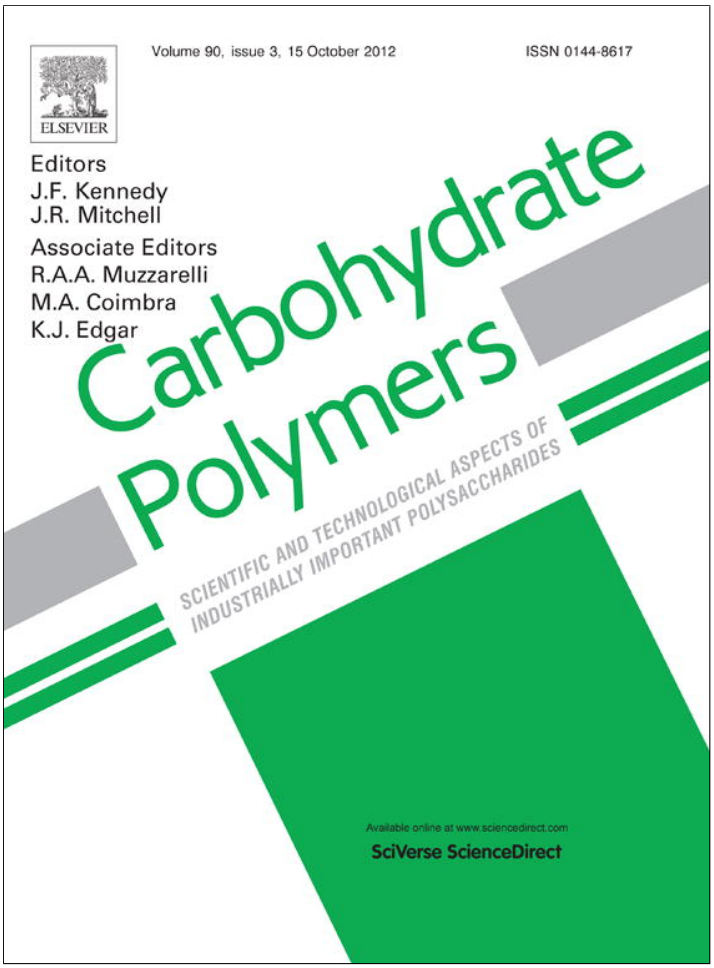

Carbohydrate Polymers 90 (2012) 1305–1313

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Fermented and enzymatic production of chitin/chitosan oligosaccharides by extracellular chitinases from *Bacillus cereus* TKU027

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ARTICLE INFO

Article history:
Received 28 May 2012
Received in revised form 25 June 2012
Accepted 27 June 2012
Available online 4 July 2012

Keywords:
Chitinase
Chitin oligosaccharides
Ahrimp heads
Bacillus cereus
Fermented
Enzymatic hydrolysis

ABSTRACT

Two chitinases, Chi I and Chi II, were purified from the culture supernatant of *Bacillus cereus* TKU027 with shrimp head powder (SHP) as the sole carbon/nitrogen source. The molecular masses of Chi I and Chi II determined using SDS–PAGE were approximately 65 kDa and 63 kDa, respectively. Chi I toward various surfactants showed high stability, such as SDS, Tween 20, Tween 40 and Triton X-100, and these surfactants were stimulator of Chi I chitinase activity. Concomitant with the production of Chi I and Chi II, chitin oligosaccharides were also observed in the culture supernatant, including chitobiose, chitotriose, chitotetrose and chitopentose at concentrations of 0.44 mg/mL, 0.08 mg/mL, 0.09 mg/mL and 0.43 mg/mL, respectively. Chitosan with 60% deacetylation was degraded by TKU027 crude enzyme to prepare chitooligosaccharides. MALDI-TOF MS analysis of the enzymatic hydrolyzates indicated that the products were mainly chitooligosaccharides with degree of polymerization (DP) in the 4–9 range.

1. Introduction

Chitin is one of the most widespread bio-polymer in nature (Muzzarelli et al., 2012). Among the natural chitinous resources, fishery wastes (shrimp and crab shells) especially have the highest content. In addition to edible parts, wastes amount to as high as 60–80% of the whole shrimp and crabs (Wang, Liang, & Yen, 2011). These wastes sometimes become an environmental threat due to their accumulation and slow degradation. Therefore, organisms that produce chitinases can not only solve environmental problems but also decreases the production cost of microbial chitinases.

Recent studies on chitosan/chitin have attracted interest for converting these species into oligosaccharides because the oligosaccharides not only are water-soluble but also possess versatile functional properties such as antitumor activity and antimicrobial activity (Liang, Chen, Yen, & Wang, 2007; Wang, Chen, & Wang, 2008; Wang, Lin, Yen, Liao, & Chen, 2006; Wang, Lin, Liang, et al., 2008). Traditionally, chitin oligosaccharides were processed using chemical methods in industries. Many problems existing in these chemical processes, such as the production of a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides, the high cost of separation and environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost and reproducibility, enzyme hydrolysis has become more popular in recent years (Wang, Chen, et al., 2008; Wang, Lin, et al., 2008). However, in our work, this investigation documents the purification and characterization of two novel chitinases from *B. cereus* TKU027 for the fermented and enzymatic production of chitin oligosaccharides. The fermented production of chitin oligosaccharides has advantage of omitting the procedure for purifying enzymes and helping decrease the cost of chitin oligomers production. Nevertheless, the enzymatic hydrolysis could obtain the chitin oligosaccharides with broader distribution range than the fermented method. The above technologies facilitate the potential use of this process in industrial applications and functional foods.

