African Journal of Biotechnology Vol. 10(13), pp. 2476-2485, 28 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1029
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Purification and characterization of a novel extracellular chitinase from thermophilic *Bacillus* sp. Hu1

De-hui Dai[1]*, Wei-lian Hu[1], Guang-rong Huang[2] and Wei Li[3]

[1]School of Biological and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou, Zhejiang 310035, China.
[2]College of Life Sciences, China Jiliang University, Hangzhou, Zhejiang 310018, China.
[3]Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang 310012, China.

Accepted 24 January, 2011

The purification and characterization of a new thermophilic chitinase from thermophilic *Bacillus* sp. HU1, originally isolated from a soil sample collected from hot spring of XinJiang Province, China, is presented in this paper. The purification steps included ammonium sulfate precipitation, with columns of DEAE-Sepharose anion exchange chromatography and Sephacryl S-400 high resolution gel chromatography on AKTA purifier 100 protein liquid chromatography. The method gave a 5.6 fold increase of the specific activity and had a yield of 15%. The molecular weight of the chitinase was found to be around 80.8 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. The optimal pH and optimal temperature of the protease were at pH 6.5 and 60°C, respectively. The thermostable chitinase still retained the activity after incubation for 100 min at 60°C and it was increased about 1.31 times than that of the control (before heating) when the enzyme solution heated at 60°C for 60 min. The chitinase activity was inhibited by Cu^{2+}, dithiothreitol (DTT), Na_2.EDTA and activated by Mg^{2+}, Ca^{2+} and Zn^{2+} whereas, Na^{+}, K^{+} and Fe^{3+} did not show significant inhibitory effects against the chitinase. Substrates specificity tests indicated that, colloid chitin was the best substrate among the seven substrates tested (colloid chitin, particle chitinase, chitosan (80% degree of deacetylation, chitosan (95% degree of deacetylation), carboxymethyl cellulose (CMC), cellulose and soluble starch. It was concluded that the chitinase have high specificity in hydrolyzing glycosidic bond between GlcNAc-GlcNAc.

Key words: Thermostable chitinase, purification, characterization, thermophilic *bacillus* sp.

INTRODUCTION

Except for cellulose, chitin is the most abundant natural biopolymer, formed from β-1,4-links of N-actyl-D-glucosamine (GlcNAc) (Guo et al., 2008; Haki and Rakshit, 2003). It is widely distributed in the coatings of many species, being a part of the cell wall of most fungi (Bartnicki and Nickerson 1962), the microfilaria sheath of parasitic nematodes, the exoskeleton of all types of arthropods and the gut lining of many insects (Gomes et al., 2001). So far, it has been found that it has very little large-scale industrial use because of its extreme insolubility; cannot be absorbed or digested directly in the gastrointestinal tract (Kwang and Ji, 2001; Natasa et al., 2006).

*Corresponding author. E-mail: daidehui@163.com. Tel: +86 571 85070370.

Chitin oligosaccharides are one of the degradations of chitin, that have been of interest in the past few decades due to their broad range of medical, agricultural and industrial applications. This potential is associated with its characteristics being reported as antibacterial, antifungal, hypercholesterolemia, antihypertensive, antitumor agents and elicitors as food quality enhancer (Muzzarelli 1996; Patil et al., 2000; Purwani et al., 2004). They have historically been produced by acid hydrolysis of chitin with concentrated HCl at high temperature (above 80°C). However, the production of these chitin oligosaccharides by chemical process causes many problems, such as a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides, high cost in separation and also environmental pollution (Wang et al., 2009).

Therefore, the focus has shifted to enzymatic hydrolysis as a more appropriate method of chitin oligosaccharides

production from chitin recently although, the chitin-hydrolyzing enzyme are produced by a wide range of organisms concluding numerous bacteria (Wu et al., 2001; Morimoto et al., 1997; Guo et al., 2004), fungi (Mario and Everardo, 1991; Sherief et al., 1991), insects (Karl and Subbaratnam, 1998), plants (Gomes et al., 1996; Sahai and Manocha, 1993; Leah et al., 1991), animals (Mana et al., 2009; Matsumiya et al., 1998; Matsumiya et al., 2002) and even human (Barnard and Gé,1994). Most of them cannot be applied wildly due to the high cost of isolation of enzymes from natural resources, their instability and activity within narrow temperatured and pH ranges.

Thermostable enzymes are advantageous in some applications, because higher processing temperatures can be employed, resulting in faster reaction rates, increase in the solubility of nongaseous reactant and products and reduced incidence of microbial contamination by mesophilic organisms (Nascimento and Martins, 2004; Gomes and Steiner, 2004; Zeikus et al., 1998). As a result, scientists have focused their attention to microorganisms capable of living under extreme environment in the course of evolution, such as hot spring and geothermal areas (Purwani et al., 2004).

This work reports the recent progress on the purification and biochemical characterization of a novel chitinase from thermophilic *bacillus* sp. Hu1, which was isolated from a soil sample collected from hot spring of XinJiang Province, China. In this study, the chitinase showed a series of good characters such as high reaction temperature, high thermal stability, strong selectivity of hydrolyzing glycosidic bond between GlcNAc-GlcNAc and capacity of hydrolyzing crystalline chitin.

