Annals of Microbiology, **57 (2)** 249-257 (2007)

Biochemical characterisation of two forms of halo- and thermo-tolerant chitinase C of *Salinivibrio costicola* expressed in *Escherichia coli*

Ratchaneewan AUNPAD[1]*, David. W. RICE[2], Svetalana SEDELNIKOVA[2], Watanalai PANBANGRED[3]*

[1]Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Rangsit Campus, Pathumthani 12121, Thailand, [2]Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, England, [3]Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

Received 12 February 2007 / Accepted 30 April 2007

Abstract - Two forms of chitinase C (Chi-I and Chi-II) were purified until homogeneity from the culture supernatant of a transformant *Escherichia coli* harbouring *chitinase C* gene from the halophilic bacterium *Salinivibrio costicola* strain 5SM-1. Chi-II was derived from Chi-I by C-terminal processing. Chi-I and Chi-II showed similar salinity optimum at 1-2% NaCl and retained more than 80% of their activity at 3-5% NaCl and more than 50% residual activity at 14% NaCl. The two enzymes could also well function (activity > 95%) in the absence of NaCl. Both had highest activity at pH 7.0 and 50 ºC and both were stable over a wide range of pH (3.0-10.0). More than 50% activity remained at 80 ºC after 60 min treatment. Among 4 major cations contained in sea water, only Mg^{2+} at 10 mM increased activity about 10%. Using *p*-nitrophenyl-*N,N'*-diacetylchitobiose as substrate, Chi-I and Chi-II had K_m of 30 and 31.8 µM and V_{max} of 10 and 9.2 µmol/h/mg protein, respectively. Chi-I and Chi-II were classified as exochitinases by product analysis of the *E. coli* culture supernatant with high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC).

Key words: chitinase C, C-terminal processing, halo-tolerant, HPLC, *Salinivibrio costicola*, thermo-tolerant, TLC.

INTRODUCTION

Chitinases (EC 3.2.1.14) cleave the polymer chitin, a major component of insect exoskeletons, shells of crustaceans and fungal cell walls, to small oligosaccharides, mainly chitobiose $(GlcNAc)_2$. Dimers are further hydrolysed to the monomer of *N*-acetylglucosamine (GlcNAc) by chitobiase or β-*N*-acetylglucosaminidase (EC 3.2.1.52) (Monreal and Reese, 1969). Based on catalytic mechanism, chitinases are classified into three groups consisting of endochitinases, exochitinases or chitobiosidases and β-*N*-acetylhexosaminidases or chitobiases (Kupiec and Chet, 1998). The combined action of endochitinase and exochitinase results in the degradation of an insoluble chitin to mainly soluble, chitobioses, which are further hydrolysed to GlcNAc. The degradation products, mainly *N*-acetylglucosamine, are then taken up by the cell as carbon and nitrogen sources (Tsujibo *et al*., 2002). These enzymes have been found in a variety of organisms such as bacteria (Monreal and Reese, 1969; Suginta *et al*., 2000; Tsujibo *et al*., 2002) and plants (Collinge *et al*., 1993) that do not contain chitin. Chitin is particularly important in aquatic ecosystems and marine chitinolytic bacteria play a crucial role in its recycling (Yu *et al*., 1991). In view of the commercial significance of chitin and its derivatives, there is a need for inexpensive and active chitinases that may be used to convert shellfish waste into useful molecules in biotechnology and medicine (Suginta *et al*., 2000).

Salinivibrio costicola strain 5SM-1 is a moderately halophilic bacterium that was isolated from saline mud in Thailand (Aunpad and Panbangred, 2003). *Salinivibrio costicola* was originally isolated and described in 1938 from salted food (Mellado *et al*., 1996). Based on 16S rRNA gene sequences, strains of *S. costicola* can be differentiated from members of the genus *Vibrio* on the basis of two unique helical sequences and secondary structures between position 178 to 197 and 197 to 219 of the rRNA (Mellado *et al*., 1996).

A number of chitinases from various bacterial organisms have been purified and characterised (Okazaki *et al*., 1995; Lin *et al*., 1997; Suginta *et al*., 2000; Bendt *et al*., 2001), although relatively little is known about chitinases from halophilic bacteria. Two isoforms of chitinase from *Streptomyces* had identical N-terminal amino acid sequences and enzymatic properties but different isoelectric points (Okazaki *et al*., 1995). ChiA from *Vibrio* sp. strain Fi:7 has been cloned and overexpressed in *Escherichia coli* BL21 and two forms of ChiA with molecular masses of 80- and 82-kDa with the same N-terminal amino acid sequence were

* Corresponding author. Phone: +66 2 201-5927;
Fax: +66 2 201-5926; E-mail: scwpb@mahidol.ac.th

accumulated in the soluble fraction of the *E. coli* extract (Bendt *et al*., 2001). In this study, chitinase C (Chi-I) and its processed form (Chi-II) from recombinant *E. coli* harbouring a chitinase gene from *S. costicola* were purified until homogeneity, characterised and compared. Both forms of the enzyme tolerated high temperature and a wide salinity range. It could efficiently hydrolyse chito-oligosaccharides and crude crab chitin. These two forms of chitinases exhibit both chitobiosidase and a weak β-*N*-acetylglucosaminidase functions. Hence, chitinase from *S. costicola* is of pivotal use in a chitin digestion and variety of applications particularly in the treatment of shellfish wastes and recycles of chitin in the marine environment.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* DH5a (pCHISI) harbours a chitinase C (ChiC) gene (Genbank accession number AF261749) from the marine bacterium, *Salinivibrio costicola* strains 5SM-1 (Aunpad and Panbangred, 2003). Recombinant (transfromant) *E. coli* were cultured in Luria-Bertani-medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) containing 50 µg/ml ampicillin at 37 ºC under shaking at 200 rpm (New Brunswick Scientific, NJ, USA).

