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ORIGINAL PAPER

Biodegradation of shellfish wastes and production of chitosanases by a squid pen-assimilating bacterium, *Acinetobacter calcoaceticus* TKU024

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Abstract Two chitosanases (CHSA1 and CHSA2) were purified from the culture supernatant of *Acinetobacter calcoaceticus* TKU024 with squid pen as the sole carbon/nitrogen source. The molecular masses of CHSA1 and CHSA2 determined by SDS-PAGE were approximately 27 and 66 kDa, respectively. The optimum pH, optimum temperature, pH stability, and thermal stability of CHSA1 and CHSA2 were (pH 6, 50°C, pH 4–10, <90°C) and (pH 7, 60°C, pH 6–11, <70°C), respectively. CHSA1 and CHSA2 had broad pH and thermal stability. CHSA1 and CHSA2 were both inhibited by EDTA and were inhibited completely by 5 mM Mn^{2+}. CHSA1 and CHSA2 degraded chitosan with DD ranging from 60 to 98%, and also degraded some chitin. The most susceptible substrate was 60% deacetylated chitosan. Furthermore, TKU024 culture supernatant (1.5% SPP) incubated for 5 days has the most reducing sugars (0.63 mg/ml). With this method, we have shown that shellfish wastes may have a great potential for the production of bioactive materials.

Keywords Chitosanase · Squid pen wastes · *Acinetobacter calcoaceticus* · Reducing sugar

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Introduction

Squid pen (SP) contain chitin, protein and inorganic compounds, which are mainly composed of calcium carbonate. The production of chitin and its hydrolyzates, such as *N*-acetylglucosamine and chitooligosaccharides, from waste of the shellfish industry has been limited due to the high cost of chitinase and the SP pretreating process. The oligosaccharides of chitin, prepared by hydrolyzing chitin with chitinase or chitosanase, have various potential applications in the fields of food, agricultural, and pharmaceutical industries (Wang et al. 2006a).

Chitosanases have been found in abundance in a variety of bacteria (Chiang et al. 2003; Gao et al. 2008; Kurakake et al. 2000; Zhu et al. 2003). Almost all of the chitosanase-producing strains used colloidal chitosan or chitosan as a major carbon source, such as *Acinetobacter* sp. C-17 (Zhu et al. 2003), *Bacillus cereus* D-11 (Gao et al. 2008), *B. cereus* S1 (Kurakake et al. 2000) and *Aspergillus* sp. CJ22-326 (Chen et al. 2005).

However, preparation of chitin/chitosan involves demineralization and deproteinization of shellfish waste by the use of strong acids or bases (Liang et al. 2007; Wang et al. 2006b). The utilization of squid pen wastes not only solves environmental problems but also decreases the production cost of microbial chitosanases. Among these published chitosanase producing strains, few have been found to utilize marine wastes as carbon/nitrogen source. The production of inexpensive chitosanase is an important element in the process.

Reports about production of chitosanases by *Acinetobacter* species seem to be scarce (Zhu et al. 2003). In this study, we attempted to optimize the culture conditions of *A. calcoaceticus* TKU024 for maximal chitosanase productivity by using cheap carbon/nitrogen source of squid pen powder. In addition, two chitosanases from *A. calcoaceticus* TKU024 were also purified, characterized, and compared with chitosanases isolated from other bacterial sources.

Materials and methods

Materials

The squid pen powder (SPP) used in these experiments was prepared as described earlier (Wang et al. 2006a). SPP was purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). DEAE-Sepharose CL-6B, Phenyl Sepharose and Sephacryl S-100 were purchased from GE healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). Weak-base anion-exchanger Macroprep DEAE was from Bio-Rad (Hercules, CA, USA). The standard proteins (Geneaid, Taiwan) used for calibration by sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) 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). All other reagents used were of the highest grade available.

Isolation and screening of chitosanase producing strains

Microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 1% SPP, 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 1% SPP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) in shaking flasks at 30°C on a rotary shaker (150 rpm, Yih Der LM-570R). After incubation for 2 days, the culture broth was centrifuged (4°C and 12,000×*g* for 20 min, Kubota 5922) and the supernatants were collected for measurement of chitosanase activity using the procedure described below. The strain TKU024 that showed the highest chitosanase activity was isolated, maintained on SPP agar, and used throughout the study.

