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Expression and characterization of *Bacillus licheniformis* chitinase (ChiA), suitable for bioconversion of chitin waste

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ABSTRACT

Chitinase (EC 3.2.1.14) is an enzyme with multiple industrial applications. These include bioconversion of chitin waste, a highly resistant and abundant biopolymer from crustacean food industry, into glucosamine and chito-oligosaccharide value-added products. This paper reports on the expression of endochitinase (ChiA) from *Bacillus licheniformis* strain DSM8785 in *E. coli* and characterization of the recombinant enzyme. Recombinant ChiA could efficiently convert colloidal chitin to *N*-acetyl glucosamine and chitobiose at pH 4.0, 6.0 and 9.0 at 50 °C and retained its activity up to 3 days under these conditions, suggesting that this enzyme is suitable for bioconversion of chitin waste.

1. Introduction

Chitin (β-1,4 linked *N*-acetylglucosamine) is the major structural polysaccharide in insects, crustaceans, and fungi; therefore, it is one of the most abundant biopolymers on earth. Each year, a vast amount of chitin waste is released from the aquatic food industry in Thailand, where crustaceans (prawn, crab, shrimp and lobster) constitute one of the main agricultural products. This creates a serious environmental problem, because chitin is very difficult to biodegrade (Hayes et al., 2008a,b). Chitinases (EC 3.2.1.14) are enzymes that hydrolyze chitin by cleaving its β-1,4 *N*-glycosidic bond (Coutinho and Henrissat, 1999; Fujita et al., 2006), and endo-chitinases cleave randomly at internal sites of chitin, generating soluble low mass multimers of GlcNAc such as chitotetraose, chitotriose, and chitobiose (Howard et al., 2003). One of the potential applications of these types of enzymes is for the bioremediation and bioconversion of chitin wastes from food processing industry into pharmacological active products, namely *N*-acetylglucosamine (NAG) and chito-oligosaccharides (Hayes et al., 2008a,b; Horn et al., 2006). Production of chitin derivatives with suitable enzyme is more appropriate for a sustainable environment than using chemical reactions. In addition, they can be used as anti-fungal agents (Tsujibo et al., 2003) and for the preparation of protoplasts of filamentous fungi (Dahiya et al., 2006). Potential roles of chitinase in bio-control of insects and mosquitoes and in production of single cell protein (SCP) have also been suggested (Dahiya et al., 2006; Hayes et al., 2008a,b). Thus, there have been many reports on cloning, expression and characterization of chitinases from various organisms, including bacteria, fungi, plant and animals (Dahiya et al., 2006; Howard et al., 2003).

B. licheniformis strain DSM 8785 has been used extensively in industry for the production of various enzymes and metabolites (Schallmey et al., 2004; Veith et al., 2004); however, the chitinases from this bacterium have apparently not been studied. This article describes the cloning; expression and characterization of recombinant chitinase from *B. licheniformis* strain DSM8785 using an *Escherichia coli* expression system. The N-terminus of the mature enzyme was fused with the *E. coli* OmpA signal peptide, allowing the secretion of the enzyme into the periplasmic space; its C-terminus was fused with a hexa-histidine tag to facilitate affinity purification using immobilized metal affinity chromatography (IMAC). Characterization of its biochemical properties suggested that this recombinant *B. licheniformis* chitinase is appropriate for various industrial applications, including bioconversion of colloidal chitin into *N*-acetyl glucosamine and chitobiose.

2. Methods

2.1. Bacterial strains and growth conditions

B. licheniformis DSM8785, which was used as the source of chitinase gene, was obtained from DSMZ; German Collection of

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Microorganisms and Cell Culture (Braunschweig, Germany). *E. coli* strain TOP10 (Invitrogen) was used as a host for cloning and protein expression. *E. coli* was cultured in Luria–Bertani (LB) medium at 37 °C with shaking.

2.2. Preparation of colloidal chitin

Colloidal chitin was prepared according to the modified method of Shimahara and Takiguchi (1988). Ten grams of chitin flakes from crab shells (Sigma–Aldrich, C9213) were mixed with 200 ml concentrated HCl on ice with vigorous stirring for 3–4 h and continued incubation on ice overnight. Then, the mixture was filtered through cheesecloth and dropped slowly into 600 ml of 50% ice-cold ethanol with rapid stirring on ice. Then, the colloidal chitin was collected by centrifugation at 8000*g*, for 30 min at 4 °C and washed several times with tap water until the pH was neutral (pH 7.0). Alternatively the filtrate was re-filtered with suction through Whatman No. 1 filter paper and washed with water until the washing solution was neutral. The colloidal chitin was kept at 4 °C until used.