Enzyme assay. Colorimetric measurement of chitinase was based on the determination of the amount of *N*-acetylglucosamine or its equivalent released as a product of enzymatic reaction according to the method of Reissig *et al*. (1955). The assay mixture consisted of 200 µl enzyme extract (i.e. the culture supernatant) in 50 mM phosphate buffer pH 7.0 and an equal volume of 5% colloidal chitin as substrate suspended in the same buffer. This mixture was incubated at 37 ºC for 30 min, and then was centrifuged (Eppendorf, Germany) at 10000 *x g* at 4 ºC for 10 min. The supernatant (200 µl) was removed and mixed with 40 µl of 0.8 M potassium tetraborate pH 9.1 and boiled for 3 min followed by immediate cooling. A solution of 1% p-dimethylamino-benzaldehyde (1.2 ml) was added and the obtained mixture was further incubated at 37 ºC for 30 min. The purple colour that developed was measured with a spectrophotometer at 585 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmole of GlcNAc equivalent from the substrate in 1 minute under the above conditions. All chitinase activity determinations were carried out in three replicates. The enzyme concentration and specific activity was reported in mg/ml and µmol/h/mg protein, respectively. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin.

Enzyme purification. Chitinase C from *E. coli* harbouring pCHISI was actively secreted into the supernatant. After 24 h of cultivation in Luria-Bertani medium, the culture supernatant (1.5 l) containing chitinase C was collected after centrifugation (Sorvall, USA) at 6000 *x g* for 20 min. Ammonium sulphate was added into the culture supernatants to obtain 65% final concentration and then kept at 4 ºC for overnight. The precipitate was collected by centrifugation at 10000 *x g* for 20 min, dissolved in 15 ml of 25 mM Tris-HCl buffer pH 8.0 (buffer A) and dialysed against the same buffer (dialysis tubing, M.W. cut off at 12000) at 4 ºC for 24 h with three buffer changes. The dialysate was adjusted with Tris-HCl buffer to give a final volume of 18 ml and then recentrifuged (Sorvall) at 10000 *x g* for 20 min to remove insoluble and denatured proteins. The dialysed enzyme solution was loaded onto a DEAE-Sepharose prepacked in an HR column 10/20 (Amersham Pharmacia Biotech, Sweden) equilibrated with buffer A. The enzyme was eluted with a linear gradient of sodium chloride from 0.075 to 0.5 M NaCl in buffer A. The active fraction eluted at 0.35 M NaCl, was loaded onto a hydrophobic interaction column of Butyl-Toyopearl 650 S (Amersham Pharmacia Biotech), previously equilibrated with 2 M potassium chloride in Buffer A. The enzyme was eluted with a step-down linear gradient of potassium chloride from 2 M to 0 M KCl. The two active peaks showing enzymatic activity eluted at 0.4 M and 0.8 M KCl, respectively, (Chitinase I, Chi-I and chitinase II, Chi-II) were collected and pooled. These 2 active fractions of Chi-I and Chi-II (4 ml each) were concentrated to 100 µl using 20 ml Vivaspin concentrators (Vivascience, Germany) and individually loaded onto gel filtration columns of Superdex 200 HR 10/30 (Amersham Pharmacia Biotech) with 24 ml bed volume equilibrated with 50 mM phosphate buffer pH 7.0. Active fractions were collected, pooled and stored at 4 ºC.

Gel electrophoresis and N-terminal amino acid sequencing. Protein samples from each purification step were run on 10% polyacrylamide gel electrophoresis following the method of Laemmli (1970). Protein bands were visualised by staining with 0.5% final concentration of Coomassie Brilliant Blue R250. The N-terminal amino sequences of recombinant Chi-I and Chi-II electroblotted onto a polyvinylidene difluoride (PVDF) membrane were analysed with protein sequencer (Applied Biosystems protein sequencer model 476A) using a standardised protocol by Bio-technology Service Unit, NSTDA, Ministry of Science and Technology, Thailand.

Optimum pH and pH stability. Optimum pH of Chi-I and Chi-II was determined in a wide range of pH, varying from 3 to 10 using 50 mM glycine HCl (pH 3), 50 mM acetate buffer (pH 4-5), 50 mM sodium phosphate buffer (pH 6-7), 50 mM Tris-HCl pH (8-9) and 50 mM glycine-NaOH (pH 10). The enzyme and colloidal chitin substrate were diluted or suspended in each buffer, mixed and assayed as described above. For determination of pH stability, the enzyme was kept at various pH (3-10) at 4 ºC for 60 min. After incubation, the pH of the enzyme solution was adjusted to pH 7.0 by diluting with 50 mM phosphate buffer pH 7.0 and the residual activity was determined.

Optimum temperature and thermostability. To measure optimum temperature, colloidal chitin (5%) in 50 mM phosphate buffer pH 7.0 (200 µl) was preincubated at various temperatures (10-80 ºC) for 10 min before appropriately diluted enzyme (about 10 mU in 200 µl) solution was added and further incubated at different temperatures for 30 min. To measure thermostability, enzyme solutions in 50 mM phosphate buffer pH 7.0 were incubated at various temperatures (10-80 ºC) for 60 min. After incubation, solutions were immediately cooled on ice before residual activity was determined by incubation with 5% colloidal chitin at 37 ºC for 30 min.

TABLE 1 - Purification of Chi-I and Chi-II of *Salinivibrio costicola* in recombinant *Escherichia coli*

Purification step	Total volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification fold
Culture supernatant	1500	690	7000	10.0	100	1.0
65% saturated ammonium sulphate	18	198	4860	24.5	69.4	2.5
DEAE-Sepharose	6.5	71.5	2242	31.4	32.0	3.1
Butyl-Toyopearl 650S Chi-I	4.0	22,6	1013.6	44.9	14.5	4.5
Butyl-Toyopearl 650S Chi-II	4.0	19.7	1065.6	54.1	15.2	5.4
Superdex 200 HR Chi-I	5.0	9.7	820.0	84.5	11.7	8.5
Superdex 200 HR Chi-II	5.0	10.2	936.0	91.8	13.4	9.1

Effect of NaCl, metal ions and other reagents on chitinase activity. The effect of NaCl on chitinase-activity was evaluated with 1-14% NaCl (final concentration) in the assay solution. Equal amounts of appropriately diluted enzyme were incubated with 5% (w/v) colloidal chitin in the presence of either metal ions (1 or 10 mM final concentration) or chemical reagents (1, 10, 20 and 50%). Chitinase activity was then measured by the standard method.

Kinetic parameters of recombinant chitinase. Kinetic constants (K_m and V_{max}) were determined using *p*-nitrophenyl-*N,N'*-diacetylchitobiose (Sigma Aldrich, USA) as a substrate with concentration varying from 5 to 30 µM. Velocities were calculated from the slope of the absorbance-time curves at OD_{410} over 40 min using the kinetics measurement mode. K_m and V_{max} were determined from the Lineweaver-Burk (linear transformation of Michaelis-Menten equation).

