World Journal of Microbiology & Biotechnology (2005) 21:733–738
DOI 10.1007/s11274-004-4797-1

Purification and characterization of a thermostable chitinase from *Bacillus licheniformis* Mb-2

Aris Toharisman[1], Maggy Thenawidjaja Suhartono[2,*], Margarethe Spindler-Barth[3], Jae-Kwan Hwang[4] and Yu-Ryang Pyun[4]
[1]*Indonesian Sugar Research Institute, Jalan Pahlawan 25 Pasuruan 67126, Indonesia*
[2]*Research Center for Biotechnology, Bogor Agricultural University, Jln. Puspa, Kotak Pos 1 Darmaga Campus, Bogor 16180, Indonesia*
[3]*Department of General Zoology and Endocrinology, University of Ulm, Albert Einstein Alle 11* D*-89081 Ulm, Germany*
[4]*Bioproducts Resource Center, Yonsei University, South Korea*
**Author for correspondence: Tel.: +62-251-625965, Fax: +62-251-621724, E-mail: xanthomb@telkom.net*

Keywords: *Bacillus licheniformis* MB-2, characterization, geothermal areas, purification, thermostable chitinases

Summary

A chitinase produced by *Bacillus licheniformis* MB-2 isolated from Tompaso geothermal springs, Indonesia, was purified and characterized. The extracellular enzyme was isolated by successive hydrophobic interaction, anion exchange, and gel filtration chromatographies. The purified enzyme was a monomer with an apparent molecular weight of 67 kDa. The optimal temperature and pH of the enzyme were 70 °C and 6.0, respectively. It was stable below 60 °C for 2 h and over a broad pH range of 4.0–11.0 for 4 h. The enzyme was resistant to denaturation by urea (1 M), Tween-20 (1%) and Triton-X (1%), but unstable toward organic solvents such as dimethyl sulphoxide, DMSO, (5%) and polyethylene glycol, PEG, (5%) for 30 min. The enzyme hydrolysed colloidal chitin, glycol chitin, chitosan, and glycol chitosan. The first 13 N-terminal amino acids of the enzyme were determined as SGKNYKIIGYYPS, which is identical to those in chitinases from *B. licheniformis* and *B. circulans*.

Introduction

Chitin, an insoluble polysaccharide consisting of β-(1-4)-linked *N*-acetyl-D-glucosamine (GlcNAc) units, is abundant in the marine environment. It is a major structural component of mollusks, insects, crustaceans, fungi, algae and marine invertebrates (Shaikh & Despande 1993). The chemical and physical properties of chitin and its derivatives are suited for a wide range of different biotechnological applications of commercial interest. Currently they are used as immunoadjuvants, flocculants of wastewater sludge, agrochemicals, drug delivery systems, dietary fibre, and in wound healing (Muzzarelli 1996).

Biodegradation of chitin is performed by chitinases and appears to occur in two steps. An endochitinase (EC 3.2.1.14) degrades the polymer to oligomers, which are subsequently degraded to monomers by exochitinase (β-*N*-acetylhexosaminidase [EC 3.2.1.52]). These enzymes are found in a wide variety of organisms such as bacteria, fungi, insects, plants and animals (Sakai *et al.* 1998). Chitinases are also synthesized by some protozoans, coelenterates, nematodes, mollusks and arthropods (Muzzarelli 1996).

During the last decade, chitinases have received remarkable attention due to their wide range of applications. Efforts are going on throughout the world to enhance the production and purity of bacterial chitinases. Some novel characteristics of bacterial chitinases have been reported (Park *et al.* 1997; Wang & Chang 1997). However, only a few thermostable chitinolytic enzymes from bacteria are known (Gomes *et al.* 2001; Wen *et al.* 2002).

This paper describes the purification and characteristics of an endochitinase produced by a thermophilic bacterium isolated from the Tompaso hot springs, North Sulawesi, Indonesia. The enzyme is unique because it is stable over a broad range of pH values and towards some denaturants, while it is relatively stable at elevated temperature.