Identification of strain TKU024

The bacterial strain TKU024 was identified on the basis of morphological, physiological and biochemical parameters as well as on the basis of 16S rDNA based sequence analysis after PCR amplification with primers and cloning. Nucleotide bases of the DNA sequence obtained were compiled and compared with sequences in the GenBank databases using BLAST program. Strain TKU024 was further characterized and identified using standard morphological, physiological and biochemical plate and tube tests and API 20NE system (ATB system, bioMérieux SA, Marcy-I'Etoile, France). API 20NE strips were incubated for 24 h at 30°C. The identification of strain TKU024 was carried out according to the API 20NE identification manual.

Purification of the chitosanases

Production of chitosanases

For the production of chitosanases, *A. calcoaceticus* TKU024 was grown in 50 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 1.5% SPP, 0.1% K_2HPO_4, 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 4 days at 37°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.

DEAE-Sepharose CL-6B chromatography

To the culture supernatant (870 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 (50 ml) was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). One chitosanase (CHSA1) was washed from the column with the same buffer and another chitosanase (CHSA2) was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions of the two peaks containing the chitosanase activity were respectively pooled and concentrated by ammonium sulfate precipitation. The resultant precipitates were collected by centrifugation and dissolved in 5 ml of 50 mM sodium phosphate buffer (pH 7).

Phenyl Sepharose chromatography

The obtained enzyme solution (the unadsorbed chitosanase fractions from DEAE-Sepharose CL-6B column) was then chromatographed on a column of Phenyl Sepharose (1.3 cm × 20 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7) containing 1 M $(NH_4)_2SO_4$. The chitosanase was eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$ in the same buffer. The chitosanase fractions were collected and the enzyme activity was measured. Fractions with confirmed enzyme activity were pooled, dialyzed overnight at 4°C against 50 mM sodium phosphate buffer pH 7, and lyophilized.

Macro-prep DEAE chromatography

The obtained enzyme solution (the adsorbed chitosanase fractions from DEAE-Sepharose CL-6B column) was then chromatographed on a column of Macro-prep DEAE (12.6 mm × 40 mm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7). The chitosanase was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions containing the chitosanase activity were pooled and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 50 mM sodium phosphate buffer (pH 7).

Sephacryl S-100 chromatography

These two resultant enzyme solutions were respectively loaded onto a Sephacryl S-100 gel filtration column (2.5 cm × 120 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7), then eluted with the same buffer. One peak exhibiting chitosanase activity for each enzyme solution was obtained and pooled fractions for each enzyme solution were used as a purified preparation.

Protein determination

Protein content was determined by the method of Bradford 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 (Wang et al. 2006a).

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 (Kiotec Co., Hsinchu, Taiwan; with 60% deacetylation) 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 (Wang et al. 2008). Specific activity was expressed as units per mg protein (U mg^{-1} protein) of the enzyme extract.

Determination of molecular mass

The molecular masses of the purified chitosanases (CHSA1 and CHSA2) were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

(SDS-PAGE) (Laemmli 1970) using 12.5% acrylamide and 2.67% methylene bis acrylamide in 0.375 M Tris–HCl buffer (pH 8.8) with 0.1% (w/v) SDS. Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercaptoethanol. The electrode buffer was 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS (pH 8.3). Electrophoresis was performed at a constant current of 70 mA through the stacking gel and 110 mA through the resolving gel. After electrophoresis, 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 masses of CHSA1 and CHSA2 in the native form were determined by a gel filtration method. The sample and standard proteins were applied to a Sephacryl S-100 column (2.5 cm × 120 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 (Wang et al. 2008).

Mass spectrometry and protein identification

Bands of interest on SDS-PAGE gel were excised and in-gel digested with trypsin. The identification of CHSA1 and CHSA2 was determined by using liquid chromatography–tandem mass spectrometry (LC-MS/MS) by the Mission Biotech, Taiwan. Fragment spectra were searched against the NCBI non-redundant protein database. Database searches were carried out using the MASCOT search engine.