2.3. Molecular cloning of *B. licheniformis ChiA* gene

The oligonucleotide primers for the cloning of the ChiA gene from *B. licheniformis* strains DSM8785 were designed from the genomic database of *B. licheniformis* strains DSM13 (Rey et al., 2004), according to the DNA sequence of gene, YvbX (NCBI Accession Number AAU21943.2), which are hypothetical protein for Glycoside Hydrolase (GH) family 18. To clone the gene of mature ChiA, a single colony of *B. licheniformis* strain DSM8785 was boiled in ultra pure water and used as the template for amplification. The gene was amplified by *Pfu* DNA polymerase using chiHind3 (5′-CTG TGC AAG CTT TTG TCA TGT TGC TGA GCT TGT CAT TTG-3′) and chi6HX-ho (5′-GCA CAG CTC GAG TCA GTG GTG GTG GTG GTG GTG TTC GCA GCC TCC GAT CAG CCG CC-3′) as primers. The reverse primer contains a hexa-histidine tag for affinity purification of the recombinant ChiA. The PCR thermal profile consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 45 s, 56 °C for 55 s, and 72 °C for 2 min, followed by final extension step at 72 °C for 10 min. The gene was then cloned into the *Hind* III and *Xho* I sites of the pFLAG-CTS vector (Sigma, St. Louise, USA) such that the native signal peptide of *B. licheniformis* ChiA was replaced by the *E. coli* OmpA signal peptide to facilitate the secretion of the recombinant enzyme as previously described (Yamabhai et al., 2008). The integrity of the construct was confirmed by automated DNA sequencing (Macrogen, Korea). The construct was transformed into *E. coli* strain Top 10 for expression of the gene in the next step.

2.4. Structural based sequence alignment

Multiple sequence alignment was done by CLUSTALW (Larkin et al., 2007) followed by ESPript (Gouet et al., 2003) to display the secondary structure of the template selected.

2.5. Protein expression and purification

To express the recombinant *B. licheniformis* ChiA, a single colony of *E. coli* Top10 harbouring recombinant plasmid was grown at 37 °C in Luria Bertani (LB) medium containing 100 μg/ml ampicillin until OD_{600} reached approximately 1.5. After that, the culture was cooled down to 25 °C before chitinase expression was induced by the addition of 1 mM isopropyl thio-β-D-galactoside (IPTG). After continuing incubation at 25 °C for 18 h the cell pellet was collected by centrifugation at 4500*g* for 45 min. The freshly-prepared cell pellet was resuspended in 10 ml of lysis buffer (20 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1.0 mg/ml lysozyme), then lysed on ice using an Ultrasonic Processor; 60 amplitude, pulser 6 s, for 2 min. Unbroken cells and cell debris were removed by centrifugation at 12,000*g* for 30 min. The supernatant was immediately applied to a Ni–NTA agarose affinity column containing 1–2 ml of bed volume (QIAGEN GmbH, Hilden, Germany), and the chromatography was carried out gravitationally at 4 °C, following the Qiagen's protocol. The column was washed two times with 50 ml of wash buffer (20 mM Tris–HCl buffer, pH 8.0 and 150 mM NaCl) containing 5 mM and 20 mM imidazole, respectively. Ni–NTA-bound enzyme was eluted with 250 mM imidazole in the same buffer. The eluated fractions were then centrifugally dialyzed using Vivaspin-20 ultrafiltration membrane concentrators (M_r 10,000 cut-off, Vivascience AG, Hannover, Germany) to remove imidazole. The enzyme was stored at −25 °C in the presence of 20% glycerol until used. The level of purification was calculated from the specific activities (Unit/mg protein) of the purified enzyme and crude enzyme preparation, using *p*-NP-chitobiose as substrate (Howard et al., 2003).

2.6. Determination of protein concentration

Protein concentrations were determined by the method of Bradford (Bradford, 1976), according to the manufacturer's protocol (Biorad). The standard calibration curve was constructed from 0 to 10 μg of Bovine serum albumin (BSA).

2.7. SDS–PAGE analysis and Zymogram

Ten microliter of purified enzyme (3 μg) was analyzed on a 12% SDS–PAGE, according to the method of Laemmli (1970). The protein samples were mixed with gel loading buffer containing β-mercaptoethanol before loading. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The molecular weight markers were from Amersham Pharmacia Biotech (Amersham Biosciences, Piscataway, NJ, USA). Zymogram was performed as previously described (Yamabhai et al., 2008).

2.8. Chitinase activity assay using p-NP-chitobiose (p-NP-$(GlcNAc)_2$)

The relative activity of the chitinase was determined by preincubating 0.5 mM of *p*-NP-chitobiose in 0.1 M phosphate buffer, pH 6.0 at 37 °C (or 50 °C) for 5 min with constant agitation in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany). After adding 1 μg of the purified enzyme (0.015 nmol), the reaction was incubated at 37 °C with shaking for 30 min (standard assay conditon) or at 50 °C for 10 min, then terminated by adding 50 μl of 1 M NaOH into 100 μl of enzyme reaction at 50 °C or 37 °C, depending on the assay condition. The amount of *p*-nitrophenol released from *p*-NP-$(GlcNAc)_2$ was detected by measuring the absorbance at 405 nm, using a standard calibration curve constructed with varying concentration of *p*-NP from 0 to 30 nmol.

One unit of enzyme is defined as the amount of enzyme that liberates 1 μmol *p*-NP per minute under the experimental conditions.

2.9. Effect of pH and temperature on enzyme activity

The optimal pH of the chitinase activity was measured between pH 2.0–12.0 under standard assay conditions, using 100 mM of each buffer: phosphoric–phosphate (pH 2.0–3.0), glycine–HCl (pH 2.0–4.0), acetate (pH 4.0–6.0), potassium phosphate (pH 6.0–8.0), Tris–HCl (pH 7.0–9.0) and glycine-NaOH (pH 9.0–12.0). To determine the pH stability of chitinases, the enzyme samples were incubated at various pH values using the same buffer system at 20 °C

for 30 min or 18 h, and then the remaining enzyme activity was measured at 37 °C under the standard assay condition.

