the FEBS Journal

Enzymatic properties of wild-type and active site mutants of chitinase A from *Vibrio carchariae*, as revealed by HPLC-MS

Wipa Suginta[1], Archara Vongsuwan[1], Chomphunuch Songsiriritthigul[1,2], Jisnuson Svasti[3] and Heino Prinz[4]

1 School of Biochemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
2 National Synchrotron Research Center, Nakhon Ratchasima, Thailand
3 Department of Biochemistry and Center for Protein Structure and Function, Faculty of Science, Mahidol University, Bangkok, Thailand
4 Max Planck Institut für Molekulare Physiologie, Dortmund, Germany

Keywords
chitinase A; chitooligosaccharides; quantitative HPLC-MS; transglycosylation; *Vibrio carchariae*

Correspondence
W. Suginta, School of Biochemistry, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
Fax: + 66 44 224185
Tel: + 66 44 224313
E-mail: wipa@ccs.sut.ac.th

(Received 13 January 2005, revised 21 March 2005, accepted 6 May 2005)

doi:10.1111/j.1742-4658.2005.04753.x

The enzymatic properties of chitinase A from *Vibrio carchariae* have been studied in detail by using combined HPLC and electrospray MS. This approach allowed the separation of α and β anomers and the simultaneous monitoring of chitooligosaccharide products down to picomole levels. Chitinase A primarily generated β-anomeric products, indicating that it catalyzed hydrolysis through a retaining mechanism. The enzyme exhibited endo characteristics, requiring a minimum of two glycosidic bonds for hydrolysis. The kinetics of hydrolysis revealed that chitinase A had greater affinity towards higher M_r chitooligomers, in the order of $(GlcNAc)_6$ > $(GlcNAc)_4$ > $(GlcNAc)_3$, and showed no activity towards $(GlcNAc)_2$ and pNP-GlcNAc. This suggested that the binding site of chitinase A was probably composed of an array of six binding subsites. Point mutations were introduced into two active site residues – Glu315 and Asp392 – by site-directed mutagenesis. The D392N mutant retained significant chitinase activity in the gel activity assay and showed ≈20% residual activity towards chitooligosaccharides and colloidal chitin in HPLC-MS measurements. The complete loss of substrate utilization with the E315M and E315Q mutants suggested that Glu315 is an essential residue in enzyme catalysis. The recombinant wild-type enzyme acted on chitooligosaccharides, releasing higher quantities of small oligomers, while the D392N mutant favored the formation of transient intermediates. Under standard hydrolytic conditions, all chitinases also exhibited transglycosylation activity towards chitooligosaccharides and pNP-glycosides, yielding picomole quantities of synthesized chitooligomers. The D392N mutant displayed strikingly greater efficiency in oligosaccharide synthesis than the wild-type enzyme.

Chitin is a homopolymer of β(1,4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues and a major structural component of bacteria, fungi, and insects. In the ocean, chitin is produced in vast quantities by marine invertebrates, fungi, and algae [1]. This highly insoluble compound is utilized rapidly, as the sole source of carbon and nitrogen, by marine bacteria such as *Vibrio* spp. [2,3]. Two types of enzymes are required for the hydrolysis of chitin. The first, chitinases, are the major enzymes, which degrade the chitin polymer into chitooligosaccharides and subsequently into the disaccharide, $(GlcNAc)_2$. $(GlcNAc)_2$ is then

Abbreviations
GlcNAc, *N*-acetyl-D-glucosamine; $(GlcNAc)_n$, β1–4 linked oligomers of GlcNAc residues where n = 2–6; pNP, *p*-nitrophenol; pNP-$(GlcNAc)_n$, pNP-β-glycosides; SIM, single ion monitoring.

further hydrolyzed by the second type of enzymes – β-glucosaminidases – to yield GlcNAc as the final product. Chitin catabolism through the carbohydrate catabolic cascade has rather complex signal transduction pathways and has been studied extensively in *Vibrio furnissi* [4–7].

Chitinases (EC 3.2.1.14) are classified into glycosyl hydrolase families 18 and 19, depending on their amino acid sequences [8–11]. All the known bacterial chitinases belong to the family 18 glycosidase. Structural data [12,13] and stereochemical studies of chitin hydrolysis [14–16] have revealed a substrate-assisted catalytic mechanism that involves substrate distortion, leading to glycosidic bond cleavage, to yield an oxazolinium intermediate and to retention of anomeric configuration in the products. Detailed characterization and kinetic analyses of chitinases, using chitin as a substrate, have been limited because enzyme-catalyzed reactions produce more than one species of oligosaccharide intermediate. Most kinetic studies of chitinases were obtained by using chitooligomers [$GlcNAc_n$, n = (2–6)] [16–20] or short chitooligomers coupled with *p*-nitrophenyl or 4-methylumbelliferyl groups [21–23].

We described, in a previous publication, the isolation of chitinase A from a marine bacterium, *V. carchariae* [24]. Chitinase A is highly expressed upon induction with chitin and is active as a monomer of M_r 62 700. Analysis of chitin hydrolysis by using the viscosity assay and HPLC-ESI MS suggested that the newly isolated chitinase acts as an endochitinase [25]. We also reported isolation of the gene encoding chitinase A and functional expression of the recombinant enzyme in an *Escherichia coli* system. In the present study, the hydrolytic activity of chitinase A resulting in the production of a broad range of chitooligosaccharide products was measured simultaneously by means of quantitative HPLC-ESI MS. Site-directed mutagenesis was also employed to elucidate the catalytic role of two active site residues. The hydrolytic and transglycosylation activities of the mutated enzymes were studied in comparison with the recombinant wild-type enzyme.

Results

Characterization of chitooligosaccharide products

Colloidal chitin was hydrolyzed by native chitinase A at 20 °C. After different reaction times, the reaction products were analyzed by using HPLC-ESI MS. Figure 1 shows an HPLC-MS chromatogram of chitooligosaccharide products after 2 h of reaction time. The mono-deacetylated dimer (*m/z* 383), trimer (*m/z* 586) and tetramer (*m/z* 789) were detected. Partial deacetylation typically occurred when chitin was prepared by treatment with acids [26]. Note that the mono-deacetylated trimer appeared at three different elution times. This corresponds to three different isomers (e.g. GlcNAc.GlcNAc.GlcN, GlcNAc.GlcN. GlcNAc, and GlcN.GlcNAc.GlcNAc), in accordance with the location of three acetyl moieties.