Chito-oligomers digestion by Chi-I and Chi-II and product analysis by HPLC. The end products of chitin and chito-oligomer hydrolysis by Chi-I and Chi-II were analysed by High Performance Liquid Chromatography (HPLC). Enzyme solution (100 µl containing 30 mU of activity) was incubated with 2% (w/v) of colloidal chitin or 8 mM of each of the chito-oligomers from $(GlcNAc)_2$ to $(GlcNAc)_6$ (Sigma Aldrich) in the reaction mixture at 37 °C for 30 min. After centrifugation (Eppendorf) at 12000 *x g* for 15 min, the supernatant containing sugar products was analysed by HPLC using a Hypersil Model APS 25U aminopropyl column (Hypersil, England) with the dimensions of 250 x 4.6 mm using acetonitrile:water (70:30 by volume) as the mobile phase with a flow rate of 1 ml/min at 40 °C. The changes in refractive index (RI) were detected by refractoMonitor@IV (LDC analytical, USA).

Crude chitin digestion by Chi-I and Chi-II and products analysis by thin layer chromatography (TLC). Crude chitin substrates were prepared by suspending either of the two types of crude chitin [a coarse flake and powdered chitin from crab shell (Sigma Aldrich)] in 50 mM phosphate buffer pH 7.0 to give a final concentration of 2% (w/v) and the solution was then autoclaved at 121 °C for 15 min. The reaction mixture comprised an equal volume of each of the purified enzyme (containing 30 mU of activity) and 2% (w/v) suspension of the coarse flake or the powdered chitin. Then the mixture was incubated at 37 °C for 30 min to 48 h. The supernatant was assayed as described above after centrifugation at 12000 *x g* for 10 min. Crude chitin hydrolytic products were determined by thin layer chromatography using 10 µl aliquots of the hydrolysis mixture removed at 0, 0.5, 1, 2, 3, 4, 5, 6, 24 and 48 h. These were analysed on silica gel 60 (Merck, Germany) plates using butanol:glacial acetic acid:water (12:3:5 by volume) (Suginta *et al*., 2000) as the solvent. The TLC plate was run twice and the products were visualised by spraying with freshly prepared 20% H_2SO_4 in ethanol, followed by heating at 150 °C for 10 min. Standards comprised a mixture of 0.5% (w/v) each of N-acetylglucosamine (GlcNAc), chitobiose $(GlcNAc)_2$ and chitotriose $(GlcNAc)_3$.

RESULTS AND DISCUSSIONS

Purification of Chi-I and Chi-II

The chitinase C (ChiC) of *S. costicola* is classified in family 18 of glycosyl hydrolases based on the amino acid sequence comparison. ChiC from *E. coli* transformants was secreted into culture supernatant showing the functional signal peptide in both bacteria. The results of the purification are summarised in Table 1. Two forms of chitinase, Chi-I and Chi-II, from *E. coli* transformants harbouring *chiC* were purified to homogeneity. Starting with 1.5-l culture, Chi-I and Chi-

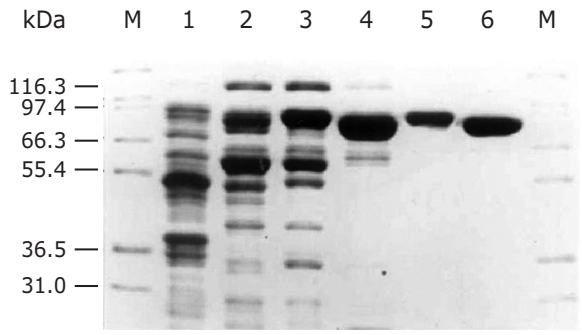

FIG. 1 - SDS-PAGE of purified Chi-I and Chi-II. Lane M, Molecular weight marker, lanes 1-6, crude enzyme, fractions of DEAE-Sepharose, fractions of Chi-I from BTP-Toyopearl 650S, fractions of Chi-II from BTP-Toyopearl 650S, fractions of purified Chi-I from Superdex 200 HR, fractions of purified Chi-II from Superdex 200 HR, respectively. The masses of molecular weight marker proteins (in KiloDaltons, kDa) are indicated on the left margin.

II were purified 8.5-fold with 11.7% yield and 9.1-fold with 13.4% yield, respectively (Table 1). As shown in Fig. 1 lanes 5 and 6, the purified chitinases were detected as a single band by Coomassie Brilliant Blue staining compared to crude protein in other fractions (Fig. 1 lanes 1-4).

ChiC processing in recombinant *Escherichia coli*

By amino acid sequence comparison, it is found that the full length ChiC enzyme structure consists of a 22 amino acid signal sequence, a catalytic domain, a cadherin-like domain and two putative chitin-binding domains (Fig. 2). The molecular mass of full length ChiC calculated from the deduced amino acid sequence was 94,634 Da. The molecular masses of Chi-I and Chi-II were determined to be 92424.00 and 87643.59 Da, respectively, by Mass spectroscopy using Q-Tof, Micromass UK Ltd. (data not shown). Both Chi-I and Chi-II had the same N-terminal amino acid sequence (APSTPSL) indicating that the putative signal peptide cleavage site was between alanine residues 22 and 23 and that processing of the enzyme occurred at the C-terminus in the *E. coli* transformant. The difference in molecular mass between Chi-I and Chi-II is 5,581 Da corresponded to about 38 amino acids, which were cleaved from the C terminal sequence of Chi-I to obtain Chi-II as calculated by Vector NTi program (InforMax Inc., USA). Two units of chitin-binding domains were found at C-terminus with 41.5% sequence homology to each other. The expected cleavage site was putatively between alanine 832 and asparagines 833. Hence, two and a single chitin binding domains were existed in Chi-I and Chi-II, respectively. The role of cadherin-like domain in chitinase remained unclear (Morimoto *et al*., 1997). Cadherins are transmembrane proteins that mediate adhesion between cells of vertebrates and many invertebrates (Boggon *et al*., 2002). Cadherin adhesion is also Ca^{2+} dependent. Cadherin-like domains in ChiC chitinase might act as a linker to maintain optimal distance and orientation between the catalytic and chitin binding domain as proposed in chitinase from *Clostridium paraputrificum* (Morimoto *et al*., 1997). In other *Bacillus* chitinase, other types of cell adhesion proteins called fibronectin-like domain (Fn 3) were found (Watanabe *et al*., 1994, Thamthiankul *et al*., 2004). Fn3 is known for its Ca^{2+} independent cell adhesion (Watanabe *et al*., 1994, Morimoto *et al*., 1997).