Materials and methods

Chemicals

Glycol chitosan, *N*-acetyl D-glucosamine, chitosan, chitin, 4-methylumbelliferyl *N*′-acetyl-β-D-glucosami-

nide [MUF(GlNAc)], 4-methylumbelliferyl *N*′,*N*′-diacetylchitobioside [MUF(GlcNAc)$_2$], 4-methylumbelliferyl *N*′,*N*′,*N*′-triacetylchitotrioside [MUF(GlcNAc)$_3$], and phenyl-Sepharose CL-4B resin were purchased from Sigma-Aldrich Inc. Other chemicals were obtained from local supplier (Merck, Difco, Oxoid and Boehringer). Glycol chitin was prepared from glycol chitosan by the method of Trudel & Asselin (1989) with reacetylation using acetic anhydride. Regenerated chitin and colloidal chitin were prepared from chitosan by the method of Molano *et al.* (1977) and Roberts & Selitrennikoff (1988), respectively.

Microorganism

Bacillus licheniformis MB-2 was isolated from the Tompaso hot spring in North Sulawesi, Indonesia using agar plates containing colloidal chitin. The cells were cultured on LB medium supplemented with 0.3% colloidal chitin and incubated at 55 °C for 48 h in a rotary shaker at 200 rev min^{-1}. Ten milliliters of bacterial broth was used as an inoculum and added onto 90 ml of medium with the following composition: 0.5% colloidal chitin, 0.7% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract and 1.5% agar (pH 7.0) and incubated at 55 °C on a rotary shaker at 180 rev min^{-1}. The culture was centrifuged at 10,000 × *g* (4 °C) for 20 min. The supernatant was collected for further purification.

Enzyme purification

Free-cell supernatant (250 ml) was heated at 80 °C for 5 min and centrifuged at 10, 000 × *g* for 5 min to remove the heat-labile proteins. The supernatant was mixed with $(NH_4)_2SO_4$ to a final concentration of 1 M and the pH was adjusted to 8.0 with 1 M NaOH. The mixture was loaded on to a phenyl-Sepharose CL-4B column (1.6 × 15 cm) equilibrated with 20 mM Tris–HCl buffer, pH 8.0 (buffer A) containing 1 M $(NH_4)_2SO_4$. After washing with 200 ml of the same buffer, the enzyme was eluted with a combination of a linear descending gradient between 1.0 and 0 M ammonium sulphate in buffer A and a linear gradient ascending gradient of Tween-20 between 0 and 1% (v/v) in the same buffer at a flow rate of 10 ml h^{-1} (2 ml per tube). The enzyme activity appeared in fractions between 113 and 150 that were eluted with 0 M ammonium sulphate and 0.5% Tween 20. Fractions were pooled, concentrated by Amicon ultrafiltration (NMWL 10 kDa, Millipore) and subjected to DEAE-Sephacel column (1.6 × 15 cm) equilibrated with 10 mM Tris–HCl, pH 8.0 (buffer B). Bound proteins were eluted using a combined linear ascending gradient of 0–0.5 M NaCl in buffer B (Figure 1, line A, B) and 0 to 1% (v/v) Tween-20 in the same buffer (Figure 1, line C, D). Most activity eluted at 0.1–0.2 M NaCl and 0.05–0.1% Tween-20, and the corresponding fractions were pooled and concentrated as above. The concentrated fractions were applied to a HiLoad Superdex 75 column (1.6 × 60 cm) equilibrated with buffer B at a flow rate of 0.1 ml min^{-1} (1 ml per tube), and eluted with the same buffer. Active fractions were pooled, concentrated and used for further study. All purification steps were performed at ambient temperature except for gel filtration chromatography (4 °C).