Effect of various chemicals and surfactants on chitosanase activities

The effects of metal ions (5 mM) were investigated using Mg^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, and Ba^{2+}. The effects of chitosanase inhibitors were studied using phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA). The effects of surfactants were also studied using SDS, Tween 20, Tween 40, and Triton X-100. Enzyme was pre-incubated with various chemicals and surfactants for 30 min at 37°C and then the residual activity was tested using water soluble chitosan as substrate.

Results and discussion

Identification of the strain TKU024

To characterize strain TKU024, 16S rDNA and phylogenetic analyses were utilized. According to the analysis of 16S rDNA gene sequence, TKU024 was most closely aligned to *Acinetobacter* sp. with 99% similarity. To characterize strain TKU024 further, standard morphological, physiological and biochemical plate and API 20NE analyses showed that strain TKU024 was Gram-negative, rod-shaped, and without a flagellum. It was negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, β-galactosidase, esculin hydrolysis gelatin hydrolysis, and oxidase assays. Strain TKU024 was positive for the assimilation of arabinose, *N*-acetyl-glucosamine, capric acid, adipic acid, malate, and trisodium citrate; negative for the assimilation of glucose, mannose, mannitol, maltose, potassium gluconate, and phenylacetic acid. According to the API 20NE identification, TKU024 was most close to *Acinetobacter baumannii* and *Acinetobacter calcoaceticus* with 98.9% similarity. However, strain TKU024 could not grow at 44°C. This characteristic of strain TKU024 was most close to *Acinetobacter calcoaceticus*. The phylogenetic identification and API 20NE analysis indicated that the strain TKU024 belongs to *Acinetobacter calcoaceticus*.

Culture conditions and enzyme production

In our preliminary experiments, we found 50 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 chitosanase by strain TKU024 at 30°C. To study the effect of carbon/nitrogen sources on the production of chitosanase, growth was carried out in 50 ml of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing additional carbon/nitrogen sources of 0.5–2% (w/v) SPP. The result showed that 1.5% SPP was more suitable as an inducer for chitosanase production than others (data not shown). Therefore, in the following experiments, 50 ml of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing additional carbon/nitrogen source of 1.5% (w/v) SPP was used. To study the time course of cultivation, 50 ml of the

media (1.5% SPP contained basal medium, pH 7) was used, and the relationship between incubation time (1–6 days), chitosanase activity was investigated. As shown in Fig. 1, maximum activity of chitosanase (0.39 U/ml) was found at the fourth day and then decreased gradually.

Isolation and Purification

The purification of the TKU024 chitosanases from the culture supernatant (870 ml) was described under *Materials and Methods*. First, the supernatant was submitted to ion exchange chromatography showing two peaks, CHSA1 and CHSA2 that both display chitosanase activity. After DEAE-Sepharose CL-6B chromatography, one protein peak containing the chitosanase activity (CHSA1) was washed from the DEAE-Sepharose CL-6B column, and another peak with chitosanase activity (CHSA2) was eluted from the column. By further purification, the purified CHSA1 (Fig. 2) and CHSA2 (Fig. 3) were obtained. As shown in Table 1, the purification steps were combined to give an overall purification of about 130.0-fold for CHSA1 and 140.5-fold for CHSA2. The overall activity yields of the purified CHSA1 and CHSA2 were 10 and 20% respectively, with specific chitosanase activities of 5.20 U/mg (CHSA1) and 5.62 U/mg (CHSA2). The final amounts of TKU024 chitosanases obtained were 5.0 mg (CHSA1) and 9.4 mg (CHSA2). In the enzyme purification processes, the recovery of the chitosanase activity decreased gradually. This may be due to the removal of some fractions containing chitosanase with lower specific activity during column chromatography. It is also possible that the chitosanase was unstable on a series of purification. CHSA1 and CHSA2 were also confirmed to be homogeneous by SDS-PAGE (Fig. 4a, b). The molecular masses of CHSA1 and CHSA2 were determined by SDS-PAGE and gel filtration were approximately 27/25 and 66/67 kDa, respectively.