MATERIALS AND METHODS

Bacterial strain

Thermophilic *bacillus* sp. Hu1 exhibiting strong extracellular chitinase activity was isolated from soil samples collected from hot spring in XinJiang Province, China. The strain was maintained at 45℃ on double-layer medium. Up-layer medium contained 8.0 g/l chitin and 15.0 g/l bacto agar. Down-layer medium contained (g/l): KH_2PO_4 0.5, $MgSO4{\cdot}7H_2O$ 0.4, NaCl 0.5, $CaCl_2{\cdot}2H_2O$ 0.05, NH_4Cl 0.5, peptone 5.0, yeast extract 1.0 and $FeCl_2{\cdot}4H_2O$ 0.0005. After 2 days, the slants were kept at 4℃ and thereafter sub-cultured every 60 days, or at -18℃ as glycerol stock solutions (40%, v/v) for long-term maintenance.

Enzyme production and purification

Thermophilic *bacillus* sp. Hu1 was cultivated under aerobic conditions at 45℃ for 24 h in a seed medium containing (g/l): chitin 8.0, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.4, NaCl 0.5, $CaCl_2{\cdot}2H_2O$ 0.05, NH_4Cl 0.5, peptone 5.0, yeast extract 1.0, and $FeCl_2{\cdot}4H_2O$ 0.0005, solution adjusted to pH 7.0. 5% of the seed culture was transferred into the same medium and grown for 72 h at 45℃ under aerobic conditions (a continuous stir at 180 rev/min). The culture broth was centrifuged for 20 min at 12,000×g at a cool temperature of 4℃ (Hitach CF15R, Hitachi Koki Co. Ltd., Japan). The supernatant was used for enzyme purification. The supernatant was brought to 80% saturation with solid ammonium sulfate and left standing overnight at 4℃. The precipitate was collected by centrifugation at 12,000×g (4℃) for 20 min and re-dissolved in 50 mM pH 4.2 sodium-acetate buffer. The solution was dialyzed for the removal of ammonium sulfate in the same buffer for 12 h at 4℃. The dialyzed sample was passed through a DEAE-sepharose fast flow column (1.6×45 cm, Amersham Biosciences) pre-equilibrated with 50 mM pH 4.2 sodium-acetate buffer. First, the column was washed with five column volumes of the same eluting buffer and then, the enzymes were fractionated with a linear gradient of 0-1 M NaCl in 50 mM pH 4.2 sodium-acetate buffer at 0.4 ml/min for 500 min. The fractions with chitinase activity were pooled and precipitated again with 80% saturation of ammonium sulfate solution. The mixture was left overnight at 4℃ and then, centrifuged at the same conditions as stated earlier. The collected precipitates were dissolved in 50 mM pH 7.2 Tris-HCl buffer. The obtained solution was applied on a sephacryl S-400 high resolution column (1.6×60 cm, Amersham Biosciences) pre-equilibrated with 50 mM pH7.2 Tris-HCl. Then, the column was washed with the same buffer at the flow rate of 0.4 ml/min. The fractions with chitinase activity were collected and dialyzed against 50 mM pH 7.2 Tris-HCl buffer for the determinations of purity and characterization.

Enzyme assay

Chitinase activities were measured with colloidal chitin as substrate. Colloidal chitin 1% (w/v) was prepared and modified according to the method of Lee et al. (2009): 1 g chitin powder was added slowly to 20 ml of concentrated HCl and left at 4℃ overnight with vigorous stirring. The mixture was then added to 200 ml of ice-cold 95% ethanol with rapid stirring and kept overnight at 4℃. The precipitate was collected by centrifugation at 5,000×g for 20 min at 4℃ and then, washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Subsequently, the final volume was raised to 100 ml by adding 50 mM pH 6.5 sodium phosphate buffer. The reaction mixture consisted of 1ml 1% (w/v) colloidal chitin and 1 ml enzyme solution. After incubation at 60℃ for 45 min, the reaction was stopped by heating in boiling water for 20 min. Then, the mixture was centrifuged immediately at 10,000×g for 5 min. The supernatant was used for reduced sugar analysis using the dinitrosalicylic acid (DNS) method. One unit (U) of chitinase activity was defined as the amount of enzyme required to release 1 µmol GlcNAc per minute under the earlier mentioned conditions (Nawani and Kapadnis, 2005)

Determination of protein concentration

Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Determination of the chitinase molecular weight

The molecular weight of the chitinase was determined by SDS-PAGE. It was carried out with12% (w/v) isolation gel and 5% (w/v) concentration gel according to the method of Laemmli (1970). Before electrophoresis, proteins were exposed overnight to 50 mM pH 7.2 Tris-HCl buffer containing β-mercatpethanol. The gels were stained with Coomassie brilliant blue R-250 in methanol-acetic acid-water (5:1:5, v/v) and decolorized in 7% acetic acid. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the R_f value (relative mobility) on Minibis Bioimaging system (DNR Bio-Imaging

Systems Ltd., Israel) and the following proteins were used as standards: phosphorlase b (molecular mass, 97.4KDa), BSA (66.2KDa), ovalbumin (43 KDa), carbonic anhydrase (31 KDa), trypsin inhibitor (20.1 KDa) and α-lactalbumin (14.4kDa).

Effects of temperature and pH on chitinase activity

In order to determine the optimum temperature, chitinase activity was examined at various temperatures (35 to 80°C) in 50 mM pH 7.2 Tris-HCl buffer (10 U/ml). Thermal stability of the chitinase was measured in terms of residual activity after incubation of the purified chitinase at various temperatures ranged from 50 to 70°C for 30 to 240 min and from 80 to 90°C for 10 to 60 min.

