Appl Biochem Biotechnol (2009) 157:23–35
DOI 10.1007/s12010-008-8328-7

Purification and Characterization of Thermostable Chitinase from *Bacillus licheniformis* SK-1

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Received: 14 January 2008 / Accepted: 17 July 2008 /
Published online: 4 February 2009

Abstract Chitinase was purified from the culture medium of *Bacillus licheniformis* SK-1 by colloidal chitin affinity adsorption followed by diethylamino ethanol-cellulose column chromatography. The purified enzyme showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular size and p*I* of chitinase 72 (Chi72) were 72 kDa and 4.62 (Chi72) kDa, respectively. The purified chitinase revealed two activity optima at pH 6 and 8 when colloidal chitin was used as substrate. The enzyme exhibited activity in broad temperature range, from 40 to 70°C, with optimum at 55°C. It was stable for 2 h at temperatures below 60°C and stable over a broad pH range of 4.0–9.0 for 24 h. The apparent K_m and V_{max} of Chi72 for colloidal chitin were 0.23 mg ml^{-1} and 7.03 U/mg, respectively. The chitinase activity was high on colloidal chitin, regenerated chitin, partially *N*-acetylated chitin, and chitosan. N-bromosuccinamide completely inhibited the enzyme activity. This enzyme should be a good candidate for applications in the recycling of chitin waste.

Keywords *Bacillus licheniformis* SK-1 · Characterization · Chitinase · *N*-bromosuccinamide · Purification

Introduction

Chitinase, a group of enzymes capable of degrading chitin to low-molecular-weight products, have been shown to be produced by a number of microorganisms. The production of inexpensive chitinolytic enzymes is an important element in the utilization of shellfish

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wastes that not only solves environmental problems but also promotes the economic value of marine products [1]. Thus, chitinolytic enzymes have been studied and purified from many microorganisms, and their enzymatic properties have been investigated [2–9]. *Bacillus* spp. is regarded as a group of bacteria particularly efficient in the breaking down of chitin [5]. However, only a few thermostable chitinolytic enzymes from bacteria have been reported [6–9]. Previously, thermostable chitinases have been isolated from *Bacillus licheniformis* MB-2 [8] and *Streptomyces thermoviolaceus* OPC-520 [9].

Previously, we have isolated *B. licheniformis* SK-1 that produces a particular high activity of thermostable chitinase when cultured in the medium containing colloidal chitin as the sole carbon source. We were able to use crude chitinase from this microorganism to prepare *N*-acetyl-D-glucosamine and *N*-*N*′-diacetylchitobiose from crystalline chitin [10]. This paper describes the purification, characterization, and some properties of chitinases from the culture medium of *B. licheniformis* SK-1.

Materials and methods

Chemicals

The *p*-nitropheny-®-*N*-acetylglucosaminide, *N*-acetylglucosaminide, glycol chitin, and caucofluor white M2R were purchased from Sigma Chemical Co. (St. Louis, USA). All of the other chemicals and reagents that were used were grade commercially available.

Microorganism and culture

SK-1 was isolated from soil in Angthong Province, Thailand. SK-1 was cultured at 50 °C on 0.02% colloidal chitin minimum medium (CCMM) agar plate, 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.03% $MgSO_4$, 0.6% KH_2SO_4 and 1.0% K_2HPO_4, pH 7.5, and 2% agar. For liquid culture, the medium contained 0.1% CCMM. The microorganism was incubated with shaking at 50 °C in CCMM for 3–4 days.

Morphological and biochemical characterization

The exponential phase culture, cultivated in nutrient agar (NA) after 2–4 days of incubation at 37 °C was examined for cell form, cell arrangement, spore forming, and gram staining for SK-1 using light microscope. SK-1 cells were observed by scanning electron microscopy (SEM; JSM-35CF). The organism, grown on colloidal chitin agar medium, was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 at 25 °C for 2 h. After being washed with buffer, the samples were treated with 1% osmium acid in buffer then dehydrated by using series of ethanol (35, 70, 95, and 100%) and freeze-dried. The sample was then coated with gold (JFC-1100) and analyzed by SEM, JEOL model JSM-5800LL.