The optimum temperature of chitinase activity was studied by incubating the enzyme samples with the substrate at temperature ranging from 0 to 100 °C in 100 mM phosphate buffer pH 6.0. Thermal stability of the enzyme was determined by incubating the enzyme samples in 100 mM phosphate buffer, pH 6.0 at various temperatures ranging from 0 to 100 °C for 30 min, then the remaining enzyme activities were measured under standard assay conditions.

In addition, the residual enzyme activities after incubation in 100 mM phosphate buffer, pH 6.0 at 50 °C without substrate at 0, 0.5, 3, 6, 24, 48, 96 and 120 h were assayed at 37 °C under standard conditions.

2.10. Chitinase activity assay using colloidal chitin

To determine chitinase activity using colloidal chitin as substrate, the reaction containing 300 μl of 10 mg/ml colloidal chitin in 0.1 M phosphate buffer pH 6.0 was mixed with 75 μl of the buffer and pre-incubated for 30 min (at 37 °C or 50 °C). Then, 25 μl of 1.0 mg/ml of purified enzyme was added and further incubated at 37 °C or 50 °C for 30 and 10 min, respectively. The reaction was terminated by boiling at 100 °C for 10 min. Then the reaction mixture was centrifuged at 12,000*g* for 5 min to precipitate the remaining chitin. The reducing sugar liberated in the enzyme reaction was assayed by mixing 100 μl of the supernatant with 100 μl of 3,5-dinitrosalicylic acid (DNS) solution, boiling at 100 °C for 20 min, cooling on ice and measuring the absorbance at 540 nm, using a standard curve constructed with varying concentration of *N*-acetylglucosamine (NAG, or G1) or (di-*N*-acetyl chitobiose, or G2).

2.11. Kinetic analysis

Enzyme kinetic parameters were determined by setting up reaction mixture consisting of 0.4 μg pure enzyme and 0.008–0.7 mM of *p*-NP-$(GlcNAc)_2$ and assayed under standard condition at 37 °C as previously described (Songsiriritthigul et al., 2009). The kinetic constants were calculated by nonlinear regression, and the obtained data were fitted to the Henri–Michaelis–Menten equation (GraphPad Prism 5).

2.12. Hydrolytic analysis by TLC

Hydrolysis of chito-oligosaccharides (G2–G6) was carried out in a 30-μl of reaction mixture, containing 10 nmol substrate and 500 ng of purified enzyme in 0.1 M phosphate buffer pH 6.0. The reaction was incubated at 37 °C with shaking for 10 min, 1 h and 18 h, and then terminated by boiling for 10 min. To analyze the product by TLC, each reaction mixture was applied five times (1 μl each) onto a Silicagel 60 F_{254} aluminum sheet (6.0 × 10.0 cm) purchased from Merck (Damsladt, Germany) and chromatographed two times (1 h each) in a mobile phase containing n-butanol: methanol: 28% ammonia solution: H_2O (10:8:4:2) (v/v). The products were detected by wiping the TLC plate with a cotton ball soaked with 5% sulphuric acid followed by baking at 180 °C for 3 min. The standard mixture of 10 nmol chito-oligosaccharides (G2–G6) was used. To analyze the hydrolysis products using colloidal chitin as substrate, 3 mg of colloidal chitin suspended in 0.1 M phosphate buffer pH 6.0 was pre-incubated for 30 min at 37 °C before 25 μg of purified enzyme was added and incubated at 37 °C. The actual reaction comprised 75 μl of 0.1 M phosphate buffer pH 6.0, 300 μl of 10 mg/ml colloidal chitin in the same buffer, and 25 μl of 1.0 mg/ml enzyme. The sample was taken for TLC analysis at various time points (2, 5, 10, 15, 30 and 60 min).

Product analysis when colloidal chitin was used as substrate at different pHs was done in reaction mixtures containing 30 mg of colloidal chitin suspended 0.1 M of each buffer (glycine–HCl, pH 4.0; potassium phosphate buffer, pH 6.0 and Tris–HCl, pH 9.0). After pre-incubation at 50 °C for 30 min, 50 μg of chitinase was added and further incubated at 50 °C for different durations (1, 3, 6, 12, 24, 48 and 72 h) prior to termination by boiling for 10 min. The actual reaction comprised 150 μl of 0.1 M buffer (glycine–HCl buffer pH 4.0, phosphate buffer pH 6.0 and Tris–HCl pH 9.0), 600 μl of 50 mg/ml colloidal chitin, and 50 μl of 1.0 mg/ml enzyme. Products released from the reactions were subsequently analyzed by TLC as described above.

3. Results and discussion

3.1. Amino acid sequence analysis of chitinase (ChiA) from *B. licheniformis* strain DSM8785

The *chiA* gene from *B. licheniformis* strain DSM8785 (GenBank Accession Number FJ465148) and *B. licheniformis* strain DSM13 (GenBank Accession Number AAU21943) (Rey et al., 2004) are highly similar (99% identity) with only five base pairs and three amino acid differences. Comparison of the specific activity and expression level of the two recombinant enzymes indicated that the enzyme from strain DSM 8785 was slightly better (data not shown); therefore ChiA from *B. licheniformis* strain DSM8785 was selected for further study. Amino acid sequence alignment between these two strains of *B. licheniformis* can be found in the previous publication (Songsiriritthigul et al., 2009).

