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Purification and characterization of a chitosanase from *Serratia marcescens* TKU011

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Abstract—A chitosanase was purified from the culture supernatant of *Serratia marcescens* TKU011 with shrimp shell wastes as the sole carbon/nitrogen source. Zymogram analysis revealed the presence of chitosanolytic activity corresponding to one protein, which was purified by a combination of ion-exchange and gel-filtration chromatography. The molecular weight of the chitosanase was 21 kDa and 18 kDa estimated by SDS–PAGE and gel-filtration, respectively. The optimum pH, optimum temperature, pH stability, and thermal stability of the chitosanase were 5, 50 °C, pH 4–8, and <50 °C, respectively. The chitosanase was inhibited completely by EDTA, Mn^{2+}, and Fe^{2+}. The results of peptide mass mapping showed that three tryptic peptides of the chitosanase were identical to a chitin-binding protein Cbp21 from *S. marcescens* (GenBank accession number gi58177632) with 63% sequence coverage.

Keywords: *Serratia marcescens*; Chitosanase; Chitosan; Chitin; Shrimp shell wastes

1. Introduction

Chitin is the second-most abundant polysaccharide with an annual production of 10^{10}–10^{11} tons per annum, which is only next to that of cellulose. Chitin and chitosan are commercially obtained from shrimp and crab shells from the fishing industry. Chitosan, a D-glucosamine polymer, is a totally or partially deacetylated derivative of chitin. It is usually obtained by the artificial deacetylation of chitin in the presence of alkali.[1] Recent studies on chitin and chitosan have attracted interest for their conversion to oligosaccharides, because these oligosaccharides are not only water-soluble, but also possess versatile functional properties such as antitumor and antimicrobial activities.[1–3] Traditionally, chitosan oligosaccharides were processed by chemical methods in the industry. There are many problems existing in these chemical processes, such as the production of a large amount of short-chain oligosaccharides, low yields of oligosaccharides, high cost in separation, and also environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost, and reproducibility, chitosanase hydrolysis becomes more and more popular in recent years.[4,5]

Most chitosanases are found in microorganisms while a few are found in plants.[4,5] However, to date, there is no paper that reports on the production of a chitosanase by *Serratia marcescens*. Almost all of the chitosanase-producing strains 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.[1,6] The utilization of shellfish waste not only solves environmental problems, but also decreases the production cost of microbial chitosanases. The production of an inexpensive chitosanase is an important element in the process.

Bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes. To further enhance the utilization of

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chitin-containing marine crustacean waste, we have previously investigated the bioconversion of shrimp- and crab-shell powder of marine waste for the production of proteases[7] and chitinases.[1,6]

In this study, shrimp-shell powder was used as a substrate for isolating and screening of chitosanase-producing strains. The potential strain TKU011 was isolated from the soil in Taiwan and identified as *S. marcescens*, which displayed chitosanase activities when cultured in a shrimp-shell powder medium. Considering the potential of such an enzyme that could be used in biotechnological processes, together with the fact that the extracellular chitosanase of *S. marcescens* had not been isolated and purified, the current work presents the purification, characterization and identification of a chitosanase obtained from *S. marcescens* TKU011. The enzyme is compared with chitosanases isolated from other microbial sources.

2. Materials and methods

2.1. Materials

The squid pen powder (SPP), shrimp-shell powder (SSP), shrimp- and crab-shell powder (SCSP) used in these experiments were prepared as described earlier.[8] Squid pens, shrimp shells, and crab shells were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). Katsuobushi from macherel (KM) and bonito (KB) were purchased from Yi-Fu Food Co. (I-Lan, Taiwan). In the preparation of chitosanase, the squid pens, shrimp shells, and crab 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 chitosanase production. DEAE-Sepharose CL-6B and Sephacryl S-100 were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). All other reagents used were of the highest grade available.