2. Materials and methods

2.1. Materials

The shrimp head powder (SHP) used in these experiments was prepared as described previously (Wang et al., 2006). The shrimp heads were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). During the preparation of the SHP, the shrimp heads were washed thoroughly with tap water and then dried. The dried materials obtained were milled to powders for use as the carbon source for chitinase production. The DEAE-Sepharose CL-6B and Sephacryl S-100 were purchased from GE healthcare UK Ltd.

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http://dx.doi.org/10.1016/j.carbpol.2012.06.077

(Little Chalfont, Buckinghamshire, England). The standard proteins (Geneaid, Taiwan) used for the calibration of the sodium dodecyl sulfate–polyacrylamide 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-lactalbumin (14.4 kDa). All other reagents used were of the highest grade available.

2.2. Isolation and screening of chitinase producing strains

The microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 1% SHP, 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. The organisms obtained from this screening were subcultured in liquid media (containing 1% SHP, 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 the measurement of chitinase activity using the procedure described below. The TKU027 strain that showed the highest chitinase activity was isolated, maintained on SHP agar and used throughout the study.

2.3. Identification of strain TKU027

The bacterial strain TKU027 was identified on the basis of morphological, physiological and biochemical parameters as well as on the basis of a 16S rDNA-based sequence analysis after PCR amplification with primers. The nucleotide bases of the DNA sequence obtained were compiled and compared with sequences in the GenBank databases using the BLAST program. Further identification of strain TKU027 was performed using the analytical profile index (API).

Strain TKU027 grew on nutrient agar plates. The bacteria which grew on the surface of agar plate were suspended by gentle mechanical agitation in 2 mL of sterile distilled water. This bacterial suspension was used to inoculate 50 CHB API strips (ATB System, bioMérieux SA, Marcy-l'Etoile, France) following the manufacturer's instruction. The strips were incubated at 30 °C and observed after 16, 24, 40 and 48 h and compared to the API identification index and database.

2.4. Purification of the chitinases

2.4.1. Production of chitinase

For the production of chitinase, *B. cereus* TKU027 was grown in 50 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SHP, 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 37 °C and pH 7.2 (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 using chromatography.

2.4.2. DEAE-Sepharose CL-6B chromatography

To the culture supernatant (940 mL), ammonium sulfate was added (608 g/L). The resultant mixture was stored at 4 °C overnight and the precipitate that 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 (55 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). One chitinase (Chi I) was washed from the column with the same buffer and another chitinase (Chi II) was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions of the two peaks containing the chitinase activity were independently pooled and concentrated using ammonium sulfate precipitation. The resultant precipitates were collected by centrifugation and dissolved in 5 mL of 50 mM sodium phosphate buffer (pH 7).

2.4.3. Sephacryl S-100 chromatography

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

2.5. Protein determination

The protein content was determined using the Bradford method with a 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., 2006).

2.6. Measurement of chitinase activity

The colloidal chitin (1.3% in 50 mM phosphate buffer) was used as the substrate for the measurement of chitinase activity. The mixture of enzyme solution (0.5 mL) and substrate (1 mL) was incubated at 37 °C for 30 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita (1971) with *N*-acetylglucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min.

2.7. Determination of molecular mass

The molecular masses of the purified chitinases (Chi I and Chi II) were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) with 12.5% acrylamide and 2.67% methylene bis acrylamide in a 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 and 0.1% (w/v) SDS (pH 8.3). The 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 a methanol–acetic acid–water (5:1:5, v/v/v) solution and decolorized in 7% acetic acid. The molecular masses of Chi I and Chi II in the native form were determined using a gel filtration method. The sample and standard proteins were applied to a Sephacryl S-100 column (2.5 cm × 100 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 (Wang et al., 2006).

2.8. Mass spectrometry and protein identification

The bands of interest were excised from the SDS–PAGE gel and in-gel digested by trypsin. The identity of the enzyme was determined by using liquid chromatography–tandem mass spectrometry (LC–MS/MS) performed by Mission Biotech, Taiwan. The fragment spectra were searched against the NCBI non-redundant

Table 1
Purification of two chitinases (Chi I and Chi II) from *B. cereus* TKU027.

Step	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification fold	Yield (%)
Culture supernatant	10466.0	11.2	1.1	1.0	100
$(NH_4)_2SO_4$ ppt	1305.0	6.8	5.2	4.7	61
DEAE-Sepharose					
Chi I	106.9	3.4	31.8	28.9	30
Chi II	250.0	3.4	13.6	12.4	30
Sephacryl S-100					
Chi I	3.8	0.3	78.9	71.7	3
Chi II	4.4	0.2	45.5	41.4	2

B. cereus TKU027 was grown in 50 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SHP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$ in a shaking incubator for 2 days at 37 °C.

protein database. The database searches were performed using the MASCOT search engine.

2.9. Effect of pH and temperature on enzyme activities

The optimum pHs of Chi I and Chi II were studied by assaying the samples at different pH values. The pH stability of Chi I and Chi II was determined by measuring the residual activity at pH 7 as described above after the sample had been dialyzed against a 50 mM buffer solution of various pH values (pH 3–11) in seamless cellulose tubing (Sankyo). The buffer systems used were glycine HCl (50 mM, pH 3), acetate (50 mM, pH 4–5), phosphate (50 mM, pH 6–8), and Na_2CO_3–$NaHCO_3$ (50 mM, pH 9–11). To determine the optimum temperatures for Chi I and Chi II, the activity values of the samples were measured at various temperatures (25–90 °C). The thermal stability of Chi I and Chi II was studied by incubating the samples at various temperatures for 60 min. The residual activity was measured as described above.

