Identification of chitin binding proteins and characterization of two chitinase isoforms from *Vibrio alginolyticus* 283

Wipa Suginta *

School of Biochemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

Received 18 September 2006; received in revised form 21 December 2006; accepted 3 January 2007

Abstract

This study reports the isolation of chitin binding proteins secreted from a highly chitinase producing bacterium, *Vibrio alginolyticus* strain 283. Tryptic peptide mass analysis by HPLC–ESI/MS identified four proteins that bound specifically to chitin. Submission of mass fingerprinting data for database search identified the 90, 65, and 47 kDa proteins as chitinases. On the other hand, the 38 kDa protein was compatible with a sugar-inducible porin. The 90 and 65 kDa proteins, later designated Chi-90 and Chi-65 respectively, were further co-purified using Sephacryl 200 HR gel filtration chromatography and their enzymatic properties relatively studied. Kinetically, Chi-65 displayed 2.3 folds greater catalytic efficiency (k_{cat}/K_m) towards *p*NP-diNAG than Chi-90. Investigation of the chitinase activity as a function of pH revealed that both enzymes worked best at pH of 6.5. At this pH, Chi-65 revealed almost seven folds higher activity than Chi-90. The similarity in peptide mass fingerprinting data, together with the equivalence of the optimal pH value and the resemblance in their hydrolytic patterns towards soluble chitooligosaccharides and insoluble chitin suggested that Chi-65 may be derived from the Chi-90 precursor.

Keywords: Chitin binding proteins; Chitinases; *Vibrio alginolyticus* 283; Thin layer chromatography; Chitooligosaccharide; Chitin

1. Introduction

Chitin, a linear β-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc or G1), is one of the three most abundant polysaccharides in nature, in addition to cellulose and starch. Chitin is found naturally in the shells of crustaceans, such as crab, shrimp and lobster, as well as in the exoskeleton of marine zoo-plankton, including coral and jellyfish. Insects, such as butterflies and ladybugs, have chitin in their wings and the cell walls of yeast, mushrooms and other fungi also contain this natural polymer. In oceans, insoluble chitin is an important nutrient source for maintaining the ecosystem in the marine environment. Although 10^{11} metric tonnes are produced annually in the aquatic biosphere alone, there is no substantial accumulation of chitin in ocean sediments [1]. This is because a bioconversion process is naturally driven by chitinolytic marine bacteria, especially *Vibrio* species [2,3]. These bacteria utilize chitinous materials very efficiently by converting them into organic compounds that then can be used as nitrogen and carbon sources. The enzymes that directly perform chitin degradation are located extracellularly, in the cell envelope, the periplasmic space, the inner membrane and the cytoplasm of the marine bacteria. They can be divided into two major groups, which are the chitinases (EC 3.2.1.14) and the β-*N*-acetylglucosaminidases (GlcNAcases; EC 3.2.1.30). Chitinases hydrolyze chitin to soluble oligosaccharides, mainly *N*,*N*′-diacetylchitobiose (G2), which is further hydrolyzed to G1 by GlcNAcases. The short-chain hydrolytic products, both G1 and G2, are then taken up by the bacterial cells initially through an outer membrane protein (chitoporin) [4], followed by specific cytoplasmic membrane transporters [5–7]. The catabolic cascade of chitin utilization by marine bacteria has been suggested to comprise a large number of genes and proteins [8] and is controlled by complex signal transduction systems [4] that influence important steps such as: (1) chitin sensing, (2) substrate attachment, (3) chitin degradation into oligosaccharide fragments, (4) transport of the chitooligosaccharides, mainly G2, to the cytoplasm, and (5) catabolism of the transport products to fructoses-phosphate, acetate and NH_3.

Abbreviations: Gn, β-1-4 linked oligomers of GlcNAc residues where $n = 1–6$; *p*NP-$(GlcNAc)_2$, 4-nitrophenyl *N*,*N*′-diacetyl-β-D-chitobioside; TLC, thin-layer chromatography; HPLC–ESI/MS, high performance liquid chromatography electrospray mass spectrometry

* Tel.: +66 44 22 4313; fax: +66 44 22 4193.
E-mail address: wipa@sut.ac.th.

0141-0229/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2007.01.006

Chitin and its derivatives have many properties that make them very attractive for applications in food and nutrition technology, cosmetics, biomedicine, agriculture as well as environmental ecology [9]. Antibacterial, anti-fungal and anti-viral activities, together with their highly biocompatible quality, make the chitin derivatives particularly useful for biomedical applications, such as wound healings, cartilage tissue engineering, drug delivery, and nerve generation [10–13]. Chitin's biodegradable and anti-fungal properties are also useful for environmental and agricultural uses [14–16]. Chitooligosaccharides, which are the sugar intermediates released during chitin hydrolysis, also are pharmaceutically important. For example, G1 is known as an effective anti-inflammatory agent [17] or glucosamine acts as an anti-tumor drug [18].

Because of the immense economic potential behind chitin exploitation, many attempts have been made to efficiently convert this insoluble polysaccharide to commercially valuable products using chitinases as a highly selective bioengineering tool [17,19]. We previously reported isolation of the *chitinase A* gene from *V. carchariae* [20] and the enzymatic properties of the gene product (chitinase A) have been studied extensively [21]. Based on a chitinase activity screening of 12 *Vibrio* species, *Vibrio alginolyticus* strain 283 was found to secrete the highest level of chitinases with double the activity of *V. carchariae* after 3 days of growth [3]. An immunoblotting that displayed the cross-reactivity of several secreted components with anti-chitinase A polyclonal antibodies prepared from chitinase A antigen of *V. carchariae* strongly suggested that *V. alginolyticus* 283 accumulated more than one chitinolytic enzymes for a competent action on chitin. In the present study, we aim at identifying the important elements that are responsible for the effectiveness of *V. alginolyticus* 283 in chitin exploitation. Chitin binding proteins that are potentially important in chitin consumption were isolated from the bacterium using chitin affinity chromatography and identified using HPLC–ESI/MS. Two of the identified proteins were subsequently purified and displayed significant activity towards *p*NP-glycoside, chitooligosaccharides and chitin. The kinetic data and the effect of pH on the chitinase activity are discussed along with TLC analysis that displayed the hydrolytic patterns of the isolated enzymes towards chitin oligosaccharides.