Reports about production of chitosanases by *Acinetobacter* species seem to be scarce (Zhu et al. 2003). To our knowledge, only *Acinetobacter* sp. C-17 that produced chitosanase was reported in 2003 (Zhu et al. 2003). Another one *Acinetobacter* sp. that produced chitosanase was reported in 1995 (Shimosaka et al. 1995), but it was reidentified and renamed as a *Burkholderia gladioli* strain in 2000 (Shimosaka et al. 2000). The molecular mass of CHSA1 (27 kDa) and CHSA2 (66 kDa) was obviously different from *Acinetobacter* sp. C-17 chitosanase (35.4 kDa) (Zhu et al. 2003).

Effect of pH and temperature on the enzyme activity

The effect of pH on the catalytic activity was studied by using soluble chitosan as a substrate under the standard assay conditions. The pH activity profile of CHSA1 and CHSA2 were with maximum values at pH 6 and pH 7, respectively. Compared with

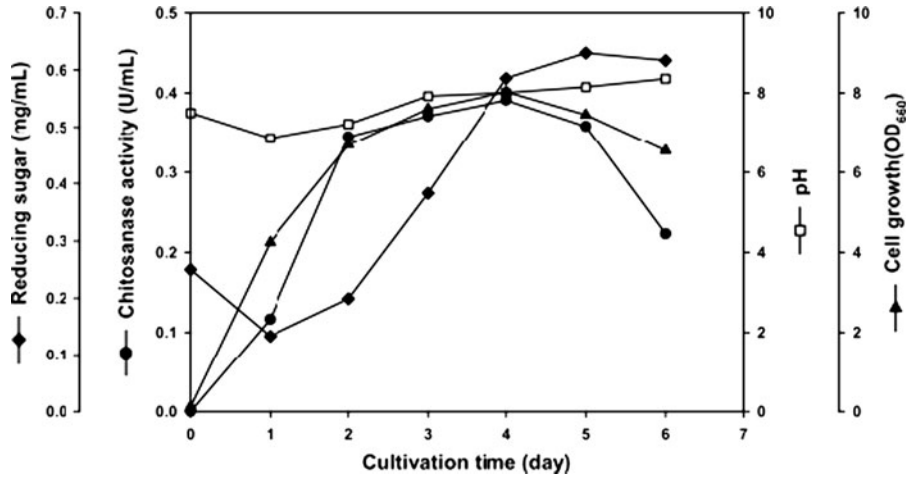

Fig. 1 Time courses of chitosanase production in a culture of *A. calcoaceticus* TKU024 on squid pen containing media: (*filled circle*) chitosanase activity (U/ml); (*filled triangle*) cell growth; (*filled diamond*) reducing sugar (mg/ml); (*open square*) pH

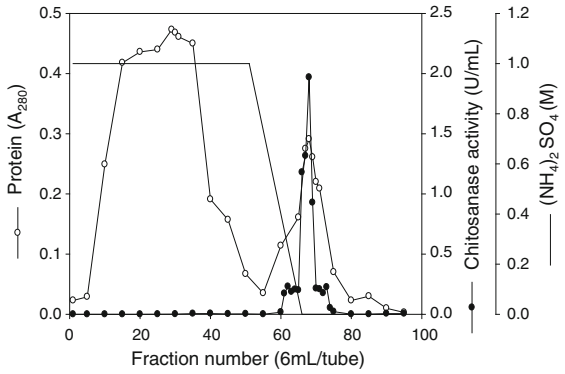

Fig. 2 Elution profile of TKU024 chitosanase (CHSA1) on Phenyl Sepharose 6 Fast Flow: (*open circle*) absorbance at 280 nm; (*filled circle*) chitosanase activity (U/ml)