The hydrolysis of skim milk and starch were carried out by adding 1% skim milk and starch in Luria–Bertani (LB). Catalase production was determined by bubble formation with 3% H_2O_2. Oxidase test and carbohydrate fermentation test were performed.

16S rRNA gene sequence comparison

The partial 16S rRNA gene (1.5-kb fragment) of SK-1 was amplified by polymerase chain reaction (PCR) with forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ (8F) and reverse primer 5′-AAGGAGGTGATCCAGCCGCA-3′ (1542R). The amplified PCR

product was purified by Qiagen quick Gel Extraction Kit (Qiagen, Germany) and sequences by the dideoxy-chain termination method with fluorescent primer [11].

The 16S rDNA sequence was aligned with other 16S rDNA bacterial sequence obtained from GenBank by basic local alignment search tool (BLAST) program [12, 13].

Nucleotide sequence accession number

The partial 16S rDNA sequence determined for strain SK-1, identified as *B. licheniformis*, was deposited in the GenBank database under accession number AF411341.

Purification of chitinase

SK-1 was cultured in an orbital shaker for 4 days in 0.1% CCMM (2 l), at 50 °C, 250 rpm. The cells were removed by centrifugation (8,000×*g*; 20 min) to obtain culture supernatant. For affinity adsorption, the amount of total protein in the culture supernatant was determined, colloidal chitin was added (10 mg/mg of protein), and stirred gently overnight at 0 °C [14]. The colloidal chitin was then washed three times with 10 mM potassium phosphate buffer (KPB, pH 6.0) and collected by centrifugation. The precipitated colloidal chitin was resuspended in 20 ml of KPB and incubated at 50 °C overnight to digest the colloidal chitin. The digested solution was dialyzed against 25 mM Tris–HCl buffer, pH 7.5 to remove the hydrolytic product. The dialyzed enzyme was applied to a diethylamino ethanol (DEAE)-cellulose column, previously equilibrated with 25 mM Tris–HCl buffer, pH 7.5, and eluted with 0–1.0 M NaCl gradient.

Enzyme assay

Chitinase activity was assayed in a 1.5-ml reaction mixture containing 0.1% colloidal chitin in 0.1 M phosphate buffer, pH 6.0, and 0.1 ml of enzyme solution. After incubation at 50 °C for 10 min, the reaction was stopped by boiling and then centrifuged. Reducing sugar produced was measured by the modified Schales method [15]. One unit of chitinase activity was defined as the amount of enzyme that liberates reducing sugar corresponding to 1 μmol of *N*-acetyl-D-glucosamine per minute.

Protein measurement

Protein measurement was performed by the Bradford method [16] using bovine serum albumin as the standard. For chromatographic profile, the protein concentration was estimated by measuring the absorbance at 280 nm.

Determination of the p*I* of the enzyme

Isoelectric focusing (IEF) was performed according to Robertson et al. [17] with ampholine carrier ampholytes pH 3.0–10.0 at final concentration of 1%. After electrofocusing, the gel was stained with Coomassie brilliant blue R-250 to visualize the protein.

Determination of molecular weight and activity staining

The molecular weight of the enzyme was determined on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and standard proteins were used. The

activity of chitinase in SDS-PAGE was detected according to the method of Trudel and Asselin [18].

Optimum temperature and pH

The optimum temperature and pH were measured by a colorimetric method, using colloidal chitin as a substrate. The enzyme was assayed for 30 min at temperatures 40–70 °C and pH 3.0–10.0, 0.1 M citrate buffer at pH 3.0–6.0, phosphate buffer at pH 6.0–8.0, and Tris–HCl buffer at pH 8.0–10.0 were used.

Enzyme stability and substrate specificity

The thermal stability was investigated by incubating the enzyme for 12 h at temperatures 40, 50, and 60 °C in phosphate buffer pH 6.0. Aliquots were taken every 2 h, and residual activity of the enzyme was determined under standard assay condition. The pH stability was determined by incubation in buffers with the pH range of 3.0–10.0, at 4 °C, for 24 h. Substrate specificity was determined on various chitin 1.0% *w*/*v* [colloidal chitin, regenerated chitin, *p*-nitropheny-β-*N*-acetylglucosaminide, chitobiose, 50% degree of deacetylation (DD) partially *N*-acetylated chitin (PNAC), 80%DD chitosan, powdered chitin and flaked chitin] in phosphate buffer pH 6.0 for 30 min at 55 °C. The enzyme was assayed colorimetrically as previously described.

