completely inhibited by Cu^{2+} and Fe^{2+}. Inhibitor of serine protease (phenylmethanesulfonyl fluoride [PMSF]) had no significant effect on the activity of the purified TKU011 protease. EDTA, a chelator of divalent cations, was a strong inhibitor, indicating that the TKU011 protease was a metalloenzyme.

The inhibition of TKU011 protease by Cu^{2+} and Fe^{2+} was obviously different from most of the other *Serratia* proteases. In contrast with our study, studies by Khardenavis, Kapley, and Purohit (2009) showed that 1 mM concentrations of Fe^{2+} resulted in enhancement in *Serratia* sp. HPC1383 protease activity by 26% and no inhibition of the enzyme was found to occur in presence of Cu^{2+}. Studies by Kwak et al. (2007) showed that *S. proteamaculans* HY-3 protease was inhibited by Cu^{2+}, but Fe^{2+} showed approximately 15% increases in relative activity.

Table 2
Effects of various chemicals on protease activity of TKU011.

Chemicals	Concentration (mM)	Relative activity (%)
None	0	100
PMSF	10	98
EDTA	10	0
Mg^{2+}	5	109
Cu^{2+}	5	0
Fe^{2+}	5	0
Ca^{2+}	5	71
Zn^{2+}	5	67
Mn^{2+}	5	51
Ba^{2+}	5	102

Purified enzyme was preincubated with the various reagents at 37 °C for 30 min and residual protease activity were determined as described in the text. One hundred percent was assigned to the activity in absence of reagents.

3.6. Effects of various solvents

Enzymes are usually inactivated by the addition of organic solvents to the reaction solution. The effect of different organic solvents (20%, v/v) on stability of the purified TKU011 protease was also studied. Purified protease preparation was incubated with solvents (20%, v/v) at 37 °C for 30 min and the remaining enzymatic activity was determined under normal assay conditions. The enzyme activity of the sample without any solvent (control) was taken as 100%. The TKU011 protease was more stable in the cases of toluene and ethyl ether, but the protease activity was inhibited in the case of ethyl acetate and butanol. The solvent stable proteases were also reported in the cases of purified *Pseudomonas aeruginosa* PseA protease (Gupta, Roy, Khare, & Gupta, 2005), *Bacillus* sp. TKU007 protease (Wang & Yeh, 2006) and *Bacillus* sp. TKU004 protease (Wang, Kao, et al., 2006). Among them, the TKU011 protease was more stable (85%) than *Bacillus* sp. TKU004 metalloprotease (Wang, Kao, et al., 2006) in the case of toluene.

3.7. Organic solvent stability

Several reports showed that peptide synthesis could be enhanced by the addition of organic solvents in the reaction mixture (Gupta et al., 2005). However, enzymes are usually inactivated or denaturated in the presence of organic solvents. In this study, phosphate buffer (pH 7) was used as control and the effects of various organic solvents (such as methanol, ethanol, toluene, acetonitrile, acetone, isoamyl alcohol, and isopropyl alcohol) on the stability of TKU011 protease were investigated. After being exposed in phosphate buffer (50 mM) (control) at 4 °C and 25 °C for 10 days, the remaining protease activities in the culture supernatant were 0.225 U/mL and 0.04 U/mL, respectively. In addition, in the presence of 25% (v/v) tested organic solvents, the protease retained more than 90% of its activity after keeping at 4 °C for 10 days. However, after keeping at 25 °C for 10 days, the protease retained lower than 30% its initial activity (data not shown).

Different from other reported protease-producing strains, this research aimed for the microbial reclamation of shrimp processing wastes. Shrimp shells were used as the sole carbon/nitrogen source to screen the protease-producing bacteria. The medium for TKU011 is obviously much simpler and cheaper. Considering the production cost and the reutilization of bioresources, utilizing TKU011 on the microbial reclamation of food processing wastes such as shrimp shell wastes for the production of proteases seems to provide a promising approach.

3.8. Identification of TKU011 protease by LC–MS/MS analysis

To identify the protease activity appearing as a prominent 50 kDa band on SDS–PAGE gel, the band was excised and analyzed after tryptic digestion. The 50 kDa band from SDS–PAGE gel was subjected to electrospray tandem mass spectrometry analysis. The fragment spectra were evaluated and submitted to the bacteria subset of NCBInr database of GenBank. The spectra matched eleven tryptic peptides (data not shown) that could be correlated to a protein of unknown function (GenBank Accession No. gi999638) from *Serratia* protease (E.C.3.4.24.40) with 33% sequence coverage. The sequence of the unknown protein gave a calculated nominal mass of 50,424 Da, similar to the experimental values obtained with the purified protease.

