Structures of the cloned chitinase and its truncant from *A. caviae* *

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Abstract *Aeromonas caviae* can secrete several chintinases with different molecular weights. One chitinase gene *chi1* has been cloned and sequenced. It encodes a protein of 865 amino acids with a signal peptide at the N-terminus, a polycystic kidney disease(PKD)-like domain, a triosephosphate isomerase(TIM) catalytic region, a receptor for egg jelly(REJ)-like domain and two tandem chitin binding domains (ChBDs). The entire chitinase degrades colloid chitin both endolytically and exolytically into *N*-acetylglucosamine, chitobiose and chitotriose. When removing the 302 amino acids at C-teminus its activity remains but the degraded products are chitobiose, chitotriose and chitotetraose. The study shows that for the full-length chitinase, its substrate with the shortest length is chitotriose while in its truncated form, it is chitotetraose.

Keywords: chitinase, chitin binding domains, sequence analysis, spatial structure.

Chitin, a carbohydrate polymer composed of alternating β-1, 4-linked *N*-acetylglucosamine residues, is the second most abundant organic compound in nature next to cellulose. Chitinases, which are able to break down the glycosidic bonds of Chitin, are widespread in organisms including bacteria, fungi, plants, invertebrates (mainly nematodes, insects and crustaceans) and all classes of vertebrates (even humans). Up to date, a number of chitinase genes have been cloned and sequenced. Based on the amino acid sequence similarity of their catalytic domains, chitinases are classified into two families, family 18 and family 19, in the classification system of glycosyl hydrolases[1]. The majority of bacterial chitinases belong to family 18, with some exceptions in *Streptomyces*[2].

The three-dimensional structures of three bacterial family 18-type chitinases have recently been determined. They are chitinase A(Chi A)[3] and chitinase B(Chi B)[4] from *Serratia marcescens* and chitinase A1(ChiA1)[5] from *Bacillus circulans*. Chi A has two domains, an N-terminal domain and a catalytic $(\beta/\alpha)_8$ barrel domain. Chi B comprises a catalytic $(\beta/\alpha)_8$ barrel domain and a C-terminal chitin-binding domain (ChBD). The structures of these two *Serratia* chitinases are both resolved by X-ray crystallography, but the structures of different domains of *Bacillus* Chi A1 are determined separately, namely an N-terminal catalytic domain (CatD) by X-ray crystallography and two Fibronectin type IIIs (Fn III) and one C-terminal ChBD by NMR. A catalytic model for *Bacillus circulans* Chi A1 according to its spatial structure, enzyme characteristics and some experiments on mutants[5] has been proposed. However, the limited information on the 3D-structures has hindered the elucidation of their catalytic mechanism. In addition, functions of some domains such as Fn III -like domain, PKD-like domain, etc. are still unclear. Thus in the near future, clarification of respective roles of these distinct domains will not only assist in deeper apprehension of chitinase reaction mechanism but serve as a guidance to its application.

Aeromonas caviae CB101 is one of the bacteria secreting mutiple chitinases, it utilizes chitin as its sole carbon and nitrogen source. In this study, we cloned and sequenced one of the chitinase genes from *Aeromonas caviae* CB101, named *chi 1*. It encodes a protein of 865 amino acids and the protein can catalyze chitin in both intact and truncated forms.

1 Materials and methods

1.1 Bacteria and plasmids

A. caviae CB101, a chitinolytic strain with a

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high-catalytic activity, was isolated from soil samples collected from the coast of Xiamen. pBluescriptII KS was used as the cloning vector, and the plasmid pQE-70 (Qiagen) as the expression vector. pQE-70-563aa and pQE-70-865aa containing a 1.7 kb or 2.6 kb insert respectively were both recombinant plasmids derived from pQE-70. All strains harboring recombinant plasmids were grown in LB medium with 100 μg/mL ampicillin.

1.2 DNA manipulation

DNA extraction from *A. caviae* CB101 or from plasmids, transformation of *E. coli* with the recombinant plasmids, and electrophoresis of DNA fragments were performed as described in Ref. [6].