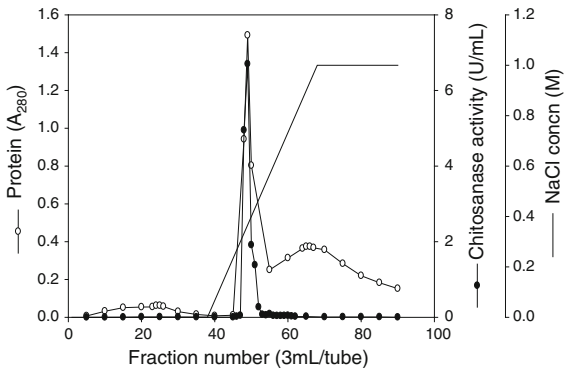

Fig. 3 Elution profile of TKU024 chitosanase (CHSA2) on Macro-prep DEAE: (*open circle*) absorbance at 280 nm; (*filled circle*) chitosanase activity (U/ml)

Acinetobacter sp. C-17 chitosanase, similar optimal pHs were obtained with pH 7 (Zhu et al. 2003). The pH stability profile of the chitosanase activity was determined by the measurement of the residual

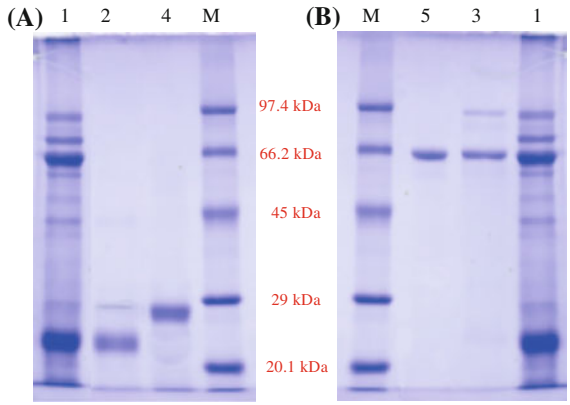

Fig. 4 SDS-PAGE analysis of CHSA1 (**a**) and CHSA2 (**b**) produced by strain TKU024. *Lanes*: M, molecular markers; 1, crude enzyme; 2, the unadsorbed chitosanase fractions after DEAE-Sepharose CL-6B chromatography; 3, the adsorbed chitosanase fractions after DEAE-Sepharose CL-6B chromatography; 4, CHSA1 (purified by Phenyl Sepharose); 5, CHSA2 (purified by Macro-Prep DEAE chromatography). The molecular mass markers 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)

activity at pH 7 after incubation at various pH values at 37°C for 60 min. The chitosanase activity of CHSA1 and CHSA2 were stable at pH 4–10 and pH 6–11, respectively (Fig. 5a). The pH stability profile of CHSA1 and CHSA2 was broader than *Acinetobacter* sp. C-17 chitosanase which was stable over the pH range of 5–9 (Zhu et al. 2003).

The effect of temperature on the activity of chitosanase was studied with soluble chitosan as a substrate. The optimum temperature of CHSA1 and CHSA2 were 50 and 60°C, respectively (Fig. 5b).

Table 1 Purification of chitosanases (CHSA1 and CHSA2) from *A. calcoaceticus* TKU024

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture supernatant	6177.0	270.0	0.04	1.0	100
$(NH_4)_2SO_4$ ppt	2214.6	260.0	0.12	3.0	96
DEAE-Sepharose					
CHSA1	134.8	59.5	0.44	11.0	22
CHSA2	212.5	110.6	0.52	10.4	41
Phenyl Sepharose					
CHSA1	5.0	26.0	5.20	130.0	10
Macro-prep DEAE					
CHSA2	9.4	52.8	5.62	140.5	20