Comparison of amino acid sequence of *B. licheniformis* ChiA with other chitinases revealed that the N-terminal catalytic domain of *B. licheniformis* ChiA (pFchi8785) has highest identity (48.80%) to catalytic domain (CatD) of chitinase A1 (ChiA1) from *Bacillus circulans* WL-12; followed by chitinase B1 (ChiB1) from *Aspergillus fumigatus* (24.79%); chitinase from the pathogenic fungus *Coccidioides immitis* (23.42%); chitinase B (ChiB) from *Serratia marcescens* (22.42%); human chitotriosidase (21.05%); chitinase A (ChiA) from *Vibrio harveyi* (20.28%), chitinase A (ChiA) from *Serratia marcescens* (19.80%) and acidic mammalian chitinase (18.74%). The enzyme belongs to glycosyl hydrolase (GH) family 18, according to the CAZy (Carbohydrate-Active enZYmes) system (Coutinho and Henrissat, 1999) and shows the TIM $(\beta/\alpha)_8$-barrel architecture that is typical to this class of hydrolases. It comprises N-terminal catalytic domain, one fibronectin type III (FnIII)-like domain and one C-terminal chitin binding domain (ChBD).

The gene was cloned into the expression vector such that the hypothetical native signal peptide was replaced with the *E. coli* OmpA signal peptide that was included in the pFLAG–CTS vector. In addition, the DNA sequence encoding hexa-histidine and stop codon was incorporated into the reverse primers to create His-tagged fusion enzyme for further purification step. The integrity of the construct was confirmed by automated DNA sequencing.

3.2. Enzyme expression and purification

The gene of the recombinant *B. licheniformis* ChiA was under the control of *tac* promoter and could be efficiently expressed after induction with 1 mM IPTG. The enzyme could be purified to apparent homogeneity as seen as a band of approximately 66 kDa on SDS–PAGE (Fig. 1A) and Zymogram analysis (Fig. 1B). These results correspond to hypothetical M_r of 66.801 kDa as predicted by Scansite program (Obenauer et al., 2003).

The mature ChiA gene was fused with *E. coli* OmpA signal peptide for efficient secretion using *E. coli* expression system as has been previously described (Yamabhai et al., 2008). In this report,

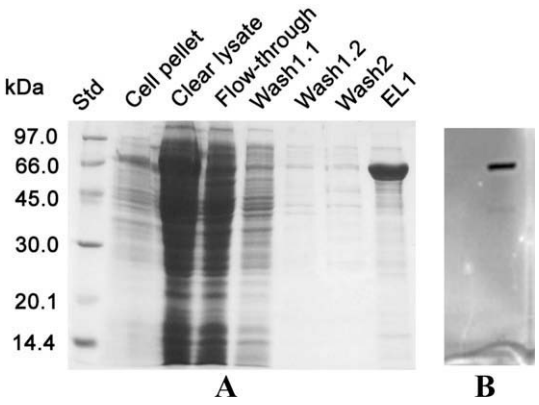

Fig. 1. Expression and purification of recombinant *B. licheniformis* ChiA. Panel A illustrates SDS–PAGE analysis of recombinant ChiA from various purification steps. Approximately 10 μl of samples were loaded onto each lane and stained by Coomassie brilliant blue. Zymogram analysis of purified enzyme is shown in panel B.

we harvested the recombinant enzyme from both cytosol and periplasm after induction with 1 mM IPTG for 18 h. This is the optimized condition for a small-scale preparation as performed in this study. Routinely we obtain about 20 mg of purified enzyme from 1-liter culture. The purification fold was 5.4 (this value was calculated from the specific activity, using *p*-NP-chitobiose as substrate, which was 0.054 and 0.294 U/mg for crude extract and purified enzyme, respectively). We have found that the enzyme could be kept in 20% glycerol at −25 °C for as long as one year with more than 80% residual activity, when compared to freshly-prepared enzyme. For a large-scale production, further optimization is needed and it may be more convenient to harvest the enzyme from culture medium or periplasmic space (Pines and Inouye, 1999). Since the C-terminus of the enzyme was fused with hexa-histidine tag, the enzyme can be easily purified to apparent homogeneity by affinity chromatography such as Immobilized metal ion affinity chromatography (IMAC). For industrial scale, it might be more cost-effective to purify the enzyme from periplasmic extract, by ammonium sulfate precipitation and anion-exchange chromatography, as has been previously described (Chen et al., 2004).

3.3. Effect of pH and temperature

The relative activities of the enzyme at various pHs were measured at 37 °C after incubation for 30 min (standard assay condition) The optimal pH of ChiA was 6.0 (Fig. 2A). The enzyme is more active when using glycine–HCl than acetate buffer at pH 4.0. The enzyme was stable within pH 4–11 after incubation for 30 min to 18 h without substrate (Fig. 2B). At pH 12, its remaining activity was decreased to 68% after incubation for 18 h. The residual activity of the enzyme at pH 3.0, after incubation for 0.5 h, was slightly (and non-significant) better that that at pH 4.0, whereas the residual activity at pH 3.0, after incubation for 18 h, was much