Effects of chemical reagent

The enzyme was preincubated with each chemical reagent [*N*-ethylmaleimide (NEM), dithiothreitol (DTT), 2,4,6-trinitrobenzenesulfonic acid (TNBS), phenylmethylsulfonyl fluoride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), diethylpyrocarbonate, *N*-bromosuccinimide (NBS), and *N*-acetylimidazole] at 1 mM, in 0.1 M phosphate buffer (pH 6.0) at 50 °C for 30 min. The residual activity was measured using the standard assay conditions.

Results

Identification of strain SK-1

Bacteria strain SK-1 was identified by morphological and biochemical characteristics listed in Table 1. This strain was a Gram-positive rod shape, size 0.4×1.3 μM, which formed subterminal ellipsoidal endospores as shown in Fig. 1. These bacteria grew both aerobically and anaerobically on LB agar. The limiting temperature for growth was 60 °C. The colonies on NA were reddish-brown in color and had a rough surface with hair-like outgrowths. This bacterium was highly active in producing acids from sugars and in degrading macromolecule such as starch. From these results, strain SK-1 was identified as *B. licheniformis*.

The identification of strain SK-1 as *B. licheniformis* was further confirmed by 16S rDNA sequence comparison. BLAST analysis of 16S rDNA nucleotide sequence of SK-1 revealed close matches to members of different genera of the family *Bacillus* sp. on the basis of the nucleotide sequence of 16S rDNA, within 1,533 bp that was sequenced and

Table 1

Characteristics
Morphological characteristics
Shape: bacilli
Mobility: motile, with peritrichous figella
Spores: Subterminal ellipsoidal endospores formed
Gram stain: positive
Culture characteristics
Nutrient agar colony: circular, flat, opaque with rough surface, hair-like outgrowths
Physiological characteristics
Growth at 60 °C: positive
pH for growth 5-9, optimum 7.5
Biochemical characteristics
Growth in anaerobic condition: positive
Growth in 10% NaCl: positive
Phenylamine deaminase: positive
Alkaline phosphatase: positive
Acid from Ribose: positive
Manitol: positive
Cellobiose: positive
Lactose: positive
Sucrose: positive
Hydrolysis of starch: positive
Casein: positive
Antibiotic resistance
Ampicillin: positive

compared. SK-1 was identified as *B. licheniformis*. SK-1 was most similar to *B. licheniformis* B (AF276309), with 97% identity.

Therefore, from morphological and biochemical characteristics and 16S rDNA sequence comparison, isolated SK-1 was designed as *B. licheniformis*. The nucleotide sequence of the 16S rDNA of SK-1 was submitted to GenBank (accession number AF411341).

Production of chitinase from *B. licheniformis*

SK-1 grew well on CCMM and gave a wide clear zone in 2 days, at 50 °C. The optimum temperature for maximum chitinase production in colloidal chitin broth was 50 °C. The relationship between cell growth and total chitinase activity in the culture medium was shown in Fig. 2. These data demonstrated that enzyme production of SK-1 in CCMM corresponded with cell growth.

Purification of chitinase from *B. licheniformis*

With colloidal chitin as the sole carbon source, *B. lichenifomis* SK-1 produced chitinases in the culture medium. The Chi72 was purified using colloidal chitin adsorption techniques, DEAE-cellulose ion exchanger chromatography (Fig. 3). The protein was eluted with 0.45 M NaCl collected and selected to further characterization. The results of chitinase purification are summarized in Table 2. The Chi72 was purified 11.5-fold with a yield of 35%. The final preparation gave a single band, which had a molecular size 72 kDa in SDS-PAGE, and isoelectric point of Chi72 was 4.62 (Fig. 4).