2. Materials

2.1. Bacterial strains and chemicals

The marine bacterium *V. alginolyticus* strain 283 (LMG4408T) was a gift from Dr. Peter Robertson, Department of Biological Sciences, Heriot Watt University, Edinburgh, UK. All chemicals and reagents were of analytical grade and purchased from the following sources: reagents for bacterial media were from Scharlau Chemie S.A. (Barcelona, Spain); flake chitin (crab shell), chitooligosaccharides and *p*NP-$[GlcNAc]_2$ were from Sigma–Aldrich Pte., Ltd. (Citilink, Warehouse Complex, Singapore); SDS/PAGE chemicals were from Amersham Pharmacia Biotech Asia Pacific Ltd. (Bangkok, Thailand) and from Sigma–Aldrich Pte., Ltd.; Sephacryl S200HR resin was from Amersham Biosciences (Piscataway, NJ, USA); and chemicals and reagents for protein preparation were from Sigma–Aldrich Pte., Ltd. and from Carlo Erba Reagenti (Milan, Italy). Aluminum sheets (Silica gel $6OF_{254}$, 20 cm × 20 cm) for thin-layer chromatography (TLC) were products of Merck Co. (Berlin, Germany).

3. Methods

3.1. Screening on chitin agar plates

The overnight bacterial culture was stabbed into agar plates prepared from marine medium 2216E [22] in the presence of 3.0% (w/v) colloidal chitin prepared according to Hsu and Lockwood [23]. The plates were incubated for 3 days at 30 °C, and a clear zone around the stab sites showed the presence of chitinase activity. The plates could be stored after the initial incubation for up to 4 weeks at 4 °C, during which time the zone became clearer and larger.

3.2. Preparation of chitin binding proteins by chitin affinity chromatography

An overnight culture (10 mL marine medium, 30 °C) from a single colony of *V. alginolyticus* 283 was used to inoculate three flasks, each containing 500 mL of marine medium with colloidal chitin (2.5%, w/v). After about 72 h of incubation at 30 °C, the cells and chitin were removed by centrifugation (2795 × *g*, 15 min, 4 °C), and the supernatant (fraction GM, growth medium) was collected. A further 20 g of colloidal chitin was added to the suspension, stirred at 4 °C for 60 min, and then centrifuged for 20 min (2795 × *g*, 4 °C). The sample of precipitated chitin and bound proteins were combined, and washed by centrifugation with 100 mM sodium carbonate buffer, pH 8.5, until the A_{280} was less than 0.1, followed by washing with 100 mM sodium acetate buffer, pH 5.5, until the A_{280} was ca. 0. Guanidine HCl (2 M, 50 mL) was added and the suspension stirred at 4 °C for 30 min. Then, the chitin was removed by centrifugation (2795 × *g*, 30 min, 4 °C) and the guanidine-eluted proteins were dialyzed extensively overnight against 20 mM sodium phosphate buffer, pH 7.0 (fraction CA, chitin affinity).

3.3. Co-purification of Chi-90 and Chi-65

The dialyzed CA fraction obtained from chitin affinity step was precipitated overnight by 0–70% saturated ammonium sulfate and collected by centrifugation (16,099 × *g*, 45–60 min, 4 °C), and then dissolved in 2.0 mL of 20 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl. This solution was applied to a Sephacryl S200 HR (1.5 cm × 120 cm) column under the same buffer system. Fractions that gave high A_{280} peaks were analyzed on SDS/PAGE for purity confirmation. The fractions that contained the 90-kDa band were pooled separately from the fractions that contained the 65-kDa band. These two fractions, later designated as Chi-90 and Chi-65, respectively, were immediately subjected to chitinase activity assay and biochemical characterization or stored at −30 °C until used. A final concentration of the protein was determined by Bradford's method [24] using a standard calibration curve constructed from BSA (0–25 μg).

3.4. Chitinase activity assay

Chitinase activity was determined in a 96-well microtiter plate and a 100-μL assay mixture contained protein sample (10 μL), 1 mM *p*NP-$(GlcNAc)_2$ (25 μL), and 100 mM sodium acetate buffer, pH 5.0 (65 μL). The reaction mixture was incubated at 37 °C for 10 min with constant agitation, then the enzymatic reaction was terminated by the addition of 50 μL 1.0 M Na_2CO_3. The amount of *p*-nitrophenol (*p*NP) released was determined spectrophotometrically at 405 nm in a microtiter plate reader (Applied Biosystems, Foster City, CA, USA). The molar concentrations of *p*NP were calculated from a calibration curve constructed with *p*NP concentrations varying from 0 to 30 nmol.

3.5. SDS/PAGE following immunoblotting

Antisera against chitinase A were prepared with the purified chitinase A isolated from *V. carchariae* as described previously [3]. The purified proteins (2 μg) were electrophoresed on a 12% SDS/PAGE gel following the Laemmli system [25], and subsequently transferred onto a nitrocellulose membrane using Trans-Blot® Semi-Dry Cell (Bio-Rad Laboratories Ltd., Bangkok, Thailand). Immunodetection was carried out using enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech Asia Pacific Ltd.,

Thailand), according to manufacturer's instructions. The primary antibody was anti-chitinase A polyclonal antibody (1:2500 dilution) and the secondary antibody was anti-rabbit IgG conjugated with horse radish peroxidase (1:5000 dilution).

3.6. Protein identification and peptide mass analysis by HPLC–ESI/MS

The protein bands from SDS gels (see above) were excised, destained, reduced, alkylated with iodoacetamide and digested with Promega sequencing-grade trypsin (Bio-Active Co., Ltd., Bangkok, Thailand) following a standard protocol [26]. After overnight digestion at 37 °C, the peptides were extracted and dried in a SpeedVac vacuum centrifuge, and then separated by a C_{18} column connected to an Agilent 1100 HPLC using a 0–40% linear gradient of acetronitrile containing 0.1% acetic acid, then detected directly by a Thermo Finnigan LCQ Deca electrospary ionization mass spectrometer (ESI/MS). The obtained masses (*m/z* 500–2000) were subjected to "Sequest search" (http://fields.scripps.edu/sequest/index.html) for protein identification.