Chitinase assay

For isolation and substrate specificity experiments, chitinase activity was measured by a colorimetric method using colloidal chitin as substrate (Wang & Chang 1997). The enzyme solution (100 μl) was mixed with 300 μl of 0.3% (w/v) colloidal chitin dissolved in McIlvaine buffer (pH 6.0) and incubated at 60 °C for 30 min. The control was prepared by adding inactivated enzyme after incubation. The solution was then centrifuged at 10,000 × *g*, 4 °C for 5 min. The amount of reducing sugars produced was determined at 420 nm by

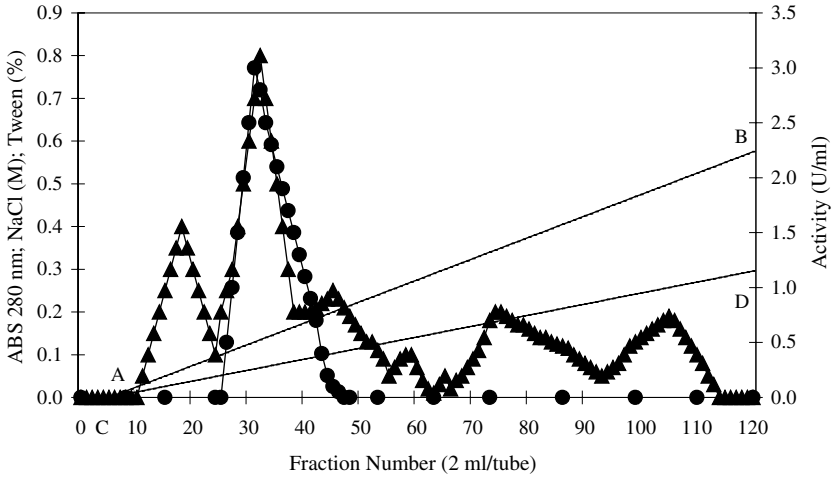

Figure 1. Elution profile of chitinase from DEAE-Sephacel column chromatography with gradients of NaCl at pH 8.0 (A, B) and increasing Tween-20 (C, D); (▲) absorbance 280 nm; (●) endochitinase activity.

a modified Schales method (Ueda *et al*. 1996). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar as *N*-acetylglucosamine (GlcNAc) equivalent per minute.

For other studies, chitinase activity was measured by a fluorimetric assay using one of the following substrates: 1 mM MUF(GlNAc), 250 μM MUF$(GlNAc)_2$, and 250 μM MUF$(GlNAc)_3$ (Spindler 1997). The enzyme (20 μl) was mixed with 50 μl substrate and 30 μl McIlvaine buffer (pH 6.0), and incubated at 60 °C for 30 min. The reaction was stopped with 100 μl of 1 M glycine–NaOH, pH 11.0. The amount of 4-methylumbelliferone released from the substrate was measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm with a Cytofluor Multi-Well Plate Reader series 4000 (PerSeptive Biosystems).

Protein determination

Protein was determined by measuring the absorbance at 280 nm during chromatographic separation. Protein was also determined by the Bradford method with bovine serum albumin fraction V as a standard.

Optimum temperature and pH

The optimum temperature and pH of the enzyme were determined with the fluorimetric assay. The enzyme was assayed for 30 min at various temperatures (40–80 °C) and pHs (pH 2.0–12.0). The pH was varied using the following buffers at a concentration of 0.2 M: glycine–HCl (pH 2.0–3.0), citrate phosphate buffer (pH 3.0–7.0), phosphate buffer (pH 7.0–8.0), Tris–HCl buffer (pH 8.0–9.0), and glycine–NaOH buffer (pH 9.0–12.0).

Enzyme stability and substrate specificity

The thermal stability was investigated after incubating the enzyme for 2 h at different temperatures in McIlvaine buffer, pH 6.0. The pH stability was determined after incubation of the enzyme at room temperature for 4 h at pH range of 2.0–12.0. Aliquots of the enzyme were sampled at 30 min intervals and residual activity of the enzyme was determined under standard assay conditions. For substrate specificity study, the enzyme was assayed colorimetrically with various chitin substrates (0.5%) in McIlvaine buffer (pH 6.0) for 30 min at 60 °C.