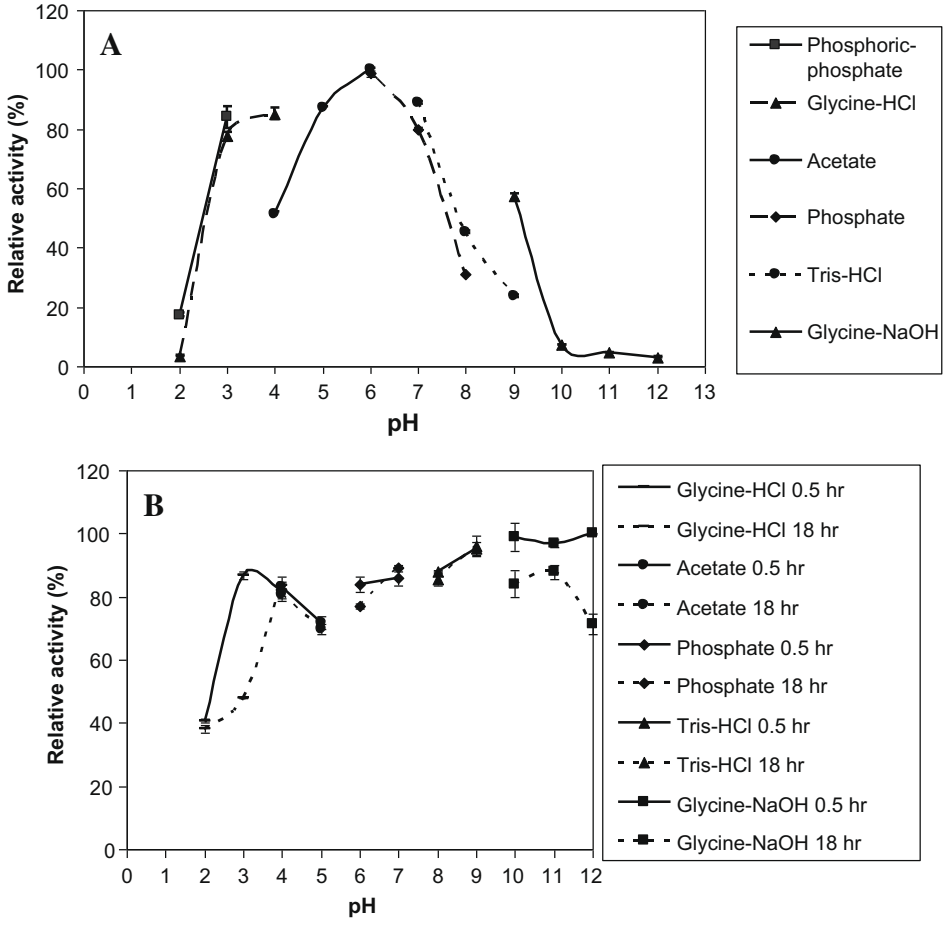

Fig. 2. Effect of pH on catalytic activity. (A) The optimal pH was determined at 37 °C in 100 mM of different buffers as indicated under the standard assay condition. (B) The pH stability was determined by measuring the residual activity of ChiA after incubation at various pHs at 20 °C for 30 (solid line) min or 18 h (broken line) without substrate.