The signal-to-noise ratio improved significantly when the mass spectra were recorded in the single ion monitoring (SIM) mode corresponding to selected masses of reaction products [GlcNAc to $(GlcNAc)_6$]. The signal of ion clusters and deacetylated oligomers were thus excluded from the analysis. Figure 2 shows

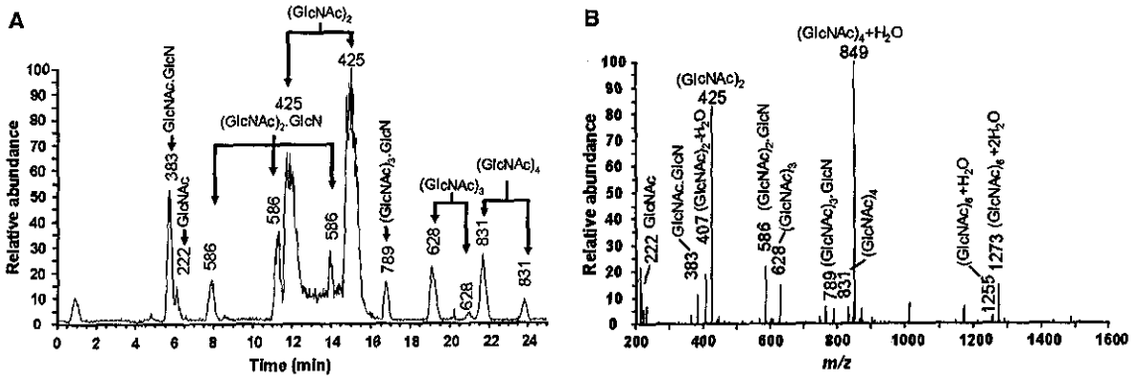

Fig. 1. Chitinase A catalyzes chitin hydrolysis. Native chitinase A (5 μg) was added to 10 mg·mL^{-1} colloidal chitin and incubated at room temperature (20 °C) for 2 h. Ten microlitres of the sample was added to a Hypercarb® column and eluted at 250 μL·min^{-1} with a linear gradient of 5–40% (v/v) acetronitrile into an LCQ ESI mass spectrometer. (A) An HPLC chromatogram representing chitin hydrolysis by chitinase A. The chitooligosaccharide masses are indicated on the corresponding peaks. (B) The mass spectrum averaged over the time range of the chromatogram in Fig. 1A is shown between 200 and 1600 *m/z*. All peaks are singly charged, as deduced from their isotope pattern. All clusters (two or three oligosaccharides with one proton) map to the chromatographic peaks of the respective molecules.

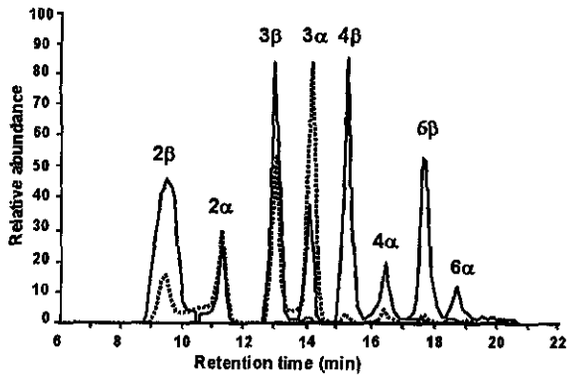

Fig. 2. Stereochemistry of chitin hydrolysis. Native chitinase A (75 ng) was added to 400 μg·mL^{-1} colloidal chitin and incubated at 20 °C for 5 min (solid line) and 60 min (dotted line). Ten microlitres of the sample was subjected to HPLC-MS. The signal was recorded in the single ion mode set for the masses 222, 425, 628, 831, 1034 and 1237. The relative intensity of the base peaks is plotted as a function of the elution time. Numbers indicate the amount of 2-amino-2-*N*-acetylamino-D-glucose (GlcNAc) units in an oligomer; β and α indicate their isoform.

the elution profile of the selected reaction products after 5 min (solid line) or 1 h (dotted line). Clearly, the longer oligomers are formed only transiently within the initial time of reaction, and then subsequently degraded over a longer incubation time.

Stereochemistry of chitin hydrolysis

Hydrophobic stationary phases of HPLC have been shown to bind preferentially to the α anomer, allowing both isomers to be separated and identified. The cleavage pattern was assessed from a previously published separation profile of chitooligosaccharides obtained by using reverse-phase HPLC and ^{1}H NMR [14,15]. The earlier peak represented the β anomer and the later peak corresponded to the α anomer of the oligomeric products obtained at initial stage of reaction (Fig. 2, solid line). In order to evaluate which anomer was initially produced by chitinase A, we determined the peak ratio of oligomers immediately after hydrolysis of chitin and at equilibrium. The HPLC column was run at 10 °C and the sample was immediately loaded onto the column after 10 min of hydrolysis at 20 °C to minimize isomerization. Note that the peak ratio is related to the concentration ratio by a factor C [i.e. $(\beta/\alpha)_{concentrations} = C \times (\beta/\alpha)_{peaks}$], but this factor C disappears when ratios of ratios are calculated. The peak ratio β/α 'immediately' after hydrolysis divided by the peak ratio β/α at equilibrium was 6.9 for the dimer, 4.3 for the trimer, and 5.4 for the tetramer.

Quantitative analysis of chitooligosaccharide hydrolysis by native chitinase A

The hydrolysis of short chitooligosaccharides [$(GlcNAc)_n$, $n = 2$, 3, 4 and 6] and colloidal chitin was studied further. The reaction was quenched by the addition of acetic acid, so that substrate decrease and product formation could be monitored at various time-points. Quantification of the reaction products shown in Fig. 3 was obtained by means of separate calibration experiments using known concentrations of the oligomers, as described in the 'Experimental procedures'. This was mandatory, even for these chemically similar compounds, because MS ion counts were generally higher for longer oligomers than for shorter ones.

When chitinase A was incubated with $(GlcNAc)_2$, neither a decrease in $(GlcNAc)_2$ nor an increase in GlcNAc was observed upon incubation up to 57 h (Fig. 3A). In contrast, when $(GlcNAc)_3$ was the substrate, a slow decrease in $(GlcNAc)_3$ concentrations was already detected within the first 15 min of reaction (Fig. 3B). After 57 h, hydrolysis was complete, with

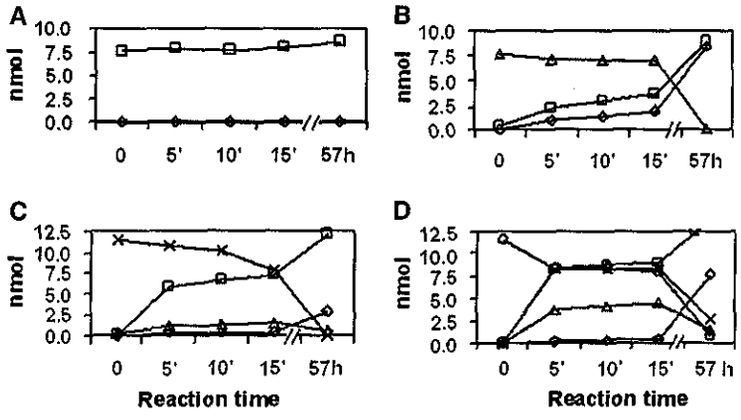

Fig. 3. Quantitative analysis of chitooligosaccharide hydrolysis. Native chitinase A (75 ng) was incubated at 20 °C with 2 mM of (A) $(GlcNAc)_2$, (B) $(GlcNAc)_3$, (C) $(GlcNAc)_4$, and (D) $(GlcNAc)_6$. The reaction was quenched by the addition of acetic acid to 10% and then applied to HPLC-ESI MS. For calibration of the HPLC peaks (α and β anomers) recorded at different masses in the single ion mode, mixtures of the same chitooligosaccharides and the monomer were applied at known concentrations. The calculated amounts of GlcNAc (◇), $(GlcNAc)_2$ (□), $(GlcNAc)_3$ (△), $(GlcNAc)_4$ (×), and $(GlcNAc)_6$ (○) are shown as a function of reaction times.