2.10. Effect of various chemicals and surfactants on enzyme 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 enzyme 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. The enzyme was pre-incubated with various chemicals and surfactants for 30 min at 25 °C and the residual chitinase activities were then tested.

2.11. Fermented and enzymatic production of the chitin oligosaccharides

For the fermented production of the chitin oligosaccharides, *B. cereus* TKU027 was grown in 50 mL of liquid medium containing 1% SHP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$ for 2 days at 37 °C. For the enzymatic production of the chitin oligosaccharides, chitosan with 60% deacetylation (0.5%, w/v in 50 mM phosphate buffer) was used as the substrate. The mixture of TKU027 crude enzyme solution (1 mL) and substrate (1 mL) was incubated at 37 °C for 2 h. After incubation, the fermented culture supernatant and the enzymatic hydrolysates were collected, respectively, for further preparation of the chitin oligosaccharides. These solutions were concentrated to about 1/5 of the original volume with a rotary evaporator under diminished pressure and followed by adding methanol with final methanol concentration of 90% (v/v). Yellow agglomerates were formed in the solution. The agglomerates were concentrated with a rotary evaporator under diminished pressure and were collected after drying in vacuum. The supernatant was concentrated to about 1/10 of the original volume with a rotary evaporator under diminished pressure. Then, it was precipitated by adding acetone with final acetone concentration of 90% (v/v). The precipitates were collected after drying in vacuum.

2.12. MALDI-TOF MS analysis

An amount of 1 μL of the sample solution (2 mg/mL) was mixed on the target with 1 μL of a solution of 2,5-dihydroxybenzoic acid as a matrix (15 mg/mL) in H_2O–ACN–TFA solution (50/50/0.1%, v/v/v). Positive ion MALDI mass spectra were acquired with MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser emitting at 337 nm operating in linear mode. Each mass spectrum was the accumulating data of approximately 30–50 laser shots. External 3-points calibration was used for mass assignment.

2.13. HPLC analysis

HPLC analysis of the chitin oligosaccharides was performed on a Hitachi L-7000 apparatus (column, Nucleosil 5 NH_2 4.6 mm × 250 mm; mobile phase, acetonitrile/water = 70/30, v/v; flow rate = 1.0 mL/min; detection, RI). The samples were analyzed by HPLC, which was used for quantification of $(GlcNAc)_{1-6}$. The amounts of $(GlcNAc)_{1-6}$ were estimated with the calibration curve of standard 5 mg/mL $(GlcNAc)_{1-6}$. The yield of $(GlcNAc)_{1-6}$ was calculated by the following equation.

$$\text{The concentration of sample (mg/mL)} = 5 \times \frac{\text{the area of sample}}{\text{the area of standard}}$$

3. Results and discussions

3.1. Isolation and identification of chitinase producing strain

Microorganisms isolated from soils collected at different locations in midland Taiwan were screened on agar plates containing 1% SHP, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, and 1.5% agar powder (pH 7.0). The plates were incubated at 30 °C for 2 days. Those organisms obtained from the screening were subcultured in liquid media (containing 1% SHP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$) in shaken flasks at 30 °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 chitinase activity using the procedure described below. The strain TKU027 that showed the highest chitinase activity was isolated, maintained on nutrient agar, and used throughout the study.

Strain TKU027 is a Gram-positive and endospore-forming bacillus, with catalase but without oxidase, which grows in both aerobic and anaerobic environments. According to the result of 16S rDNA partial base sequence (approximately 1.5 kbp) analysis, strain TKU027 is most close to *Bacillus* sp. According to the API identification, strain TKU027 was most close to *B. cereus* with 95.2% similarity. Therefore, the isolate was identified as *B. cereus*.

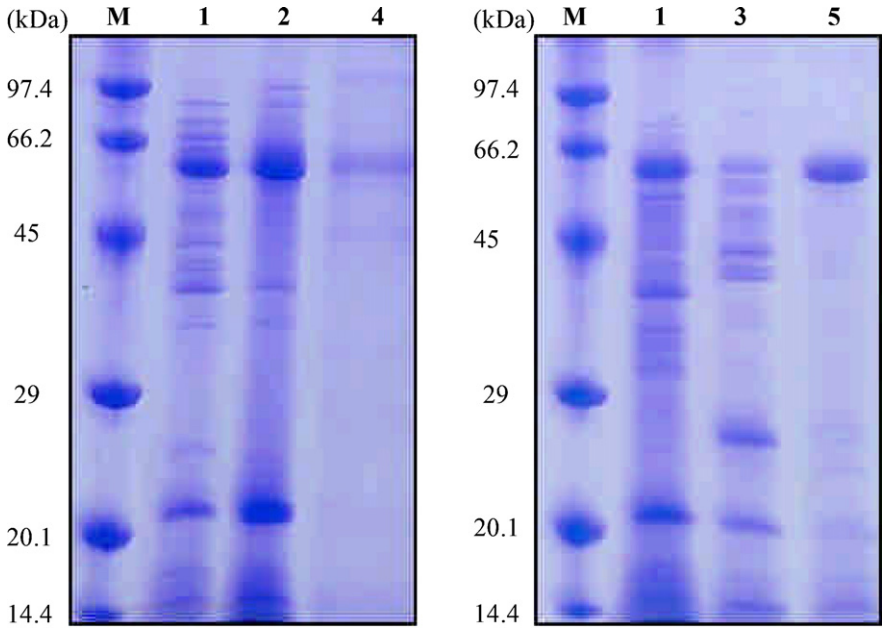

Fig. 1. SDS–PAGE analysis of Chi I and Chi II produced by *B. cereus* TKU027. Lanes: M, molecular markers; 1, crude enzyme; 2, the unadsorbed chitinase fractions after DEAE-Sepharose CL-6B chromatography; 3, the adsorbed chitinase fractions after DEAE-Sepharose CL-6B chromatography; 4, Chi I (purified by Sephacryl S-100); 5, Chi II (purified by Sephacryl S-100). 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).