Effects of NaCl on chitinase activity

Both forms of chitinase were able to tolerate up to 14% NaCl, retaining more than 50% residual activity (data not shown). Chi-I and Chi-II could function well at 1-2% NaCl with a salinity tolerant range from 1-5% to 1-6%, respectively, over which more than 80% of activity was retained. In the absence of NaCl, both were able to digest colloidal

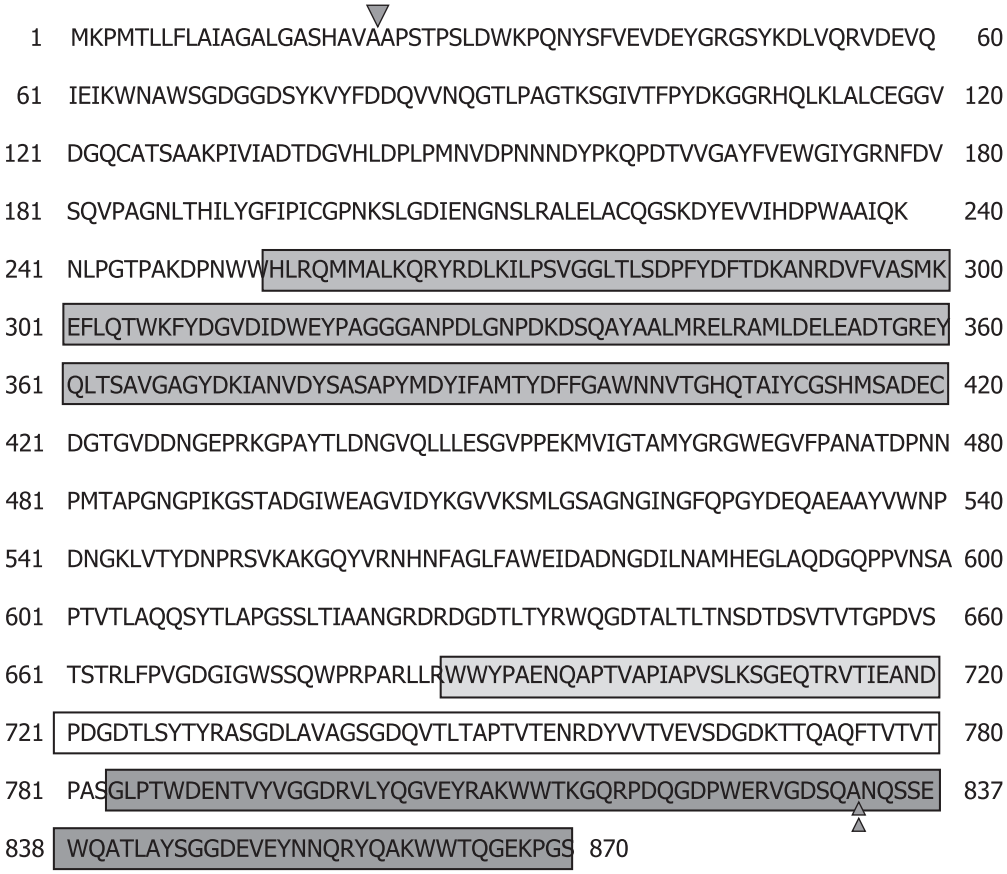

FIG. 2 - Deduced amino acid sequence of ChiC from *Salinivibrio costicola*. Grey, white and black boxes indicate catalytic, cadherin-like and two chitin binding domain. Data from Mass spectrometry analysis showed that Chi-I and Chi-II in *Escherichia coli* transformants have two and a single chitin binding domains, respectively. Vertical arrow indicates cleavage site of signal peptide. Double vertical arrows indicate the putative processing site of Chi-I to Chi-II.

chitin with relative activity more than 90% (data not shown). Similar findings have been reported for chitinases from other halophilic bacteria. The chitinases from marine bacteria *Aeromonas caviae*, *Vibrio parahemolyticus* and *Clostridium* sp. strain E-16, were not only able to function in the absence of NaCl (Zhu *et al*., 1992; Lin *et al*., 1997; Konagaya *et al*., 2006) but also chitinases from the two latter bacteria were also resistant to denaturation due to NaCl at concentrations up to 10% (Zhu *et al*., 1992; Konagaya *et al*., 2006). These enzymes as well as ChiC from *S.costicola* can be regarded as salt tolerant marine chitinase. Thus, given their wide range of salt tolerance, Chi-I and Chi-II might be used not only under low salt conditions but also under reaction conditions which take place at high saline concentrations, for example in the treatment of seafood waste.

Optimum pH and stability

The optimum pH for Chi-I and Chi-II activity with colloidal chitin as substrate was neutral at pH 7.0 (Fig. 3A). Both enzymes showed some acid tolerance with 80% residual activity at pH 6.0 while activities decreased to about 50% at pH 8.0. Most marine bacterial chitinases work better at alkaline pH, around 8.0, which is the normal pH of seawater (Tsujibo *et al*., 1992; Ueda *et al*., 1995; Bendt *et al*., 2001). However, some marine chitinases such as recombinant ChiA from *A. caviae* exhibited maximum activity at pH 6.2-6.5 (Lin *et al*., 1997), which is slightly acidic. Chi-I and Chi-II were stable and retained more than 60% of their original activities from pH 3.0 to 10.0 (Fig. 3A). They showed a broad range of pH stability comparable to that of ChiA from *A. caviae*, which was stable over a pH range of 5.0-7.0 (Lin *et al*., 1997).