dimers and monomers being produced in equal amounts as the final products. Figure 3C represents the hydrolysis of $(GlcNAc)_4$. The enzyme hydrolyzed the tetramer mainly in the middle, so that dimers were formed. Trimers were also produced but in comparatively lower quantities (< 20% of the dimers at 15 min of reaction) and were degraded into dimers and monomers towards the end of the reaction. No monomer was detectable at the very early stages, but ≈20% of monomers were obtained after the reaction was complete. The hydrolysis of $(GlcNAc)_6$ yielded predominantly $(GlcNAc)_4$ and $(GlcNAc)_2$ (Fig. 3D). The amount of transiently formed $(GlcNAc)_3$ was more than double that observed for tetramer hydrolysis. Tetramers and trimers were further hydrolyzed, again giving dimers and monomers as the end products.

The hydrolytic activity of chitinase A against colloidal chitin was also studied at various incubation times. All chitooligosaccharides, from monomers to hexamers, were observed, but dimers dominated the population of reaction intermediates. The monomer, GlcNAc, only appeared after a lag time of ≈30 min, and the larger oligomers – $(GlcNAc)_4$ and $(GlcNAc)_6$ – were only observed transiently within the first hour, with the levels of $(GlcNAc)_6$ being too low to be calculated. In contrast to these, the trimer $(GlcNAc)_3$ produced was rather stable and only further hydrolyzed after a few hours.

Steady-state kinetics of chitinase A with various substrates

HPLC-MS is a relatively complex technique compared to well-established colorimetric assays. In order to relate our findings to this standard methodology, hydrolysis of *p*-nitrophenol substrates was studied by using both methods. As with $(GlcNAc)_2$, chitinase A did not hydrolyze pNP-GlcNAc, but hydrolyzed pNP-$(GlcNAc)_2$ mainly into pNP + $(GlcNAc)_2$ (> 99%). For quantitative analysis, product concentrations were calculated directly by means of a pNP calibration curve in the case of the colorimetric assay, or by using a $(GlcNAc)_2$ calibration curve in the case of quantitative HPLC-MS. If pNP is used for monitoring the hydrolysis of pNP-$(GlcNAc)_2$, the other product will be $(GlcNAc)_2$, so that the results with both assays should be identical. Using linear regression plots, the K_m and k_{cat} values determined for the spectroscopic assay were 1.04 ± 0.10 mM and 5.78 ± 0.58 s^{-1}, and for the LC-MS assay were 1.05 ± 0.03 mM and 5.73 ± 0.16 s^{-1} (Table 1). The correlation coefficient between the two data sets was 0.997. The close similarity between the K_m and k_{cat} values obtained from the two methods confirms that the ESI MS assay is a reliable method for using to determine the kinetic parameters of chitinase A.

Table 1. Kinetic parameters of chitinase A with various substrates. The hydrolysis of chitooligosaccharides and colloidal chitin at substrate concentrations of 0–2 mM was carried out with 75 ng of native chitinase A in 0.1 M ammonium acetate buffer (pH 7.1) at 20 °C for 5 min and quenched with 10% (v/v) acetic acid. The terminated reactions were then analyzed by using quantitative HPLC-MS. Kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) were obtained from Lineweaver–Burk plots, which were assessed by using a standard linear regression function. $(GlcNAc)_n$, β1–4 linked oligomers of GlcNAc residues where n = 2–6; $(GlcNAc)_n$-pNP, *p*-nitrophenol β-glycosides.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
GlcNAc-pNP	No reaction	–	–
$(GlcNAc)_2$-pNP[a]	1.04 ± 0.10	5.78 ± 0.58	5.29×10^3
$(GlcNAc)_2$-pNP[b]	1.05 ± 0.03	5.73 ± 0.16	5.84×10^3
$(GlcNAc)_2$	No reaction	–	–
$(GlcNAc)_3$[b]	10.54 ± 1.40	9.71 ± 1.29	9.21×10^2
$(GlcNAc)_4$[b]	2.17 ± 0.29	0.63 ± 0.08	2.89×10^2
$(GlcNAc)_6$[b]	0.19 ± 0.01	5.81 ± 0.19	3.06×10^4
Chitin[b]	0.10 ± 0.02 mg·mL^{-1}	0.07 ± 0.006	–

[a] Determined by colorimetric assay, [b] determined by HPLC-ESI MS.

Having established confidence in the validity of the method, we systematically investigated, by using ESI MS, the kinetic properties of chitinase A with pNP-glycosides, chitooligosaccharides, and chitin. The initial velocity of the enzyme for concentrations of the substrates ranging from 0 to 2.0 mM was determined after 5 min of reaction. Given the fact that chitinase A produced $(GlcNAc)_2$ as the major end product, the initial velocity of all the substrates was calculated based on the release of $(GlcNAc)_2$.

Kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) were obtained from linear regression plots, as shown in Table 1. For chitooligomers, the K_m values decreased with increased length of oligomers [the K_m values for $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_6$ were 10.54 ± 1.40 mM, 2.17 ± 0.29 mM, and 0.19 ± 0.01 mM, respectively], indicating that the enzyme had greater affinity towards the higher M_r substrates.

The catalytic efficiency constant (k_{cat}/K_m) of pNP-$(GlcNAc)_2$ (5.84×10^3 $s^{-1}\cdot M^{-1}$) was higher than that of $(GlcNAc)_3$ (9.21×10^2 $s^{-1}\cdot M^{-1}$) or $(GlcNAc)_4$ (2.89×10^2 $s^{-1}\cdot M^{-1}$), but lower than that of $(GlcNAc)_6$ (3.06×10^4 $s^{-1}\cdot M^{-1}$). K_m and k_{cat} values for chitin were 0.10 ± 0.02 mg·mL^{-1} and 0.07 ± 0.006 s^{-1}. These values were similar to those measured for glycol-chitin with the 65 kDa chitinase from *Bombyx mori* (K_m, 0.13 mg·mL^{-1}; k_{cat}, 0.08 s^{-1}) [17].