3.2. Production and purification of extracellular chitinases by B. cereus TKU027

The production of chitinase by strain TKU027 was investigated during 5 days of cultivation in the production medium. The 50 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing 1% SHP was the most suitable for the production of chitinase by strain TKU027 at 37 °C. After a 6-h lag phase, exponential growth was observed for 3 days, and stationary phase was reached after 3 days. The highest chitinase activity of *B. cereus* TKU027 was detected in the culture on the second day of bacterial growth (data not shown).

The purification of *B. cereus* TKU027 chitinase from the culture supernatant (940 mL) was described in Section 2. Two chitinases (Chi I and Chi II) were purified, respectively, from *B. cereus* TKU027 cultures by a three-step purification procedure presented in Table 1. As shown in Table 1, the purification steps were combined to give an overall purification of about 71.7-fold and 41.4-fold for Chi I and Chi II, respectively. The overall activity yield of Chi I and Chi II was 3% and 2%, with specific chitinase activity of 78.9 mU/mg and 45.5 mU/mg, respectively. The final amount of Chi I and Chi II obtained was 3.8 mg and 4.4 mg, respectively. Chi I and Chi II were confirmed to be homogeneous by SDS–PAGE with a molecular weight of around 65 kDa and 63 kDa, respectively (Fig. 1). The molecular mass of Chi I (65 kDa) and Chi II (63 kDa) was obviously different from some of the other *Bacillus* chitinases, such as, *B. cereus* (36 kDa) (Wang et al., 2001), *B. circulans No.4.1* (45 kDa) (Wiwat, Siwayaprahm, & Bhumiratana, 1999) and *Bacillus* sp. NCTU2 (36.5 kDa) (Wen, Tseng, Cheng, & Li, 2002).

3.3. Effect of pH and temperature

The pH activity profiles of Chi I and Chi II were with maximum values both at pH 6 (Fig. 2a). The pH stability profiles of Chi I and Chi II were determined by the measurement of the residual activity at pH 7 after incubation at various pH values at 37 °C for 60 min. The Chi I was stable at pH 5–8 and Chi II was stable at pH 3–10 (Fig. 2a). In the present study, Chi II was demonstrated to exhibit broad

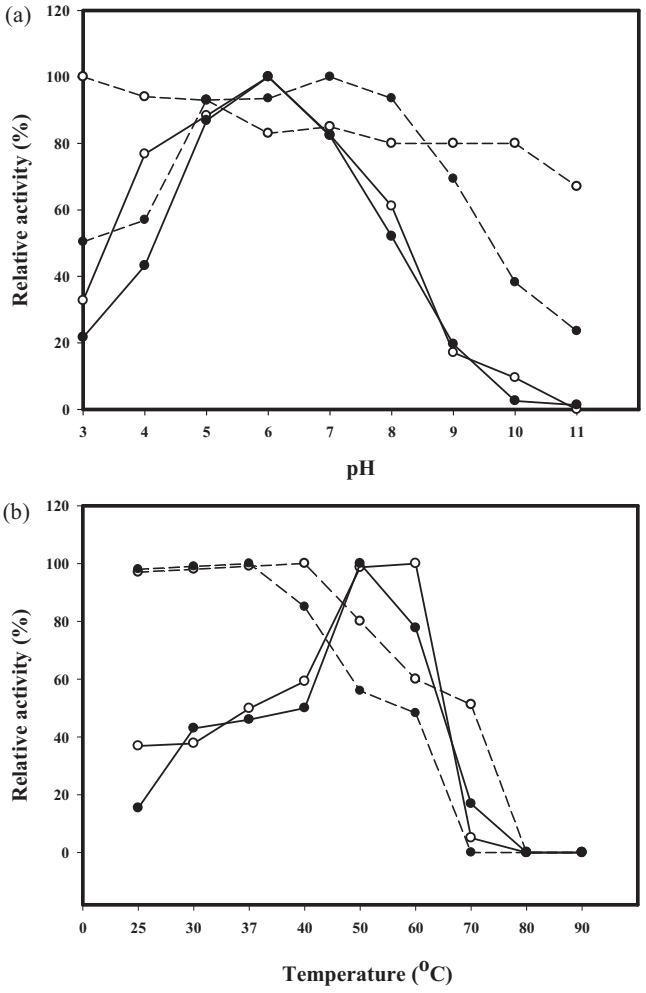

Fig. 2. Effect of pH (a) and temperature (b) on the activity (solid line) and stability (dashed line) of Chi I and Chi II: (●) Chi I; (○) Chi II.

Table 2
Substrate specificities of Chi I and Chi II from *B. cereus* TKU027.