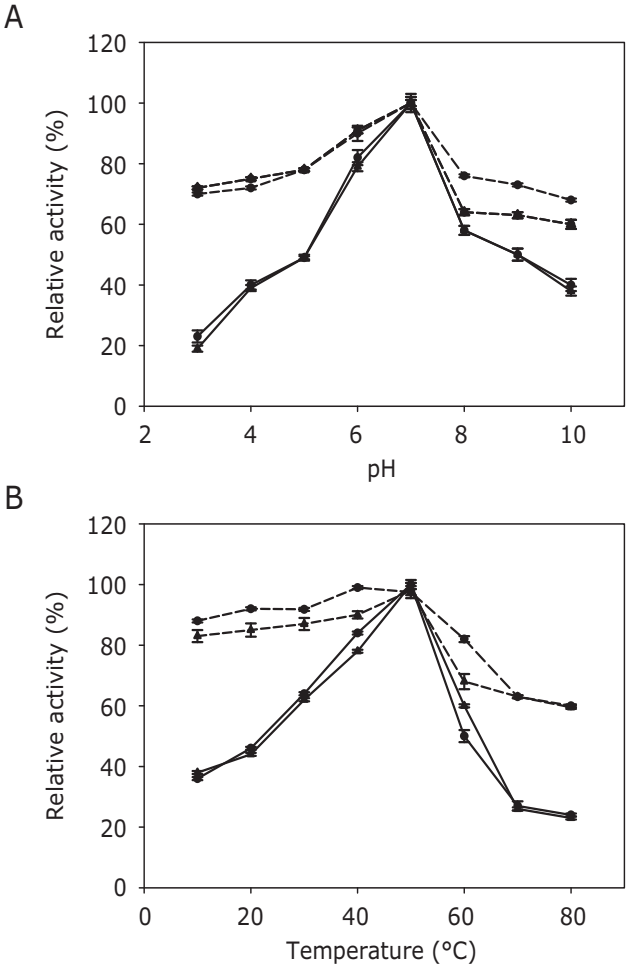

FIG. 3 - Effect of pH and temperature on the activity and stability of purified Chi-I and Chi-II. A: optimum pH (solid line) and pH stability (broken line); B: optimum temperature (solid line) and temperature stability (broken line). Chi-I (●); Chi-II (▲). Bars indicate standard deviation.

Optimum temperature and thermostability

The optimum temperature for the Chi-I and Chi-II activity was 50 ºC (Fig. 3B). This characteristic was similar to chitinases from other marine bacteria such as ChiA from *Alteromonas* (Tsujibo *et al*., 1992), ChiC3 from *Vibrio alginolyticus* (Ohishi *et al*., 1996) and chitinases from *Aeromonas* sp. No. 10S-24 (Ueda *et al*., 1995). When preincubated for 1 h from 10 to 50 ºC, more than 85% of Chi-I and Chi-II activity remained, while 60% activity remained after heating at 80 ºC for 60 min (Fig. 3B). This revealed remarkable thermostability that was almost comparable to that of thermostable chitinases (chitinase II-IV) from *Bacillus licheniformis* X7U, where more than 75% chitinase activity remained after heating (Takayanagi *et al*., 1991), and markedly higher than the thermal stabilities observed from other marine chitinases (Tsujibo *et al*., 1992; Ueda *et al*., 1995; Ohishi *et al*., 1996; Lin *et al*., 1997).

Effect of metal ions and other reagents on chitinase activity

As shown in Table 2, Hg^{2+} and IO_3^- (1 and 10 mM), dithiothreitol (DTT, 10 mM) and 2-mercaptoethanol (1%) completely inhibited the activity of both Chi-I and Chi-II. Complete inhibition by Hg^{2+} is similar to that of other chitinases from both marine (Ohishi *et al*., 1996; Lin *et al*., 1997) and non marine organisms (Okazaki and Tagawa, 1991). Inhibition by Cu^{2+} (10 mM) was 90%. This ion also gave 80% inhibition with two chitinases derived from *Vibrio* sp. (Ohtakara *et al.*, 1979). However, activity of chitinases from *A. caviae* (Lin *et al*., 1997) and *Pseudomonas* (Wang and Chang, 1997) was enhanced by the addition of Cu^{2+}. Of the 4 major cations contained in sea water (Na^+, Mg^{2+}, Ca^{2+} and K^+), 1 mM of Mg^{2+}, Ca^{2+} and K^+ very slightly stimulated Chi-I and Chi-II activity. At 10 mM, Mg^{2+} enhanced activity of both enzymes by approximately 10%. In contrast, the same amount of K^+ inhibited Chi-I and Chi-II by approximately 10 and 5%, while Ca^+ inhibited Chi-I and Chi-II by about 20 and 40%, respectively. The effect of Na^+ at 1 and 10 mM was not determined, since both enzymes had previously been shown to tolerate a wide range of NaCl 1-14% with activities ranging from 100-50%. Chitinase A from *Alteromonas* sp. was enhanced (1.6-fold higher) by 1-40 mM of Mg^{2+} (Tsujibo *et al*., 1992). In contrast chitinase from *A. caviae* was inhibited around 10% by these four cations (Lin *et al*., 1997). Nearly all of the tested chemical reagents except EDTA drastically inhibited chitinase activity. Dithiothreitol (1 mM) inhibited Chi-I and Chi-II activity by approximately 36 and 29%, respectively. This was similar to results with ChiA from *Aeromonas* (Lin *et al*., 1997) whilst the chitinase from *Pseudomonas* (Wang and Chang, 1997) was completely inhibited by 1 mM of dithiothreitol. This inhibition was also observed in chitinas-

TABLE 2 - Effect of metal ions, chemical reagents and detergents on chitinase activity

Reagents	Final concentration	Relative activity (%)	
		Chi-I	Chi-II
None		100	100
$CaCl_2$	1 mM	102.8	105
	10 mM	78.6	62
$CoCl_2$	1 mM	86.1	99.2
	10 mM	69.6	73.8
$CuSO_4$	1 mM	90.8	97.7
	10 mM	9.7	10.1
$FeCl_3$	1 mM	97.5	104.1
	10 mM	7.3	11.7
$MgSO_4$	1 mM	101.5	104.2
	10 mM	108.3	110
$MnSO_4$	1 mM	96.5	89.7
	10 mM	32	31.7
K_2SO_4	1 mM	100.6	100.4
	10 mM	89.3	95.3
KIO_3	1 mM	0	0
	10 mM	0	0
$HgCl_3$	1 mM	0	0
	10 mM	0	0
NH_4NO_3	1 mM	100.1	100.2
	10 mM	88.1	96.4
$ZnSO_4$	1 mM	92.4	96.1
	10 mM	54.5	58.5
EDTA	1 mM	93.3	95
	10 mM	99	99.8
Dithiothreitol	1 mM	64.2	71.3
	10 mM	0	0
SDS	1%	66	72.1
Triton X-100	1%	94	93.1
2-mercaptoethanol	1%	0	0
Dimethylformamide	1%	66	67
Methanol	10%	48	45.6
	20%	26.7	25.1
	50%	0	0
Ethanol	10%	61.9	58.6
	20%	30.1	29.6
	50%	0	0
Acetonitrile	50%	17	19.5
Acetone	50%	10	21.8