The activity of the purified chitinase was also measured at different pH values. The pH was adjusted using the following buffers: 50 mM sodium acetate buffer (pH 4.0 to 5.0), 50 mM sodium phosphate buffer (pH 5.5 to 7.0) and 50 mM Tris-HCl (pH 7.5 to 9.0). The reactions were incubated at 60°C for 45 min and the activity of the enzyme was assayed to measure the optimum pH.

Effects of metal ions and other reagents on chitinase activity

The effects of different metal cations on chitinase activity were measured by the addition of the corresponding ion at a concentration of 4.0 mM to the reaction mixture and the assay was performed under standard conditions. The tested cations included the following corresponding salts: KCl (K^+), NaCl (Na^+), $FeCl_3$ (Fe^{3+}), $MgSO_4$ (Mg^{2+}), $CaCl_2$ (Ca^{2+}), $CuSO_4$ (Cu^{2+}) and $ZnSO_4$ (Zn^{2+}). The effects of 4.0 mM Na_2.EDTA and DTT on chitinase activity were also determined under the same conditions as mentioned earlier.

Substrate specificity

The purified chitinase was incubated with various substrate (1.0%, w/w), such as powder chitin, colloidal chitin, chitosan (80% degree of deacetylation), chitosan (95% degree of deacetylation), carboxymethyl cellulose(CMC), cellulose and soluble starch in 50 mM pH 6.5 sodium phosphate buffer at 60°C for 45 min and then, their hydrolysis activity was assayed.

RESULTS AND DISCUSSION

Purification of the chitinase

To purify the crude enzyme to homogeneity, the enzyme purification procedure included the following three steps: Firstly, the supernatant from the cell culture was precipitated with 80% saturation of $(NH_4)_2SO_4$, then the precipitates were dissolved and dialyzed in 50 mM pH 4.2 sodium-acetate buffer. There were three protein peaks and one chitinase activity peaks (Figure 1) when the dialyzed chitinase was applied on a DEAE-sepharose fast flow column. The chitinase activity peak was eluted at 0.8 M NaCl. The fractions of the last protein peak on the DEAE-sepharose fast flow column was collected and precipitated with 80% saturation of $(NH_4)_2SO_4$, was dissolved and dialyzed in 50 mM pH 7.2 Tris-HCl buffer for 24 h at 4°C. Lastly, the solution was applied on a Sephacryl S-400 high resolution column. Only one strong chitinase activity peak was detected, although, there appeared three protein peaks (Figure 2). The protease activity peak fractions was collected and run in a SDS-PAGE gel. A single band was obtained with a molecular mass of approximately 80.8 kDa (Figure 3). A considerable variation in the molecular weight of chitinase had been earlier reported: 55 kDa for *Sanguibacter antarcticus* KOPRI 21702^T (Park et al., 2009); 61 KDa for *Cellulosimicrobium cellulans* 191 (Fleuri et al., 2009); 47 kDa for *Penicillium* sp. LYG 0704 (Lee et al., 2009); 72 kDa for *Bacillus licheniformis* SK-1(Kudan and Pichyangkura, 2009) 70 KDa for *Streptomyces* RC1071 (Gomes. et al., 2001); 36 kDa for *Aeromonas* sp. DYU-Too7 (Lien et al., 2007); 53 KDa for ASCHI53 and 61KDa for ASCHI61 form *Aeromonas schubertii* (Liu et al., 2009). Generally, the molecular weight of chitinase was ranged from 20 to 80 kDa. Sometimes, several chitinases was secreted from one strain, whereas, there are visible variation in their molecular weight and enzyme activity (Sakai et al., 1998).

The purification procedures of the protease secreted by the thermophilic *Bacillus* sp. Hu1 are summarized in Table 1. The results showed that, the enzyme was purified 1.9-fold with a specific activity of 20.6 U/mg protein after ammonium sulfate fractionation. The enzyme was then purified with DEAE-sepharose fast flow and showed 4.4-fold enzyme purification with a specific activity of 49.2 U/mg protein. The final purification step presented 5.6-fold enzyme purification with a specific activity of 62.4 U/mg protein. These results indicated that the the effectiveness of the purification method applied in this research and the protease was purified to 5.6-fold with a 15% yields (Table 1).

Effect of pH and temperature on chitinase

To determine the optimum temperature of the chitinase, enzyme reaction was performed at various temperatures (35 to 80°C) in 50 mM pH 7.2 Tris-HC for 45 min, using colloidal chitin as substrate as shown in Figure 4. The chitinase activity was active at 55 to 70°C but low active in the ranges of 35 to 50°C and 75 to 85°C, respectively. The optimum temperature of chitinase was 60°C. When the reaction temperature reached higher than 70°C, there was a sharp decrease and only 42% of the maximum activity at 80°C was observed (Figure 4). Similar studies were described for other thermophilic chitinase. For example, the optimum temperature of chitinase from *Microbispora* sp. V2 was 60°C (Nawani et al., 2002), and from *Aeromonas* sp. DYU-too7 (Lien et al., 2007), *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997), *Cellulomonas flavigena* NTOU 1 (Chen et al., 1997) and *Bacillus* sp. BG-11 was 50°C (Bhushan and Hoondal, 1998).

For determination of the thermostability of the purified chitinase, enzyme solution was incubated for 20 to 120 min at 50 to 70°C or 10 to 50 min at 80 to 90°C. The pH was kept at 7.2. The residual chitinase activity was

Table 1. Purification of thermal chitinase from thermophilic *Bacillus sp.* Hu1.