1.3 Cloning and sequencing of Chitinase1(Chi 1)

Two primers were designed according to the chitinase gene sequence in GenBank (accession no. U09139), which included an *Eco*RI or a *Hin*d III site respectively. The primer sequences are 5'CTCTGAATTCATGTTAAGTCCAAAACTTTC3' (N-terminus) and 5' TCTCAAGCTTCAGTTGCAGCTCGCCGCGGCCTGAT3' (C-terminus). A 30-cycle of PCR was performed with the following profile: denaturation at 94 ℃ for 1 min, annealing and extension at 68 ℃ for 4 min except for the final cycle where extension continued for additional 8 min. The amplified product and the cloning vector pBluescriptII KS were ligated and transformed into *E. coli* XL1-Blue. Transformants were screened on LB agar plates in the presence of ampicillin (100 μg/mL), isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mmol/L), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 40 μg/mL). The positive colonies were selected for the subsequent subcloning experiment for acquiring the full-length DNA sequence.

1.4 Construction of the recombinant expression plasmids

Three primers, all with a *Sph*I site at 5' ends were designed based on the above full-length sequence. N1 primer (5'TTCGCATGCTAAGTCCAAAACTTTCCCTG3') came from the N-terminus of the signal peptide sequence; C1 primer (5' TGTGCATGCTCAGCGTACCCTCGCCGTG3') came from the C-terminus but without the stop codon; C2 primer (5' TGTGCATGCTGTTGCAGCTCGCCGCGC3') was from the sequence encoding for 558～563 amino acids.

PCR was performed as indicated in Sec. 1.3. The products amplified by primers N1 and C2, or primers N1 and C1 were digested with *Sph*I and then inserted into the dephosphorylated *Sph*I site of pQE-70 to yield the recombinant plasmids expressing the entire Chi1 (pQE-70-865aa) or the truncated Chi1$^-$ with only the first 563 amino acids (pQE-70-563aa), both with a six-His tag at their C-termini.

1.5 Expression and purification of the recombinant chitinases

The transformants carrying plasmids pQE-70-865 and pQE-70-563 were cultured at 37 ℃ in LB until OD_{600nm} reached about 0.6. IPTG was added to a final concentration of 0.5 mmol/L. Cultivation was continued for another 6 h before harvest. The cells were then centrifuged at 8000 g for 5 min. The periplastic proteins were prepared by the osmotic shock method and purified through an Ni-NTA agarose matrix according to the manufacturer's instruction (Qiagen).

1.6 Thin layer chromatography (TLC) assay

Into 20 μL of 25 mmol/L sodium phosphate buffer (pH6.0) containing 10 μg $(GlcNAc)_{3-6}$ (Sigma) or 500 μg colloid chitin, 0.5 μg recombinant chitinase with 865 or 563 amino acids was added and the mixture was incubated at 37 ℃. Released chitooligomers were separated by TLC on silica gel 60 plates (Merck) using 1-butanol/ethanol/water (5∶3∶2) as solvent. Sugars were detected on the TLC plate by rapid immersion into the staining agent of diphenylamine/aniline/orthophosphoric and by the following heating at 120 ℃ for 10 min.

1.7 Homology modeling

Homology modeling was done by Swiss-Pdb-Viewer software on computer, using *Serratia marcescens* ChiA and ChiB as the structural templates.

2 Results

2.1 Cloning of the chitinase gene from *A. caviae* and analysis of its protein product

A 2.6 kb PCR product was amplified from *A. caviae* CB101 chromosomal DNA, and it was confirmed to be a chitinase gene (named *Chi1*) by sequencing. The gene is 2598 bp in length, encoding a protein of 865 amino acids with molecular weight of

91.5 kD(Fig. 1).