Protein expression and hydrolytic activity of the wild-type chitinase A and mutants

We recently reported cloning and expression of the recombinant wild-type chitinase A as a $(His)_6$-tagged fusion protein [25]. As judged by a colorimetric assay using pNP-$(GlcNAc)_2$ as the substrate, the recombinant enzyme exhibited 117% of the specific activity of the native enzyme. The Quickchange Site-directed Mutagenesis Kit was used to generate three active site mutants using the clone carrying the recombinant wild-type DNA as template. The three mutated clones had changes of two amino acids, namely Glu315→Met (mutant E315M), Glu315→Gln (mutant E315Q), and Asp392→Asn (mutant D392N). Using the same expression and purification systems, the mutated and the wild-type enzymes were expressed in equivalent amounts, yielding $\approx 70\ mg \cdot L^{-1}$ of purified protein. SDS/PAGE analysis followed by staining with Coomassie blue showed single bands for the wild-type and mutants D392N and E315M, migrating with an M_r of $\approx 63\,000$ (Fig. 4A). In the case of the E315Q mutant, an additional faint band was also seen at an M_r of $\approx 43\,000$. This band appeared as a degradation product during freezing and thawing of the protein that was stored at −30 °C. As revealed by immunoblotting, all the mutants, as well as the wild-type, strongly reacted with polyclonal anti-(chitinase A) Ig (Fig. 4B), confirming that the expressed proteins were chitinase A. A gel activity assay using glycol-chitin displayed chitinase activity only for the wild-type and for the D392N mutant, with the mutant having much less activity. The E315Q and E315M mutants, by contrast, completely lacked hydrolytic activity (Fig. 4C).

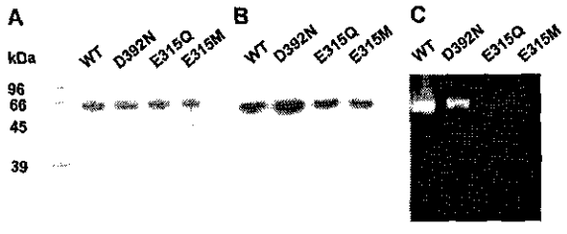

Fig. 4. SDS/PAGE analysis of the recombinant chitinase A and mutants. Purified chitinases (2 μg) were electrophoresed through a 12% (w/v) SDS polyacrylamide gel. After electrophoresis, protein bands were (A) stained with Coomassie blue, (B) immunoblotted and detected with polyclonal anti-(chitinase A), and (C) stained for chitinase activity with glycol-chitin using fluorescent Calcoflour white M2R. The tracks represent the following samples: 1, low-M_r standard proteins; 2, recombinant wild-type protein; 3, D392N mutant; 4, E315Q mutant; and 5, E315M mutant.

The products of chitooligosaccharide and colloidal chitin hydrolysis generated by recombinant wild-type and mutants were further analyzed as a function of time. No detectable products were seen when the chitin polymer was incubated with the mutants E315Q and E315M, even after 60 min. On the other hand, the D392N mutant was able to hydrolyze chitin with ≈20% residual activity. As with the wild-type chitinase A, the D392N mutant released multiple species of hydrolytic products, varying from GlcNAc to $(GlcNAc)_6$.

After adjusting the concentration of the enzymes to yield similar activity, the hydrolytic activities of the wild-type protein and of the D392N mutant were assayed with $(GlcNAc)_{2-6}$. As expected, the enzymes failed to hydrolyze $(GlcNAc)_2$ and showed very low activity towards $(GlcNAc)_3$. With $(GlcNAc)_4$ as the substrate, both enzymes recognized the middle glycosidic bond of the tetrameric chain, releasing $(GlcNAc)_2$ as a major product (Fig. 5A). $(GlcNAc)_3$ appeared in small amounts only after $(GlcNAc)_2$ had accumulated. With $(GlcNAc)_5$ as the substrate, $(GlcNAc)_2$ and $(GlcNAc)_3$ were formed as the primary products, with the hydrolytic rate of the D392N mutant being much slower than that of the wild type. $(GlcNAc)_4$, measured in trace amounts, was probably formed through the reaction intermediates.

Both wild-type and D392N mutant cleaved $(GlcNAc)_6$ asymmetrically, mainly releasing $(GlcNAc)_2$ and $(GlcNAc)_4$ in equal amounts, followed by $(GlcNAc)_3$, and $(GlcNAc)_5$. At 60 min of reaction time, the yields of the trimer and the pentamer compared to the dimer were 34% and 30% for the wild-type, but 66% and 47% for the D392N mutant (Fig. 5B).

Oligosaccharide synthesis by chitinase A

Direct detection of molecular mass by HPLC-MS instantly identified higher M_r intermediates occurring in the course of hydrolysis. This transglycosylation was observed immediately with chitooligosaccharides, as well as with pNP-glycosides. Figure 6 demonstrates the quantitative analysis of polymerized (transglycosylation) products of $(GlcNAc)_4$ hydrolysis.

The transglycosylation reaction took place as early as 2 min after initiation, yielding picomole quantities of the elongated oligomers. The maximum yields of $(GlcNAc)_5$ and $(GlcNAc)_6$, synthesized relatively to the hydrolytic product, $(GlcNAc)_2$, were 3% and 9% for the wild-type enzyme and 11% and 12% for the D392N mutant. The synthesis of $(GlcNAc)_8$ was also detected, but with lower yields (< 1%). All synthe-

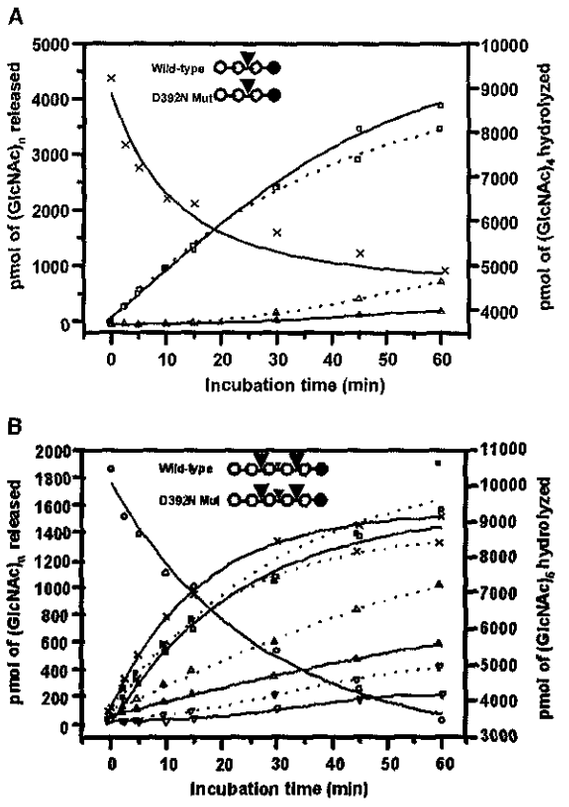

Fig. 5. Hydrolytic activity of the wild-type chitinase A and D392N mutant. The purified recombinant chitinase A (100 ng) or D392N mutant (500 ng) was added to a reaction mixture containing 1 mM $(GlcNAc)_n$ in 50 mM ammonium acetate buffer, pH 7.1. The reaction was quenched after the indicated reaction times at 20 °C by the addition of acetic acid to 10% and applied to calibrated HPLC-MS. For each substrate, the calculated concentrations of the products formed by the wild-type (solid line) and D392N mutant (broken line) are shown. (A) Hydrolysis of $(GlcNAc)_4$ and (B) hydrolysis of $(GlcNAc)_6$. □, $(GlcNAc)_2$; △, $(GlcNAc)_3$; ×, $(GlcNAc)_4$; ▽, $(GlcNAc)_5$; and ○, $(GlcNAc)_6$. The inset schematically shows the chitooligomers with the proposed cleavage sites (▼). The GlcNAc units at the reducing end are represented with filled circles (●).