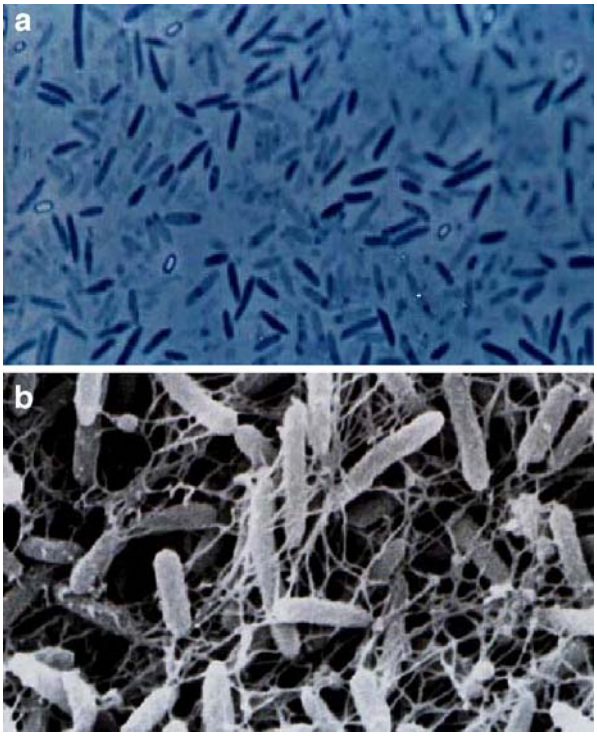

Fig. 1 Microscopic morphological **A** phase contrast light microscope, ×1,000 and **B** scanning electron microscopic, SEM ×15,000, of *B. licheniformis* SK-1 as described in "Materials and Methods"

Enzyme characterization

The enzyme showed optimal activity at two pHs: 6 and 8 (Fig. 5). The enzyme was remarkably stable at temperatures below 60 °C (Fig. 6). The half-life at 60 °C was about 90 min, whereas at 50 and 40 °C, no significant activity lost was observed within 2 and 4 h, respectively.

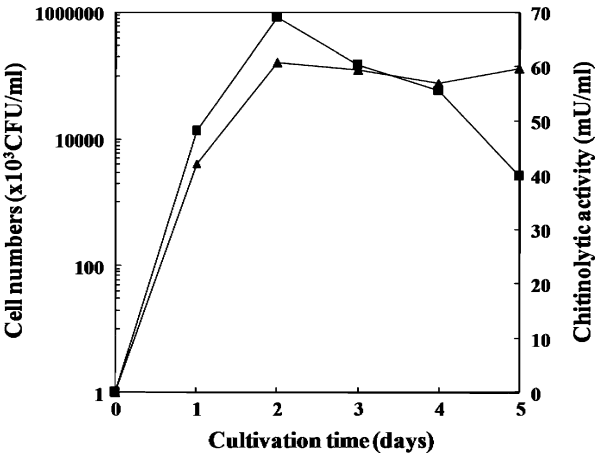

Fig. 2 The relationship between cell growth and extracellular chitinolytic activity of *B. licheniformis* SK-1; *square* cell number; *triangle* chitinase activity. Cells were grown in colloidal chitin minimum medium with shaking at 50 °C, 250 rpm. Cell density was measured by colony count and the chitinase activity in the culture supernatant was determined as described in "Material and methods"

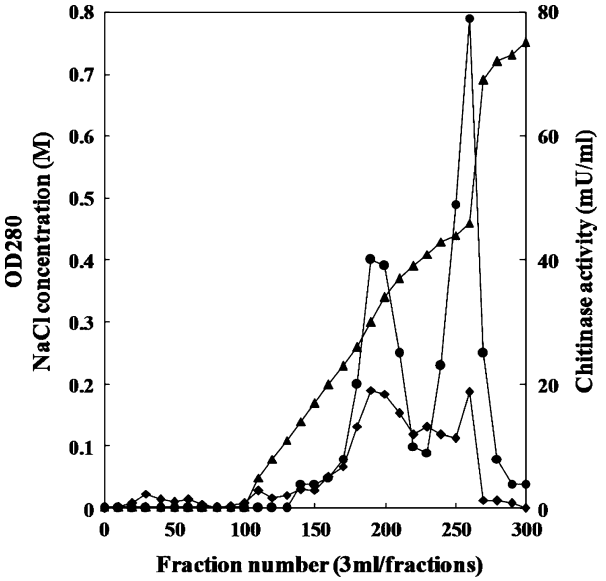

Fig. 3 Chromatogram of chitinase from *B. licheniformis* SK-1 purification by DEAE cellulose column. The enzyme from colloidal chitin adsorption was loaded onto the equilibrated DEAE-cellulose column with 25 mM Tris-HCl buffer pH 7.5. The column was washed with equilibrated buffer until A_{280} was negligible, then the column was eluted with 0–1.0 M NaCl linear gradient. *Diamond* Absorbance 280 nm, *circle* chitinase activity, and *triangle* NaCl concentration