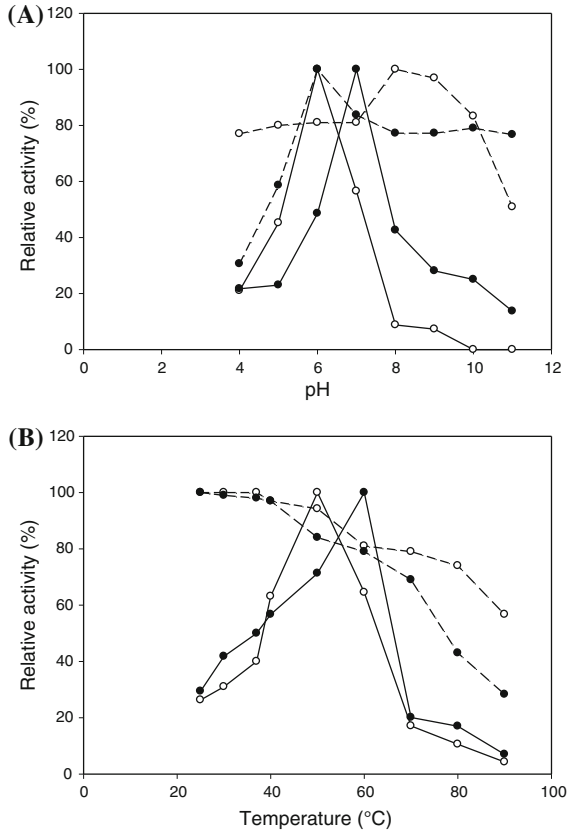

Fig. 5 Effect of pH (**a**) and temperature (**b**) on the activity (*solid line*) and stability (*dashed line*) of CHSA1 and CHSA2. (*open circle*) CHSA1; (*filled circle*) CHSA2

Compared with *Acinetobacter* sp. C-17 chitosanase, the optimum temperature of CHSA1 and CHSA2 were higher than *Acinetobacter* sp. C-17 chitosanase (36°C) (Zhu et al. 2003). To examine the heat stability of the chitosanases, 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. CHSA1 maintained its initial activity at less than 50°C and still maintained 60% of its initial activity at less than 90°C (Fig. 5b). CHSA2 maintained 70% of its initial activity at less than 70°C but was inactivated at higher than 80°C (Fig. 5b). However, *Acinetobacter* sp. C-17 chitosanase was stable only at low temperature. When *Acinetobacter* sp. C-17 chitosanase was kept at 40°C for 60 min, 27% of the activity was lost, and when the enzyme was kept at 60°C for 20 min, only 7% activity was detected (Zhu et al. 2003). The results showed that CHSA1 and CHSA2 had high thermal stability.

Table 2 Substrate specificities of CHSA1 and CHSA2 purified from *A. calcoaceticus* TKU024

Substrate	Relative activity (%)	
	CHSA1	CHSA2
Soluble chitosan (95% DD)	12	21
Soluble chitosan (85% DD)	3	18
Soluble chitosan (82% DD)	4	15
Soluble chitosan (73% DD)	5	0
Soluble chitosan (60% DD)	100	100
Colloidal chitin	60	48
Chitin (α-type)	18	8
Chitin (β-type)	16	8
CMC	0	0
Glycol chitosan	0	0

Substrate specificity

For the substrate specificity of CHSA1 and CHSA2, chitin, and chitosan with degree of deacetylation (DD) ranging from 60 to 95% were used as substrates, as summarized in Table 2. These enzymes could hydrolyze chitin and chitosan, but exhibited no activity on glycol chitosan and CMC. The chitosanases from *Amycolatopsis* sp. CsO-2 (Okajima et al. 1994), *Nocardioides* sp. K-01 (Okajima et al. 1995), *Bacillus circulans* MH-K1 (Yabuki et al. 1988), *Bacillus* sp. PI-7S (Seino et al. 1991), and *Bacillus* sp. CK4 (Yoon et al. 2001) were most active on approximately 100% deacetylated chitosan. The chitosanases from *Acinetobacter* sp. CHB101 (Shimosaka et al. 1995) and *Bacillus* sp. P16 (Jo et al. 2003) were most active on approximately 80% deacetylated chitosan. The chitosanase from *Penicillium islandium* (Fenton and Eveleigh 1981) was less active in hydrolyzing chitosan that less than 40% or more than 70% DD. The most susceptible substrates for TKU024 chitosanases (CHSA1 and CHSA2) were both 60% deacetylated chitosan, suggesting that CHSA1 and CHSA2 have specificity to the linkages of GlcN-GlcN and GlcNAc-GlcN and/or GlcN-GlcNAc, and the *N*-acetylglucosamine residues are important in the recognition and reaction mechanism of the substrate by these enzymes (Jo et al. 2003).