3.7. N-terminal protein sequence analysis

The purified Chi-90 and Chi-65 were applied on a 12% SDS-PAGE gel, which was pre-run overnight, then transferred on to BioTrace™ polyvinylidene fluoride (PVDF) membrane (Pall Corporation, Pensacola, FL, USA) for 30–45 min with a constant voltage of 20 V using Trans-Blot® Semi-Dry Cell (Bio-Rad Laboratories Ltd., Bangkok, Thailand). The blotted membrane was thoroughly washed with deionized water for 5 min, and then stained with a freshly prepared 0.1% Coommassie blue. After destaining with methanol:glacial acetic acid:water (50:10:40, v/v), the protein bands of interest were excised and subjected to Edmand degradation reaction for determination of the N-terminal amino acids using automatic amino acid sequencer (Scientific Equipment Center, Prince of Songkla University, Songkla, Thailand).

3.8. Kinetic measurements

Kinetic studies of Chi-90 and Chi-65 were performed by colorimetric assay in a microtiter plate reader (Applied Biosystems, USA). The reaction mixture (100 μL), containing 0–500 μM *p*NP-(G1cNAc)$_2$, dissolved in 100 mM sodium acetate buffer, pH 5.0, and dH_2O, was pre-incubated at 37 °C for 10 min. After the enzyme (400 ng) was added, the reaction was continued for additional 10 min at 37 °C, and then the reaction was terminated with 50 μL of 1 M Na_2CO_3. Release of *p*-nitrophenol (*p*NP) was monitored at A_{405}, which was subsequently converted to molar quantities using a calibration curve of *p*NP (0–30 nmol). The kinetic values (K_m, V_{max}, and k_{cat}) were evaluated from three independent sets of data by a nonlinear regression function in the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

3.9. Effects of pH on chitnase activity

The hydrolytic activity of Chi-90 and Chi-65 towards *p*NP-[GlcNAc]$_2$ was investigated at a broad pH range from 3.0 to 10.0. To maintain the buffering capacity at a particular pH, different buffer systems were used as follows: 100 mM potassium phosphate buffer for pHs 3.0 and 4.0; 100 mM sodium acetate buffer for pHs 4.5, 5.0, and 5.5; 100 mM MES buffer for pH 6.0; 100 mM sodium phosphate buffer for pHs 6.5 and 7.0; 100 mM Tris/HCl for pHs 8.0 and 9.0; and 100 mM CAPS buffer for pH 10.0. Each reaction was carried out under the same conditions as described for the standard chitinase assay. Release of *p*NP was quantified using the same standard curve.

3.10. Product analysis by thin-layer chromatography

Hydrolysis of chitooligosaccharides (G2-G6) by Chi-90 and Chi-65 was carried out in a 20-μL reaction mixture, containing 100 mM sodium acetate buffer, pH 5.0, 2.5 mM substrate and 200 ng purified enzyme. The reaction was incubated at 37 °C with shaking for 0, 2.5, 5, 10, 30, 60 min and overnight, and then terminated by boiling for 5 min. For product analysis, 5 μL of each reaction mixture was applied slowly to a silica TLC plate (5.0 cm × 6.0 cm), and then chromatographed twice (30 min each) in a mobile phase containing *n*-butanol:methanol: 30% ammonia solution:H_2O (10:8:4:2) (v/v), followed by spraying with aniline–diphenylamine reagent and baking at 120 °C for 5–10 min [27]. The TLC analysis of the hydrolytic products generated from colloidal chitin was carried out as described for chitooligosaccharide hydrolysis with a single time point of 60 min.

4. Results

4.1. Isolation of chitin binding proteins and chitinase expression

We previously reported expression levels of chitinases from twelve strains of marine *Vibrios* [3]. It was found that *V. alginolyticus* strain 283 secreted highest level of chitin hydrolyzing enzymes to the growth medium containing chitin. When the crude enzymes were prepared by this bacterium, a number of proteins were found to cross-react with anti chitinase A polyclonal antibodies prepared from chitinase A antigen of *V. carchariae*. In the present study, we employed chitin affinity chromatography to obtain chitin binding proteins that may potentially involve in chitin degradation and chitin uptake of this bacterium. Fig. 1A shows the clear zones that appeared around four stabbed sites of the *V. alginolyticus* 283 after a prolonged storage of the chitin plate for 4 weeks at 4 °C. The result re-confirmed the production of the chitinolytic enzymes by *V. alginolyticus* 283. After the bacterium was grown in chitin-containing broth for 72 h at 30 °C, the growth medium was collected and applied batch-wise to chitin resin. As seen on SDS/PAGE (Fig. 1B), four major chitin binding proteins specifically eluted from chitin resin by 2 M guanidine HCl.

The tryptic peptides of these proteins prepared as mentioned in Methods were resolved on a C_{18} column and analyzed by electrospray MS. The peptide mass data obtained from HPLC–MS was subjected for the NCBI non-redundant protein database search. The Sequest search results showed that four peptides from the 90-kDa and from 47-kDa bands, and three peptides from the 65-kDa band were identical to a chitinase from *V. parahaemolyticus* RIMD 2210633 (accession number 28899910) (Table 1). In contrast, two tryptic peptides of the 38-kDa band were compatible with a maltose-inducible porin from *Aeromonas salmonicida* (accession number 398211).

4.2. Purification of Chi-90 and Chi-65

An attempt was made to purify all the chitin binding proteins using various types of chromatography, however with partial success. We could not obtain the 47 and 38 kDa proteins for further functional characterization. However, when Sephacryl S200 HR gel filtration was used, plotting the A_{280} values of individual fractions versus the fraction number revealed two peaks with the earlier peak having maximum absorbance at fraction 37 and the latter peak at fraction 46 (Fig. 2A). Fractions 36–38 were pooled as pool 1 (P1), while fractions 45–47 were pooled as pool 2 (P2). With SDS-PAGE analysis, P1 gave a single band of 90 kDa and P2 of 65 kDa (Fig. 2B) and according to their resultant masses the corresponding proteins were designated as Chi-90 and

