微生物学报
Acta Microbiologica Sinica
2016, 56(7): 1089-1104
http://journals.im.ac.cn/actamicrocn
DOI: 10.13343/j.cnki.wsxb.20150410

Research Paper 研究报告

Characterization of an exo-chitinase from a *Citrobacter* strain isolated from the intestine content of large yellow croakers

Jie Xu[1,2#], Yalin Yang[2#], Yang Liu[2], Chao Ran[2], Juan Li[2], Suxu He[2], Li Xu[2], Chunxiang Ai[1*], Zhigang Zhou[2*]

[1] College of Ocean & Earth Sciences, Xiamen University, Xiamen 361005, Fujian Province, China

[2] Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China

Abstract: [**Objective**] We isolated bacterial strains with chitin-degrading activity from the digesta of large yellow croakers (*Pseudosciaena crocea*) fed with chitin-enriched trash fish, and characterized potential chitinases thereof. [**Methods**] Chitin-degrading strains were screened with colloidal chitin agar from the digesta of *P. crocea* fed with trash fish. The chitinase gene (*chi-X*) was cloned and expressed in *Escherichia coli*, and the enzymatic properties of the chitinase (CHI-X) were characterized. [**Results**] A *Citrobacter freundii* strain with chitin-degrading activity was isolated. The chitinase gene encodes a protein containing 493 amino acid residues, with a proposed glycoside hydrolase family-18 catalytic domain. CHI-X could hydrolyze colloidal chitin. The optimal pH for CHI-X was 4.0 at optimal temperature (60 °C). CHI-X was active over a broad pH range, with around 90% of the activity maintained after incubation at pH between 3.0 and 11 for 1 h. The enzymatic activity of CHI-X was stimulated by Mn^{2+}, Li^{+}, and K^{+}, but inhibited by Ag^{+}. The enzyme was stable after treatment by proteases and grouper intestinal juice. CHI-X hydrolyzes colloidal chitin into GlcNAc and $(GlcNAc)_2$. Furthermore, an synergic effect was observed between CHI-X and ChiB565 (a chitinase from *Aeromonas veronii* B565) on colloidal chitin. [**Conclusion**] CHI-X from intestinal bacterium may be potentially used as feed additive enzyme for warm water marine fish.

Keywords: chitinase, *Citrobacter freundii*, *Pseudosciaena crocea*

Supported by the Ocean Industry Research Special Funds for Public Welfare Projects (201305022-6), by the National Science and Technology Support Program Project of China (2012BAD25B02, 2013BAD10B01-2-3), by the Beijing Earmarked Fund for Modern Agro-industry Technology Research System (SCGWZJ20121104-4), by the Special Fund for Basic Research of the Chinese Academy of Agricultural Sciences (2013ZL043) and by the Central Public-Interest Scientific Institution Basal Research Fund (2013ywf-sl-4)

[*]Corresponding authors. Chunxiang Ai, Fax: +86-592-2186397, E-mail: chunxai@xmu.edu.cn; Zhigang Zhou, Tel: +86-10-82106073, Fax: +86-10-82106054, E-mail: zhouzhigang03@caas.cn

[#]These authors contributed equally to this work.

Received: 10 September 2015; Revised: 5 December 2015; Published online: 11 December 2015

Chitin is a polysaccharide composed of β-(1, 4)-*N*-acetylglucosamine (GlcNAc) units. It is the second most abundant biomass next to cellulose, and represents an important carbon and nitrogen source for marine organisms[1]. Chitin is widely distributed in organisms, mainly as structural components such as arthropod exoskeletons, mollusk shells, and fungal cell walls. Most of the chitin that exists in the natural world has α- or β-chitin crystalline structure[2–3].