Parameter	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude enzyme	78.8	876	11.1	100	1
Ammonium sulfate (80%)	30.6	631	20.6	72	1.9
DEAE-sepharose fast flow	6.4	315	49.2	36	4.4
Sephacryl S-400 high resolution	2.1	131	62.4	15	5.6

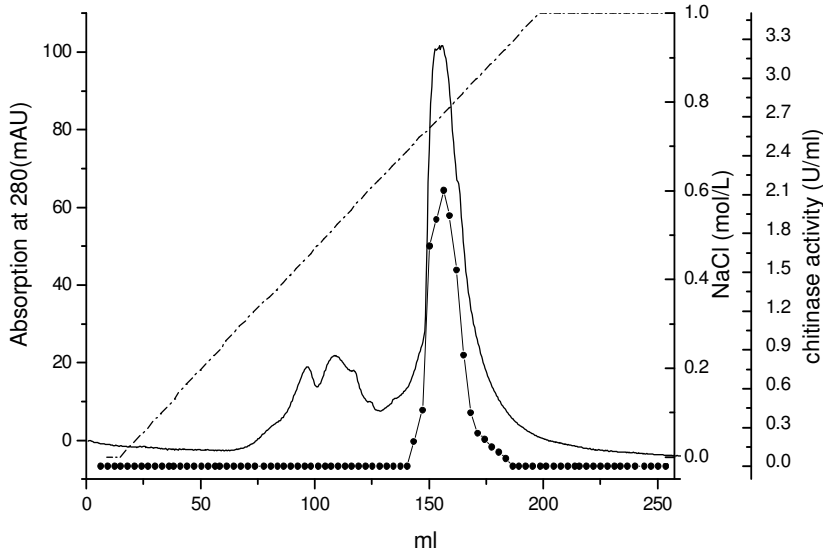

Figure 1. Chromatograph of the thermophilic chitinase by 1.6×45 cm column with DEAE-Sepharose fast flow at 0.4 ml/min. 2 ml fractions were collected and analyzed for chitinase activity. ●, Chitinase activity; (---), NaCl gradient; (–), protein concentration with UV280.

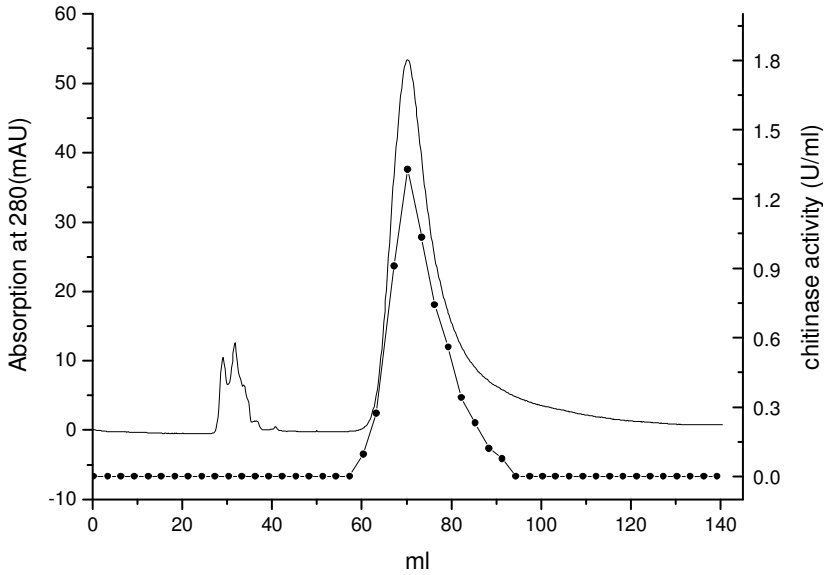

Figure 2. Chromatograph of protease by 1.6 × 60 cm column with Sephcryl S-400 high resolution at 0.4 ml/min. 3 ml fractions were collected and analyzed for chitinase activity. (●), Chitinase activity; (–), protein concentration with UV280.

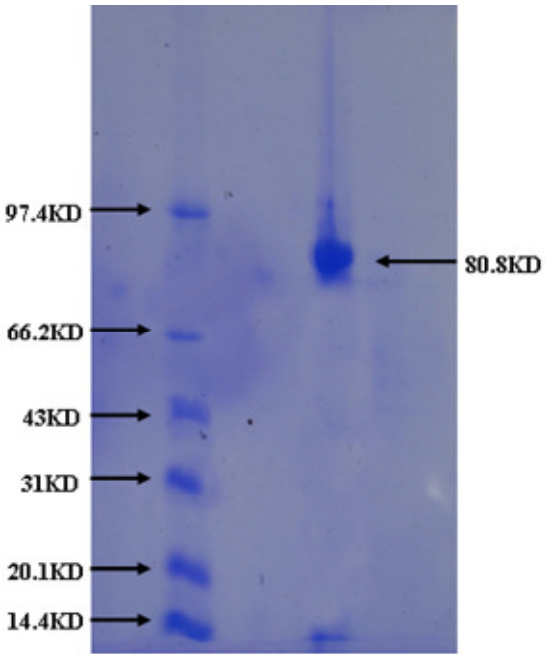

Figure 3. SDS-PAGE chromatograph of the thermophilic neutral chitinase. Marker: standard molecular protein; chitinase, active fractions eluted from the Sephcryl S-400 high resolution column.