Substrate	Relative activity (%)	
	Chi I	Chi II
Colloidal chitin	100	100
Chitin (α-type)	115	61
Chitin (β-type)	153	104
Chitosan (α-type; 98% DD)	4	8
Chitosan (β-type; 98% DD)	13	26
Chitosan (95% DD)	23	58
Chitosan (80% DD)	5	0
Chitosan (60% DD)	102	40
CMC	0	0
Glycol chitosan	0	0

pH stability. Several chitinases have broad pH stability, including the chitinase of *B. licheniformis* Mb-2 (pH 4–11) (Toharisman, Suhartono, Spindler-Barth, Hwang, & Pyun, 2005) and a chitinase from *Pseudomonas aeruginosa* strain 385 (pH 5–10) (Thompson, Smith, Wilkinson, & Peek, 2001). Compared with the previously reported chitinases, however, the Chi II of *B. cereus* TKU027 has a broader pH range, pH 3–10.

The effect of temperature on the activity of Chi I and Chi II was studied, respectively. The optimum temperature for Chi I and Chi II were 50 °C and 50–60 °C, respectively (Fig. 2b). To examine the thermal stability of Chi I and Chi II, 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. Chi I maintained its initial activity from 25 to 37 °C and had 56% of its activity at 50 °C but was completely inactivated at 70 °C. Chi II maintained its initial activity from 25 to 40 °C and had 51% of its activity at 70 °C but was completely inactivated at 80 °C (Fig. 2b).

3.4. Substrate specificity

For the substrate specificity of Chi I and Chi II, chitin and chitosan with degree of deacetylation (DD) ranging from 60% to 98% were used as substrates, as summarized in Table 2. The two enzymes could hydrolyze chitin and chitosan, but exhibited no activity on CMC and glycol chitosan. The Chi I was most active on β-chitin, followed by α-chitin, 60% deacetylated chitosan and colloidal chitin. However, the Chi II was less susceptible for 60% deacetylated chitosan. These results indicate that the physical form of the substrate affect the rate of hydrolysis.

Table 3
Effects of various chemicals and surfactants on the activities of Chi I and Chi II from *B. cereus* TKU027.

Chemicals	Concentration (mM)	Relative activity (%)	
		Chi I	Chi II
None	0	100	100
PMSF	5	47	98
EDTA	5	74	24
Mg^{2+}	5	109	114
Cu^{2+}	5	69	29
Fe^{2+}	5	57	97
Ca^{2+}	5	98	73
Zn^{2+}	5	111	86
Ba^{2+}	5	106	105
Mn^{2+}	5	39	0
SDS	0.5/1/2	118/108/110	97/96/83
Tween 20	0.5/1/2 (%)	108/109/109	91/86/81
Tween 40	0.5/1/2 (%)	109/108/117	92/84/55
Triton X-100	0.5/1/2 (%)	121/123/117	96/94/93

Purified enzyme was preincubated with the various reagents at 25 °C for 30 min and residual chitinase activity were determined as described in the text. One hundred percent was assigned to the activity in absence of reagents.

3.5. Effects of various inhibitors and metal ions

To further characterize Chi I and Chi II, 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 25 °C and then measuring the residual chitinase activity. The results were summarized in Table 3. Chitinase activities of Chi I and Chi II were not affected by some metal ions. However, there were 43% and 31% reduction in chitinase activities of Chi I in the presence of 5 mM Fe^{2+} and Cu^{2+}, respectively. There were 71%, 27% and 14% reduction in chitinase activities of Chi II in the presence of 5 mM Cu^{2+}, Ca^{2+} and Zn^{2+}, respectively. Chitinase activities of Chi I and Chi II were both inhibited completely by 5 mM Mn^{2+}. Identical to our results,

Table 4
Identification of Chi I and Chi II by LC–MS/MS.

Protein	Peptide sequence	Identified protein	Accession number
Chi I	^{55}IVGYFPSWGIYGRNYQVADIDASKLTHLNYAFADICWNGK^{94}	Chitinase C[a]	gi229171229
	^{123}EVPNGTLVLGEPWADVTKSYPGSGTTWEDCDK^{154}		
	^{176}TIISVGGWTWSNR^{188}		
	^{200}KVFAESTVAFLR^{211}		
	^{241}QNFTLLLQDVR^{251}		
	^{264}QYLLTIASGASQR^{276}		
	^{327}FYVDGAIDIYTNEGVPADKLVLGVPFYGR^{355}		
	^{385}GTWDDYSTGDTGVYDYGDLAANYVNK^{408}		
	^{421}VPYLYNATTGTFISYDDNESMK^{442}		
	^{452}GLSGAMFWELSGDCR^{466}		
	^{485}ELLGGPITQK^{494}		
	^{540}WSTTTNSITIK^{550}		
	^{572}SQPTSIIVK^{580}		
Chi II	^{55}IVGYFPSWGIYGR^{67}	Chitinase C[b]	gi229028231
	^{176}TIISVGGWTWSNR^{188}		
	^{201}VFAESTVAFLR^{211}		
	^{241}QNFTLLLQDVR^{251}		
	^{264}QYLLTIA SGASQR^{276}		
	^{346}LVLGVPFYGR^{355}		
	^{359}SCGKENNGQYQPCKPGSDGK^{378}		
	^{452}GLSGAMFWE LSGDCR^{466}		

Peptide fragments were identified by LC–MS/MS and by database searching.
[a] Chitinase C from *B. cereus* MM3.
[b] Chitinase C from *B. cereus* AH1271.