Effect of denaturants, organic solvents and others

The enzyme was preincubated for 30 min at room temperature with various concentrations of denaturing reagents (1 M), detergents (1%), organic solvents (5%) and other reagents such as PMSF (5 mM), EDTA (1 mM), and allosamidin (25 μM). The enzyme activity was measured using a fluorimetric assay with MUF$(GlNAc)_2$ as a substrate.

Electrophoresis and zymogram

SDS-PAGE was performed by the method of Laemmli (1970). After electrophoresis, gels were stained either with silver nitrite or with Coomassie brilliant blue R-250. The chitinolytic activities were also detected on gels after SDS-PAGE or PAGE (zymogram) by using 0.1% glycol chitin or 300 μg ml^{-1} of MUF$(GlcNAc)_3$ (Spindler & Rapp 1997).

N-terminal amino acid sequencing

The enzyme was fractionated by SDS-PAGE and transferred onto a polyvinylidene flouride (PVDF) membrane (Immobilon-P, Millipore) at a constant current of 250 mA for 1 h. The N-terminal amino acid sequence was conducted by stepwise Edman degradation in an automated protein sequencer (Appl. Biosystems Co.).

Results and discussion

Morphology and characteristics of MB-2

We have screened and isolated many chitin-degrading bacteria from the Tompaso hot spring (Indonesia) and the MB-2 isolate was one which formed a significant clear zone when grown on solid medium containing colloidal chitin as a carbon source. The 16S rRNA (1 478 bases) sequence of MB-2 showed high similarity to *Bacillus licheniformis* (99% homology). Therefore, it was named *B. licheniformis* MB-2. The bacterium was gram-positive, rod shaped and spore-forming. It was a facultative anaerobe, grew in the temperature range of 37–60 °C and hydrolysed gelatine, mannitol, maltose, and starch.

MB-2 produced five extracellular chitinases continuously during 5 days when grown at 55 °C in medium containing 0.5% colloidal chitin. Their molecular masses were 78, 67, 61, 50 and 47 kDa and they were tentatively named Chi-78, -67, -61, -50 and -47, respectively. The major chitinase produced by MB-2 i.e. the chitinase-67 kDa (Chi-67) was further studied. This enzyme was the most heat stable and present in the highest concentrations in the culture medium.

Multiform chitinases in the range of 30–81 kDa produced by bacteria have been reported previously. In the case of *Bacillus* MH-1, three isoform chitinases corresponding to 71, 62 and 53 kDa were found (Sakai *et al*. 1998). The bacteria produced several chitinases, probably to hydrolyse the diversed chitin substrates found in nature. Chitin molecules can vary depending on by the arrangement of *N*-acetylglucosamine strands, degree of deacetylation, and the presence of cross-linked structural components such as protein and glucans.

Purification of chitinase Chi-67

Chi-67 was purified from the extracellular culture by heat treatment, and successive chromatographic steps

including hydrophobic interaction, anion exchange and gel filtration chromatography. Chitinase was separated from heat-unstable proteins by heating at 80 °C and applied to hydrophobic interaction phenyl–Sepharose chromatography. Our initial study indicated that elution of the enzyme from hydrophobic chromatography using gradient ammonium sulphate from 1 to 0 M preduced only a minute amount of chitinase. Addition of Tween-20 to the elution buffer dramatically increased the yield. In fact, the addition of Tween-20 was also useful for elution from the anion exchanger DEAE-Sephacel. In both separation methods, chitinase activity was detected in the low-salt fraction, suggesting that the enzyme surface is rather hydrophobic.

Elution with Tween-20 resulted in three protein peaks containing endochitinase activities. One major peak containing the highest endochitinase activity was collected and concentrated, then loaded onto to a DEAE-Sephacel column. The enzyme was eluted with a gradient of 0.1–0.2 M NaCl and 0.3–0.4% Tween 20 (Figure 1). The endochitinase-active fractions (fractions 26–45) was collected, concentrated, and run on SDS-PAGE. Some bands was seen on SDS-PAGE (Figure 2, lane 5), indicating that the fraction was not pure. The next purification step with gel filtration produced a major active fraction which was subjected to SDS-PAGE resulting in a single band at 67 kDa (Figure 2, lane 6 and 8). Therefore, the enzyme was named tentatively Chi-67.