It has been reported that chitosanase was produced by using various carbon sources, for instance chitosan (Shimosaka et al. 1995; Chen et al. 2005; Gao et al. 2008), wheat bran (Chiang et al. 2003), glucose (Kurakake et al. 2000), and shrimp shell (Wang et al. 2008). Most chitosanases exhibit a wide range of substrate specificities (Fenton and Eveleigh 1981). In this study, CHSA1 and CHSA2 degraded chitosan specifically and cleaved chitosan with maximal activity on polymers of 60% DD. Thus, both glucosamine and *N*-acetylglucosamine are necessary for optimal activity. The chitin and lowly acetylated chitosans appear to be poor substrates because they contain either insufficient glucosamine or insufficient *N*-acetylglucosamine residues for optimal activity.

How does one define chitosanase and distinguish it from chitinase? Chitinases have been defined as enzymes which act on chitin and yield products with *N*-acetylglucosamine terminal residues. The initial simple concept of a chitosanase was a glycosidase that would hydrolyse chitosan to yield glucosamine terminal residues (Fenton and Eveleigh 1981). However, CHSA1 and CHSA2 do not fit this concept completely, and they showed activity toward both chitosan and chitin, although they reacted slowly with chitin. They may be chitosanase-chitinase enzymes with broad specificity, but showed maximal activity on chitosan of 60% DD. Therefore, it is fitting that CHSA1 and CHSA2 should be chitosanases. The further study of this interesting categorizing of enzymes should be done to determine their site of cleavage.

Effects of various chemicals on the enzyme activity

Metal ions had different impacts on chitosanases. It was reported that Ca^{2+} triggered the refolding of chitosanase from *B. subtilis* GM9804 (Colomer-Pallas et al. 2003). Therefore, we hypothesized that different metal ions might affect CHSA1 and CHSA2 activity through influencing the structure of the protein. EDTA was an activator of *Bacillus* sp. R-4 chitosanase (Somashekar and Joseph 1996). But to CHSA1 and CHSA2, it was an inhibitor. Therefore, CHSA1 and CHSA2 are metalloenzymes, the presence of metal ions non-covalently bound to the enzyme is an important means to influence its catalytic activity. To further characterize CHSA1 and CHSA2, 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 soluble chitosan (DD 60%) as substrate. The results showed that CHSA1 was completely inactivated by Mn^{2+} and significantly inactivated by $Zn^{2+}$and Cu^{2+} at 5 mM concentration (Table 3). CHSA2 was also completely inactivated by Mn^{2+} and significantly inactivated by Zn^{2+} and Ca^{2+} at 5 mM concentration (Table 3). However, the other ions little affected these enzymes activities. Moreover, CHSA1 and CHSA2 were still active when the metal ions were removed. This result was similar to those for other chitosanolytic enzymes and suggested the tested metal ions were not essential for the catalytic action of the enzymes.

Effect of various surfactants on the enzyme activity

Enzymes are usually inactivated by the addition of surfactants to the reaction solution because the structure of the enzymes might be influenced. Moreover, certain surfactants affected the chitooligosaccharides synthesis by chitosanase (Hsiao et al. 2008). Therefore, the effect of different surfactants (2%, v/v) on stability of CHSA1 and CHSA2 were also studied. CHSA1 and CHSA2 were incubated with surfactants (0.5-2%, v/v) at 37°C for 30 min and the remaining enzymatic activity were determined under normal assaying conditions. The chitosanase 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 CHSA1 and CHSA2 retained more than 86% of its original activity. At the presence of 2 mM SDS (anionic surfactant), the activity of CHSA1 was inhibited and CHSA2 retained about 90% of its original activity (Table 3). These differences between both enzymes might be related to the dissimilarity of the ratio of their hydrophobic and hydrophilic amino acids. Surfactants such as Tween 20 and Tween 40 had stimulatory effect on CHSA1 and CHSA2 chitosanase activity which may be due to change in the conformation of these enzymes thus increasing the substrate