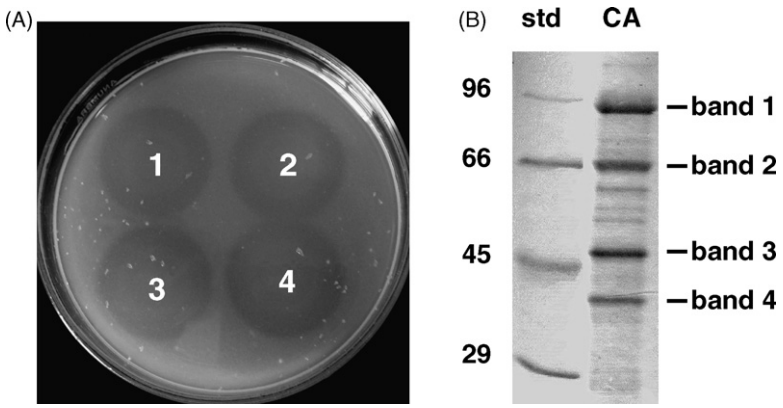

Fig. 1. Identification of chitin binding proteins from *V. alginolyticus* 283. (A) Expression of chitin hydrolyzing enzymes from *V. alginolyticus* 283. Numbers indicate stab sites of the bacterium grown on a chitin agar plate. After overnight incubation at 30 °C, the development of the clear zones was observed at day 30 at 4 °C. (B) SDS/PAGE analysis of chitin binding proteins. The growth medium prepared from a 1.50-L culture of *V. alginolyticus* 383 was stirred in a beaker with colloidal chitin for 60 min at 4 °C. After washing, chitin binding proteins (CA fraction) were eluted with 2 M guanidine HCl, dialyzed thoroughly, and then analyzed on a 12% SDS/PAGE.

Table 1
Chitin binding proteins identified by HPLC–ESI/MS

Band number (#)	Monoisotopic mass (MH+)	Charge, *Z*	Peptide sequence	Identified protein	Accession number
1. 90 kDa	1404.68	3	R. TTGELITFDDHR. S	Chitinase from *Vibrio parahaemolyticus* RIMD 2210633	28899910
	1724.81	2	K. GSTAQGVWEDGVIDYK .G		
	2148.98	2	K. WWTQGDDPSKSGEWGVWK .E		
	2186.97	2	K. FYDGVDIDWEFPGGGGAAADK .G		
2. 65 kDa	1561.77	2	K. SFPQAGHEYSTPIK .G	Chitinase from *V. parahaemolyticus* RIMD 2210633	28899910
	1404.68	2	R. TTGELITFDDHR .S		
	1724.81	2	K. GSTAQGVWEDGVIDYK .G		
3. 47 kDa	1561.77	2	K. SFPQAGHEYSTPIK .G	Chitinase from *V. parahaemolyticus* RIMD 2210633	28899910
	1404.68	2	R. TTGELITFDDHR .S		
	1516.75	2	R. TYELTSAIGVGYDK .I		
	1724.81	2	K. GSTAQGVWEDGVIDYK .G		
4. 38 kDa	1345.66	2	K. TVLQYGTEGYSK .T	Maltose-inducible porin from *Aeromonas salmonicida*	398211
	864.47	2	K. VSFAWVR .T		

Peptide fragments from gel bands 1–4 (see Fig. 1b) were identified by exact mass analysis using HPLC–ESI/MS and by database searching. Monoisotopic masses of the identified bands gave highest scores in the FASTA search.

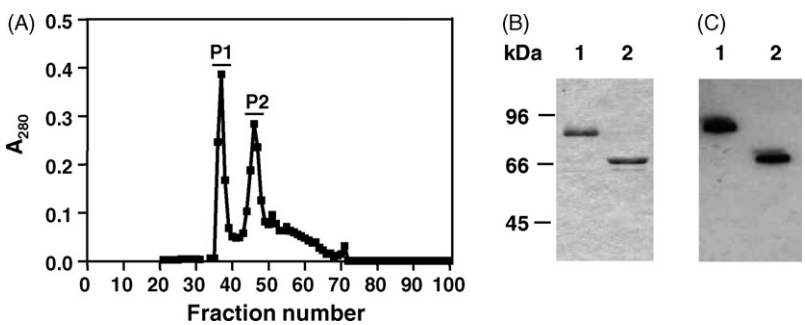

Fig. 2. Purification of two chitinase isoforms. (A) A plot of A_{280} vs. fraction number obtained from Sephacryl S200 HR chromatogrpahy. Fractions 36–38 were pooled as pool 1 (P1), while fractions 45–47 were pooled as pool 2 (P2). (B) P1 and P2 were electrophoresed on a 12% SDS/PAGE gel, and stained with Coommassie blue. (C) Immunoblotting of P1 and P2 using anti-chitinase A polyclonal antibodies and anti-IgG conjugated with horse radish peroxidase as primary and secondary antibodies, respectively. Lane 1: 10 μL of P1; lane 2: 10 μL of P2.

Table 2
Purification of the 90 and 65 kDa chitinases from *V. alginolyticus* 283

Purification step	Total protein (mg)	Total activity (U)[a]	Specific activity (U mg^{-1})	Fold purification	Activity recovery (%)
Growth medium (GM)	2054.0	142.76	0.07	–	100
Chitin affinity (CA)	29.5	5.40	0.18	2.6	3.8
Sephacryl 200 (S200)					
Pooled 90 kDa fractions (P1)	1.05	0.68	0.65	9.3	0.48
Pooled 65 kDa fractions (P2)	1.45	1.47	1.01	14.4	1.03

V. alginolyticus 283 was grown in a 1.5-L marine medium, containing 3% (w/v) colloidal chitin at 30 °C. After 72 h of incubation, the growth medium was collected and applied batch-wise to chitin resin. The chitin affinity fraction (CA) was ammonium sulfate precipitated, then further purified by gel filtration using Sephacryl S200-HR column. Fractions containing a single protein band of 90 kDa were pooled as P1 and fractions containing a single protein band of 65 kDa were pooled as P2.

[a] One unit is defined as 1 μmol of *p*NP released from *p*NP-[GlcNAc]$_2$ per minute at 30 °C.

Chi-65. The molecular weights of Chi-90 and Chi-65 as determined on SDS/PAGE were equivalent to the ones determined by gel filtration, indicating that both proteins are monomeric proteins.

Additional analysis using immunological technique revealed a strong cross-reactivity of Chi-90 and Chi-65 with anti-chitinase A polyclonal antibodies prepared from *V. carchariae* chitinase A antigen (Fig. 2C). This verified that the purified proteins were indeed chitinase homologues.

Table 2 represents the purification of Chi-90 and Chi-65 using chitin affinity chromatography, followed by Sephacryl S200 HR gel filtration. Based on the applied protocol, the specific hydrolyzing activity of the purified proteins was increased, giving 9.3- and 14.4-fold purification for Chi-90 and Chi-65 after gel filtration step. The final yield obtained from a 1500-mL culture was 1.05 mg of highly purified Chi-90 and 1.45 mg of highly purified Chi-65.