The yield and purity for each purification step are summarized in Table 1; a 3.56% yield with 15.33-fold purification was obtained. Although the yield of enzyme was relatively low for the commercial production, the method gave sufficient pure enzyme for initial characterization studies. Furthermore, this system of purification was reproducible as the pattern of elution was nearly the same when the fractionation was repeated.

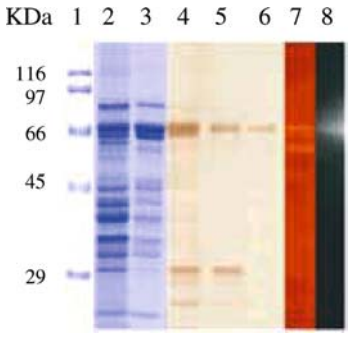

Figure 2. SDS-PAGE of peak fractions from different purification steps. Lane 1, molecular weight markers; lane 2, crude extract enzymes; lane 3, proteins derived from heating 80 °C for 5 min; lane 4, proteins from hydrophobic interaction chromatography; lane 5, proteins from anion exchanger; lane 6, protein from gel filtration; lane 7, zymogram analysis with 0.1% glycol chitin of band from lane 2; lane 8, zymogram analysis with $MUF(GlNAc)_3$ of band from lane 6.

Table 1. Purification steps of chitinase produced by *Bacillus licheniformis* MB-2.

Purification step	Total protein (mg)	Total activity (U)	Spec. activity (U/mg)	Yield (%)	Purification (-fold)
Cell free supernatant	21.50	3 750	174	100.00	1.00
Heat treatment (80 °C, 5 min)	12.80	3 050	238	81.34	1.37
Phenyl Sepharose CL-4B	0.86	672	782	17.94	4.49
DEAE Sephacel	0.44	560	1 274	14.94	7.30
Superdex 75	0.05	267	2 673	3.56	15.33

Enzyme characteristics

Chitinolytic enzymes can be grouped into three types (Shaikh & Despande 1993). Endochitinases (EC 3.2.1.14) are defined as enzymes catalysing the random hydrolysis of 1,4-β linkages of GlNAc at internal sites over the entire length of the chitin microfibrils. The products are soluble, low-molecular mass oligomers of GlNAc such as chitotetraose, chitotriose and diacetylchitobiose. Exochitinases catalyse the successive release of diacetylchitobiose units in a stepwise fashion as the sole product from chitin. The third type is *N*-acetyl-β-1,4-D-glucosaminidase (EC 3.2.130), a chitinolytic enzyme which also acts in exo-splitting mode on diacetylchitobiose and higher analogs. Based on this nomenclature, Chi-67 can be classified as an endochitinase. It produced a fluorescent product from $MUF(GlNAc)_2$ and $MUF(GlNAc)_3$, but it did not hydrolyse MUF from MUF(GlNAc).

The optimum temperature of the enzyme was 70 °C (Figure 3) which coincides with that of thermostable chitinases from *B. licheniformis* X-70 (Takayanagi *et al.* 1991). The enzyme had almost no activity at 30 °C, but the activity increased from 35 to 70 °C and decreased above 75 °C. These temperature optima are a common feature of chitinases independent of the habitat of the organisms. Even among fungi or crustaceans (Spindler & Buchholz 1998) found in the Antarctic, chitinase temperature optima of 55 °C were also found. The enzyme was remarkably stable at temperatures below 60 °C (Figure 4). The half-life at 60 °C was about

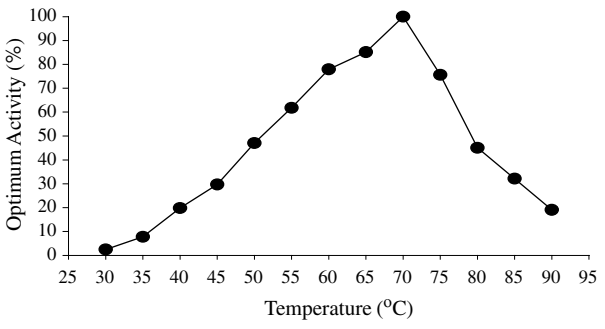

Figure 3. Influence of temperature on chitinase activity from MB-2.