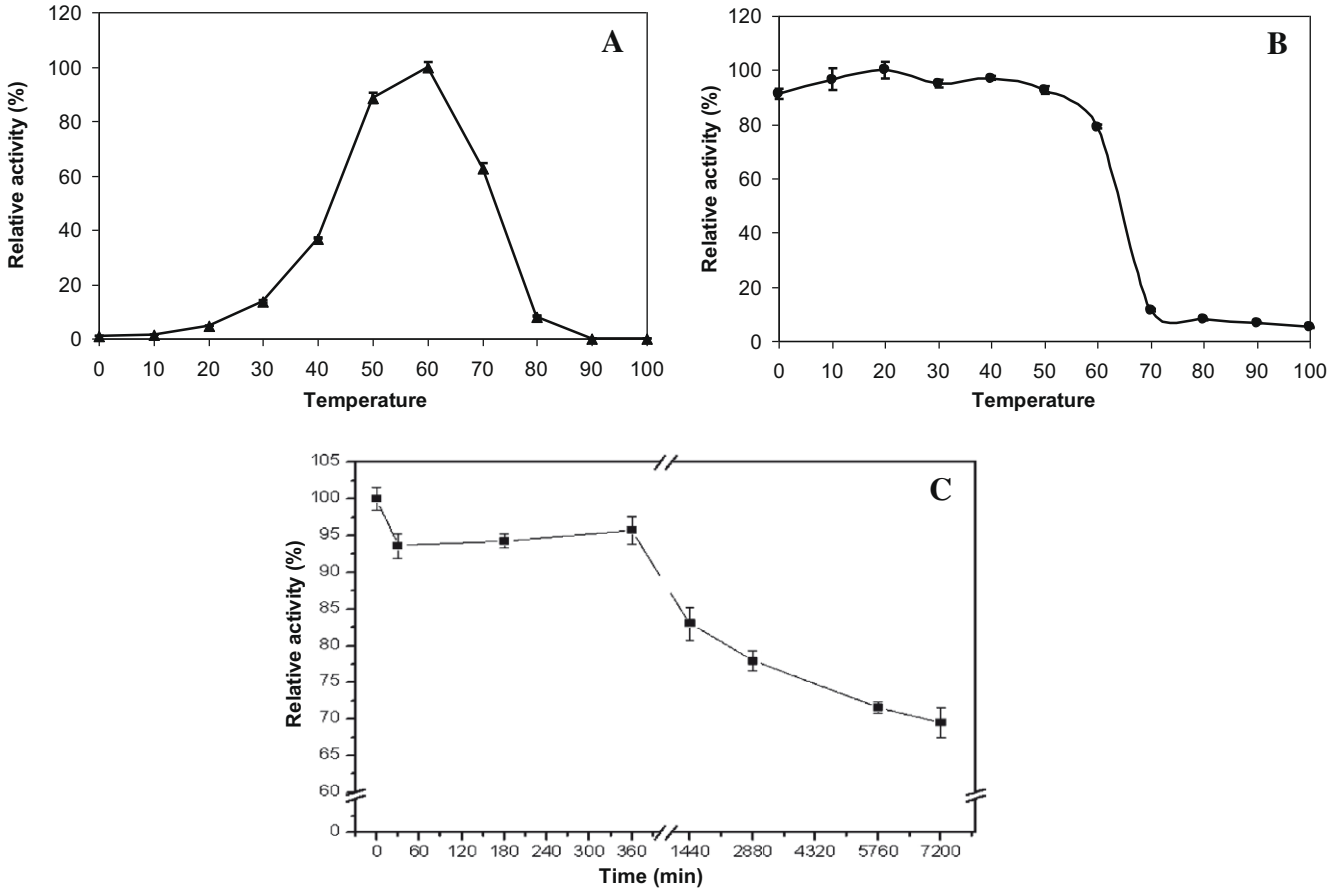

Fig. 3. Effect of temperature on catalytic activity. (A) The optimal temperature was determined under standard assay conditions. (B) The temperature stability was determined by measuring the remaining activity after incubation at various temperatures at pH 6.0 for 30 min without substrate. (C) The remaining activities after incubation at 50 °C without substrate at various time points were reported.

worse (significant) than that at pH 4.0. We have used two buffer systems to test enzyme stability at pH 4.0, i.e. glycine–HCl and acetate buffers. The actual relative activity of the enzyme when incubated at pH 4.0 using acetate buffer after 0.5 h was 82.93 ± 3.48. This value overlapped with the relative activity of the enzyme when incubate at pH 3.0 using glycine–HCl buffer after 0.5 h, which was 86.72 ± 1.329. Thus, we conclude that the stability of the enzyme after incubation for 0.5 h was the same (not significantly different) at pH 3.0 or pH 4.0, whereas at 18 h after incubation, the enzyme was more stable at higher pH (4.0). The optimal temperature for ChiA1 was 60 °C (Fig. 3A). It was stable up to 60 °C (more than 80% residual activity) after incubation for 30 min at pH 6.0 (Fig. 3B) without substrate. After incubation at 50 °C for 5 days without substrate, approximately 70% of the activity was retained (Fig. 3C).

Analysis of biochemical properties of the purified enzyme as described above revealed that the *B. licheniformis* ChiA is relatively thermostable when compared to other bacterial enzymes that have been suggested to be appropriate for bioconversion of colloidal chitin (Dahiya et al., 2006; Kudan and Pichyangkura, 2009; Lee et al., 2007; Yuli et al., 2004). Moreover, it showed the widest pH stability, ranging from pH 4.0–12.0, (Dahiya et al., 2006; Kudan and Pichyangkura, 2009; Yuli et al., 2004). Purification and characterization of wild-type chitinase from *B. licheniformis* Mb-2, which has identical N-terminal amino acid sequence and same molecular weight to our recombinant enzyme, has previously been reported (Toharisman et al., 2005). The properties of the wild-type and recombinant ChiA were similar except that the optimal temperature of the wild-type ChiA was 70 °C, instead of 60 °C for recombinant enzyme. However, this different might be because of the different in the method of enzyme assay.

3.4. Enzyme activity and kinetic analysis

Specific activities of recombinant *B. licheniformis* ChiA, when using *p*-NP-chitobiose and colloidal chitin as substrates at 37 °C or 50 °C, are reported in Table 1. In addition, Michaelis–Menten type kinetic parameters were determined using 0.008–0.18 mM of *p*-NP-chitobiose as a substrate. This substrate concentration range was used because substrate inhibition became apparent at higher substrate concentration as previous shown for this type of chitinases (Brurberg et al., 1996; Synstad et al., 2004). Kinetic analysis revealed that K_m, V_{max}, and k_{cat} values of recombinant *B. licheniformis* chitinase were 0.03 ± 0.003 mM, 0.28 ± 0.063 mM/min, and 0.31 ± 0.070 s^{-1}, respectively. The overall catalytic efficiency, k_{cat}/K_m of the enzyme was 10.30 ± 3.236 s^{-1}/mM.

Table 1
Specific activities of recombinant *B. licheniformis* ChiA.

Substrate	Specific activity (Unit/mg)*	
	37 °C	50 °C
p-NP-chitobiose[a]	0.294 ± 0.009	0.946 ± 0.030
Colloidal chitin[b,c]	1.50 ± 0.20 (4.84 ± 0.04)	5.11 ± 0.20 (15.67 ± 0.37)

[a] Unit means micromole of ρNP released per minute.
[b] Unit means micromole of G1 (in parenthesis) released per minute.
[c] Unit means micromole of G2 (in parenthesis) released per minute.
* The enzyme assay was done at two temperatures as described in Section 2.