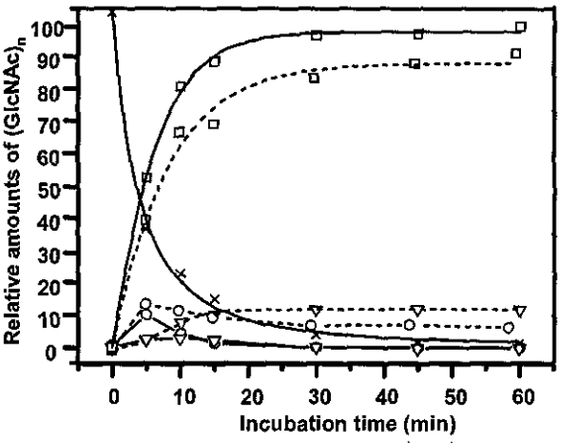

Fig. 6. Transglycosylation activity of the wild-type chitinase A and of the D392N mutant. The recombinant wild-type (100 ng) or the D392N mutant (500 ng) was added to a reaction mixture containing 1 mM $(GlcNAc)_4$ substrate in 50 mM ammonium acetate buffer, pH 7.1. The reaction was quenched after the indicated reaction times at 20 °C by the addition of acetic acid to a final concentration of 10% and the glycosylation products were analyzed by using the calibrated HPLC-MS. The chitooligomers formed by the wild-type enzyme are shown by solid lines and those formed by the D392N mutant in broken lines. □, $(GlcNAc)_2$; ×, $(GlcNAc)_4$; ▽, $(GlcNAc)_5$; and ○, $(GlcNAc)_6$.

sized oligomers were present only as reaction intermediates, which were utilized further within 30 min; $(GlcNAc)_5$, obtained with the D392N mutant, was the only exception – its concentration remained relatively steady up to 60 min. Similar patterns were also seen with other substrates. For instance, the tetramers, pentamers, and hexamers were formed during $(GlcNAc)_3$ hydrolysis, while hexamers, heptamers, and octamers were formed during $(GlcNAc)_5$ hydrolysis. Transglycosylation activity of chitinase A was also observed with pNP-glycosides, where $(GlcNAc)_3$ and $(GlcNAc)_4$ were detected during pNP-$(GlcNAc)_2$ hydrolysis and $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ were found during pNP-$(GlcNAc)_3$ hydrolysis.

Discussion

We have demonstrated the power of quantitative HPLC-MS when an enzymatic reaction with a large variety of products is investigated, such as the enzymatic reaction with chitinase A from *V. carchariae*. A combination of HPLC and ESI MS allowed the separation of α and β anomers and all chitooligosaccharide products to be monitored simultaneously. At the initial stage of reaction and low temperature, the enzyme yielded predominantly β anomers. The anomeric configurations gradually reached mutarotation equilibrium, where the ratio of β/α anomer peaks was similar among the different oligosaccharides. This clearly indicates that chitinase A has a stereo-selectivity for β anomers over α anomers. Chitinase A cleaved β-glycosidic linkages, retaining the anomeric form of the resulting products, which supports the substrate-assisted mechanism as described for family 18 chitinases [12].

Chitinase A had greater affinity towards higher M_r chitooligomers. The increase in affinity with chain

length of chitooligomers implies that the binding site must be composed of an array of subsites, probably six GlcNAc subsites. This corresponds to the structural data obtained for *Serratia marcescens* ChiA and the hevamine chitinase [12,15], in which a substrate-binding site extends over six GlcNAc subsites designated from −4 to +2.

Quantitative analysis showed that $(GlcNAc)_2$ was the main product of the hydrolytic reactions. The smallest substrates for chitinase A were trimers [$(GlcNAc)_3$ and pNP-$(GlcNAc)_2$], suggesting that the cleavage site is located asymmetrically in the substrate recognition sites, two of which form the product site. Hydrolysis of pNP-$(GlcNAc)_2$ with the chromophore attached at the reducing end of the sugar chain yielded > 99% $(GlcNAc)_2$, indicating that chitinase A cleaves the second bond from the nonreducing end. When $(GlcNAc)_3$ was produced as a reaction intermediate, it was relatively stable because its low affinity prevented rapid hydrolysis. Apparently, all monomers found as end products arose from these intermediate trimers. Indeed, the bond cleavage in the middle of $(GlcNAc)_6$, which produced two molecules of $(GlcNAc)_3$ in significant amounts, suggested that the catalytic cleft of the *Vibrio* enzyme has an open structure at both ends, giving long sugars access in a flexible manner. Such a feature can be expected from an enzyme with endo characteristics. The endo property of chitinase A is further verified by the formation of reaction intermediates of varying length (Fig. 2).

The role of two catalytic amino acid residues (Glu315 and Asp392) in the enzyme catalysis was also investigated. Of three newly generated chitinase A variants, the hydrolytic activity of E315M and E315Q mutants was entirely abolished. Apparently, structural modification of the carboxylate side-chain of Glu315 led to a loss of the hydrolytic activity, providing evidence that Glu315 is essential for catalysis. The catalytic role of the equivalent glutamic acid has been well demonstrated by the 3D structures of ChiA from *S. marcescens* [12,27] and of CiX1 from the pathogenic fungus *Coccidioides immitis* [28]. The D392N mutant retained significant hydrolytic activity with the tested substrates, implying that Asp392 does not have a direct catalytic function.

When the hydrolytic activity of the wild-type enzyme and the D392 mutant was investigated at various substrate concentrations, almost identical K_m values, but greatly decreased V_{max} values, were obtained for the D392N mutant. This may reflect influences of the mutation on the catalytic process, but not on the substrate-binding process. Site-directed mutagenesis and the 3D structures of other chitinases showed that the equivalent Asp392 residues take part in the catalytic process by stabilizing the transition states flanking the oxazolinium intermediate and subsequently assisting the correct orientation of the 2-acetamido group in catalysis [13,29–31].

Chitooligosaccharide hydrolysis, as a function of time, revealed some differences between the nonmutated and mutated enzymes. As with native chitinase A, $(GlcNAc)_2$ did not act as a substrate and $(GlcNAc)_3$ was a poor substrate for both enzymes. These small M_r sugars are more likely to be generated as reaction products than to act as substrates. As judged by the patterns of the product formation, the release of dimers, trimers and tetramers from the hexamer was considered to result from direct action of the enzymes. On the other hand, the pentamer appeared to be formed by the condensation of smaller intermediates. Note that the wild-type enzyme prefers to degrade chitooligosaccharides, yielding direct formation of the primary products, while the mutant enzyme acted more efficiently on the transiently formed secondary products (Fig. 5).