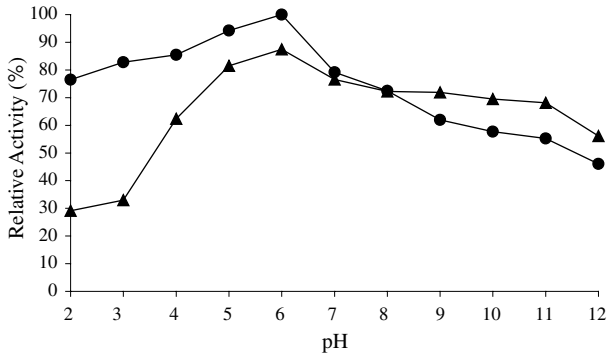

Figure 4. Influence of pH on chitinase activity (●) and pH stability of the enzyme (▲). The pH was adjusted by the following buffers at a concentration of 0.2 M: glycine–HCl (pH 2.0–3.0), citrate phosphate (pH 3.0–7.0), phosphate (pH 7.0–8.0), Tris–Cl (pH 8.0–9.0), and glycine–NaOH buffer (pH 9.0–12.0).

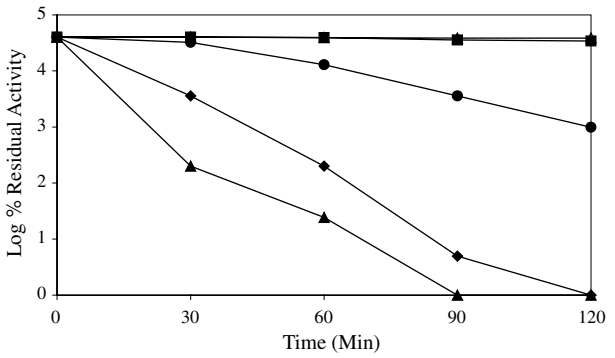

Figure 5. Heat stability of the enzyme. The purified enzyme was incubated separately at (△) 40 °C (■) 50 °C (●) 60 °C, (♦) 70 °C and (▲) 80 °C.

80 min, whereas at 50 °C no significant activity lost was observed within 120 min.

Chi-67 has an optimum pH of 6.0 (Figure 5). The enzyme was extremely stable at pH 4.0 to 11.0 (Figure 5). At least 70% of the enzyme activity was retained at pH 4.0 and 11.0. Incubation of the enzyme at pH 4.0 or pH 11.0 for 4 h resulted in only 30% reduction of the activity. This is so far the first report of a bacterial chitinase, which is stable at this broad range of pH, although the molecular basis for the high pH stability of the enzyme remains to be elucidated. A thermostable chitinase from *S. thermoviolaceus* OPC-520 was stable at pH 6.0–8.0 (Tsujibo *et al.* 2000), from *Streptomyces* RC1071 at pH 4.0–9.0 (Gomes *et al.* 2001), and from *Bacillus* sp. NCTU2 at pH 6–8 (Wen *et al.* 2002). Some other characteristics of bacterial chitinases are compared in Table 2.