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1   MLSPKLSLLA LLVGGLCTTS AFAAAPGKPT IGSGPTKFAI VEVNQAASAY NQLVTVHKDG
61  APVSVTWNLW SGDVGQTAKV LLDGKEVWSG PASAAGTANF KVTKGGRYQM QVALCNADGC
121 TLSDKKELMV ADTDGSHLAP LNAPLKENNK PYANKSGKVV GAYYVEWGVY GRKFTVDKIP
181 AQNLTHILYG FTPICGGNGI NDSLKEISGS FEALQRSCAG REDFKVSIHD PWAAIQMSQG
241 NLSAWDEPYK GNFGNLMALK QAHPDLKILP SVGGWTLSDP FYFLGDKTKR DTFVASVKEF
301 LQTWKFFDGV DIDWEFPGGQ GANPSLGGPN DGATYVVLMK ELRAMLDELE AETGRQYELT
361 SAISAGGDKI AKVDYQAAQQ YMDHIFLMSY DFSGAFDLTN LAHQTNLFAS SWDPATKYTT
421 DKGVKALLGQ GVTPGKIVVG AAMYGRGWTG VKNYQAGNPF TGTATGPVSG TWENGVVDYR
481 DIVNNRMGAG WEQGYDESAE APYVFKASSG DLITFDNDRS VKAKGQYVLA NQLGGLFAWE
541 IDADNGDILN AMHEGLGHGE GTLPPVNKPP VANAGSDLSA TGPAEVTLNG SASHDPENGA
601 LTYSWKQVSG PQASLLDATQ AKARVVLDAV SSDINLVFEL TVTDDQGLSA KDQVVVTNKA
661 PQPNLPPVVS VPASATVEAG KQVSIKATAS DPNGDALSYQ WTVPAGLSAT GLDSATLVVT
721 GSNVTSDTAY DLTLVVTDGA LDATAVTRLT VKPASTGGGC EASDPDAANH PAWSAGTVYN
781 TNDKVSHKQL VWQAKYWTQG NEPSRTADQW KLVSQVQLGW DAGVVYNGGD VTSHNGRKWK
841 AQYWTKGDEP GKAAVWVDQG AASCN
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Fig. 1. The amino acid sequence of Chi1 from *A. caviae* CB101.

2.2 Domains of Chi1

A typical signal peptide of 23 amino acids is located at the N-terminus of Chi1, showing a positive-charged region (Lys-5), an α-helix segment, a little longer hydrophobic fragment (six-Leu, two-Ala as well as one-Val) and a short noncharged hydrophilic part (Thr, Ser) in turn. By searching on www.ncbi.nlm.nih.gov, four conserved domains were found, namely in order from N- to C-terminus, a polycystic kidney disease (PKD)-like domain, once considered to be an Fn-III-like motif, a catalytic domain classified into the family-18 type glycosyl hydrolases, a receptor for egg jelly (REJ)-like domain and two tandemly-repeated chitin-binding domains (ChBDs). All these different domains had their parellels in many other chitinases (Plate I (a)). The PKD domain and the REJ domain are somehow analogous, both supposed to function in the interaction between proteins and other macromolecules. Two ChBDs are highly related and had counterparts in many cellulases and xylanses, which also are involved in the binding of other insoluble substrates[7]—cellulose or xylan.

2.3 Homology modeling of Chi1

For up to 74.29% identity between the N-terminal 563 amino acids sequence of Chi1 and the crystallized *Serratia marcenscens* ChiA (PDB no. 1EDQ), it was possible to construct a partial 3D-structure of Chi1 by homology modeling. The model demonstrated the existence of three domains, an all β strand PKD at N-terminus (residues 24 ~ 137) connecting through a hinge region to the main triosephosphate isomerase (TIM) catalytic barrel (residues 159 ~ 563) (Plate I (b)). The TIM barrel was a tight structure of eight β-strand inner barrels surrounded by eight-α-helix outer barrels forming a long grooves for holding chitin chain (Plate I (c)), yet interrupted by an almost all β-strand of 74 amino acids (residues 443 ~ 516) which appeared to be a slightly-distorted plane in supersecondary structure. In the PDB database, similar distorted planes merely with different numbers of β-strands could be found in the crystal structures of xylases, cellulases as well as some other chitinases. Interestingly, *S. m*arcescens ChiB (PDB no. 1E15B) was almost able to be precisely interposed onto *S. m*arcescens ChiA only with exception of the lackage of the N-terminal all β-sheet domain and the additional C-terminal extension. Within the C-terminal extension fragment of *S. m*arcescens ChiB, there were two discrete parts related to the rest 302 amino acids at C-terminus of Chi1. One was a flexible linker and the other was just one ChBD. So Chi1 seemed to be the combination of ChiA and ChiB of *S. m*arcescens. The C-terminal 302 amino acids may play certain roles in the binding or recognizing of chitin.