3.9. N-terminal amino acid analysis

The first 18 N-terminal amino acid residues of the purified alkaline protease were identified as AATTGYDAVDDLLHYHER. The N-terminal amino acid sequence of TKU011 protease was analyzed using a BLAST search against GenBank. The comparison of N-terminal amino acid sequence of the protease from strain TKU011 with other metalloproteases from *Serratia* sp. and *Erwinia* sp. are listed in Table 3. The first 18-N-terminal amino acid sequence shows 100% identity to 1st–18th amino acid sequence of *Serratia* sp. metalloprotease (Katsuya et al., 1985), 17th–34th amino acid sequence of *S. marcescens* SM6 metalloprotease (Braunagel & Benedik, 1990), and 34th–51st amino acid sequence of *Serratia* sp. E-15 metalloprotease (Hamada et al., 1996). Besides, homology search revealed 77% of homology with 34th–51st amino acid sequence of *S. proteamaculans* metalloprotease and 23rd–40th amino acid sequence of *Serratia* sp. KCK metalloprotease. The 3rd, 4th, 5th, and 7th amino acids of TKU011 metalloprotease (T, T, G, and D, respectively) differed from those of *S. proteamaculans* (S, S, A, and N, respectively) and *Serratia* sp. KCK metalloprotease (S, S, A, and N, respectively). Tao et al. (2006) reported that the first 18 N-terminal amino acid residues of an insecticidal protein (61 kDa) from *S. marcescens* HR-3 (MQSTKKAIEITESELAAA) showed homology of 94% and 88% to 1st–18th amino acid sequence of *S. marcescens* SM6 metalloprotease (Braunagel & Benedik, 1990) and *Serratia* sp. E-15 metalloprotease (Hamada et al., 1996), respectively. Thus, the *S. marcescens* SM6 metalloprotease contains an extraordinary 33-amino acid fragment at the N-terminus relative to that of TKU011 metalloprotease, the identical region starting from the 34th residue in the *S. marcescens* SM6 metalloprotease. Nonetheless, in the *Serratia* sp. E-15 metalloprotease, the identical region starts from the 17th residue which is aligned with the 1st residue in TKU011 metalloprotease. Although several stains of *S. marcescens* have been found to be human opportunistic pathogens with a broad host range, they are also the most often reported insect pathogens (Tao et al., 2006). Considering the differences in the N-terminal 1st–34th amino acid sequence (Table 3), the differential pathogenicity among these *Serratia* metalloproteases, namely whether they act as a human opportunistic pathogen or exhibit insecticidal activity, may well be correlated to the presence or absence of the 1st–34th N-ternimal amino acid sequence.

Table 3
Comparison between the N-terminal amino acid sequence of TKU011 protease and other metalloprotease sequences.

Origin	N-terminal amino acid sequence[a]
Serratia marcescens TKU011	01**AATTGYDAVDDLLHYHER**18
Serratia sp. E-15	17**AATTGYDAVDDLLHYHER**34
Serratia sp	01**AATTGYDAVDDLLHYHER**18
Serratia marcescens SM6	34**AATTGYDAVDDLLHYHER**51
Erwinia chrysanthemi B374	17**AA**SSS**YD**S**V**Y**DLLHYHER**34
Erwinia chrysanthemi EC16	18**A**PSSS**Y**NSIY**DLLHYHER**35

[a] Identical residues at each position are highlighted using bold type.

3.10. Degradation of squid pen in liquid phase fermentation

The medium containing squid pen and small amounts of minerals was more suitable for the production of protease and chitosanase by *S. marcescens* TKU011. During 7 days fermentation, the squid pen powder decreased gradually in the medium. As to degradation of squid pen in liquid phase fermentation, the following was investigated. TKU011 chitosanase showed activities toward chitosan with different degrees of deacetylation, but no activity toward colloidal chitin and chitin as reported previously (Wang, Peng, et al., 2008). To seek the feasibility of *S. marcescens* TKU011 for β-chitin preparation from squid pen, strain TKU011 was tested for its deproteinization performance in liquid phase fermentation. As shown in Fig. 5a, after cultivation for 1 day, the deproteinization of the revovered squid pen was only 2%. However, the deproteinization of the revovered squid pen dramatically increased to 80% on the 3rd day. For the optimum media (heated at 121 °C for 120 min) of enzyme production, the protease activity approached to half of the maximum value at this time. The deproteinization might be related to the production of the protease. The percentage of protein removal reached the peak (more than 92%) after 4 days. It is possible that the structure of SP might be changed in a prolonged autoclave to make deproteinization more easily by TKU011 protease. However, as shown in Fig. 5a, the relative weight of the recovered squid pen decreased with an increase in cultivation time. The relative weight of the recovered squid pen was 44% on the 3rd day. The relative weight of the recovered squid pen decreased to 2% on the 5th day. The percentage of relative weight of the recovered squid pen reached the valley (0.1%) on the 7th day.