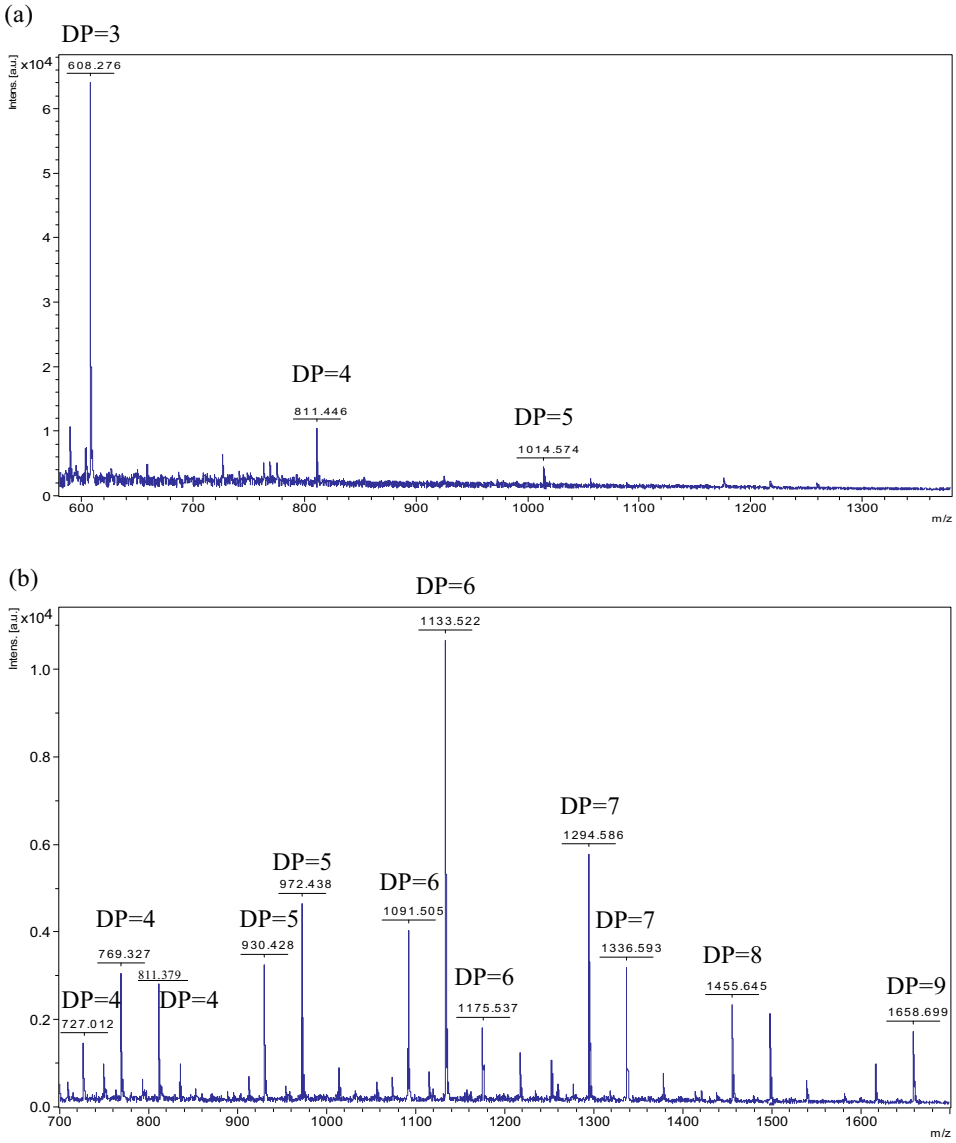

Fig. 3. MALDI-TOF MS of 90% methanol soluble/90% acetone insoluble fraction of *N*-acetyl chitooligosaccharides obtained from TKU027 culture supernatant (a) and TKU027 crude enzyme hydrolysis (b).

Mn^{2+} inhibited bacterial chitinases activity (Wang, Chen, et al., 2008; Wang, Lin, et al., 2008; Yuli, Suhartono, Rukayadi, Hwang, & Pyun, 2004) but Mn^{2+} stimulated the fungal chitinases activity (Lee, Chung, Wi, Lee, & Bae, 2009). Interestingly phenylmethanesulfonyl fluoride (PMSF) at 5 mM concentration did not inhibit the chitinase activity of Chi II significantly; therefore it could be used to control the serine proteases activity of crude extract for preventing exhaustive degradation of proteins during downstream processes.

3.6. Effect of various surfactants

Enzymes are usually inactivated by the addition of surfactants to the reaction solution. The effects of different surfactants on stability of Chi I and Chi II were also studied. Chi I and Chi II preparations were incubated with surfactants at 25 °C for 30 min and the remaining enzymatic activity were determined under normal assaying conditions. The enzyme activity of the sample without any surfactants (control) was taken as 100%. High stability was observed for Chi I toward various surfactants (Table 3). Upon incubation with 0.5–2 mM of SDS and 0.5–2% of Tween 20, Tween 40 and Triton X-100, Chi I exhibited enhanced residual activities between 108 and 123% of its original activity. However, at the presence of 2% Tween 40, the chitinase activity of Chi II retained only 55% of its original activity. It is notable that all the surface-active detergents like SDS, Tweens (20 and 40) and Triton X-100 were stimulator of Chi I chitinase activity. Perhaps these results are based on the fact that surface-active reagents might increase the turnover number of chitinase by increasing the contact frequency between the enzyme active site and the substrate which are accomplished through lowering the surface tension of the aqueous solution.