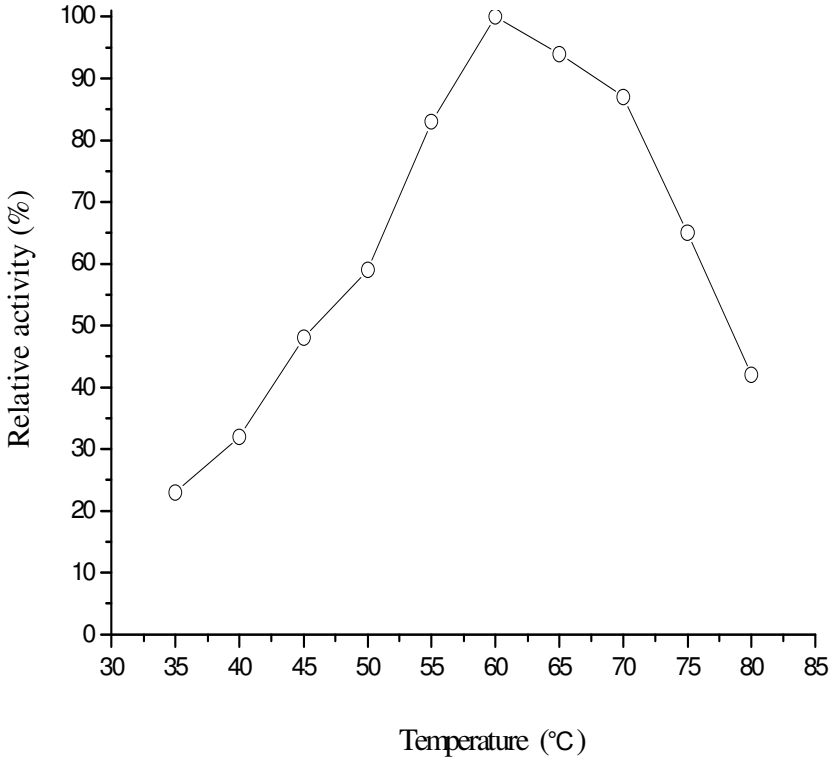

Figure 4. Effects of temperature on the chitinase activity. The activity assay was carried out at different temperatures using 1% (w/v) colloidal chitin (dissolved in 50 mM pH 7.2 Tris-HCl buffer) as substrate.

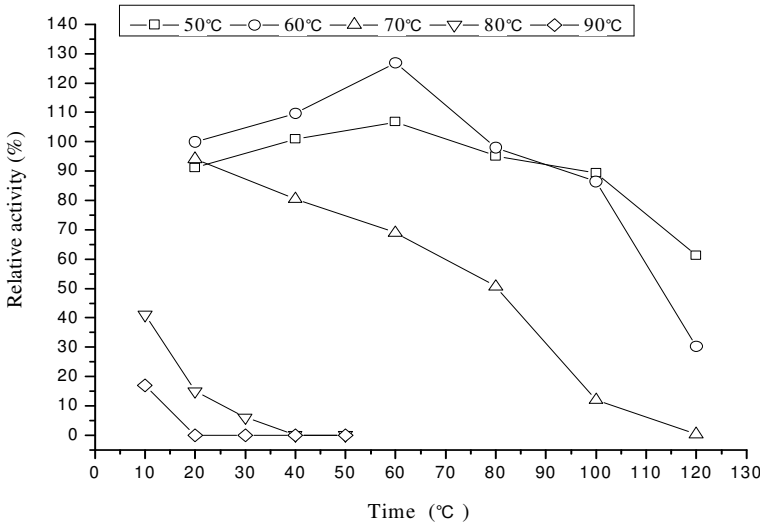

Figure 5. Effects of temperature on the stability of chitinase activities. The chitinase was pre-incubated in 50 mM pH 7.2 Tris-HCl buffer for different times at a certain temperature, then the remaining activity of the pre-incubated chitinase was assayed at 60℃ using 1%(w/v) colloidal chitin (dissolved in 50 mM pH 7.2 Tris-HCl buffer) as substrate.

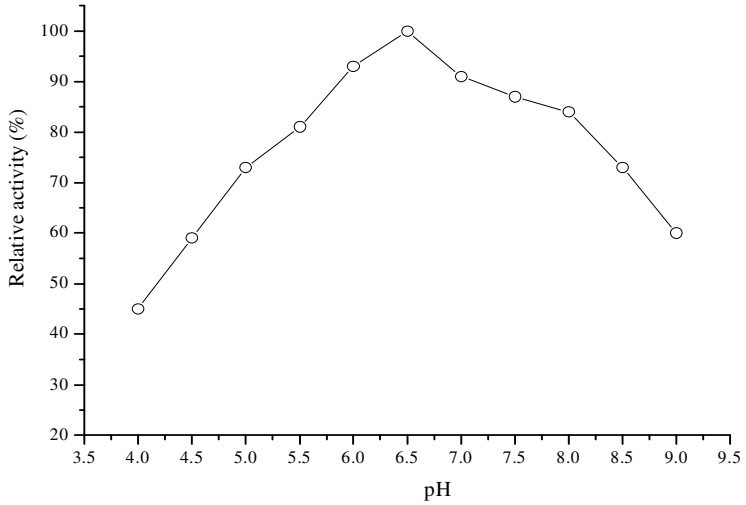

Figure 6. Effects of pH on the chitinase activity. The activity assay was carried out at 60℃ using 1% (w/v) colloidal chitin (dissolved in 50 mM different pH buffer) as substrate.

the residual relative activity of more than 89% was found within 100 min at 50 to 60℃ whereas, complete inactivation was observed when the enzyme was incubated at 70℃ for 120 min, 80℃ for 40 min and 90℃ for 20 min. So, the chitinase can be classified as thermophilic chitinase. It was interesting to know that, the thermostable enzyme from thermophilic *Bacillus* sp. Hu1 was increased about 1.31 times than that of the control (before heating) when the enzyme solution was heated at 60℃ for 60 min whereas, other heat stable chitinases activity such as a novel thermostable chitinase from *Thermomyces lanuginosus* SY2 (GUO et al., 2008), a thermostable chitinase from *Planococcus rifitoensis* strain M2-26 (Essghaier et al., 2009), were decreased at high temperature for a long time. The only research (Purwani et al., 2004) in the currently available reports also found nearly the same results: a relative activity of chitinase enzyme produced by *Bacillus* sp.13.26 were reported to be 300% after incubation of the enzyme at 65℃ for 3 h.