In the culture supernatant, the protein concentration increased quickly on the 3rd day and reducing sugar dramatically increased on the 4th day. It means that treatment of squid pen with TKU011 protease promoted the chitinolytic activity of TKU011 chitosanase, as has been reported in *Alteromonas* sp. (Miyamoto et al., 2002) and *Serratia* sp. KCK (Kim, Golyshin, & Timmis, 2007). TKU011 protease promoted chitin degradation, presumably through hydrolysis of constituent proteins on the surface of chitin cuticles and facilitates access between TKU011 chitosanase and chitin molecules by removal of sterically-hindering chitin-associated proteins in squid pen.

The identification of TKU011 chitosanase was identical to a chitin-binding protein Cbp21 from *S. marcescens* with 63% sequence coverage as reported previously (Wang, Peng, et al., 2008). Chitin binding protein, Cbp21, produced by *S. marcescens* has been reported to be an essential factor in chitin degradation (Suzuki, Suzuki, Taiyoji, Nikaidou, & Watanabe, 1998; Vaaje-Kolstad, Horn, van Aalten, Synstad, & Eijsin, 2005), and is, with chitinase A, one of the major proteins excreted into the culture supernatant when this bacteria is cultivated in the presence of chitin (Suzuki et al., 1998). Therefore, it also might induce other excreted proteins by *S. marcescens* TKU011 fermentation in squid pen include a chitin-binding protein and another protease that is able to promote chitin degradation. As for deproteinization, according to the reported results of deproteinization of shellfish chitin wastes, microbial deproteinization has better effect than enzymatic (protease) deprotenization (Gagne & Simpson, 1993; Oh et al., 2000; Yang et al., 2000; Wang, Kao, et al., 2006). This coincides with our result in this research. In other words, during microbial deproteinization, microbial strains used would be affected (induced) by chitin and protein contained in the shellfish chitin wastes and thus produced the activities of

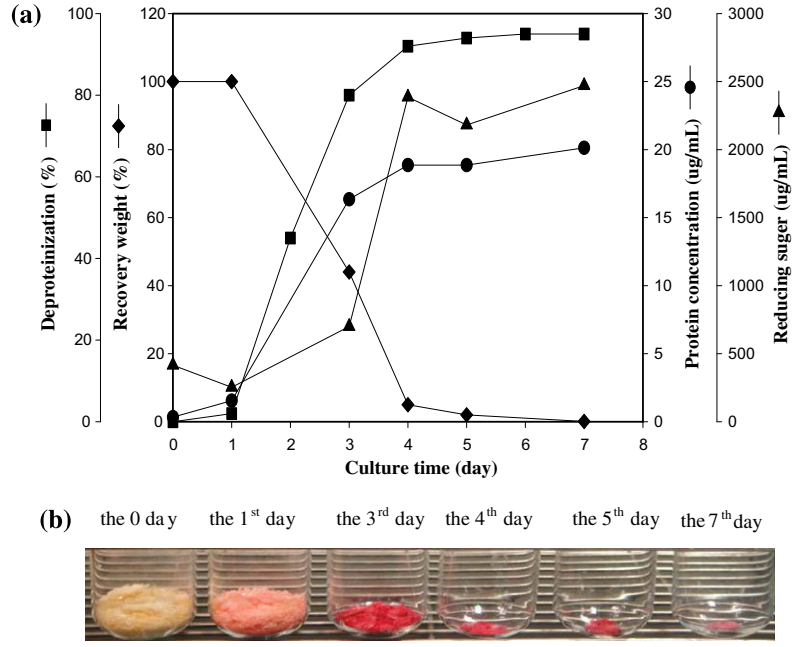

Fig. 5. (a) The degradation of squid pen in liquid phase fermentation by *S. marcescens* TKU011. *S. marcescens* TKU011 was grown in liquid medium heated at 121 °C for 120 min containing 1% squid pen powder, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ in an shaking incubator at 25 °C. The protein concentration and reducing sugar of the culture supernatant were measured. The ratio of deproteinization was calculated from the ratio of the dry weight decrease of squid pen powder after alkali treatment. (b) The photo of residual squid pen by *S. marcescens* TKU011 fermentation for 0–7 days.

protease and chitosanase at the same time. Besides, the peptides, reducing sugar and pigment (Fig. 5b) in the culture supernatant could be recovered for biological applications.

With this method, TKU011 protease and chitosanase were produced at the 5th day, deproteinization of squid pen for β-chitin and peptide preparation were at the 3rd day, the production of reducing sugar was at the 4th day. These results may be useful for biological applications in relation to enzyme production and chitin/protein degradation.

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