Effect of denaturants and various inhibitors

The enzyme was resistant to denaturation by 1 M urea, 1% Tween-20 and 1% Triton-X, partially resistant to 1% sodium dodecyl sulphate (SDS) but unstable toward organic solvents such as butanol, 2-propanol, ethanol, and DMSO at a concentration of 5%, indicating that hydrophobic interactions are significantly important for enzyme activity. The enzyme was also insensitive toward mercaptoethanol (1 mM), but the activity was diminished moderately by dithiothreitol (DTT) (10 mM), implying the reduction of S–S bridges of the enzyme. In addition, treatment of the enzyme with 25 μM allosamidin completely abolished enzyme activity, suggesting that the molecule is a member of family 18 chitinases, although a few members of family 19 are also reported to be allosamidin-sensitive. Another unique features of the enzyme is the stability toward detergents (1%) and denaturing agents (1 M). These unique characteristics have made this enzyme ideal for structure/function research and many applications in the future.

Substrate specificity of chitinase

The enzyme was highly active for substrates such as colloidal chitin, glycol chitin, chitosan and colloidal chitosan. This suggests that the chitin-binding domain possesses a wide range of binding capability for various chitinous substrates, which is similar to the chitinases

Table 2. Properties of some bacterial chitinases[a].

Sources	Molecular mass (kDa)	Optimum pH	Optimum temp. (°C)	Thermal stability (°C, min)	Substrate	References
Bacillus licheniformis MB-2	67 (SDS, GF)	6.0	70	60, 80	$(MU\text{-}(GlcNAc)_2$	This study
Streptomyces. thermoviolaceus OPC-520	30 (Chi30, SDS)	4.0	60	60, 30	Glycol chitin	Tsujibo *et al.* (2000)
Enterobacter sp. G-1	60 (SDS)	7.0	40	< 50, ni	Colloidal chitin	Park *et al.* (1997)
Pseudomona. aeruginosa K-187	30 (F1, SDS); 32 (F2, SDS)	8.0 (F1) ; 7.0 (F2)	50 (F1); 40 (F2)	50, 10 (F1 and F2)	Colloidal chitin	Wang & Chang (1997)
Bacillus MH-1	71 (L, SDS); 62 (M, SDS); 53 (S, SDS)	6.5 (L); 5.5 (M & S)	75 (L & S); 65 (M);	80, 10 (L & S); 70, 10 (M)	$PNP\text{-}(GlcNAc)_2$	Sakai *et al.* (1998)
Streptomyces RC1071	70 (GF)	8.0	40	60, 60	$(MU\text{-}(GlcNAc)_3$	Gomes *et al.* (2001)
Bacillus sp. NTCU2	36.5 (SDS)	7.0	50-60	60, > 30	Colloidal chitin	Wen *et al.* (2002)

[a] The molecular size is determined by SDS, sodium dodecyl sulphate-polyacrylamide gel electrophoresis and GF, gel filtration. ni – no information.

from *Bacillus* strain MH-1 (Sakai *et al*. 1998). This is in agreement with fact that the enzyme activity was reduced in the presence of DDT, since the typical chitin-binding domain has a consensus sequence of $CX_{11}CX_5CX_9CX_{12}CX_7C$ and the cysteines are connected by S-S- bridges (Hayashi *et al*. 1995). Chi-67 was moderately active towards regenerated chitin, chitin powder and methyl cellulose. Chi-67 degraded chitosan at a rate of about 67% as compared to the colloidal chitin. This activity against chitosan may be due to the activity toward the GlcNAc-GlcN linkage and not to the activity of hydrolysing GlN–GlN linkage retained in chitosan, as the chitosan used was 80% deacetylated.

N-terminal amino acid sequence

The N-terminal amino acid sequence of Chi-67 was determined as SGKNYKIIGYYPS. The sequence was compared with the SwissProt protein database and is identical with chitinases from *B. licheniformis* (accession no AA022144) and *B. circulans* (AAF23368).

Acknowledgements

This work was supported by a DAAD scholarship to Aris Toharisman (2003), by the Department of Zoology and Endocrinology, University of Ulm, Germany, as well as a Research Grant from Bioproducts Research Center Yonsei University, Korea.

References

Fukamizo, T. 2000 Chitinolityc enzymes: catalysis, substrate binding, and their application. *Current Protein and Peptida Sciences* **1**, 105–124.