Enzyme kinetic of Chi72

The kinetic of the enzyme was studied on colloidal chitin. The kinetics of Chi72 followed the classical Michaelis–Menten kinetics. The K_m and V_{max} value calculated from Lineweaver–Burk plots was 0.23 mg/ml and 7.03 U/mg, respectively (Fig. 7).

Substrate specificity

Table 3 shows the results of the hydrolysis of various substrates with the enzyme. The enzyme hydrolyzed the colloidal chitin, regenerated chitin, 50%DD partially *N*-acetylated chitin, 80% acetylated chitosan, powdered chitin, and flaked chitin but did not hydrolyze chitobiose, *p*-nitrophenyl-*N*-acetylglucosaminide. The relative activity, expressed as percentage, of the enzyme on 50%DD partially *N*-acetylated chitin, 80% DD chitosan, regenerated chitin, powdered chitin, and flaked chitin were 394%, 150%, 74%, 4%, and 2%, respectively, normalized to the enzymatic activity on colloidal chitin.

Chemical modification of chitinase

When Chi72 was incubated with various group-specific reagents for amino acid modification (cysteine, lysine, serine, carboxylic amino acids, histidine, tryptophan, and tyrosine residues),

Table 2 Purification of thermostable chitinase from *B. licheniformis* SK-1.

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Crude enzyme	98.0	190.00	1.95	1.0	100
Chitin adsorption	16.75	127.25	5.46	2.8	64
DEAE-cellulose	3.0	67.50	22.5	11.5	35

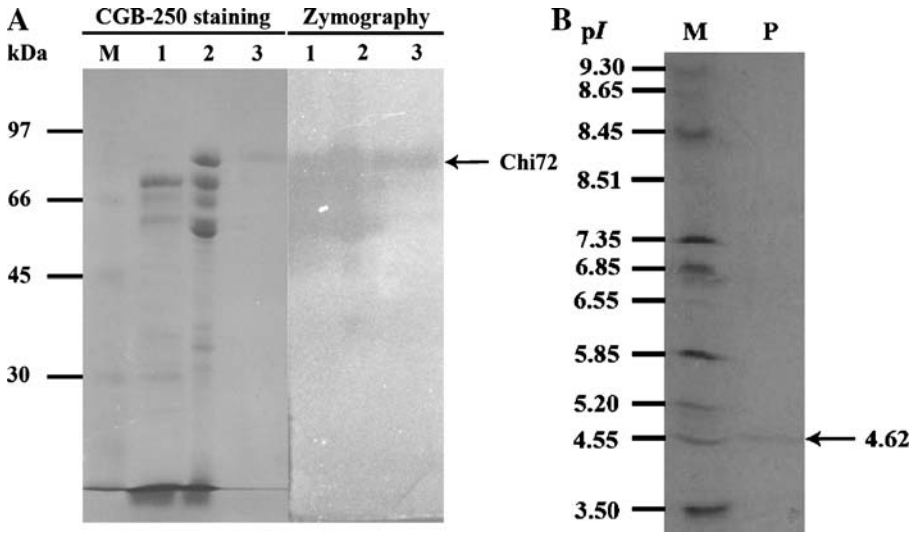

Fig. 4 SDS-PAGE analysis and native IEF pattern of the purified chitinase from *B. licheniformis* SK-1. **A** *Lane M* Molecular weight markers; *lane 1* crude enzyme (100 μg); *lane 2* chitin adsorbed protein (20 μg); *lane 3* DEAE purified chitinase (5 μg). **B** *Lane M* IEF standard marker; *lane P* purified Chi72

it was found that the activity was abolished in the presence of 1 mM NBS. Partial activity loss was observed when incubated with NEM, DTT, and TNBS (Table 4). This indicates the importance of tryptophan, cysteine, and lysine on the enzyme activity.