2.4 Expression, purification and functional comparison of the entire chitinase and its truncant

In order to study possible function distinction between the entire chitinase and the truncated form which without the C-terminus (302 amino acids), we constructed the pQE-70-derived recombinant expression plamids and purified the two chitinases by affinity chromatography from their periplasmic proteins (Fig. 2). By using colloidal chitin as the substrate, TLC assays indicated that the major products of the complete chitinase were chitobiose(80%) and rather less chitotriose. When extending the reaction time, *N*-acetylglucosamine became visible (Fig. 3, lane 10). In contrast, the truncated chitinase released much larger chitooligomers, that is, from chitobiose to chitotetraose of almost the same amount (Fig. 3, lane 9). *N*-acetylglucosamine could not be visualized even prolonging the incubation time. Further experiment with chitooligomeric substrates from chitotriose to chitohexaose revealed apparent disparity in their cuttable minimal chitooligosaccharides. In the case of full-length chitinase (Fig. 3, lane 2), it was chitotriose, while for the truncated one it was chitotetraose(Fig. 3, lanes 1 and 3). Obviously, Chi1 had both endo- and exo-chitinase activity.

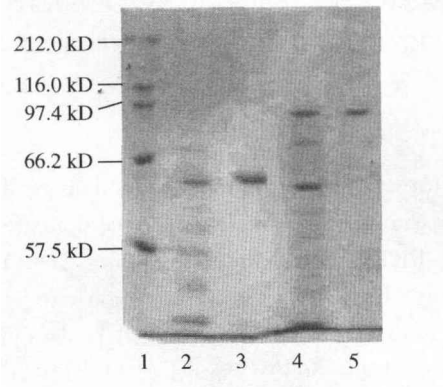

Fig. 2. SDS-PAGE of Chi1 and its truncant Chi1$^-$. Lane 1, molecular weight marker; lane 2, total periplasmic proteins from *E. coli* Xl1-Blue expressing Chi1$^-$; lane 3, purified Chi1$^-$ from Ni-NTA agarose; lane 4, total periplasmic proteins from *E. coli* Xl1-Blue expressing Chi1; lane 5, purified Chi1 from Ni-NTA agarose.

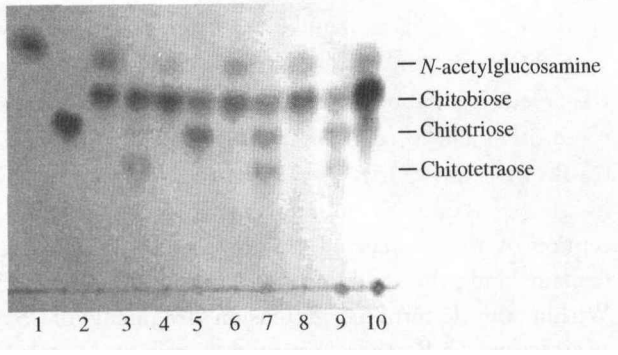

Fig. 3. TLC assay of the Chi1 and Chi1$^-$. Lanes 1, 3, 5, 7, 9 are released products with chitotriose, chitotetraose, chitopentaose, chitohexaose or colloid chitin as substrates by Chi1$^-$ (563 amino acids) treatment (24 h); lanes 2, 4, 6, 8, 10 are released products with chitotriose, chitotetraose, chitopentaose, chitohexaose or colloid chitin as substrates by Chi1(865 amino acids) treatment(24 h). Lanes 1 and 3 indicate that chitotetraose is the minimal substrate of Chi1$^-$.

3 Discussions

Chitinolytic bacteria play very important roles in natural chitin recycle. As to the extensive chitin polymorphism, they develop various complete chitin-digesting systems. Chitinases can hydrolyze chitin endolytically and exolytically, and in combination with *N*-acetylglucosaminase, they generate *N*-acetylglucosamine as the final product[8]. Chitin-binding proteins without any chitinase activity are also found involved in the digestion. Till now, many different chitinase genes have been cloned and analysed[8~12], and it is shown that chitinases are composed of one or several discrete domains. Besides the most significant catalytic segment, other domains, for example Fn III-like domain, chitin-binding domain are also found[13]. As *A. caviae* Chi1 has several of these domains even with the combinational acitivity of endo-chitinase and exo-chitinase, it could serve as a good model to study their respective functions. Considering the facts that the first 563 amino acids of Chi1 are similar to *Serratia marcescens* ChiA, and its linker and ChBD domains similar to *Serratia marcescens* ChiB, *A. caviae* Chi1 could be deduced directly by threading, editing and modifying with Swiss-Pdb Viewer software using the data from ChiA and ChiB.