Gomes, R.C., Semedo, L.T.A.S., Soares, R.M.A., Linhares, L.F., Ulhoa, C.J., Alviano, C.S. & Coelho, R.R.R. 2001 Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. *Journal of Applied Microbiology* **90**, 653–661.

Hayashi, K., Sato, S., Takanom R., Tsujibo, H., Orikoshi, H., Imada, C., Okami, Y., Inamori, Y. & Hara, S. 1995 Identification of the positions of disulfide bonds of chitinase from a marine bacterium, *Alteromonas* sp. Strain O-7. *Bioscience Biotechnology and Biochemistry* **59**, 1981–1982.

Laemmli, U.K. 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.

Molano, J., Duran, A. & Cabib, E. 1977 A rapid and sensitive assay for chitinase using tritiated chitin. *Analytical Biochemistry* **83**, 648–656.

Muzzarelli, R.A.A. 1996 Chitosan-based dietary foods. *Carbohydrate Polymers* **29**, 309–316.

Park, J.K., Morita, K., Fukumoto, I., Yamasaki, Y., Nakagawa, T., Kamamukai, M. & Matsuda, H. 1997 Purification and characterization of the chitinase (ChiA) from *Enterobacter* sp. G-1. *Bioscience Biotechnology and Biochemistry* **61**, 684–689.

Roberts, W.K. & Selitrenikoff, C.P. 1988 Plant and bacterial chitinases differ in antifungal activity. *Journal of General Microbiology* **134**, 169–176.

Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., & Moriguchi, M. 1998 Purification and characterization of three thermostable endochitinases of a noble *Bacillus* strain, MH-1, isolated from chitin-containing compost. *Applied and Environmental Microbiology* **64**, 3397–3402.

Shaikh, S.A. & Deshpande, M.V. 1993 Chitinolytic enzymes: their contribution to basic and applied research. *World Journal of Microbiology and Biotechnology* **9**, 468–475.

Spindler, K.D. 1997 Chitinase and chitosanase assays. In *Chitin Handbook*. pp. 229–235, eds. Muzzarelli, R.A.A. & Peter, M.G. European Chitin Society, Italy: Grottammare. ISBN: 88-86889-01-1.

Spindler, K.D. & Rapp, C. 1997 Detection of chitin degrading enzymes on gels. In *Chitin Handbook*. pp. 243–247, eds. Muzzarelli, R.A.A. & Peter, M.G. European Chitin Society, Italy: Grottammare. ISBN: 88-86889-01-1.

Spindler, K.D. & Buchholz, F. 1988 Partial characterization of chitin degrading enzymes from two euphausiids, *Euphausia superba* and *Meganyctiphanes norvegica*. *Polar Biology* **9**, 115–122.

Takayanagi, T., Ajisaka, K., Takiguchi, Y. & Shimahara, K. 1991 Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. *Biochimica et Biophysica Acta* **1078**, 404–410.

Trudel, J. & Asselin, A. 1989 Detection of chitinase activity after polyacrylamide gel electrophoresis. *Analytical Biochemistry* **178**, 362–366.

Tsujibo, H., Hatano, N., Endo, H., Miyamoto, K. & Inamori, Y. 2000 Purification and characterization of a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520 and cloning of the encoding gene. *Bioscience Biotechnology and Biochemistry* **64**, 96–102.

Ueda, M., Shiro, M., Kawaguchi, T. & Arai, M. 1996 Expression of the chitinase III gene of *Aeromonas* sp. No. 10S-24 in *Escherichia coli*. *Bioscience Biotechnology and Biochemistry* **60**, 162–164.

Wang, S.L. & Chang, W.T. 1997 Purification and characterization of two bifunctional chitinases/lysizymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Applied and Environmental Microbiology* **63**, 380–386.

Wen, C.M., Tseng, C.S., Cheng, C.Y. & Li, Y.K. 2002 Purification, characterization and cloning of a chitinase from *Bacillus* sp. NCTU2. *Biotechnology and Applied Biochemistry* **35**, 213–219.