2.2. Isolation and screening of protease/chitosanase-producing strains

Microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 1% SCSP, 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 2% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$) in shaking flasks at 37 °C and 150 rpm. After incubation for 2 days, the culture broth was centrifuged (4 °C and 8200*g* for 20 min), and the supernatants were collected for measurement of protease/chitosanase activity using the procedure described below. The strain TKU011 that showed the highest protease/chitosanase activity was isolated, maintained on nutrient agar, and used throughout the study.

2.3. Enzyme production and purification

2.3.1. Culture conditions. In the investigation of the culture conditions, 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 0.5–3% (w/v) of various carbon sources to be investigated. The carbon sources investigated included SPP, SSP, SCSP, KM, or KB. Various volumes of the resultant medium in a 250-mL Erlenmeyer flask were aerobically cultured at 25 °C for 1–4 days on a rotary shaker (150 rpm). After centrifugation (12,000*g*, 4 °C, for 20 min), the supernatants were collected for the measurement of chitosanase activity.

2.3.2. Production of chitosanase. For the production of chitosanase, *S. marcescens* TKU011 was grown in 50 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 milliliter of the seed culture was transferred into 50 mL of the same medium and grown in an orbital shaking incubator for 2 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.

2.3.3. DEAE-Sepharose CL-6B chromatography. To the culture supernatant (900 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 dialyzed against the buffer. The resultant dialysate (25 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) and equilibrated with 50 mM sodium phosphate buffer (pH 7). One chitosanase was washed from the column with the same buffer, and a protease was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The peak showing higher chitosanase activity (Fig. 1) was 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).

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

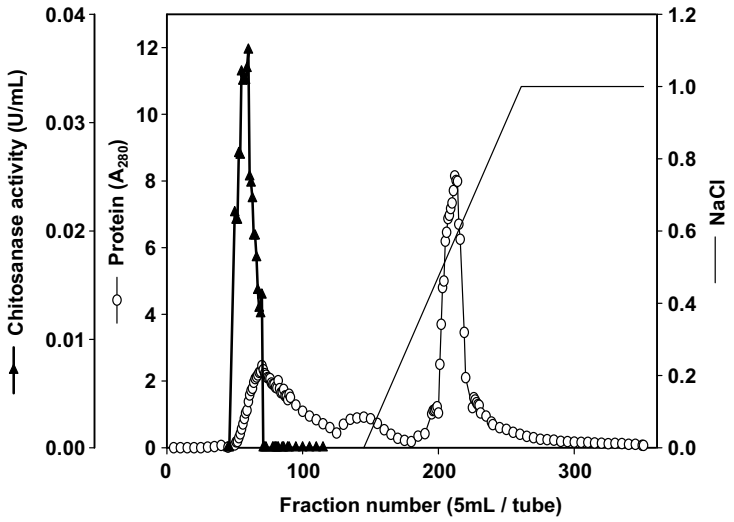

Figure 1. Elution profile of TKU011 chitosanase on DEAE-Sepharose CL-6B: (○) absorbance at 280 nm; (▲) chitosanase activity (U/mL).

2.4. Protein determination

Protein content was determined by the method of Bradford[9] 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 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[10] 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 polyacrylamide gel electrophoresis

The molecular mass of the purified chitosanase was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli.[11] 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-lactalbumin (14.4 kDa). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercaptoethanol. The protein bands on the gel after electrophoresis were visualized by silver staining. The molecular mass of the TKU011 chitosanase 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 (1.5 cm × 1.5 cm, Amersham Pharmacia) 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.

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 chitosanase was determined by using liquid chromatography–tandem mass spectrometry (LC–MS/MS) by Mission Biotech, Taiwan. Fragment spectra were searched against the NCBI non-redundant protein database. Database searches were carried out using the MASCOT search engine.