3.7. Identification of Chi I and Chi II by LC–MS/MS analysis

To identify the proteins (Chi I and Chi II) of chitinase activity appearing as prominents 65 kDa and 63 kDa bands on SDS–PAGE gel, respectively, 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. As shown in Table 4, the spectra of Chi I and Chi II matched thirteen and eight tryptic peptides, respectively, that were identical to chitinase C from *B. cereus* MM3 (GenBank

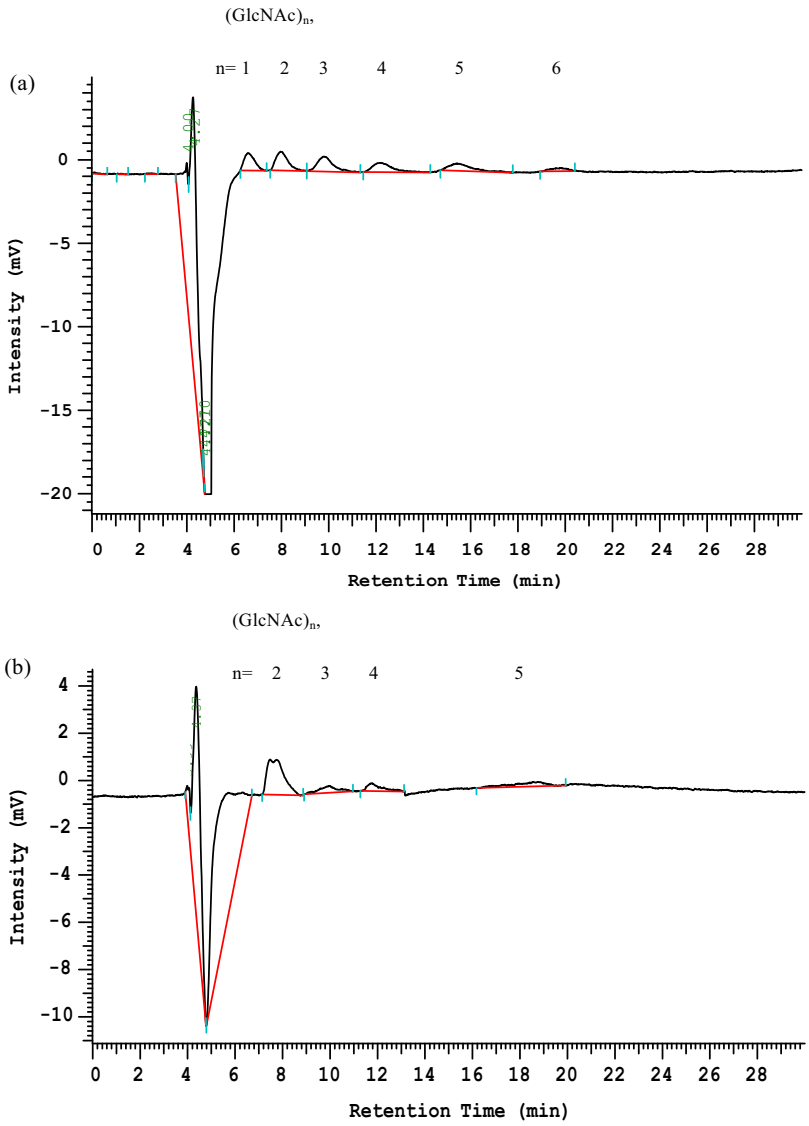

Fig. 4. A typical HPLC profile of the *N*-acetyl chitooligosaccharides obtained from the culture supernatant at 1% SHP by using *B. cereus* TKU027 fermentation for 2 days: a, standards; b, TKU027 culture supernatant.

Table 5
Assigned ion composition of MALDI-TOF MS spectra of chitin/chitosan oligosaccharides produced by *B. cereus* TKU027 fermentation and enzymatic hydrolysis, respectively.

Process	*m*/*z*	Types	Ion composition
Fermentation	608	$[M+Na]^+$	GlcN-$(GlcNAc)_2$
	811	$[M+Na]^+$	GlcN-$(GlcNAc)_3$
	1014	$[M+Na]^+$	GlcN-$(GlcNAc)_4$
Enzymatic hydrolysis	727	$[M+Na]^+$	$(GlcN)_3$-GlcNAc
	769	$[M+Na]^+$	$(GlcN)_2$-$(GlcNAc)_2$
	811	$[M+Na]^+$	GlcN-$(GlcNAc)_3$
	930	$[M+Na]^+$	$(GlcN)_3$-$(GlcNAc)_2$
	972	$[M+Na]^+$	$(GlcN)_2$-$(GlcNAc)_3$
	1091	$[M+Na]^+$	$(GlcN)_4$-$(GlcNAc)_2$
	133	$[M+Na]^+$	$(GlcN)_3$-$(GlcNAc)_3$
	175	$[M+Na]^+$	$(GlcN)_2$-$(GlcNAc)_4$
	294	$[M+Na]^+$	$(GlcN)_4$-$(GlcNAc)_3$
	336	$[M+Na]^+$	$(GlcN)_3$-$(GlcNAc)_4$
	455	$[M+Na]^+$	$(GlcN)_5$-$(GlcNAc)_3$
	658	$[M+Na]^+$	$(GlcN)_5$-$(GlcNAc)_4$

accession number gi229171229) with 35% sequence coverage and chitinase C from *B. cereus* AH1271 (GenBank accession number gi229028231) with 15% sequence coverage, respectively, and others remain unmatched. The putative conserved domains could be detected by searching the NCBI database non-redundant protein sequences (nr) using Blastp (protein–protein BLAST). These unmatched sequences when submitted to BLAST (NCBI) showed that no putative conserved domains have been detected using these sequences as search query. The identification of Chi I and Chi II was carried out by the Mission Biotech, Taiwan.