Studies of the influence of pH on the enzyme activity were also carried out and the results are shown in Figure 6. The residual relative activity of more than 80%

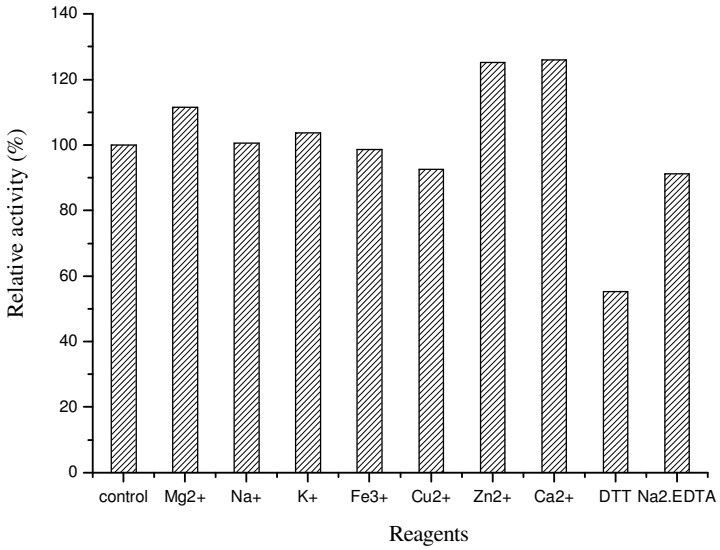

Figure 7. Effect of some reagents on the chitinase activity. The activity assay was carried out at 60℃ using 1% (w/v) colloidal chitin (dissolved in 50 mM pH 6.5 sodium phosphate buffer) as substrate.

Table 2. Chitinase activities on various polysaccharide substrates.

Substrate	Relative activity (%)
Colloid chitin	100
Powder chitin	49.8
Chitosan (80% degree of deacetylation)	3.2
Chitosan(95% degree of deacetylation)	0
Carboxymethyl cellulose	0
Cellulose	0
Soluble starch	0

was found at pH range from 5.5 to 8.0. However, the relative activity was observed to be less than 50% at pH 4.0. The optimum pH for the enzyme activity was found to be 6.5 (Figure 6), which suggested that the chitinase was a nearly neutral enzyme. Similar optimum pH was reported for other chitinase, such as *Enterobacter* SP. G-1 (Park et al., 1997) and *P. aeruginosa* K-187 (Wang and Chang, 1997) whereas, the chitinase from *T. lanuginosus* SY2 (Guo et al., 2008) had the highest activity at pH 4.5, *Aeromonas* sp. DYU-too7 (Lien et al., 2007) and Bacterium *Ralstonia* sp. A-471 (Mitsuhiro et al., 2005) at pH 5.0, *Microbispora* sp. V2 (Nawani et al., 2002*)* at pH 3.0, *Bacillus cereus* at pH 5.8 (Wang et al., 2001), *Bacillus circulans* No.4.1 at pH 8.0 and *Beauveria bassiana* at pH 9.2 (Suresh and Chandrasekaran, 1999). Therefore, the chitinase from thermophilic *Bacillus* sp. Hu1 would be very useful for industrial applications performed at nearly neutral conditions, such as production of the chitin oligosaccharide for medical purposes (Purwani et al., 2004).

Effects of metal ions and other reagents on chitinase

The effects of various metal ions and other reagents on the chitinase activity are showed in Figure 7. It was found that 4.0 mM Cu^{2+}, DTT and Na_2.EDTA only gave a relative activity of 92.5, 55.3 and 91.1%, respectively. However, the enzyme activity of chitinase was significantly increased with the addition of 4.0 mM of Mg^{2+}, Ca^{2+}, Zn^{2+}, where the relative activity was recorded to be 111.5, 126 and 125.2%, respectively. Other reagents such as Na^{+}, K^{+}, and Fe^{3+} did not show significant inhibition or activation effects on the chitinase. Report on the effect of metal ions on chitinase is quite diverse. The chitinase from *Bacillus* sp. DAU101 (Lee et al., 2007) and Indonesian *Bacillus* K29-14 (Sri et al., 2004) were increased by Co^{2+}, while the chitinases from bacterium C4 (Yong et al., 2005) and *Bacillus* MH-1 (Sakai et al., 1998) were activated by Mn^{2+}. Generally, most chitinases were inhibited by Zn^{2+} and Cu^{2+} ion. (Konagaya et al., 2006; Lee et al., 2006; Guo et al., 2005; Sri et al., 2004; Rajiv et al., 2003). It was believed that, the thermostable chitinase from the thermophilic *Bacillus* sp. Hu1 appeared to be another different type of chitinase, since it was only slightly inhibited by Cu^{2+} ion and increased significantly by Mg^{2+}, Ca^{2+} and Zn^{2+}.