4.3. Steady state kinetics and the effect of pH on Chi-90 and Chi-65 activities

When *p*NP-[GlcNAc]$_2$ was the substrate, Chi-90 displayed a specific activity of 0.69 nmol *p*NP min^{-1} μg^{-1} protein. This value was 4.2 times less than the specific activity of Chi-65 (2.91 nmol *p*NP min^{-1} μg^{-1} protein). The kinetic parameters of Chi-90 and Chi-65 were further investigated at varied concentrations of *p*NP-[GlcNAc]$_2$. A comparison of data revealed that Chi-90 displayed higher affinity of binding to the *p*NP glycoside than Chi-65 by having a two times lower K_m value (0.34 mM) compared to that of Chi-65 (0.67 mM) (Table 3). In contrast, Chi-65 exposed a 4.5 times higher rate of catalysis (k_{cat}) than the one obtained with Chi-90. Thus, the overall catalytic efficiency (k_{cat}/K_m) of Chi-65 was about 2.3 times of that of Chi-90.

Table 3
Kinetic parameters of the hydrolytic activity of Chi-90 and Chi-65 towards *p*NP-[GlcNAc]$_2$ substrate

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Chi-90	0.34 ± 0.07	1.65 ± 0.19	4.81×10^3
Chi-65	0.67 ± 0.06	7.41 ± 0.47	1.11×10^4

The 100-μL reaction mixture contained 0–500 μM *p*NP-(G1cNAc)$_2$, 100 mM sodium acetate buffer, pH 5.0 and 400 ng enzyme. The reaction was carried out for 10 min at 37 °C, and then terminated with 50 μL of 1 M Na_2CO_3. Release of *p*NP was monitored at A_{405}, which was converted to molar quantities using a *p*NP calibration curve. The kinetic values were estimated from three separate data sets using a nonlinear regression function in the GraphPad Prism software.

The effects of pH on the hydrolytic activity of Chi-90 and Chi-65 were examined within a broad pH range from pH 3.0 to 10 (data not shown). It was observed that both enzymes displayed an increase in their activity when the pH of the reaction mixture was raised. At pH 6.5 the maximum of enzyme activities was reached and activities began to decline gradually upon further increase of the pH of the reaction buffers. At optimum pH, Chi-65 was considerably 6.7 times more active than Chi-90.

4.4. Product analysis of the hydrolytic activity of Chi-90 and Chi-65 using TLC

To assess the ability and efficiency of the isolated enzymes to degrade their natural substrates, the hydrolytic products formed in the course of their action on chitin and chitooligosaccharide were analyzed via TLC. Up to 60 min of incubation, there were no hydrolytic products detected in reaction mixtures containing G2 and Chi-90 or Chi-65 (not shown). This strongly indicated that G2 was not a substrate of both enzymes. Fig. 3A and B are the TLC patterns showing the reaction intermediates formed from G3 as a function of time. It can be seen that even at 60 min of incubation, Chi-90 did not hydrolyze G3 at all, whereas G2 produced by Chi-65 appeared already at 30 min of reaction. G1 and G2 were more clearly seen at 60 min in the reactions performed with Chi-65. Although at different rates, both enzymes obviously hydrolyzed G4 (Fig. 3C and D) and again G2 was the end product with products of hydrolysis detected at 10 min of reaction incubation for Chi-90 but as early as 2.5 min for Chi-65. At 60 min, Chi-65 almost completely processed its substrates, whilst substantial amounts of G4 still remained in the reaction of Chi-90 at this point in time. Similar findings were observed when G6 was the substrate (Fig. 3E and F), where the reaction intermediates (mainly G2, G3, and G4) were detected again earlier in the case of Chi-65. At an hour of incubation, the reaction containing Chi-65 was complete, while the reaction by Chi-90 was still ongoing.

The hydrolytic activity of Chi-90 and Chi-65 was also assessed using chitin as the substrate. Fig. 4 displays the hydrolytic products formed at a single time point (60 min).