The HPLC-MS method was sensitive enough to detect the low levels of oligosaccharides synthesized from chitinase A. Under specific conditions (low temperature, short reaction time and low substrate concentrations), oligosaccharide synthesis was likely to take place through transglycosylation reactions. The higher M_r oligomers, including pentamers, hexamers and octamers, were obtained from the hydrolysis of $(GlcNAc)_4$. These oligomers presumably arose from the condensation of two reaction intermediates, namely the pentamer from $(GlcNAc)_2 + (GlcNAc)_3$, the hexamer from $(GlcNAc)_2 + (GlcNAc)_4$ or $(GlcNAc)_3 + (GlcNAc)_3$, and the octamer from $(GlcNAc)_2 + (GlcNAc)_6$ or $(GlcNAc)_3 + (GlcNAc)_5$. The rates of formation were in the order of $(GlcNAc)_6 > (GlcNAc)_5 > (GlcNAc)_8$ for both enzymes. The ratios of the maximal yields of the synthesized products obtained by the mutant over the wild-type were 285 : 1 for $(GlcNAc)_5$, 374 : 1 for $(GlcNAc)_6$ and 3.7 : 1 for $(GlcNAc)_8$. From these ratios, it was concluded that the D392 mutant was a more efficient enyzme in chitooligosaccharide synthesis.

In conclusion, we report, for the first time, the enzymatic properties of chitinase A as determined by using a suitably calibrated HPLC-ESI MS. This sensitive analytical method allowed a broad range of intermediate reaction products to be monitored simultaneously down to picomole levels and was therefore suitable for detailed characterization of chitinases, which is difficult to perform by using other methods.

Experimental procedures

Materials

The marine bacterium *V. carchariae* (LMG7890^T) 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), chitooligosaccharide substrates and pNP-glycosides were from Sigma-Aldrich Pte., Ltd (Citilink Warehouse Complex, Singapore); SDS/PAGE chemicals from Amersham Pharmacia Biotech Asia Pacific Ltd (Bangkok, Thailand) and from Sigma-Aldrich Pte., Ltd; Sephacryl S300® HR resin was from Amersham Biosciences (Piscataway, NJ, USA); chemicals for buffers and reagents for protein preparation were from Sigma-Aldrich Pte., Ltd and from Carlo Erba Reagenti (Milan, Italy); and acetonitrile (HPLC grade) was from LGC Promochem GmbH (Wesel, Germany). All other reagents for LC-MS measurements were from Sigma-Aldrich (Munich, Germany).

Instrumentation

HPLC was operated on a 150 × 2.1 mm 5 μm Hypercarb® column (ThermoQuest, Thermo Electron Corporation, San Jose, CA, USA) connected to an Agilent Technologies 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) under the control of a Thermo Finnigan LCQ DECA electrospray mass spectrometer. The proprietary program XCALIBUR (Thermo Finnigan, Thermo Electron Corporation, San Jose, CA, USA) was used to control and calibrate HPLC-MS data.

Preparation of chitinase A

Native chitinase A secreted by *V. carchariae* culture was purified by chitin-affinity binding and gel filtration chromatography following the protocol described previously [24]. Recombinant wild-type chitinase A was obtained by cloning the chitinase A gene, lacking the C-terminal proteolytic fragment, into the pQE60 expression vector and expressing the protein in *E. coli* M15 cells [25]. For preparation of the recombinant enzyme, the bacterial cells were grown at 37 °C in 250 mL of Luria–Bertani (LB) medium, supplemented with 100 μg·mL^{-1} ampicillin, to an attenuance (D), at 600 nm, of ≈0.6, then isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 0.5 mM. Incubation was continued at 25 °C overnight, with shaking, before the cells were harvested by centrifugation at 2500 *g* for 20 min. The cell pellet was resuspended in 15 mL of 20 mM Tris/HCl buffer, pH 8.0. containing 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, and 1 mg·mL^{-1} lysozyme. The suspended cells were maintained on ice and broken by using an ultrasonicator (30 s, six to eight times). Unbroken cells and cell debris were removed by centrifugation. The supernatant containing soluble chitinase A was purified by using Ni- nitrilotriacetic acid agarose chromatography, according to Qiagen's protocol (Qiagen, Valencia, CA, USA). Fractions eluted with 250 mM imidazole, which contained soluble chitinase A, were pooled, concentrated by using Vivaspin (Vivascience AG, Hannover, Germany) membrane concentrators (10 000 M_r cut-off), and further purified by gel filtration chromatography using an ÄKTA purifier system (Amersham Biosciences, Sweden) on a Superdex S-200 HR 10/30 column (1.0 × 30 cm). The running buffer was 20 mM Tris/HCl, pH 8.0, containing 150 mM NaCl. A flow rate of 250 μL·min^{-1} was applied and fractions of 500 μL were collected. Chitinase-containing fractions were combined and stored at −30 °C until use. Protein concentrations were determined by Bradford's method [32] and quantified using a standard calibration curve produced from BSA. Purity of the resultant protein was verified by SDS/PAGE operated under a Laemmli buffer system [33]. Unless otherwise stated, experiments were carried out at 4 °C throughout the purification steps.

Site-directed mutagenesis

Point mutations were introduced to the wild-type chitinase A DNA via pPCR-based mutagenesis using *Pfu* Turbo DNA polymerase (QuickChange Site-Directed Mutagenesis kit; Stratagene, La Jolla, CA, USA). Three chitinase A mutants were generated by using three sets of mutagenic oligonucleotides (Proligo Singapore Pte Ltd, Science Park II, Singapore). The forward oligonucleotide sequences designed for D392N, E315M, and E315Q mutants (sequences underlined) were 5′-CTTTGCGATGACTTAC AACTTCTACGGCGG-3′, 5′-GTAGATATTGACTGGAT GTTCCCTGGTGGCGGCG-3′ and 5′-GATATTGACTG GCAATTCCCTGGTGGCGGC-3′, and the reverse oligonucleotide sequences were 5′-CAGCCGCCGTAGAAGTT GTAAGTCATCGCAAAG-3′, 5′-CGCCGCCACCAGGG AACATCCAGTCAATATCTAC-3, and 5′-GCCGCCAC CAGGGAATTGCCAGTCAATATCTAC-3′, respectively. Confirmation of the mutated nucleotides by automated sequencing was carried out by the Bio Service Unit (BSU, Bangkok, Thailand). The oligonucleotide used for determining the nucleotide sequences of the three mutants was 5′-TTCTACGACTTCGTTGATAAGAAG-3′. The mutated proteins were expressed and purified under the same conditions as described for the wild-type enzyme.