3.5. Hydrolytic analysis by thin layer chromatography (TLC)

Product analysis by thin layer chromatography (TLC) using various substrates was performed to determine the hydrolytic activity of *B. licheniformis* ChiA. When the enzyme was incubated with G2, no detectable products were observed even after 18 h of incubation (Fig. 4), suggesting that G2 was not the substrate of this enzyme. With G3 substrate, G2 could be detected after 10 min of incubation, and after incubation for 18 h, the band of G3 disappeared, indicating that *B. licheniformis* ChiA has activity toward G3. When G4 was used as substrate, G2 was the major product, suggesting that the enzyme efficiently cleaved the middle glycosidic bond of the tetrameric chain. When longer oligomers (G4–G6) were used to study, distinct patterns of product formation

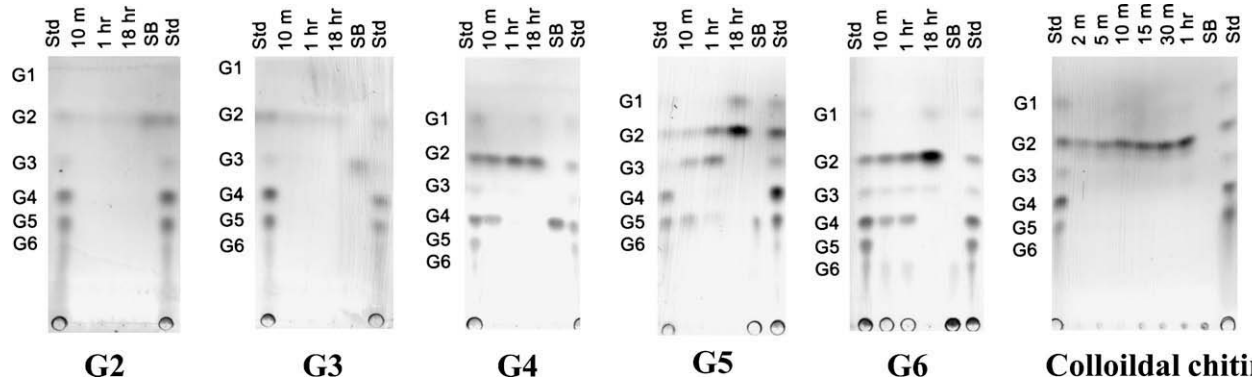

Fig. 4. TLC analysis of hydrolytic products. TLC analysis of chito-oligosaccharides (G2–G6) and colloidal chitin hydrolysis. The reaction products after incubation for different durations are indicated. Std: a standard mixture of G1–G6; SB: substrate blank; m: minute; hr: hour. The hydrolytic reaction was carried out at 37 °C for up to 18 h. The reactions comprised 100 nM chito-oligosaccharide substrates and 0.5 μg enzyme in 30-μl reaction volume, or 3 mg colloidal chitin and 25 μg enzyme in 400-μl reaction volume.