Discussion

A thermotolerant bacterium, SK-1, which has chitinolytic activity, was isolated from soil by conventional plate assay, which is simple and easy to perform. Thus, this method was selected for screening of chitinolytic producers from numbers of bacteria isolates. SK-1 can grow in LB medium at temperatures 30–60 °C, with optimum growth at 45–50 °C, indicating that it is a thermotolerant bacterium (data not shown). Morphological and physiological characteristics of strain SK-1 (Table 1 and Fig. 1) clearly demonstrated that

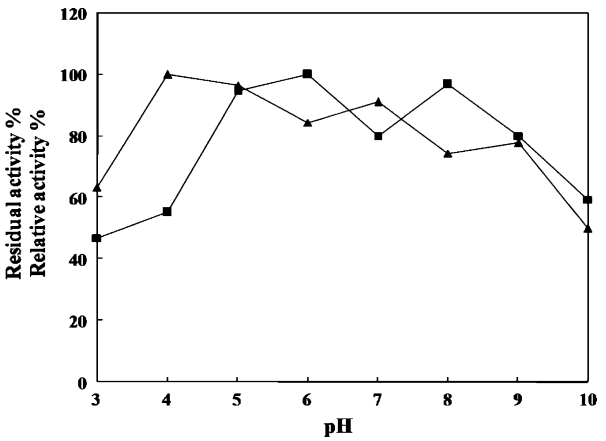

Fig. 5 Optimal pH (*square*) and pH stability (*triangle*) of purified chitinase from *B. licheniformis* SK-1

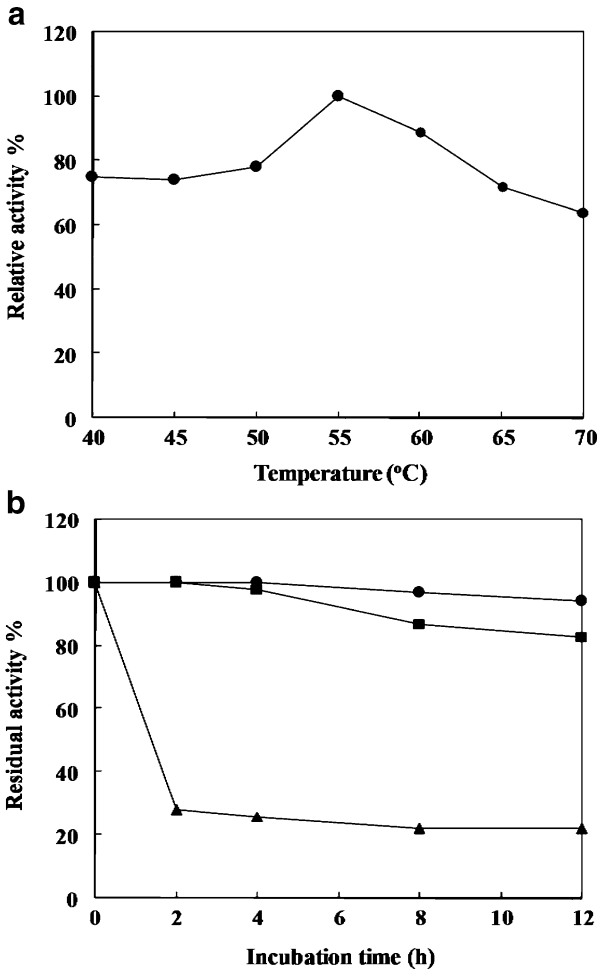

Fig. 6 Optimal temperature and thermal stability of purified enzyme from *B. licheniformis* SK-1. **A** The optimum temperature of purified chitinase from *B. licheniformis* SK-1. **B** The thermal stability of purified chitinase from *B. licheniformis* SK-1. The enzyme was incubated separately at 40 °C (*diamond*), 50 °C (*square*), and 60 °C (*circle*)

SK-1 is a to *B. licheniformis*. This classification is consistent with the result from 16S rDNA sequence comparison. From these results, the SK-1 was identified and classified as *B. licheniformis*. *B. licheniformis* has been shown to produce extracellular chitinolytic enzyme [19, 20]. Our results showed that *B. licheniformis* SK-1 was one of the strongest chitinolytic enzyme producer.