2.8. Effect of organic solvents on chitosanase stability

The organic solvent stability of TKU011 chitosanase was studied by incubating the chitosanase preparation (3 mL) with various organic solvents (1 mL) at 4 °C and 25 °C for 10 days, respectively. The organic solvent stability of the chitosanase was determined by the measurement of the residual activity.[12]

3. Results and discussion

3.1. Identification of the strain TKU011

TKU011 is a Gram-negative and non-spore-forming bacillus, with catalase but without 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, TKU011 is most closely related to *S. marcescens* subsp. *marcescens*, and the similarity of 16S rDNA partial base sequence was more than 97%. The identification of strain TKU011 was carried out by the Bioresource Collection and Research Center (Shin-Chu, Taiwan).

3.2. Culture conditions and enzyme production

In our previous experiments (data not shown), we found 50 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$, pH 7) containing 2% SSP was better for the production of protease by strain TKU011 at 25 °C for 2 days. However, at the same time, we found chitosanase activity in the culture supernatant. To study the time course of cultivation, 50 mL of the media (2% SSP contained basal medium, pH 7) was used, and the relationship between incubation time (1–4 days), chitosanase activity was investigated. As shown in Figure 2, maximum chitosanase activity (0.024 U/mL) was found at the 2nd day by using 50 mL of the media.

3.3. Isolation and purification

The purification of the TKU011 chitosanase from the culture supernatant (900 mL) was described under Section 2.3. As shown in Table 1, the purification steps were combined to give an overall purification of about 50-fold for chitosanase. The overall activity yield of the purified chitosanase was 24%, with specific chitosanase activity of 7 U/mg. The final amount of TKU011 chitosanase obtained was 69 mg. The purified enzyme migrated as a single band in SDS–PAGE, and the apparent molecular mass of the chitosanase was about 21 kDa (Fig. 3), whereas it was estimated as approximately 18 kDa by Sephacryl S-100 gel-filtration.

To date, there has been no report on a chitosanase from *S. marcescens*. However, some *Serratia* sp. chitinases were found to exhibit chitosanase activity. Different from other reported *Serratia* sp. chitinases, TKU011 chitosanase was not found to exhibit chitinase activity. The molecular mass of TKU011 chitosanase (21 kDa) was obviously different from most of the other *Serratia* sp. chitinase/chitosanase, such as, *Serratia* sp. KCK (57 kDa),[13] *S. marcescens* NK1 (57 kDa),[14] *S. marcescens* BJL200 (55.5 kDa),[15] *S. marcescens* QMB1466 (58 kDa),[16] *S. marcescens* 2170 (50 and 47 kDa),[17] and *Serratia plymuthica* HRO-C48 (60.5 and 95.6 kDa).[18] Though most *Serratia* sp. chitinase/chitosanase had molecular mass in the range of 47–60 kDa, the molecular mass of TKU011 chitosanase was smaller than these reported values. This is a novel characteristic about *Serratia* sp. chitosanase.

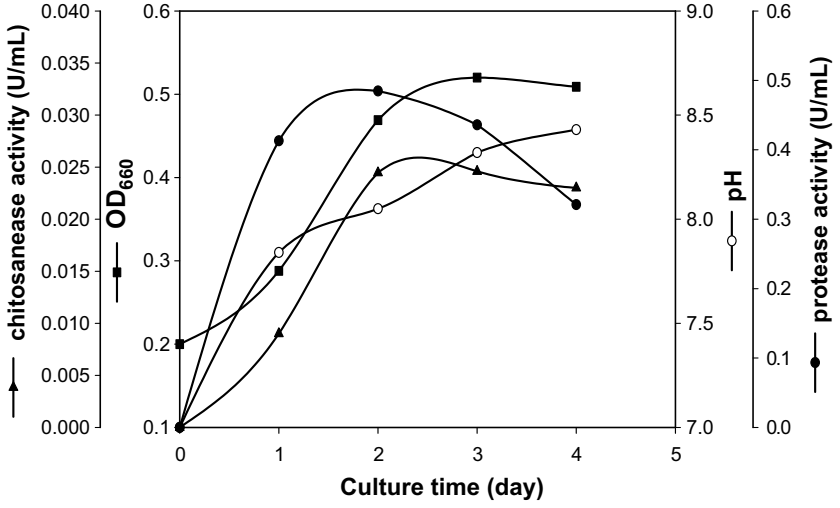

Figure 2. Time courses of chitosanase production in a culture of *S. marcescens* TKU011 on shrimp shell containing media: (●) protease activity (U/mL); (▲) chitosanase activity (U/mL); (○) pH; (■) OD_{660}.