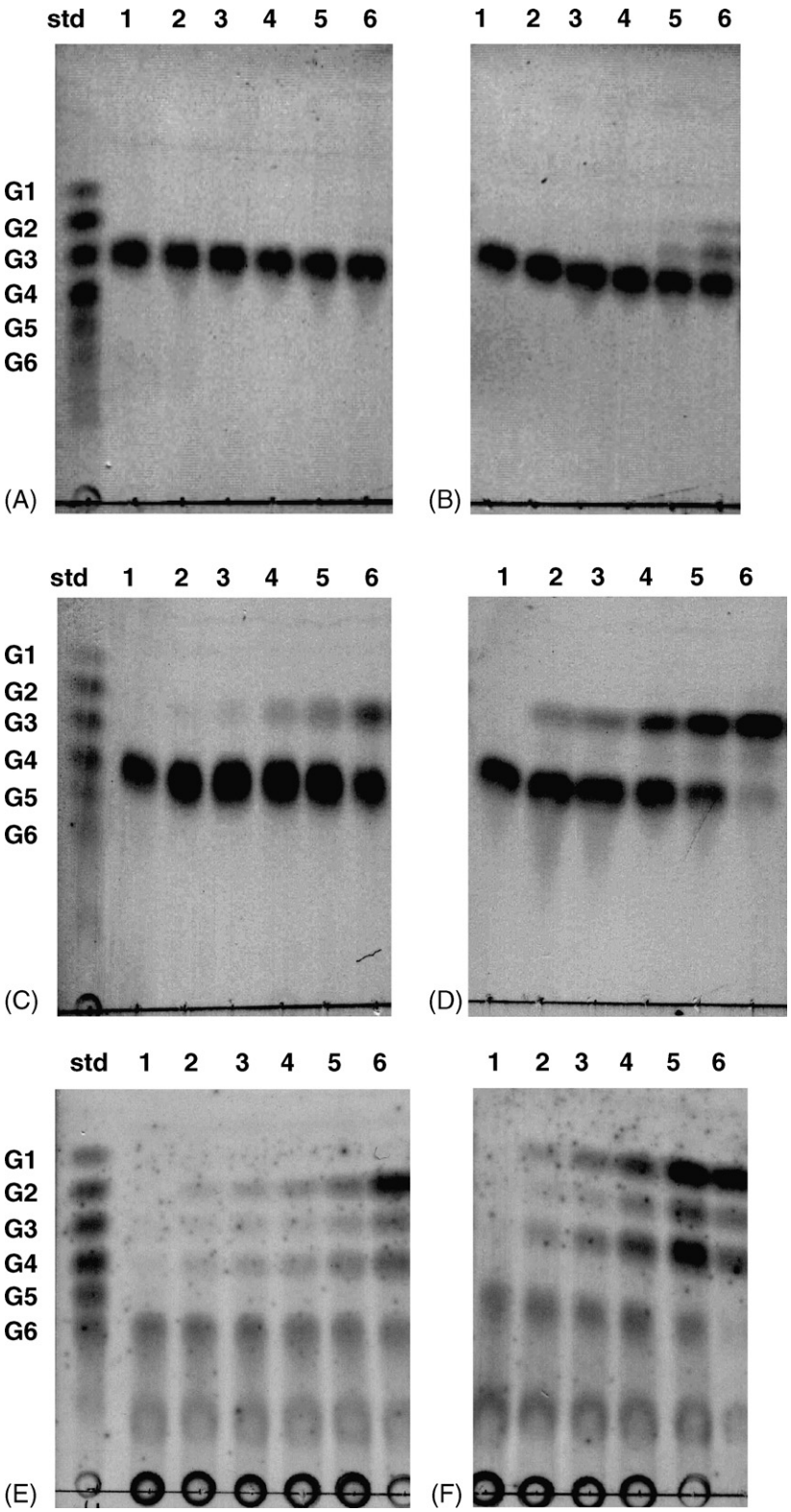

Fig. 3. Time course of chitiooligosaccharide hydrolysis of Chi-90 and Chi-65 as analyzed by TLC. A reaction mixture, containing 200 ng chitinase and 2.5 mM substrate in 100 mM sodium acetate buffer, pH 5.0, was incubated at 37 °C for varied times (0, 2.5, 5, 10, 30 and 60 min). After boiling, the reaction solution (5 μL) was analyzed on TLC and sugar products detected with aniline–diphenylamine reagent. (A and B) Hydrolysis of G3 by Chi-90 and Chi-65, (C) and (D) hydrolysis of G4 by Chi-90 and Chi-65, (E and F) hydrolysis of G6 by Chi-90 and Chi-65, respectively. Lane std, a standard mix of G1–G6; lanes 1–6, incubation times of 0, 2.5, 5, 10, 30, and 60 min.

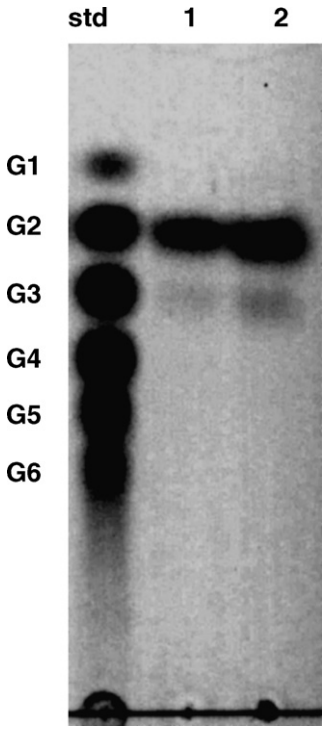

Fig. 4. TLC analysis of chitin hydrolysis by Chi-90 and Chi-65. A reaction mixture, containing 200 ng chitinase and 20 mg colloidal chitin in 100 mM sodium acetate buffer, pH5.0, was incubated at 37 °C for 60 min. After boiling, the reaction solution (5 μL) was analyzed on TLC and sugar products detected with aniline–diphenylamine reagent. Lane std: a standard mix of G1-G6; 1, hydrolytic products obtained from Chi-90; lane 2: hydrolytic products obtained from Chi-65.

It has been recognized that both enzymes hydrolyzed chitin polymer, also giving G2 as the major product. G3 was clearly found as an additional intermediate in the reaction of Chi-65 with chitin, while much less signal was seen in the reaction of Chi-90.

5. Discussion

Marine *Vibrios* are thought to play a pivotal role in the bioconversion of chitin, which is produced in a multi-million tonne quantity in the marine biosphere. The bacteria degrade chitin into small oligosaccharides, which can be metabolized and further used as a sole source of nitrogen and carbon. It has been suggested that chitin hydrolysis by these bacteria is a sequence of complex processes and engages a large number of chitin related genes [1]. A recent study by Meibom et al. [8] using DNA microarray technique identified 360 genes that were involved in regulating the growth of *V. cholerae* on chitin or chitin derivatives. We previously screened for chitinase activity from 12 species of *Vibrios* and found that, upon induction by chitin, *V. alginolyticus* strain 283 expressed highest levels of chitinases into the growth medium [3]. The cross-reactivity of a protein cluster produced by this bacterium with anti-chitinase A antibodies prepared from *V. carchariae* chitinase A antigen suggested that the bacterium secreted several chitin-degrading enzymes during growth in order to utilize the polymeric substrate most efficiently. In this study, we employed HPLC–MS to identify four proteins that specifically bound to chitin (see Fig. 1B and Table 1). It is noticeable that two of the identified peptides (TTGELITFDDHR and GSTAQGVWEDGVIDYK) were identical among the proteins of 90, 65 and 47 kDa, all unambiguously compatible with a database-search chitinase of *V. parahaemolyticus*. Like chitinases 52 and 35 kDa from *Serratia marcescens* KCTC2172 [28], these three proteins might be products of a single *chitinase* gene, which was later generated differentially during post-proteolytic modifications. Alternatively, they may be originated from different *chitinase* genes with overlapping open reading frames as described for *Alteromonas* sp. strain O-7 [29]. Employing the same proteomic tool, we also identified the smallest protein (38 kDa) to be a sugar-inducible porin. Although, to our best knowledge, only chitoporin from *V. furnissii* was isolated and preliminarily characterized [30], the basic function of this porin has been proposed to act as a specific channel allowing chitin-degradation products (mainly G1 and G2) to be transported into the bacterial cell for further metabolization. Although porins are proteins located in the outer membrane of Gram-negative bacteria, a prolonged period of cell growth (72 h) could possibly lead to the release of broken membranes of death/damaged cells to the growth medium. If the identified porin of *V. alginolyticus* 283, by all means, involved in chitooligosaccharide transport, the protein would be undoubtedly expected to form certain types of interactions with the GlcNAc moieties. Under such circumstances, it would explain the detection of the sugar-inducible porins, as part of the membrane components, in the chitin affinity fraction.