es from other organisms such as in *Pseudomonas* (Wang and Chang, 1997). It contrasts with an increase in chitinase activity upon DTT treatment of *Streptomyces* chitinase where the presence of sulphydryl groups has been proposed at active site (Gupta *et al*., 1995). Some detergents such as Triton X-100 slightly inhibited both Chi-I and Chi-II activity whereas 1% SDS decreased the activity of both up to 40%. This result was similar to that of ChiA from *Alteromonas* (Tsujibo *et al*., 1992). However, these detergents strongly inhibited chitinase A activity of *A. caviae* (Lin *et al*., 1997). Ethanol and methanol at 10% caused a drop in Chi-I and Chi-II activity to 60 and 50%, respectively, whereas at 20% caused in a further drop in activity to 30%. Both were totally inhibited by 50% ethanol and methanol. Acetonitrile and acetone at 50% inhibited Chi-I by 83 and 90% and Chi-II by 81 and 79%, respectively.

Kinetic parameters of recombinant chitinase

The chitinase activity was assayed against 5-30 µM of substrate, *p*-nitrophenyl-*N,N'*-diacetylchitobiose. At substrate concentrations higher than 30 µM, chitinase inhibition was observed. Inhibition may have been due to substrate molecules resembling to the product and binding to the enzyme in an incorrect orientation, or to other factors (Barata *et al*., 2002). Analysis of the kinetic data at lower substrate concentrations (5-30 µM) by the Lineweaver-Burk transformation indicated similar K_m values for Chi-I and Chi-II of 30 and 31.8 µM while V_{max} were 10 and 9.2 µmol/h/mg protein, respectively. K_m and V_{max} against this substrate for ChiA from *Pseudomonas* (Lee *et al*., 2000) were 1.06 mM and 44.4 µmol/h/mg protein. As a low K_m indicate a high affinity of the enzyme for its substrate, Chi-I and Chi-II from *S. costicola* showed higher affinity for the substrate than that of chitinases from *Pseudomonas*.

Product analysis of Chi-I and Chi-II by HPLC

To determine the final reaction products of both enzymes, the products from colloidal chitin and five chito-oligosaccharides treated with Chi-I and Chi-II were analysed by HPLC. Endochitinases cleave chitin randomly at internal sites generating soluble low molecular mass multimers of *N*-acetylglucosamine (GlcNAc). Exochitinases or chitobiosidases cleave chitin to release the dimer chitobiose from the non-reducing end while b-*N*-acetylhexosaminidases hydrolyse *N*-acetyl-chitooligosaccharides from the non-reducing end releasing GlcNAc (Kupiec and Chet, 1998). Both Chi-I and Chi-II released GlcNAc and chitobiose as major products from colloidal chitin and $(GlcNAc)_3$ to $(GlcNAc)_6$ substrates (Fig. 4). Trace amounts of $(GlcNAc)_3$ were detected when Chi-II hydrolysed $(GlcNAc)_5$ and $(GlcNAc)_6$ as substrates (Fig. 4). Both forms of enzymes could also hydrolyse $(GlcNAc)_2$ to a monomer of *N*-acetylglucosamine (Fig. 4). These results indicated an exo-type mode of chitinase action. Chi-I and Chi-II are bacterial chitinases that exhibit both chitobiosidase function as a major activity and also has b-*N*-acetylglucosaminidase function. Chi-I and Chi-II at high activity were able to hydrolyse the dimer or trimer to a monomer and a dimer with remaining trace amount of substrates (dimmer or trimer). ChiA from *Pseudomonas* (Lee *et al*., 2000) exhibited only chitobiosidase activity while chitinase from *V. parahaemolyticus* (Zhu *et al*., 1992) functioned as a b-*N*-acetylhexosaminidase. ChiA from *S. marcescens* exhibited both exo- and endo- chitinolytic acitivity. However, it lacked b-*N*-acetylhexosaminidase activity since it could not hydrolyse the dimer (Brurber *et al*., 1996).

Crude chitin digestion by Chi-I and Chi-II and product analysis by TLC

The crude chitin hydrolytic ability of Chi-I and Chi-II was examined using a coarse flake and powdered chitin from crab shells as a substrate. Time course hydrolysis over 48 h (Fig. 5) revealed that both Chi-I and Chi-II could hydrolyse both types of crude chitin with more hydrolysing activity toward powdered chitin, possibly due to its higher surface area. Chi-I degraded crude chitin with more activity than Chi-II as shown in Fig. 5. At 48 h, the relevant activ-