Chitinases (EC 3.2.1.14) are enzymes that can degrade chitin into *N*-acetylchito-oligosaccharides $(GlcNAc)_n$ and GlcNAc[4–5]. Chitinases are widely distributed in nature across the domains of bacteria, archaea, and eukarya, while microorganisms, especially bacteria, represent an important source of chitinases[1, 6–9]. Chitinases are present in the digestive system of many fish species, particularly crustaceans, with the physiological function as degrading chitinous substances as food[10–11]. Most of reported chitinases from the digestive tracts of fish were endogenous fish enzymes[12–15], while chitinases produced by microorganisms in the gastrointestinal tract of fish were also reported[16–18]. As examples of the involvement of microorganisms-originated chitinases in the nutrition intake of fish, chitinases produced by bacteria in the gut of *Gadusmorhua* can degrade chitin into energy sources[19]; and chitinase ChiB565 from *Aeromonas veronii* B565 improved the growth and feed conversion of tilapia[20].

The large yellow croaker *Pseudosciaena crocea* (Richardson, 1846), native to the Yellow and East China Seas, is a perciform marine and brackish-water species (Family, Sciaenidae). It is economically valuable as a major maricultured fish in China[21]. *P. crocea* mainly feed on small fish, shrimps, and crabs, which contain high amount of chitinous substances[22]. Although the gastrointestinal microbiota of fish have been suggested to be associated with the chitin digestive processes[23], no studies have investigated chitinases produced by the gastrointestinal microorganisms in large yellow croakers. In this research, chitin-degrading bacteria were screened from the intestinal samples of juvenile large yellow croaker fed with trash fish. A *Citrobacter freundii* strain with chitin-degrading activity was isolated and a new chitinase gene was cloned and expressed. The enzymatic property of chitinase was also characterized.

1 Materials and methods

1.1 Isolation and identification of chitin-degrading bacteria

The intestinal contents of fish fed with trash-fish (0.5 g) were homogenized in 500 μL phosphate buffered saline (0.01 mol/L, pH 7.4), then serially diluted to 10^{-1}, 10^{-2} and 10^{-3}. One hundred microliters of three different dilutions were individually spread onto TSA (Tryptic Soy Agar) and colloidal chitin (prepared according to Zhang et al. 2014)[20] agar. These cultures were incubated at 28 °C for 48 h. Colonies were picked and identified by PCR amplification and sequencing of the 16S rRNA genes[27].

1.2 Cloning of chi-X

The degenerate primers ChiR (5′-CCAGGC GCCGTGGARRTCRTANSWCA-3′) and ChiF (5′-CGTGGACATCGACTGGGARTWYCC-3′) were designed with CODEHOP (Consensus-degenerate hybrid oligo nucleotide primers software, http://blocks.fhcrc.org/codehop.html) according to

the conserved amino acid sequences of bacterial chitinases downloaded from NCBI. A fragment of the chitinase gene was amplified from genomic DNA of *Citrobacter freundii* and sequenced. The upstream and downstream sequences of the chitinase gene fragment were cloned with fusion primer and nested, integrated PCR (FPNI-PCR)[28]. Special nested primers designed by Primer 5.0 are listed in Table 1. The flanking sequences that most resemble those of known chitinases and the sequence of the chitinase gene fragment were assembled *in silico* by DNAstar V7.10. The open-reading frame was identified by the Open Reading Frame Finder tool (http://www.ncbi.nlm.nih.gov/projects/gorf/), and the assembled gene is named *chi-X*. PR. The open reading frame of *chi-X* was amplified with primers PF (5′-CAGCAAATGGGTCGCGGATCCGAATTCATGACTAACAGCAAACTGGTAC-3′) and PR (5′-GTGGTGGTGGTGGTGGTGCTCGAGTGCCTTCGTAATACCTTTAAAATAGAAC-3′), with *Eco*R I or *Xho* I restriction sites underlined. The PCR product was cloned into a pEASY-T3 vector to construct the plasmid *chi-X*-T3.