We have made attempts, with only partial success, to obtain all the chitin binding proteins. After various types of chromatography were tested, only Chi-90 and Chi-65 were co-purified when Sephacryl S200 HR gel filtration was used after chitin affinity chromatography. Although chitin affinity was shown to be the powerful isolating step, it appeared to bind so tightly to the proteins that the subsequent eluting step employing mild conditions was proved unsuccessful. We previously employed the chitin affinity technique to purify *V. carchariae* chitinase using several eluting conditions and eventually found that the proteins could only be eluted effectively from the resin with guanidine HCl [3] with 2 M tested to be optimal to yield highest activity. In this study, we applied the same condition as for the *V. carchariae* enzyme. However, it was found that only 3.8% activity was recovered after the removal of the guanidine HCl using dialysis technique. The relatively low yield reflects the instability of the enzyme under the condition of the guanidine HCl elution. After Sephacryl S-200 HR purification, two proteins, namely Chi-90 and Chi-65, have been identified in fractions that gave high A_{280} peaks (P1 and P2 in Fig. 2A and B) and they were found to be immunologically related, as judged by strongly interacting with anti-chitinase A polyclonal antibodies of *V. carchariae* chitinase A (Fig. 2C).

TLC analysis of the hydrolytic activity of Chi-90 and Chi-65 clearly revealed that G2 was not a substrate for both enzymes. This was not surprising since G2 is generally formed as the end product of the action of endochitinases. As expected, G3 was a poor substrate for Chi-65 and was not hydrolyzed at all by

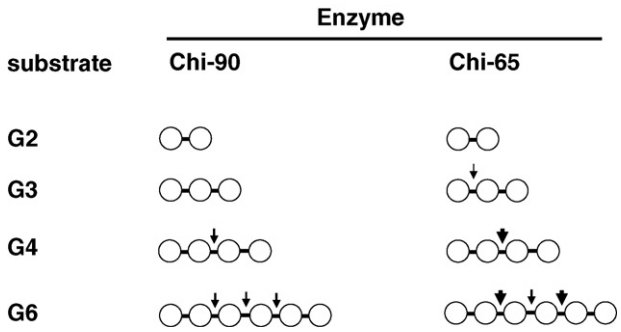

Fig. 5. A proposed action of Chi-90 and Chi-65 on chitooligosaccharides. Arrows indicate sites of cleavage. Thickness of the arrows represents the level of the product formation as observed by TLC.

the less active Chi-90. Previous reports on the 3D-structures of hevamine chitinase and *S. marcescens* chitinase A clearly suggested that the catalytic pocket of the enzymes comprised an array of at least six binding subsites [31,32]. If this would be also the case for Chi-90 and Chi-65, the binding affinity of the enzymes with G3 should not be strong because a full occupancy of binding would be only satisfied by six sugar moieties. The kinetic data of the highly related homologue, *V. carchariae* chitinase A, also suggested the higher affinity of binding with higher molecular weight substrates [21]. With a qualitative method of detection like TLC, we also observed that at equivalent time point of reaction, i.e. at 60 min, both enzymes showed a more complete degradation with longer chain substrates with the rate of degradation following the tendency G6 > G4 > G3 (see Fig. 3). In addition, a various range of oligomeric products were detected in the reaction of Chi-90 or Chi-65 with G6. For example, G2, G3, G4 and G5 were the reaction intermediates, indicating the endo characteristic of both chitinases, in which the enzymes will act randomly on internal sites of the glycosidic bonds. A proposed action of the two enzymes towards chitooligosaccharides is schematically presented in Fig. 5.

The endo activity of the two enzymes was also observed when colloidal chitin was a substrate. G2 and G3 were detected even when the reaction was incubated for time as long as 60 min.

When subjected to N-terminal amino acid sequence determination, data from definite 20 rounds of the Edman degradation could be interpreted with confidence for the Chi-65 polypeptide. The N-terminal sequence of this protein was A-P-Q-A-P-S-I-D-M-Y-T-S-N-N-L-Q-F-V-A-I. When this sequence was subjected to BLAST search, it showed highest sequence identity with the N-terminal sequences of a *chitinase* gene product of *V. alginolyticus* 12G01 (accession number Q1V7B6), a *chiK* gene product of *Vibrio* sp. (accession number Q32Y72), and chitinase A precursor of *V. carchariae* (*harveyi*) (accession number Q9AMP1). The latter sequence was a single chitinase isolated previously in our laboratory [20]. Under the same experimental conditions, only the first two amino acids from the N-terminus of Chi-90 were ambiguously identified as glutamine (Q) and glycine (G). Because of the retardation of the higher-M_r protein on SDS-PAGE gel, it was not possible to elute the protein band out of the gel, thus giving a low yield of the intact protein to be further analyzed. The non-similar properties of the two enzymes were proved by the differences in their kinetic values (K_m, k_{cat}, and k_{cat}/K_m) as shown in Table 3 and the influence of pH on their chitinase activity. Both enzyme displayed their maximal activity at pH 6.5 (see Fig. 3), however, different pH values that gave half maximal activity (pHs 4.2 and 8.5 for Chi-90 and pHs 3.5 and 7.6 for Chi-65) indicated that the two proteins possessed different ionizing groups that may be important for enzyme catalysis. Or, the two proteins may possess the relevant ionizing groups, but located in dissimilar hydrophobic environments in the catalytic cleft. At this point of study, it was not possible to judge whether the proteins were the products of the same gene or separate genes. However, taking into account that identical peptides were found for Chi-90 and Chi-65 and that the pH for optimum activity of the two were the same and the similar patterns of substrate hydrolysis, it is likely that Chi-65 may be derived from Chi-90 as a precursor. Following the cellular events of proteolytic cleavages and translational modifications, a more active chitinase (Chi-65) was created. It has been suspected that the 47 kDa chitinase, which was also identified in this study, could also have been created by a similar procedure. The rationale for the generation of several chitinases from a single gene would be making most efficient use of the surrounding chitin components with the least demand of cellular energy of the bacterial cells.