Hydrolytic action of chitinase A on chitooligosaccharides and chitin

Hydrolysis of chitooligosaccharides by native chitinase A was carried out in 50 mM ammonium acetate buffer,

pH 7.1. Reactions containing 2.0 mM chitooligosaccharides, including $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_6$, were incubated in the presence of 75 ng of purified enzyme at 20 °C with shaking. One-hundred microliter aliquots were taken at 5 min, 10 min, 15 min and 57 h and quenched with 10% (v/v) acetic acid. These terminated reaction mixtures (60 μL) were injected into a Hypercarb HPLC column, operated at 40 °C unless otherwise stated. A linear gradient of 0–40% (v/v) acetonitrile, containing 0.1% (v/v) acetic acid, was applied, and oligosaccharides separated from the column were immediately detected by ESI MS connected to the LC interface. ESI MS was conducted in positive SIM mode. The mass-to-charge ratio (*m/z*) of expected oligosaccharides were selected as follows: GlcNAc, 221.9; $(GlcNAc)_2$, 425.5; $(GlcNAc)_3$, 627.6; $(GlcNAc)_4$, 830.8; $(GlcNAc)_5$, 1034.0; $(GlcNAc)_6$, 1237.2; $(GlcNAc)_7$, 1440.0; pNP-GlcNAc, 342.3; pNP-$(GlcNAc)_2$, 545.5; and pNP-$(GlcNAc)_3$, 748.7. With chitin hydrolysis, reactions were carried out the same way as described for the hydrolysis of chitooligosaccharides, but with 200 $\mu g \cdot mL^{-1}$ colloidal chitin. The peak areas of chitinase A hydrolytic products obtained from MS measurements were quantified using the program XCALIBUR applying an MS Avalon algorithm for peak detection. A mixture of oligosaccharide containing $(GlcNAc)_n$, $n = 1$–6 was prepared by dilution in two ranges: 0–500 pmol and 50 pmol to 2 nmol. The calibration curves of each GlcNAc moiety were constructed separately and used to convert peak areas into molar quantities.

Hydrolysis of chitooligosaccharides by the recombinant wild-type and mutated chitinases A was carried out in 50 mM ammonium acetate buffer, pH 7.1. Reactions containing 1.0 mM chitooligosaccharide substrates, including $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$ and $(GlcNAc)_6$ were incubated with 50 $ng \cdot \mu L^{-1}$ enzyme at 20 °C with shaking. Aliquots of a 100 μL reaction mixture were taken at 0, 2.5, 5, 10, 30, 45 and 60 min, and quenched with 10% (v/v) acetic acid. The hydrolytic products were analyzed by HPLC-ESI MS as described for the native enzyme.

Kinetic measurements

Kinetic studies of native chitinase A using a colorimetric assay were performed in a microtiter plate reader (LabSystem, Helsinki, Finland). Reaction mixtures (100 μL) comprised 0–2 mM pNP-(GlcNAc), pNP-$(GlcNAc)_2$ and pNP-$(GlcNAc)_3$ dissolved in dH_2O, chitinase A (75 ng), and 50 mM ammonium acetate buffer, pH 7.1. Release of pNP was monitored at an absorbance (*A*) of 405 nm every 15 s for 30 min at 25 °C, using a calibration curve of pNP in the same reaction buffer. Kinetic studies of chitinase A with chitooligosaccharide by LC-MS were carried out as described for the hydrolysis of chitooligosaccharides at substrate concentrations of 0.065–2 mM. This concentration range provided data points with sufficient quality, allowing K_m and k_{cat} values to be calculated with reasonable confidence by using linear regression plots.

Kinetic parameters with pNP-$(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_6$, and chitin substrates were also determined, based on the formation of $(GlcNAc)_2$ and the initial velocity of the enzyme, at 5 min of reaction at 20 °C. Kinetic values for the recombinant wild-type and D392N mutant were obtained at chitooligosaccharide substrate concentrations of 0–1 mM, as described for the native enzyme. The enzyme concentrations used in the reaction mixture were 100 ng for the purified wild-type and 500 ng for the D392N mutant.

Stereochemistry of product anomers

As the rate of mutarotation is temperature dependent, hydrolysis of chitin suspension (100 $\mu g \cdot mL^{-1}$) by native chitinase A (75 ng) was carried out at low temperature (20 °C) in 50 mM ammonium acetate buffer, pH 7.1, with shaking. Products were monitored as quickly as possible and the reactions were quenched with 10% (v/v) acetic acid. After centrifugation at 5 °C, the supernatant containing chitooligosaccharide products formed after 5 min of incubation was immediately injected into a Hypercarb HPLC. The HPLC was operated at a particularly low temperature (10 °C) and detected by ESI MS in SIM mode with selected masses from monomer to hexamer. Identification of β and α anomers was assessed from previous experiments with equivalent reverse-phase HPLC system and ^1H NMR [14].

Transglycosylation of chitinase A

Reaction mixtures (100 μL) containing 1 mM $(GlcNAc)_4$, 100 ng of the wild-type enzyme or 500 ng of the D392N mutant, and 50 mM ammonium acetate, pH 7.1, were incubated at 20 °C. Transglycosylation activities of both enzymes were observed at 20 °C at time intervals of 0, 5, 10, 15, 30, 45 and 60 min. At the required time-points, aliquots (10 μL) were mixed with 90 μL of 20% (v/v) acetic acid, and 20 μL of the reaction mixture was then analyzed by HPLC-ESI MS. Quantification of the tranglycosylation products was conducted as described for chitinase A-catalyzed hydrolysis. Molecular ions of the products were monitored either in the scan mode (*m/z* 200–2000) or in the SIM mode with selected anticipated masses.

Immunodetection

Antisera against chitinase A were prepared with the purified chitinase A isolated from *V. carchariae*, as described previously [24]. The purified wild-type and mutated chitinase A (2 μg) were electrophoresed on a 12% (w/v) SDS/PAGE gel, then transferred onto nitrocellulose membrane using a Trans-Blot® Semi-Dry Cell (BioRad, Hercules, CA,

USA). Immunodetection was carried out using enhanced chemiluminescence (ECL; Amersham Biosciences) according to the manufacturer's instructions. The primary antibody was polyclonal anti-(chitinase A) (1 : 2000 dilution) and the secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG (1 : 5000 dilution).

SDS/PAGE following the chitinase activity assay

The purified recombinant chitinase A (2 μg of each) were treated with gel loading buffer without 2-mercaptoethanol and electrophoresed through a 12% (w/v) polyacrylamide gel containing 0.1% (w/v) glycol chitin. After electrophoresis, the gel was washed at 37 °C for 1 h with 250 mL of 150 mM sodium acetate, pH 5.0, containing 1% (v/v) Triton X-100 and 1% (w/v) skimmed milk, followed by the same buffer without 1% (w/v) skimmed milk for a further 1 h to remove SDS and to allow the proteins to refold. The gel was stained with 0.01% (w/v) Calcoflour white M2R (Sigma, USA) in 500 mM Tris/HCl, pH 8.5, and visualized under UV [34].