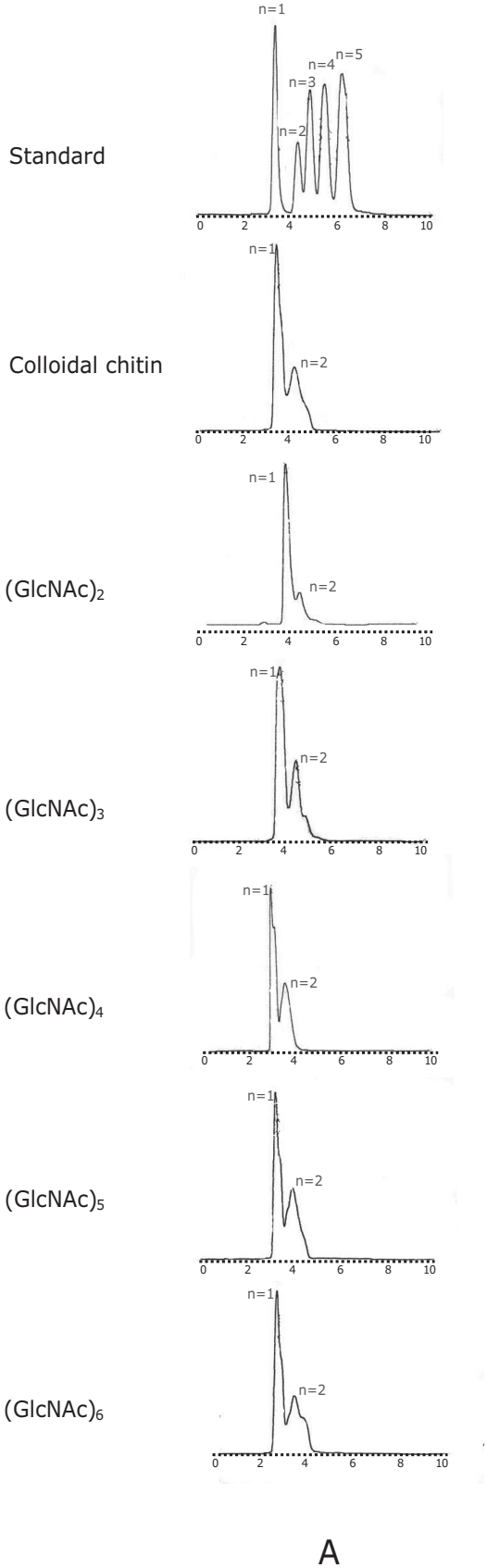

FIG. 4 - HPLC analysis of the products obtained from the hydrolysis of various chito-oligosaccharides by Chi-I (A) and Chi-II (B); n=1, n=2, n=3, n=4 and n=5 indicate *N*-acetylglucosamine, chitobiose, chitotriose, chitotetraose and chitopentaose, respectively.

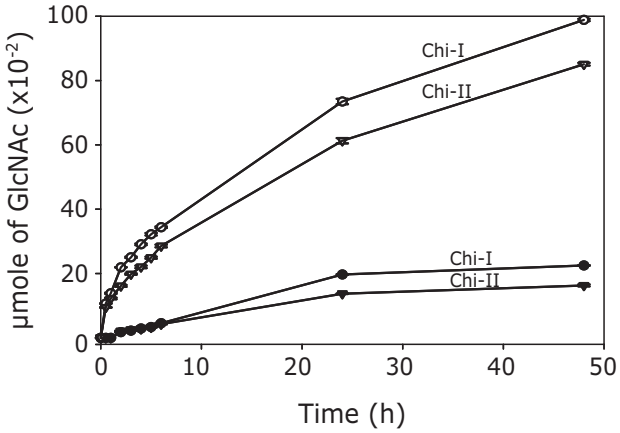

FIG. 5 - Hydrolysis of coarse flake (● and ▼) and powdered (▽ and ○) chitin by purified Chi-I and Chi-II. Bars indicate standard deviation.

ity of Chi-II toward powdered chitin and Chi-I, Chi-II toward flake chitin were 86, 23 and 16%, respectively when compared to the hydrolysis of powdered chitin by purified Chi-I (100%). A similar differential activity between coarse flake chitin and powdered chitin was observed for the recombinant chitinase A from *A. caviae* (Lin *et al.*, 1997).

By TLC analysis, it was found that the major product from crude chitin digestion by Chi-I was the dimer or chitobiose (Fig. 6A, lanes 2-10) that could be observed from as early as 0.5 h of incubation (Fig. 6A, lane 2). After 5 h incubation, the minor product, GlcNAc was also detected (Fig. 6A, lane 7). Digestion products from Chi-II were similar (Fig. 6B, lanes 2-10), although the minor product GlcNAc was not detected until 6 h of incubation (Fig. 6B,

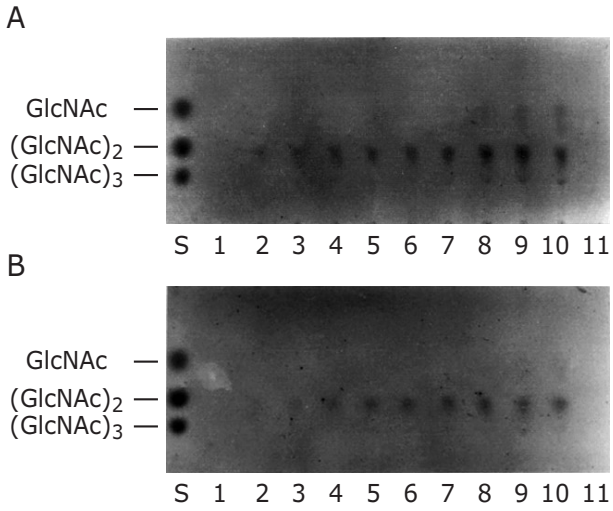

FIG. 6 - Thin layer chromatography of products from powdered chitin hydrolysis by Chi-I (A) and Chi-II (B). Lane S, a standard mixture of *N*-acetylglucosamine (GlcNAc), chitobiose $(GlcNAc)_2$ and chitotriose $(GlcNAc)_3$, lanes 1-10, 10 µl product of powdered chitin hydrolysis at 0, 0.5, 1, 2, 3, 4, 5, 6, 24 and 48h, respectively, lane 11, 10 µl of 2% crude chitin only as a control.

lane 8). These results confirmed the exo-type mechanism of both Chi-I and Chi-II as observed by HPLC analysis. Coarse flake chitin hydrolysis gave the same results (data not shown). In contrast recombinant ChiA from *A. caviae* functions as an endo-type chitinase releasing GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$ and $(GlcNAc)_4$ within 24 h with $(GlcNAc)_3$ as a major product (Lin *et al*., 1997). Chitobiose has been widely used as a starting material for synthesis of biological active compounds (Patil *et al*., 2000). The above results show that both forms of chitinase C from *S. costicola* expressed in *E. coli* can hydrolyse crude chitin without the requirement of substrate purification and in the presence of a high concentration of calcium carbonate (58%) and other components usually present in crude chitin, as reported by Kim and Park (1994) over a prolonged incubation period of 48 h. The presence of cadherin-like domain, which is Ca^{2+} dependent cell adhesion protein, might contribute in some way to this hydrolysis. However, the function of this domain in chitin hydrolysis requires further investigation. Hence, chitinase from *S. costicola* which could function in a wide range of pH and temperature together with its wide-range halotolerance; its ability to hydrolyse unprocessed chitin and releasing chitobiose as the major end product is of pivotal use in a variety of applications particularly in the treatment of shellfish wastes.

Acknowledgement

R. Aunpad received support from the Institutional Strengthening Program of the Faculty of Science, Mahidol University and a Split-mode Ph.D. scholarship from the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA). This work was partially supported by the National Research Council of Thailand (NRCT). We thank Dr. Bhinyo Panijpan and Prof. T.F. Flegel from Mahidol University for critical reading of the manuscript.