In this constructed model, the C-terminal 302 amino acids, containing a REJ-like domain and ChBDs, seems to extend the anchoring sites for chitin sugar rings (Plate I (d)), which may make substrates bind to the catalytic core more stable. The loss of REJ-like domain and ChBDs will cause that the chitotriose could not bind to the catalytic core as substrate. This truncated chitinase could only use chitotetraose as its minimal substrates instead of chitotriose. That is why the truncated chitinase has its chitin hydrolysis products changing from the major dimmer to larger oligomers (from dimmer to tetramer) and even without any *N*-acetylglucosamine at all.

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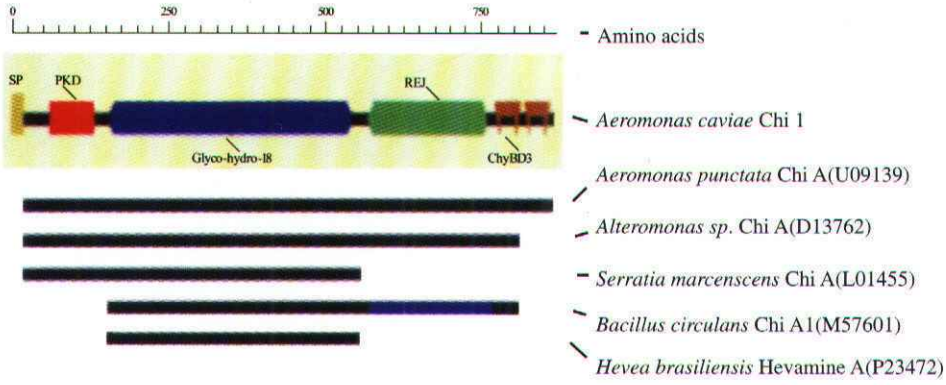
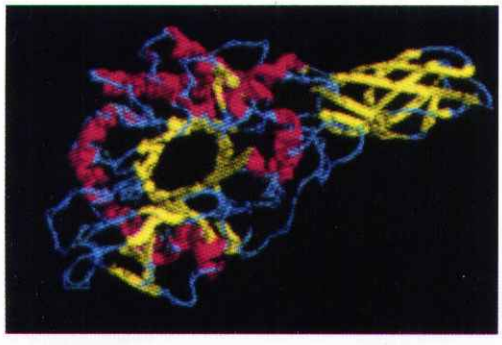
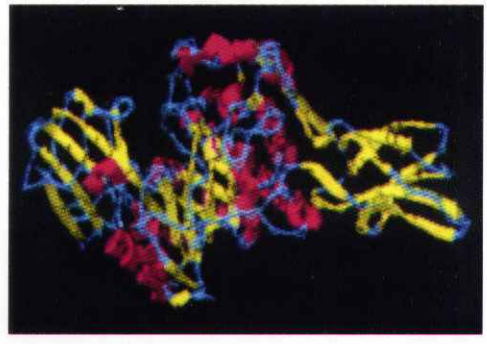
ZHOU Ying, et al. Structure of the cloned chitinase and its truncant from *A.caviae* (Plate I)



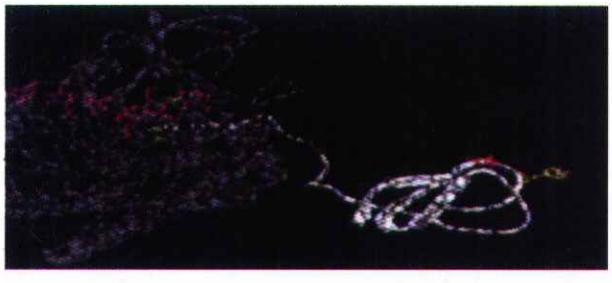
(a) Analogous domains between *A.caviae* Chi1 and other chitinases. SP stands for signal peptide; PKD, REJ, ChtBD3 are three potential domains responsible for the interaction between protein and carbonhydrate; Glyco-hydro-18 is a catalytic region. The blue region shown in *Bacillus circulans* Chi A1 is noncognate part aligned with *Aeromonas caviae* Chi1.



(b) Ribborn of the chitinase trunctant (563 amino acids). The PKD domain located at the N-terminus (right) is linked to the catalytic domain (center) through a 21 amino acids coil.



(c) Counterpart of (b) after a ninty-degree rotation round the x-axle. A groove (up-left) is a substrate binding site.



(d) Chitin binding region of Chi 1 at the C-extension (right) and the catalytic region together with chitooligomers (the magenta rings on up-left). Two catalytic residues (Glu-315 and Asp-391) are illustrated in green.