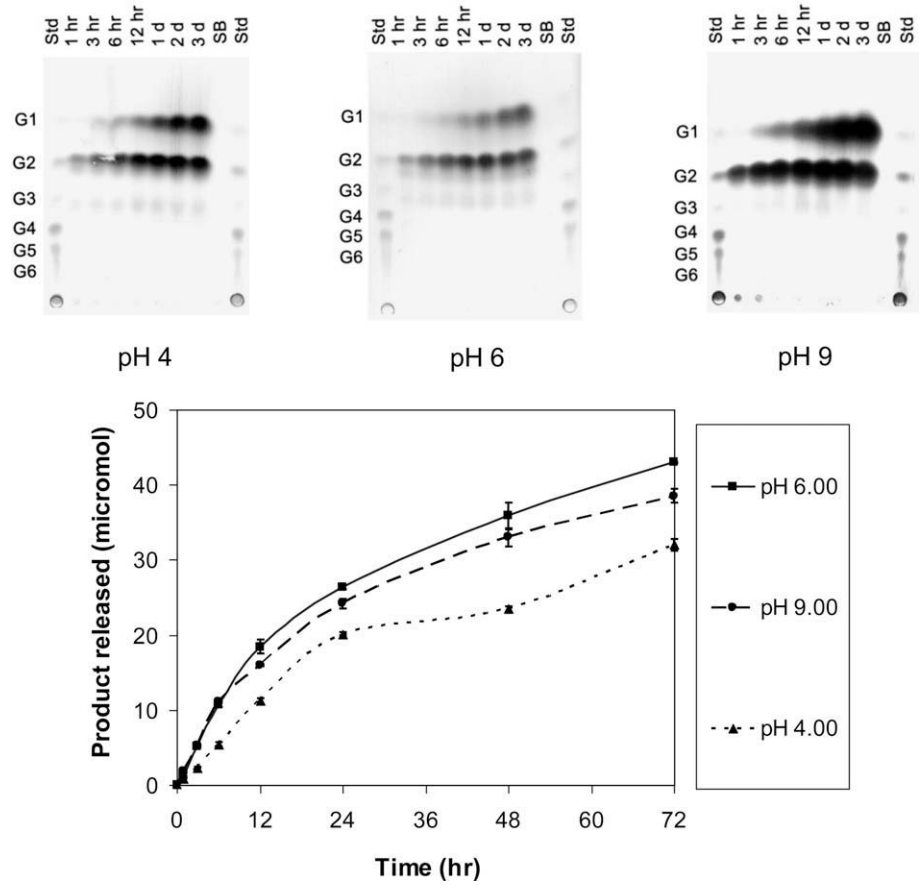

Fig. 5. Analysis of reaction products in various buffers. Upper panel illustrates TLC analysis of colloidal chitin hydrolysis by *B. licheniformis* ChiA1 at three different buffers. The hydrolytic reaction was carried out at 50 °C for up to 3 days in the presence of 30 mg colloidal chitin and 50 μg enzyme in 800-μl reaction volume. The reaction products after incubation for 1 h, 3 h, 6 h, 12 h, 1 day, 2 day and 3 day are shown. Std: a standard mixture of G1–G6; SB: substrate blank; hr: hour; d: day. The contrast and brightness of the pictures were enhanced by Image J software (Abramoff et al., 2004). Quantitative analysis of products released at various time points, determined by DNS method (Miller, 1959), is depicted in the lower panel.

could be observed. Using G5, G2 and G3 were released as intermediates, and then G3 was further hydrolyzed to G1 and G2. The hydrolysis of G6 initially yielded G2, G3 and G4. Then, G4 and G3 were further hydrolyzed to G2 and G1.

The hydrolytic activity of ChiA1 against colloidal chitin was also studied at various incubation times. The TLC result showed that G2 was rapidly formed only after 2 min of the incubation. At the end of the reaction, G2 was also observed as the major product. Taken together from the patterns of product formation, we concluded that *B. licheniformis* ChiA is an endochitinase that can efficiently cleave chitinous substrates that are equal or longer than tri-oligomers. The absence of longer lengths of chito-oligosachharide when chitin was used as substrate suggested that the enzyme could not get access to the compact structure of colloidal chitin.

3.6. Product analysis at different conditions

To determine the applicability of the recombinant enzyme as a biocatalyst for the production of *N*-acetylglucosamine (NAG) and chito-oligosaccharides, the enzyme was incubated with colloidal chitin in three different pHs at 50 °C for up to 3 days. We performed an experiment at this temperature because higher temperature will reduce the chance of microbial contaminations and the overall catalytic activity of the enzyme is higher. The reaction was sampled at various time points to measure product released. The *B. licheniformis* ChiA appeared to be stable during the course of the study. Product analysis by TLC (Fig. 5 upper panel) revealed that G1–G3 could be produced at both pH 4.0 and pH 6.0; whereas, only G1 and G2 were obtained at alkaline condition (pH 9.0). It is worthwhile to note that TLC analysis can only be used to estimate the quantity of reaction productions. Proper evaluation of hydrolytic products has to be done by more sophisticated technique such as HPLC analysis (Horn et al., 2006) (Vincent Eijsink's personal communication). In addition to determination of hydrolytic product by TLC, the amounts of reducing sugar released from various pHs were quantitatively determined as shown in Fig. 5, lower panel. The enzyme performed equally well at pH 6 and 9, and was slightly less active at pH 4, indicating that at 50 °C the enzyme was more stable at pH 9.0 than at pH 4.0. These results were consistent with the results shown in Fig. 4B, which showed that the enzyme was significantly more stable at pH 9.0 than at pH 4.0 after incubation at 20 °C for either 0.5 h or 18 h. Taken together, our results indicated that *B. licheniformis* ChiA could be efficiently used to covert colloidal chitin to G1 and G2 as major products, and G3 as a minor product. This result is similar to small-scale (50-μl reaction) hydrolysis products obtained from using chitinase from *Bacillus* sp. DAU101 (Lee et al., 2007).

Thus, TLC analysis indicated that the enzyme is an endochitinase that prefers substrate longer than tri-saccharide. When the enzyme was incubated with colloidal chitin at 50 °C in three pH conditions (pH 4.0, 6.0 and 9.0), the only 2 majors products that could be obtained were NAG and chitobiose (G2). This result supported the observation that chitin is a highly recalcitrant raw material and additional proteins are needed so that chitinase can gain access to the inside of the chitin fiber, in order to obtain a longer chito-oligosaccharide or used directly on the solid chitin (Eijsink et al., 2008). Nevertheless, the fact that the enzyme could be efficiently used for bioconversion of colloidal chitin from crab shells into NAG and chitobiose for up to 3 days at 50 °C in acidic, neutral or alkaline conditions, made this enzyme attractive for industrial applications. NAG has been shown to possess anti-inflammatory effects and has been used to treat ulcerative colitis and other gastrointestinal inflammations (Russell, 1999). In addition, it can also be used as a nutritional substrate for pediatric chronic inflammatory bowel disease (Salvatore et al., 2000). The derivative of NAG, glucosamine, has been shown to help regenerate joint cartilage and has been used extensively to treat osteoarthritis (Huskisson, 2008). Chitobiose can be used as a building block for transglycosylation reaction to generate longer chito-oligosaccharides (Kobayashi et al., 1997) or further modified to produce an enzyme inhibitor (Terayama et al., 1993). Other applications of chitinase include bio-control of plant pathogens and mosquitoes, single cell protein production and protoplast preparation (Hayes et al., 2008a,b).

4. Conclusions

In conclusion, endochitinase from *B. licheniformis* (ChiA) was cloned and efficiently produced using *E. coli* expression system. Characterization of biochemical properties suggested that the enzyme is thermo and pH-stable, suitable for various biotechnological applications, including biocoversion of colloidal chitin into NAG and chitobiose.

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