3.8. Fermented and enzymatic production of chitin oligosaccharides

Recently, most production of chitin oligomers has been reported by enzymatic hydrolysis and produced GlcNAc as a major chitinolytic product. These enzymes were derived from *Serratia marcescens* (Haynes et al., 1999), *Bacillus thuringiensis* subsp. *Pakistani* (Thamthiankul, Suan-Ngay, Tantimavanich, & Panbangred, 2001), *Aeromonas hydrophila* H-2330 (Sashiwa et al., 2002), *Trichoderma viride* and *Acremonium cellulolyticus* (Sashiwa et al., 2003),

Aeromonas sp. GJ-18 (Kuk et al., 2005) and *Paenibacillus illinoisensis* KJA-424 (Jung, Souleimanov, Park, & Smith, 2007). In our work, chitin oligosaccharides were produced by SHP fermentation and enzymatic hydrolysis of chitosan with 60% deacetylation from *B. cereus* TKU027, respectively. To obtain low DP oligomers, a selective precipitation, respectively, in 90% methanol and acetone solutions was used as described earlier (Liang et al., 2007). For SHP fermentation by *B. cereus* TKU027, the chitin oligosaccharides in the culture supernatant were recovered and lyophilized by the method described above. By MALDI-TOF analysis of the precipitation in 90% acetone solution, it appeared that chitin oligosaccharides with DP up to 5 (Fig. 3a). The higher DP chitooligomers were precipitated as a light yellow powder, leaving in the methanol solution. This culture supernatant contained *N*-acetyl chitooligosaccharides (GlcNAc-oligomers) and one GlcN-oligomer (Table 5). During *B. cereus* TKU027 fermentation, not only the *N*-acetyl linkage between residues but also the *O*-glycosidic linkage can be hydrolyzed. The chitin oligosaccharides ions present in the mass spectra were identified as sodium adducts, $[M+Na]^+$, corresponding to the following series of chitin oligosaccharides: GlcN-$(GlcNAc)_2$, GlcN-$(GlcNAc)_3$ and GlcN-$(GlcNAc)_4$ (Table 5). Since MALDI-TOF analysis is limited to molecular weights higher than 500 Da due to interference of the matrix signals, the DP < 2 oligomers could not be determined by this method. The chitin oligosaccharides obtained from the culture supernatant after 2 days of fermentation were also analyzed by HPLC and shown in Fig. 4b. The concentration of chitobiose, chitotriose, chitotetrose and chitopentose were 0.44 mg/mL, 0.08 mg/mL, 0.09 mg/mL and 0.43 mg/mL, respectively, after 2 days of fermentation. Combine Fig. 3a with Fig. 4b, it showed that chitin oligosaccharides of TKU027 culture supernatant comprise oligomers with DPs from 2 to 5.

For enzymatic hydrolysis of chitosan with 60% deacetylation from *B. cereus* TKU027, the chitosan was degraded for 2 h in the conditions of 37 °C, pH 7, 50 mM phosphate buffer by TKU027 crude enzyme. In the mass spectrum of the hydrolysates (Fig. 3b) peaks were detected corresponding to the mass numbers of $[M+Na]^+$ of tetramer to nonamer. The series of chitin oligosaccharides are shown in Table 5. The existence of different hydrolysates indicated that the chitinases produced from *B. cereus* TKU027 were both endo-chitinase and exo-chitinase.

In this study, the chitin oligomers could be produced by *B. cereus* TKU027 fermentation and enzymatic hydrolysis of TKU027 crude enzyme, respectively. Compare the difference between fermented and enzymatic production of chitin oligosaccharides from *B. cereus* TKU027, TKU027 culture supernatant combined with a selective methanol/acetone precipitation appears to be a quicker and simpler method to yield chitin oligosaccharides with low molecular weight oligomers than enzymatic hydrolysis. However, enzymatic hydrolysis could obtain the chitin oligosaccharides with broader distribution range than the fermented method. Compare with other reports, there was no production of GlcNAc by the two methods from *B. cereus* TKU027. Our method and results were obviously different from the above reports (Haynes et al., 1999; Jung et al., 2007; Kuk et al., 2005; Sashiwa et al., 2002, 2003; Thamthiankul et al., 2001). In this study, fermented production of chitin oligosaccharides means that microbial strains used would be affected (induced) by chitin contained in the shellfish chitin wastes and thus produced the activity of chitinase at the same time. Besides, the chitin oligosaccharides in the culture supernatant could be recovered for biological applications.

4. Conclusion

Different from other reported chitinase-producing strains of *Bacillus* sp., this research aimed for the microbial reclamation of shrimp processing wastes. Shrimp heads were used as the sole carbon/nitrogen source to screen the chitinase-producing strain. Consequently, although the screened TKU027 belongs to *Bacillus* sp. the same as the reported chitinase-producing strains, some properties of Chi I and Chi II were different. *B. cereus* TKU027 used 1% (w/v) SHP as the sole carbon/nitrogen source for chitinase production. The medium for TKU027 is obviously much simpler and cheaper. Besides, with this method, the chitin oligosaccharides were also produced on the 2nd day. For this result, the fermented production of chitin oligosaccharides from *B. cereus* TKU027 is a very useful procedure to prepare chitin oligosaccharides with DP 2–5, and it might be preferred over chitinase because of its economy. However, enzymatic hydrolysis by TKU027 crude enzyme could obtain the chitin oligosaccharides with DP 4–9. The multi-chitinolytic enzyme complex produced by *B. cereus* TKU027 is effective in the production of chitin oligosaccharides. These results may be useful for biological applications in relation to enzyme and bioactive materials production.

Acknowledgement

This work was supported in part by a grant of the National Science Council, Taiwan (NSC99-2313-B-032-001-MY3).

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