Table 1. Purification of chitosanase from *Serratia marcescens* TKU011[a]

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture supernatant	14,378	29	0.002	1	100
$(NH_4)_2SO_4$ ppt	1964	27	0.014	7	93
DEAE-Sepharose	431	9	0.02	10	31
Sephacryl S-100	69	7	0.1	50	24

[a] *S. 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 shaking incubator for 2 days at 25 °C.

Figure 3. SDS–PAGE analysis of the purified chitosanase produced by strain TKU011. Lanes: S, molecular markers (97.4, 66.2, 45, 29, 20.1, 14.4 kDa); 1, the purified chitosanase.

3.4. Effect of pH and temperature

The effect of pH on the catalytic activity was studied by using chitosan as a substrate under the standard assay conditions. The pH activity profile of the chitosanase was with maximum values at pH 5 (Fig. 4). The optimum pH of the chitosanase was similar to the other *Serratia* sp. chitinase/chitosanase, such as 6.2 of *S. marcescens* NK1,[14] 5–6 of *S. marcescens* BJL200,[15] 5.4 and 6.6 of *S. plymuthica* HRO-C48.[18] The pH stability profile of the chitosanase 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 chitosanase activity was stable at pH 4–8 (Fig. 4).

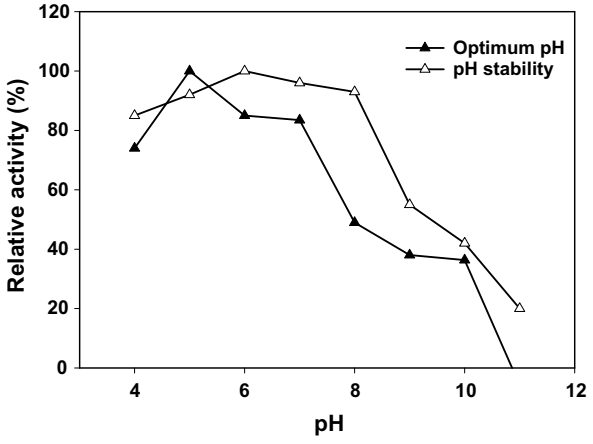

Figure 4. Effect of pH on the activity and stability of the purified chitosanase.

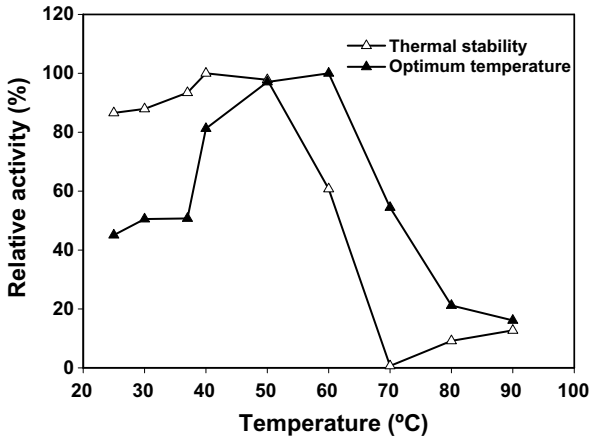

Figure 5. Effect of temperature on the activity and stability of the purified chitosanase.