Substrate specificity

The ability to hydrolyze several carbohydrates substrates is an important criterion of chitinase potency. Table 2 com-

pares the digestive capability of this chitinase on seven substrates. It is evident that the chitinase had better digestive ability on colloid chitin than other carbohydrates under the same assay condition. The relative activity of the chitinase only reached 49.8% for powered chitinase (crystalline chitin) and 3.9% for chitosan (80% degree of deacetylation) substrate of that when colloid chitin was used as the control. However, there was no hydrolysis activity when chitosan (95% degree of deacetylation), carboxymethyl cellulose, cellulose and soluble starch used as substrates. It is therefore reasonable to conclude that, the chitinase of this study have high specificity which could only hydrolyze glycosidic bond between GlcNAc-GlcNAc.

Conclusion

A new thermostable chitinase of 80.8 kDa from thermophilic *Bacillus* sp. Hu1 was purified and then, the enzymology of the chitinase was studied. The optimal pH and optimal temperature of the protease were at pH 6.5 and 60°C, respectively. The chitinase was found stable during the 100 min incubation at 60°C. The chitinase was inhibited by Cu^{2+}, DTT and Na_2.EDTA and was activated by Mg^{2+}, Ca^{2+} and Zn^{2+}, whereas Na^{+}, K^{+} and Fe^{3+} did not show significant inhibitory effects against the chitinase. Substrates specificity tests indicated that, colloid chitin was the best substrate among the seven substrates tested (colloid chitin, particle chitinase, chitosan (80% degree of deacetylation) chitosan (95% degree of deacetylation), carboxymethyl cellulose, cellulose and Soluble starch). It can be concluded that the chitinase have high specificity for hydrolyzing glycosidic bond between GlcNAc-GlcNAc.

ACKNOWLEDGEMENT

This work was supported by Zhejiang Provincial Notional Science Foundation of China. The project number was Y 3080108.

REFERENCES

Barnard O, Gé JV (1994). Human serum contains a chitinase: identification of an enzyme, formerly described as 4-methylumberyferyl-tetra-N-acetylchitotorioside hydrolase (MU-TACT hydrolase). Glycobiology, 4:797–803.

Bartnicki GS, Nickerson WJ (1962). Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. Biochim. Biophys.Acta. 58:102-119.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein dye binding. Anal. Biochem. 72:248-254.

Chen HC, Hsu MF, Jiang ST(1997). Purification and characterization of an exo-N,N′-diacetylchitobiohydrolase-like enzyme from *Cellulomonas flavigena* NTOU 1. Enzyme Microb. Technol. 20: 191–197.

Essghaier B, Rouaissi M, Boudabous A, Jijakli H, Najla SZ (2009). Production and partial characterization of chitinase from a halotolerant *Planococcus rifitoensis* strain M2-26. World J. Microbiol. Biotechnol. 26:977-984.

Gomes RC, Semedo LT, Soares RM, Soares RMA, Linhares LF, Ulhoa CJ, Alviano CS, Coelho RRR (2001). Purification of a thermostable endochitinase from Streptomyces RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. J. Appl. Microbiol. 90:653-661.

Gomes VM, Oliveira AEA, Xavier FJ (1996). A chitinase and a β-1,3-glucanase isolated from the seeds of cowpea (*Vigna unguiculata* Walp) inhibit growth of fungi and insect pests of the seed. J. Sci. Food Agric.72: 86-90.

Gomes J, Steiner W (2004). The biocatalytic potential of extremophiles and extremozymes: Food Technol. Biotechnol. 42:223-235.

Guo RF, Li DC, Wang R (2005). Purification and properties of a thermostable chitinase from thermophilic fungus *Thermomyces lanuginosus*. Acta Microbiol. Sin. 45:270-274.

Guo RF, Shi BS, Li DC, Ma W,Wei Q(2008). Purification and characterization of a novel thermostable chitinase from Thermomyces lanuginosus SY2 and cloning of its encoding Gene. Agric. Sci. in China. 7: 1458-1465.

Haki GD, Rakshit SK (2003). Developments in industrially important thermostable enzymes: a review. Bioresour. Technol. 89:17-34.

Bhushan B, Hoondal GS (1998). Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. Biotechnol. Lett. 20: 157-159.

Karl JK, Subbaratnam M (1998). Insect chitinases: Molecular biology and potential use as biopesticides. Insect Biochem. Mol. Biol. 27:887-900

Konagaya Y, Tsuchiya C, Sugita H (2006). Purification and characterization of chitinases from Clostridium sp. E-16 isolated from the intestinal tract of the South American sea lion (*Otaria flavescens*). Lett. Appl. Microbiol. 43: 187-193.

Kudan S, Pichyangkura R (2009). Purification and characterization of thermostable chitinase from *bacillus licheniformis* SK-1. Appl. Microbiol. Biotechnol. 157: 23-35.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

Leah R, Tommerup H, Svendsen I, Mundy J (1991). Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266:1564-1573.

Lee YG, Chung KC, Wi SG, Lee JC, Bae HJ (2009). Purification and properties of a chitinase from *Penicillium* sp. LYG 0704. Protein Expr. Purif. 65: 244-250.

Lien TS, Yu ST, Wu ST, Too JR (2007). Induction and purification of a thermophilic chitinase produced by Aeromonas sp. DYU-Too7 using glucosamine. Biotechnol. Bioproc. Eng. 12:610-617.