Table 3 Effects of various chemicals and surfactants on chitosanase activities of CHSA1 and CHSA2 purified from *A. calcoaceticus* TKU024

Chemicals	Concentration (mM)	Relative activity (%)	
		CHSA1	CHSA2
None	0	100	100
PMSF	5	71	55
EDTA	5	46	24
Mg^{2+}	5	76	56
Cu^{2+}	5	33	64
Fe^{2+}	5	62	89
Ca^{2+}	5	81	39
Zn^{2+}	5	18	26
Mn^{2+}	5	0	0
Ba^{2+}	5	90	51
SDS	0.5/1/2	100/95/65	111/123/90
Tween 20	0.5/1/2 (%)	169/145/126	109/93/86
Tween 40	0.5/1/2/3/4 (%)	171/179/204/267/254	147/148/146/126/134
Triton X-100	0.5/1/2 (%)	112/172/155	111/111/98

Purified enzymes were preincubated with the various reagents at 37°C for 30 min and residual chitosanase activities were determined as described in the text. 100% was assigned to the activity in absence of reagents

accessibility. The CHSA1 chitosanase activity was highly stimulated (about 2.67-fold higher) in presence of Tween 40 (3%).

Identification of CHSA1 and CHSA2 by LC-MS/MS analysis

To identify the proteins (CHSA1 and CHSA2) of chitosanase activity appearing as prominents 27 and 66 kDa bands, respectively on SDS-PAGE gel, the bands were excised and analyzed after tryptic digestion. These bands from SDS-PAGE gel were subjected to electrospray tandem mass spectrometry analysis. The fragment spectra were subjected for the NCBI non-redundant protein database search. The spectra of CHSA1 and CHSA2 matched one and two tryptic peptides, respectively that were identical to chitinase from *Cellvibrio japonicus* Ueda107 (GenBank accession number gi192361966) and *Arthrobacter* sp. (GenBank accession number gi27065204), respectively with 4 and 7% sequence coverage, respectively. The identification of TKU024 chitosanases was carried out by the Mission Biotech, Taiwan.

The production of reducing sugar in liquid phase fermentation

To increase the utilization of the chitin/protein-containing marine wastes, we incubated *A. calcoaceticus* TKU024 for 1–6 days with various concentrations (0.5–2%) of SPP under the optimal culture conditions described above (50 ml, 37°C) and analyzed the reducing sugar and enzyme activity of the culture supernatants. As shown in Fig. 1, it was found that TKU024 culture supernatant (1.5% SPP) incubated for 5 days has the most reducing sugars (0.63 mg/ml) than others. Since the medium based on the SPP, reducing sugars were mainly chitooligosaccharides. The reducing sugar content increased during initial 5 days of fermentation and reached constant values thereafter. Besides, the amount of SPP in the culture media decreased with an increase in reducing sugar content (data not shown). Comparing the difference in culture time for optimums, the highest reducing sugar content (at the fifth day) showed later than the optimal chitosanase production (at the fourth day). Conjecturing from above results, those reducing sugars in the culture supernatant might be originated from the hydrolyzates (chitooligosaccharides) from hydrolysis of squid pen chitin by chitosanases.

Conclusion

This research used SPP as the sole carbon/nitrogen sources to produce chitosanases. This is different from most other chitosanase producing strains which require chitosan as carbon/nitrogen source. In this

study, we have succeeded in developing the efficient production procedure of chitosanases by *A. calcoaceticus* TKU024 using the cheap medium based on squid pen. Besides, reports about production of chitosanases by *Acinetobacter* species seem to be scarce (Zhu et al. 2003). Although the productivity is significantly improved by the optimization of cultivation conditions, further studies on the improvement of the productivity of *A. calcoaceticus* TKU024 by mutation or genetic engineering approaches are important to develop the industrial production process of chitosanases. In addition, we also have purified and characterized the chitosanases, and found that the culture supernatant has reducing sugars as well. Our data provide a useful example of utilizing SPP biowaste materials as valuable functional ingredients. It is expected that this bioactive material rich liquor will have beneficial biological functions due to the inherent chitin hydrolysis occurring during fermentation.

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