The effect of temperature on the activity of chitosanase was studied with chitosan as a substrate. The temperature profile of chitosanase activity is presented in Figure 5. The optimum temperature for TKU011 chitosanase was 60 °C. The optimum temperature of the chitosanase was similar to the other *Serratia* sp. chitinase/chitosanase, such as *S. marcescens* NK1 (47 °C),[14] *S. marcescens* BJL200 (50–60 °C),[15] *S. marcescens* 2170 (60 °C),[17] and *S. plymuthica* HRO-C48 (55 °C).[18] To examine the heat stability of TKU011 chitosanase, 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 chitosanase maintained more than 90% of its initial activity from 25 to 50 °C, but was rapidly inactivated retaining only 60% of its initial activity at 60 °C, and was completely inactivated at greater than 70 °C. Therefore, TKU011 chitosanase was not stable under its optimum temperature. Accordingly, the temperature for determination of TKU011 chitosanase activity should be from 25 to 50 °C, not at 60 °C.

3.5. Effects of various chemicals

To further characterize the *S. marcescens* TKU011 chitosanase, 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 30 min at 37 °C and then measuring the residual chitosanase activity by using chitosan as a substrate. The results are summarized in Table 2. Zn^{2+} and Cu^{2+} inhibited the chitosanase activity by 51Mn^{2+} and Fe^{2+}. EDTA, a chelator of divalent cations, was a strong inhibitor, suggesting the metal ions were essential for the catalytic action of the enzyme. Compared with the microbial chitosanase, most of the chitosanases

Table 2. Effects of various chemicals on chitosanase activity of TKU011[a]

Chemicals	Concentration (mM)	Relative activity (%)
None	0	100
PMSF	5	27
EDTA	5	0
Mg^{2+}	5	94
Cu^{2+}	5	7.7
Fe^{2+}	5	0
Ca^{2+}	5	97
Zn^{2+}	5	51
Mn^{2+}	5	0
Ba^{2+}	5	103

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

remarkably increased activity with Mn^{2+}.[19,20] However, TKU011 chitosanase was completely inhibited by Mn^{2+}. This result was different from other microbial chitosanases.

3.6. Organic solvent stability

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, ethyl ether, toluene, ethyl acetate, acetonitrile, acetone, butanol, isoamyl alcohol, and isopropyl alcohol) on the stability of TKU011 chitosanase were investigated. After being exposed in phosphate buffer (50 mM) (control) at 4 °C and 25 °C for 10 days, the remaining chitosanase activities in the culture supernatant were 0.02 U/mL and 0.005 U/mL, respectively. In addition, in the presence of 25% (v/v) tested organic solvents, the chitosanase retained more than 50% of its activity after keeping at 4 °C for 10 days. The chitosanase was activated after keeping at 4 °C for 10 days, especially in the presence of 25% (v/v) toluene, acetonitrile, and butanol. However, after keeping at 25 °C for 10 days, the chitosanase was inactivated in the presence of 25% (v/v) ethanol and ethyl ether. But in the presence of 25% (v/v) other tested organic solvents, the chitosanase retained its original activity after keeping at 25 °C for 10 days (data not shown). From this study, it can be concluded that organic solvent stability of the enzyme depends on the nature of organic solvents. This means that replacement of some water molecules in an enzyme with organic molecules sometimes stabilizes the structure of the enzyme.

Different from other reported chitosanase-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 chitosanase-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 chitosanase seems to provide a promising approach.

3.7. Identification of TKU011 chitosanase by LC–MS/MS analysis

To identify the protein of chitosanase activity appearing as a prominent 21 kDa band on SDS–PAGE gel, the band was excised and analyzed after tryptic digestion. The 21 kDa band from SDS–PAGE gel was subjected to electrospray-ionization tandem mass spectrometric analysis. The fragment spectra were subjected for the NCBI non-redundant protein database search. The spectra matched three tryptic peptides (Table 3) that were identical to a chitin-binding protein Cbp21 from *S. marcescens* (GenBank accession number gi58177632) with 63% sequence coverage. The sequence of the chitin-binding protein Cbp21 gave a calculated nominal mass of 18782 Da and a pI of 7.11, similar to the experimental values obtained with the purified chitosanase. The identification of TKU011 chitosanase was carried out by Mission Biotech, Taiwan.