B. licheniformis SK-1 produced chitinolytic enzyme continuously during exponential phase of growth when grown in 0.02% CCMM (Fig. 2). The highest level of chitinolytic enzyme was found on the second day of cultivation. Moreover, when bacterium entered the stationary phase, the activity of chitinase in the culture medium remains constant. This suggests that chitinolytic enzyme activity from *B. licheniformis* SK-1 was stable throughout the cultivation period. Production of chitinolytic enzymes from *B. licheniformis* was studied in various medium. We found that *B. licheniformis* SK-1 grown in culture medium with colloidal chitin, CCMM, yielded the maximum chitinolytic activity of 74 mU/ml; however, a low level of expression of the enzyme, 21 mU/ml, can be found when *B. licheniformis* SK-1 is grown in the LB medium (data not shown). Although the chitinolytic enzyme could be produced constitutively by *B. licheniformis* SK-1, using chitin as the sole carbon source in the culture media greatly enhanced the enzyme production.

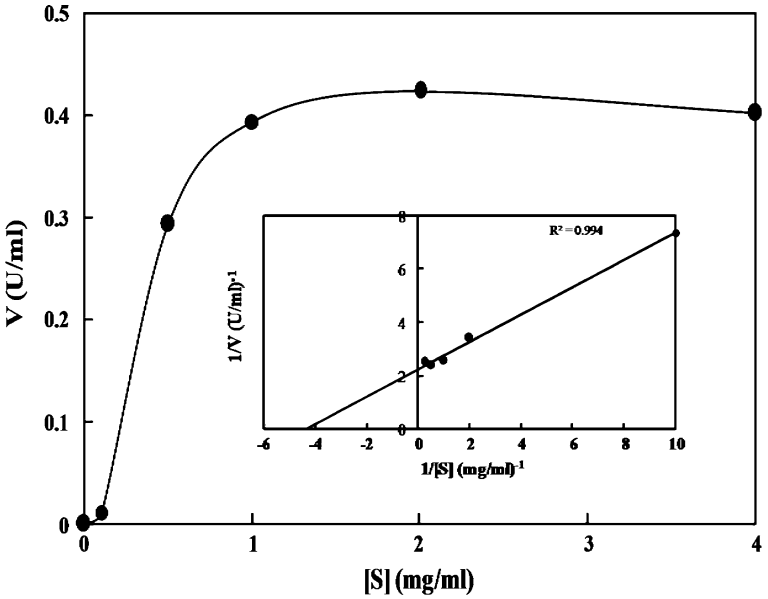

Fig. 7 Kinetic analysis of thermostable chitinase using colloidal chitin

The crude enzyme was purified by colloidal chitin affinity absorption and by DEAE-cellulose column chromatography. The result showed that Chi72 could bind with colloidal chitin, suggesting that the enzyme may have a chitin binding domain, since ChBD has been shown to be necessary for the enzyme to bind insoluble substrates. The removal of such domain eliminates the chitin binding ability of the enzyme [14, 20-21]. The molecular weight of the purified chitinase from *B. licheniformis* SK-1 was estimated by SDS-PAGE. The purified chitinase showed a molecular weight 72 kDa. The result was similar to that of the molecular mass of chitinase from *B. licheniformis* TP-1 [20]. The isoelectric point (p*I*) of purified chitinase was in the acidic range 4.62, which was found in most bacterial chitinases [14, 21].

The Chi72 showed optimum activity at two pHs: 6 and 8. This phenomena was also found in other bacterial chitinase [19, 22], using colloidal chitin as substrate. The enzyme was stable at pH 4.0 to 9.0 (Fig. 5). Previous reports have also shown that chitinase from

Table 3 Substrate specificity of purified thermostable chitinase of *Bacillus licheniformis* SK-1.

Substrate (1% *w*/*v*)	Relative activity (%)
Colloidal chitin (CC)	100
Regenerated chitin (RC)	74
p-nitropheny-β-*N*-acetylglucosaminide	0
Chitobiose	0
50% DD Partially *N*-acetylated chitin (PNAC)	394
80% DD Chitosan (CS)	150
Powdered chitin (PC)	4
Flaked chitin (FC)	2

Table 4 Effect of various group-specific reagents on the chitinase activity.