REFERENCES

Aunpad R., Panbangred W. (2003). Cloning and characterization of constitutively expressed chitinase C gene from marine bacterium *Salinivibrio costicola* strain 5SM-1. J. Biosci. Bioeng., 96: 529-536.

Barata R.A., Andrade M.H.G., Rodrigues R.D., Castro I.M. (2002). Purification and characterization of an extracellular trypsin-like protease of *Fusarium oxysporum* var. *lini*. J. Biosci. Bioeng., 94: 304-308.

Bendt A., Huller H., Kammel U., Helmke E., Schweder T. (2001). Cloning, expression and characterization of a chitinase gene from the Antarctic psychrotolerant bacterium *Vibrio* sp. strain Fi:7. Extremophiles, 5: 119-126.

BoggonT.J., Murray J., Chappuis-Flament S., Wong E., Gumbiner B.M., Shapiro L. (2002). C-cadherin ectodomain structure and implications for cell adhesion mechanisms. Science, 296: 1308-1313.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Brurber M.B., Nes I.F., Eijsink V.G.H. (1996). Comparative studies of chitinases A and B from *Serratia marcescens*. Microbiology, 142: 1581-1589.

Collinge D.B., Kragh K.M., Mikkelsen J.D., Nielsen K.K., Rasmussen U., Vad K. (1993). Plant chitinase. Plant J., 3: 31-40.

Gupta R, Saxena R.K., Chaturvedi P., Virdi J.S. (1995). Chitinase production by *Streptomyces viridificans*. J. Appl. Bacteriol., 78: 378-383.

Kim S.B., Park T.K. (1994). Isolation and characterization of chitin from crab shell. Korean J. Biotech. Bioeng., 9: 174-179.

Konagay, Y., Tsuchiy, C., Sugit, 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.

Kupiec R.C., Chet I. (1998). The molecular biology of chitin digestion. Curr. Opin. Biotech., 9: 270-277.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 227: 680-685.

Lee H.S., Han D.S., Choi S.J., Choi S.W., Kim D.S., Bai D.H., Yu J.H. (2000). Purification, characterization and primary structure of a chitinase from *Pseudomonas* sp. YHS-A2. Appl. Microbiol. Biotech., 54: 397-405.

Lin C., Chen H., Lin F. (1997). Expression and characterization of the recombinant gene encoding chitinase from *Aeromonas caviae*. Enzyme Microbiol. Technol., 21: 472-478.

Mellado E., Moore E.R., Nieto J.J., Ventosa A. (1996). Analysis of 16S rRNA gene sequences of *Vibrio costicola* strains: description of *Salinivibrio costicola* gen. nov., comb. nov. Int. J. Syst. Bacteriol., 46: 817-821.

Monreal J., Reese E.T. (1969). The chitinase of *Serratia marcescens*. Can. J. Microbiol., 15: 689-696.

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

Ohishi K., Yamagishi M., Ohta T., Suzuki M., Izumida H., Sano H., Nishijima M., Miwa T. (1996). Purification and properties of two chitinases from *Vibrio alginolyticus* H-8. J. Ferment. Bioeng., 82: 598-600.

Ohtakara A., Mitsutomi M., Uchida Y. (1979). Purification and some properties of chitinase from *Vibrio* sp. J. Ferment. Technol., 57: 169-173.

Okazaki K., Tagawa K. (1991). Purification and characterization of a chitinase from *Streptomyces cinereoruber*. J. Ferment. Bioeng., 71: 237-238.

Okazaki K., Kato F., Watanabe N., Yasuda S., Masui Y., Hayakawa S. (1995). Purification and properties of two chitinases from *Streptomyces* sp. J-13-3. Biosci. Biotechnol. Biochem., 59: 1586-1597.

Patil R.S., Chormade V., Desphande M.V. (2000). Chitinolytic enzymes: an exploration. Enzyme Microbiol. Technol., 26: 473-483.

Reissig J.L., Strominer J.L., Leloi L.F. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem., 27: 959-966.

Suginta W., Robertson P.A.W., Austin B., Fry S.C., Fothergill-Gilmore L.A. (2000). Chitinases from *Vibrio*: activity screening and purification of ChiA from *Vibrio carchariae.* J. Appl. Microbiol., 89: 76-84.

Takayanagi T., Ajisaka K., Takiguchi Y., Shimahara K. (1991). Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. Biochem. Biophys. Acta, 1078: 404-410.

Thamthiankul S., Moar W.J., Miller M.E., Panbangred W. (2004). Improving the insecticidal activity of *Bacillus thuringiensis* subsp. *aizawai* against *Spodoptera exigua* by chromosomal expression of a chitinase gene. Appl. Microbiol. Biotechnol., 65: 183-192.

Tsujibo H., Yoshida Y., Miyamoto K. (1992). Purification, properties and partial amino acid sequence of chitinase from a marine *Alteromonas* sp. strain O-7. Can. J. Microbiol., 38: 891-897.

Tsujibo H., Orikoshi H., Baba N., Miyahara M., Miyamoto K., Yasuda M., Inamori Y. (2002). Identification and characterization of the gene cluster involved in chitin degradation in a marine bacterium, *Alteromonas* sp. strain O-7. Appl. Environ. Microbiol., 68: 263-270.

Ueda M., Fujiwara A., Kawaguchi T., Arai M. (1995). Purification and some properties of six chitinases from *Aeromonas* sp. No. 10S-24. Biosci. Biotech. Biochem., 59: 2162-2164.

Wang S., Chang W. (1997). Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. Appl. Environ. Microbiol., 63: 380-386.

Watanabe T., Ito Y., Yamada T., Hashimoto M., Sekine S., Tanaka H. (1994). The role of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. J. Bacteriol., 176: 4465-4472.

Yu C., Lee A.M., Bassler B.L., Roseman S. (1991). Chitin utilization by marine bacteria. A physiological function for bacterial adhesion to immobilized carbohydrate. J. Biol. Chem., 266: 24260-24267.

Zhu B.C.R., Lo J., Li Y., Li S., Jaynes J.M., Gildemeister O.S., Laine R.A., Ou C. (1992). Thermostable, salt tolerant, wide pH range novel chitobiase from *Vibrio parahemolyticus*: Isolation, characterization, molecular cloning and expression. J. Biochem., 112: 163-167.