3.8. Substrate specificity

The activities of the TKU011 chitosanase with various substrates are summarized in Table 4. The enzyme showed activities toward chitosan with different degrees of deacetylation, but no activity toward colloidal chitin and chitin. The most susceptible chitosans were 85–95% deacetylated. Moderate susceptible chitosans were 73–82% deacetylated. A preparation of 60% deacetylated chitosan was less susceptible to the action of the enzyme.

Table 3. Identification of TKU011 chitosanase by LC–MS/MS[a]

Peptide sequence	Identified protein	Accession number
LQLNTQCGSVQYEPQSVEGLKGFPQAGPADGHIASADKSTFFELDQQTPTR	Cbp21[b]	gi58177632
TGPNSFTWK	Cbp21[b]	gi58177632
YFITKPNWDASQPLTRASFDLTPFCQFNDGGAIPAAQVTHQCNIPADR	Cbp21[b]	gi58177632

[a] Peptide fragments were identified by LC–MS/MS and by database searching.
[b] Chain A, crystal structure of the *Serratia marcescens* chitin-binding protein.

Table 4. Substrate specificity of TKU011 chitosanase

Substrate	Relative activity (%)
Chitosan (95% DD)	100
Chitosan (85% DD)	59
Chitosan (82% DD)	49
Chitosan (80% DD)	34
Chitosan (73% DD)	38
Chitosan (60% DD)	33
Colloidal chitin	0
Chitin (α-type)	0
Chitin (β-type)	0

In this study, we employed LC–MS/MS to identify the TKU011 protein (which had chitosanase activity) that specifically bound to chitin (Table 3). In the past study, almost all of the chitin-binding proteins from microorganism had chitinase activity, such as *S. marcescens* 2170, *S. marcescens* BJL200, *Vibrio alginolyticus* 283, and *Pseudomonas aeruginosa*.[21–23] The TKU011 chitosanase was not bound by chitin and showed no enzymatic activity against colloidal chitin and chitin. (Table 4).

3.9. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals

It has been reported that chitin and chitosan has antioxidative[24,25] and anticarcinogenic[3,6] properties. To increase the utilization of these chitin-containing shrimp shell wastes, we incubated *S. marcescens* TKU011 for two days under the optimal culture conditions described above (2% SSP, 25 °C) and analyzed the antioxidant activity of the culture supernatant. The antioxidant activity assayed was the scavenging ability on DPPH. Another chitinase/protease-producing strain (*Serratia ureilytica* TKU013) in our laboratory can also use shrimp shell wastes as the sole carbon/nitrogen source. In this study, strain TKU013 was incubated for four days under optimal conditions (1.5% squid pen powder, 25 °C), which is optimal for chitinase production. The antioxidant activity of the culture supernatant was analyzed and compared with that of strain TKU011. The results showed that the scavenging ability of the culture supernatant of *S. marcescens* TKU011 and *S. ureilytica* TKU013 was about 22% and 72% per mL, respectively. In further investigations on the effect of different carbon/nitrogen (crab-shell powder, chitin powder, chitosan powder) on the production of antioxidant materials by TKU011 and TKU013, no increase on the antioxidant activity was found (data not shown). Both the species studied here, *S. marcescens* TKU011 was a chitosanase/protease-producing strain, while *S. ureilytica* TKU013 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 and strain TKU013 chitinase, which is the same as the other reported *Serratia* chitinase, does not have chitosanase.

The different results of antioxidant productivity might be related to the substrate specificity of the two enzymes.

In conclusion, we have purified and characterized a chitosanase from the culture supernatant of *S. marcescens* TKU011 using shrimp shells as the sole carbon/nitrogen source. In addition, it was also found that the culture supernatant has antioxidant activity as well.

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