Reagent (1 mM)[a]	Amino acid involved	Residual activity (%)
None	–	100
N-ethylmaleimide (NEM)	Cysteine	88.9
Dithiothreitol (DTT)	Cysteine	81.2
2,4,6-Trinitrobenzenesulfonic acid (TNBS)	Lysine	88.1
Phenylmethylsulfonyl fluoride (PMSF)	Serine	92.1
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	Carboxylic amino acids	97.3
Diethylpyrocarbonate (DEP)	Histidine	95.0
N-bromosuccinimide (NBS)	Tryptophan	0
N-acetylimidazole (NAI)	Tyrosine	93.4

[a] Incubation with 20 μg/ml enzyme at 50 °C and pH 6.0 for 30 min

Aeromonas sp. no. 10S-24 [23] and *Vibrio* sp. chitinase [24] also show similar pH stability range. At least 50% of the enzyme activity was retained at pH 3.0 to 10.0. Incubation of the enzyme at pH 3.0 or 10.0 for 24 h resulted in only 50% reduction of the activity. Only few reports have shown that bacterial chitinases is stable at broad range of pH. The molecular basis for retaining of activity and stability at high pH of the enzyme remains to be elucidated [6–9]. The optimum temperature for the enzyme activity was found to be 55 °C at pH 6.0 (Fig. 6). The enzyme exhibited activity in a broad temperature range, from 40 to 70 °C. Furthermore, the enzyme was found to be stable at temperature below 60 °C (Fig. 6). The half-life at 60 °C was about 90 min, whereas at 50 °C, no significant activity lost was observed within 2 h, a 20% reduction of the activity was observed within 12 h. The high temperature optimum and the thermal stability of the chitinase from *B. licheniformis* are particularly advantageous for its applicability in recycling chitin wastes. The characterization of the chitinolytic system at molecular level would extend the prospects for the enzyme, particularly due to its extreme pH and temperature optima and relatively good stability.

The enzyme exhibited high activity toward PNAC, chitosan, colloidal chitin, and regenerated chitin compared to powdered chitin and flaked chitin. The enzyme did not have activity toward chitobiose, *p*-nitrophenyl-*N*-acetylglucosaminide, suggesting that it does not process *N*-acetylglucosaminidase activity (Table 3). Colloidal chitin and regenerated chitin are useful substrates for enzyme assays of endotype chitinase [25]. The product hydrolysis of the purified enzyme on colloidal chitin was *N*-*N*′-diacetylchitobiose as a major products and a minor of GlcNAc (data not shown). The apparent K_m and V_{max} for colloidal chitin of the purified chitinase was 0.023% (*w*/*v*, 0.23 mg/ml) and 7.03 U/mg, respectively. Chitinases reported in each bacterium seemed to be very different from this study. We found that the K_m of Chi72 was less than other bacterial chitinase [26, 27]. The K_m value implies that Chi72 has higher activity than other bacterial chitinase at low-substrate concentration.

There are very few reports in the literature on the effect of group-specific reagents on chitinase activity. Nevertheless, we have shown that *N*-bromosuccinamide at 1 mM concentration, which modifies tryptophan residues, completely inhibited the enzyme activity, while 90% inhibition was reported in *Pseudomonas* sp. YHS-A2 [28]. NEB inhibition suggested that tryptophan residue plays an important role in the activity of enzyme. Previous reports have demonstrated that exposed tryptophan residues on the surface of bacterial chitinases plays an important role in binding and guiding the substrate into the catalytic cleft on the enzyme, which has an important role on the activity of the enzyme [29, 30]. Family 18 bacterial chitinases contain consensus residues, DXXDXDXE,

and have conserved glutamic acid residues in the active site [31, 32]. EDC did not inhibit the activity of the enzyme, suggesting that the glutamic acid residue in the active site was not accessible to EDC. NEM and DDT inhibition suggested the role of cysteine in enzyme substrate interaction or in enzymatic catalysis. It has been reported that S-S bridge was found in chitin binding domain of *Alteromonas sp. strain* O-7 [5, 33].

Acknowledgments The research work was supported by the graduate school foundation, Chulalongkorn University, Thailand. We would like to thank Dr. Tipaporn Limpaseni for critically reading and giving valuable suggestion on this manuscript.

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