Liu CL, Shen CR, Hsu FF, Chen JK, Wu PT, Guo SH, Lee WH, Yu FW, Mackey ZB, Turk J, Gross ML (2009). Isolation and identification of two novel SDS-Resistant secreted chitinases from *Aeromonas schubertii*. Biotechnol. Progr. 25:124-131

Mana I, Kouji M, Atsushi Mi, Masahiro M (2009). Purification and characterization of chitinase from the stomach of silver croaker *Pennahia argentatus*. Protein Expression Purif. 65:214-222.

Mario P-R, Everardo L-R (1991). Detection of Nine Chitinase Species in Germinating Cells of *Mucor rouxii*. Curr. Microbiol. 22: 43-46.

Matsumiya M, Miyauchi K, Mochizuki A (1998). Distribution of chitinase and β-N-acetylhexosaminidase in the organs of a few squid and a cuttlefish. Fisheries Sci. 64:166–167.

Matsumiya M, Miyauchi K, Mochizuki A (2002). Characterization of 38 kDa and 42 kDa chitinase isozymes from the liver of Japanese squid *Todarodes pacificus*. Fisheries Sci. 68:603-609.

Mitsuhiro U, Yukiko K, Aji S, Masami N, Kazutaka M (2005). Purification and Characterization of Chitinase B from Moderately *Thermophilic Bacterium Ralstonia* sp. A-471. Biosci. Biotechnol. Biochem. 69:842-844.

Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K (1997). Cloning, sequencing, and expression of the gene encoding Clostridium paraputrificum chitinase ChiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain. J. Bacteriol.179: 7306-7314.

Natasa J, Barbara K, Helena L, Katja B (2006). Purification and

molecular characterization of chitin deacetylase from *Rhizopus nigricans*. Enzyme Microb. Technol. 39:1294-1299.
Nawani NN, Kapadnis BP (2005). Optimization of chitinase production using statistics based experimental designs. Process Biochem. 40: 651-660.
Nawani NN, Kapadnis BP, Das AD, Rao AS, Mahajan SK (2002). Purification and characterization of a thermophilic and acidophilic chitinase from *Microbispora* sp. V2. J. Appl. Microbiol. 93:965-975.
Park HJ, Kim D, Kim IH, Lee CE, Kim IC, Kim JY, Kim SJ, Lee HK, Yim JH (2009). Characteristics of cold-adaptive endochitinase from Antarctic bacterium *Sanguibacter antarcticus* KOPRI 21702. Enzyme Microb. Technol. 45: 391-396.
Purwani EY, Maggy TS, Yaya R, Jae KH, Yu RP (2004). Characteristics of thermostable chitinase enzymes from the indonesian *Bacillus* sp.13.26. Enzyme Microbial. Technol. 35: 147-153.
Rajiv Vaidya, Satarupa Roy, Simone Macmil (2003) The reference in the main work does not correspond. Purification and characterization of chitinase from *Alcaligenes xylosoxydans*. Biotechnol. Lett. 25: 0141-5492.
Reetarani SP, Vandana G, Mukund VD (2000). Chitinolytic enzymes: an exploration. Enzyme Microbial. Technol. 26:473–483.
Riccardo AAM (1996). Chitosan-based dietary foods. Carbohydr. Polym. 29: 309-316.
Sahai AS, Manocha MS (1993). Chitinases of fungi and plants: their involvement in morphogenesis and host–parasite interaction, FEMS Microbiol. Rev. 11:317-338.
Sakai K, Yakota A, Kurokawa H, Mamoru W, Mitsuaki M, Moriguchi M (1998). Purification and characterization of three thermostable endochitinases of *Bacillus* noble strain MH-1 isolated from chitin containing compost. Appl. Environ. Microbiol. 64:3340-3397.
Sherief AA, El-SAawah MMA, Mohamed AA (1991). Some properties of chitinase produced by a potent *Aspergillus carneus* strain. Appl. Microbiol. Biotechnol. 35: 228-230.
Sri R, Tanuwidjaja F, Rukayadi Y, Suwanto A, Thenawidjaja SM, Jae KH, Yu RP (2004). Study of thermostable chitinase enzymes from *Indonesian Bacillus* K29-14. J. Microbiol. Biotechnol. 14 :647-652.
Suresh PV, Chandrasekaran M (1999). Impact of process parameters on chitinase production by an alkalophilic marine *Beauveria bassiana* in solid state fermentation. Process Biochem. 34:257-267.
Vaidya R, Roy S, Macmil S, Gandhi S, Vyas P, Chhatpar HS (2003). Purification and characterization of chitinase from Alcaligenes xylosoxydans. Biotechnol Lett. 25:715-717.
Wang SL, Chang WT (1997). Purification and characterization of two bifunctional chitinase/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. Appl. Environ. Microbiol. 63(2): 380-386.
Wang SL, Chao CH, Liang TW, Chen CC (2009). Purification and Characterization of Protease and Chitinase from *Bacillus cereus* TKU006 and conversion of marine wastes by these enzymes. Mar. Biotechnol. 11: 334–344.
Wang SY, Moyne AL, Thottappilly G, Wu SJ, Locy RD, Singh NK (2001). Purification and characterization of a *Bacillus cereus* exochitinase. Enzyme Microb. Technol. 28: 492-498.
Wu ML, Chuang YC, Chen JP (2001). Identification and characterization of the three chitin-binding domains within the multidomain chitinase Chi92 from *Aeromonas hydrophila* JP101. Appl. Environ. Microbiol. 67: 5100-5106.