6. Conclusions

In this study, we employed HPLC–ESI/MS to identify four promising proteins that may extracellularly be involved in chitin utilization of the highly chitinase-producing bacterium, *V. alginolyticus* 283. Two proteins, identified as chitinases Chi-90 and Chi-65, were further purified and characterized. Partial identity of their peptide sequences, an identical pH optimum as well as similar hydrolytic patterns suggested that Chi-65 may be the proteolytic, active form of Chi-90 precursor.

Acknowledgements

This research was financially supported by a Suranaree University grant (grant number SUT1-102-47-24-22) to WS. The author would like to thank PD. Dr. Heino Prinz, Max-Planck-Institut fur Molekulare Physiologie, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany, for providing the HPLC/ESI-MS facilities and Mrs. Archara Kobdej for her excellent technical support.

References

[1] Keyhani NO, Roseman S. Physiological aspects of chitin catabolism in marine bacteria. Biochim Biophys Acta 1999;1473:108–22.
[2] Hirono I, Yamashita M, Aoki T. Note: molecular cloning of chitinase genes from *Vibrio anguillarum and V. parahaemolyticus*. J Appl Microbiol 1998;84:1175–8.
[3] Suginta W, Robertson PA, Austin B, Fry SC, Fothergill-Gilmore LA. Chitinases from *Vibrios*: activity screening and purification of chi A from *Vibrio carchariae*. J Appl Microbiol 2000;89:76–84.
[4] Li X, Roseman S. The chitinolytic cascade in *Vibrios* is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. Proc Natl Acad Sci USA 2004;101:627–31.

[5] Bouma CL, Roseman S. Sugar transport by the marine chitinolytic bacterium *Vibrio furnissii*. Molecular cloning and analysis of the glucose and *N*-acetylglucosamine permeases. J Biol Chem 1996;271:33457–67.
[6] Bouma CL, Roseman S. Sugar transport by the marine chitinolytic bacterium *Vibrio furnissii*. Molecular cloning and analysis of the mannose/glucose permease. J Biol Chem 1996;271:33468–75.
[7] Keyhani NO, Wang LX, Lee YC, Roseman S. The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. Characterization of an *N*,*N*′-diacetyl-chitobiose transport system. J Biol Chem 1996;271:33409–13.
[8] Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK. The *Vibrio cholerae* chitin utilization program. Proc Natl Acad Sci USA 2004;101:2524–9.
[9] Goosen MFA, editor. Applications of chitin and chitosan. Lancaster, PA, USA: Technomic Publishing; 1997. p. 336.
[10] Shi C, Zhu Y, Ran X, Wang M, Su Y, Cheng T. Therapeutic potential of chitosan and its derivatives in regenerative medicine. J Surg Res 2006;133:185–92.
[11] Yan J, Li X, Liu L, Wang F, Zhu TW, Zhang Q. Potential use of collagen-chitosan-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. Artif Cells Blood Substit Immobil Biotechnol 2006;34:27–39.
[12] Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Rel 2004;100:5–28.
[13] Ciardelli G, Chiono V. Materials for peripheral nerve regeneration. Macromol Biosci 2006;6:13–26.
[14] Lin CC, Lin HL. Remediation of soil contaminated with the heavy metal (Cd^{2+}). J Hazard Mater 2005;122:7–15.
[15] Galed G, Fernandez-Valle ME, Martinez A, Heras A. Application of MRI to monitor the process of ripening and decay in citrus treated with chitosan solutions. Magn Reson Imag 2004;22:127–37.
[16] Rabea EI, Badawy ME, Stevens CV, Smagghe G, Steurbaut W. Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules 2003;4:1457–65.
[17] Patil RS, Ghormade VV, Deshpande MV. Chitinolytic enzymes: an exploration. Enzyme Microb Technol 2000;26:473–83.
[18] Friedman SJ, Skehan P. Membrane-active drugs potentiate the killing of tumor cells by D-glucosamine. Proc Natl Acad Sci USA 1980;77:1172–6.
[19] Dahiya N, Tewari R, Hoondal GS. Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 2006;71:773–82.
[20] Suginta W, Vongsuwan A, Songsiriritthigul C, Prinz H, Estibeiro P, Duncan RR, et al. An endochitinase A from *Vibrio carchariae*: cloning, expression, mass and sequence analyses. Arch Biochem Biophys 2004;424:171–80.
[21] Suginta W, Vongsuwan A, Songsiriritthigul C, Svasti J, Prinz H. Enzymatic properties of wild-type and active site mutants of chitinase A from *Vibrio carchariae*, as revealed by HPLC-MS. FEBS J 2005;272:3376–86.
[22] Dando RT, Young EPS. Growth media. In: The NCIMB catalogue of strains. Aberdeen: Aberdeen University Press; 1987, 172.
[23] Hsu SC, Lockwood JL. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl Microbiol 1975;29:422–6.
[24] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[25] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[26] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.
[27] Tanaka T, Fujiwara S, Nishikori S, Fukui T, Takagi M, Imanaka T. A unique chitinase with dual active sites and triple substrate binding sites from the hyperthermophilic Archaeon *Pyrococcus kodakaraensis* KOD1. Appl Environ Microbiol 1999;15:5338–44.
[28] Gal SW, Choi JY, Kim CY, Cheong YH, Choi YJ, Lee SY, et al. Cloning of the 52-kDa chitinase gene from *Serratia marcescens* KCTC2172 and its proteolytic cleavage into an active 35-kDa enzyme. FEMS Microbiol Lett 1998;160:151–8.
[29] Tsujibo H, Orikoshi H, Baba N, Miyahara M, Miyamoto K, Yasuda M, et al. Identification and characterization of the gene cluster involved in chitin degradation in a marine bacterium, *Alteromonas* sp. strain O-7. Appl Environ Microbiol 2002;68:263–70.
[30] Keyhani NO, Li XB, Roseman S. Chitin catabolism in the marine bacterium *Vibrio furnissii*. Identification and molecular cloning of a chitoporin. J Biol Chem 2000;275:33068–76.
[31] Terwisscha van Scheltinga AC, Kalk KH, Beintema JJ, Dijkstra BW. Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. Structure 1994;2:1181–9.
[32] Papanikolau Y, Prag G, Tavlas G, Vorgias CE, Oppenheim AB, Petratos K. High resolution structural analyses of mutant chitinase A complexes with substrates provide new insight into the mechanism of catalysis. Biochemistry 2001;40:11338–43.