Acknowledgements

This work was supported by the Thailand Research Fund for Young Researchers (TRG-4580058), by a grant from Suranaree University of Technology (SUT-1-102-46-48-06), and by a grant from the German Academic Exchange Service 'DAAD' (to WS). Jisnuson Svasti is a Senior Research Scholar of the Thailand Research Fund. We would like to thank Prof. Steve C. Fry, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, UK, and Dr Albert Schulte, Ruhr University of Bochum, Germany, for critical reading of the manuscript.

References

1 Gooday GW (1990) The ecology of chitin degradation. In: *Advances in Microbial Ecology* (Marshall KC, ed.), pp. 387–430. Plenum Press, New York.

2 Yu C, Lee AM, Bassler BL & Roseman S (1991) Chitin utilization by marine bacteria: a physiological function for bacterial adhesion to immobilized carbohydrates. *J Biol Chem* **266**, 24260–24267.

3 Bassler BL, Gibbons PJ, Yu C & Roseman S (1991) Chitin utilization by marine bacteria: chemotaxins to chitin oligosaccharides by *Vibrio furnissii*. *J Biol Chem* **266**, 24268–24275.

4 Keyhani NO & Roseman S (1996) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*: molecular cloning, isolation, and characterization of a periplasmic chitodextrinase. *J Biol Chem* **271**, 33414–33424.

5 Keyhani NO & Roseman S (1996) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*: molecular cloning, isolation, and characterization of a periplasmic β-N-acetylglucosaminidase. *J Biol Chem* **271**, 33425–33432.

6 Keyhani NO, Li X-B & Roseman S (2000) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*: identification and molecular cloning of chitoporin. *J Biol Chem* **275**, 33068–33076.

7 Park JK, Keyhani NO & Roseman S (2000) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*: identification and molecular cloning, and characterization of a N, N'-diacetylchitobiose phosphorylase. *J Biol Chem* **275**, 33077–33083.

8 Warren RAJ (1996) Microbial hydrolysis of polysaccharides. *Annu Rev Microbiol* **50**, 183–212.

9 Patil RS, Ghormade V & Deshpande MV (2000) Review: Chitinolytic enzymes: an exploration. *Enzyme Microb Technol* **26**, 473–483.

10 Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **280**, 309–316.

11 Henrrisat B & Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **293**, 781–788.

12 Papanikolau Y, Prag G, Tavlas G, Vorgias CE, Oppenheim AB & Petratos K (2001) High resolution structural analyses of mutant chitinase A complexes with substrates provide new insight into the mechanism of catalysis. *Biochemistry* **40**, 11338–11343.

13 Bortone K, Monzingo AF, Ernst S & Robertus JD (2002) The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism. *J Mol Biol* **320**, 293–302.

14 Armand S, Tomita H, Heyraud A, Gey C, Watanabe T & Henrissat B (1994) Stereochemical course of the hydrolysis reaction catalysed by chitinase A1 and D from *Bacillus circulans* WL-12. *FEBS Lett* **343**, 177–180.

15 Terwissha van Scheltinga AC, Armand S, Kalk KH, Isogai A, Henrissat B & Dijkstra BW (1995) Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and x-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis. *Biochemistry* **34**, 15619–15623.

16 Sasaki C, Yokoyama A, Itoh Y, Hashimoto M, Watanabe T & Fukamizo T (2002) Comparative study of the reaction mechanism of family 18 chitinases from plants and microbes. *J Biochem* **131**, 557–564.

17 Koga D, Sasaki Y, Uchiumi Y, Hirai N, Arakane Y & Nagamatsu Y (1997) Purification and characterization of *Bombyx mori* chitinases. *Insect Biochem Mol Biol* **27**, 757–767.

18 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. *Appl Environ Microbiol* **64**, 3397–3402.
19 Bokma E, Barends T, Terwisscha van Scheltinga AC, Dijkstra BW & Beintema JJ (2000) Enzyme kinetics of hevamine, a chitinase from the rubber tree Hevea brasiliensis. *FEBS Lett* **478**, 119–122.
20 Fukamizo T, Sasaki C, Schelp E, Bortone K & Robertus JD (2001) Kinetic properties of Chitinase-1 from the fungal pathogen *Coccidioides immitis*. *Biochemistry* **40**, 2448–2454.
21 Hollis T, Honda Y, Fukamizo T, Marcotte E, Day PJ & Robertus JD (1997) Kinetic analysis of Barley chitinase. *Arch Biochem Biophys* **344**, 335–342.
22 Thompson SE, Smith M, Wilkinson MC & Peek K (2001) Identification of a chitinase antigen from *Pseudomonas aeroginosa* strain 385. *Appl Environ Microbiol* **67**, 4001–4008.
23 Honda Y, Kitaoka M, Tokuyasu K, Sasaki C, Fukamizo T & Hayashi K (2003) Kinetic studies on the hydrolysis of N-acetylated and N-deacytylated derivatives of 4-methylumbelliferyl chitobioside by the family 18 chitinases ChiA and ChiB from *Serratia marcescens*. *J Biochem* **13**, 253–258.
24 Suginta W, Robertson PAW, Austin B, Fry SC & Fothergill-Gilmore LA (2000) Chitinases from *Vibrio*: activity screening and purification of Chi A from *Vibrio carchariae*. *J Appl Microbiol* **89**, 76–84.
25 Suginta W, Vongsuwan A, Songsiriritthigul C, Prinz H, Estibeiro P, Duncan RR, Svasti J & Fothergill-Gilmore LA (2004) An endochitinase A from *Vibrio carchariae*: cloning, expression, mass and sequence analyses, and chitin hydrolysis. *Arch Biochem Biophys* **424**, 171–180.
26 Roberts WK & Selitrennikoff CP (1988) Plant and bacterial chitinases differ in antifungal activity. *J Gen Microbiol* **134**, 169–176.
27 Perrakis A, Tews I, Dauter Z, Oppenheim AB, Chet I, Wilson KS & Vorgias CE (1994) Crystal structure of a bacterial chitinase at 2.3 Å resolution. *Structure* **2**, 1169–1180.
28 Hollis T, Monzingo AF, Bortone K, Ernst S, Cox R & Robertus JD (2000) The X-ray structure of a chitinase from the pathogenic fungus *Coccidioides immitis*. *Protein Sci* **29**, 544–551.
29 Watanabe T, Kobori K, Miyashita K, Fujii T, Sakai H, Uchida M & Tanaka H (1993) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J Biol Chem* **268**, 18567–18572.
30 van Aalten DMF, Komander D, Synstad B, Gaseidnes S, Peter MG & Eijsink VGH (2001) Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *Proc Natl Acad Sci USA* **98**, 8979–8984.
31 Synstad B, Gaseidnes S, van Aalten DMF, Vriend G, Nielsen JE & Eijsink VGH (2004) Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase. *Eur J Biochem* **271**, 253–262.
32 Bradford MMA (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
33 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
34 Trudel J & Asselin A (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal Biochem* **178**, 362–366.