Table 1. FP primers (no hair pin structure), universal primers and special nested primers used in FPNI-PCR

Names	Primer sequence (5′→3′)	Primer use	References
FP1:	GTAATACGACTCACTATAGGGCACGCGTGGT NTCGA STWTS GWGTT	1st step	Wang et al. 2011
FP2:	GTAATACGACTCACTATAGGGCACGCGTGGT NGTCG ASWGA NAWGAA	1st step	Wang et al. 2011
FP3:	GTAATACGACTCACTATAGGGCACGCGTGGT WGTGN AGWAN CANAGA	1st step	Wang et al. 2011
FP4:	GTAATACGACTCACTATAGGGCACGCGTGGT AGWGN AGWAN CAWAGG	1st step	Wang et al. 2011
FP5:	GTAATACGACTCACTATAGGGCACGCGTGGT NGTAW AASGT NTSCAA	1st step	Wang et al. 2011
FP6:	GTAATACGACTCACTATAGGGCACGCGTGGT NGACG ASWGA NAWGAC	1st step	Wang et al. 2011
FP7:	GTAATACGACTCACTATAGGGCACGCGTGGT NGACG ASWGA NAWGAA	1st step	Wang et al. 2011
FP8:	GTAATACGACTCACTATAGGGCACGCGTGGT GTNCG ASWCA NAWGTT	1st step	Wang et al. 2011
FP9:	GTAATACGACTCACTATAGGGCACGCGTGGT NCAGC TWSCT NTSCTT	1st step	Wang et al. 2011
FSP1:	GTAATACGACTCACTATAGGGC	2nd step	Wang et al. 2011
FSP2:	ACTATAGGGCACGCGTGGT	3rd step	Wang et al. 2011
PU1	TTTAGCCATAAAGTGGAACG	1st step	the present study
PD1	ATGAGCCAGGCGTTCCAC	1st step	the present study
PU2	CGGGAATCGGGTGAACAG	2nd step	the present study
PD2	ACCCGATTCCCGATGTTT	2nd step	the present study
PU3	GGTGCGTCAGTATCATCGT	3rd step	the present study
PD3	GATGATACTGACGCACCAAA	3rd step	the present study

1.3 Structural analysis

The nucleotide sequence of *chi-X* was translated by Translate (http://web.expasy.org/translate) in ExPASy. Amino acid composition analysis was performed by ProtParam (http://www.expasy.org/cgi-bin/protparam). The SignalP 4.1 Server (http://www.

cbs.dtu.dk/services/SignalP/) was used to search for an N-terminal signal peptide[29]. The structural domain analysis was performed by Smart (http://smart.embl-heidelberg.de/). Highly similar amino acid sequences were identified by Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with CHI-X by structure-based sequence alignment using PROMALS3D (http://prodata.swmed.edu/promals3d/promals3d.php). CHI-X active-site residues were found by Pfam (http://pfam.sanger.ac.uk/search). The three-dimensional structure of CHI-X was modeled with the SWISS-MODEL Workspace (http://swissmodel.expasy.org/workspace/)[30–32].

1.4 Expression of CHI-X in *Escherichia coli*

Plasmid *chi-X*-T3 and pET28a (+) vector were both digested with *Eco*R I and *Xho* I. The chitinase gene from *chi-X*-T3 was inserted into the linearized pET28a (+) vector with T4 ligation (New England Biolabs, USA) to construct the expression vector, which was transformed into *E. coli* BL21 (DE3) competent cells (TransGen Biotech, China). Single *E. coli* colonies harboring the recombinant expression vector or pET28a (+) (as a control) were isolated from Luria-Bertani (LB) agar (100 μg/mL kanamycin) which was cultured at 37 °C overnight. Then the colonies were inoculated in LB broth containing 100 μg/mL kanamycin and cultured at 37 °C. Isopropyl-thio-d-galactopyranoside (final concentration, 1 mmol/L) was added when OD_{600} of the LB broth reached 0.6, and the culture was further incubated at 20 °C overnight, followed by harvest of the cells by centrifugation at 12000 ×g for 10 min at 4 °C. The pellets were suspended in 20 mmol/L Tris-HCl (pH 7.0) and sonicated (work conditions: 200 w power, 30 min total working time, each work 5 s and pause 8 s) by using JY92-II N Ultrasonic Processor (BaiDian Tech, China). The lysate was centrifuged at 12000 ×g for 10 min at 4 °C, and crude CHI-X was found in the supernatant upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The abundance of CHI-X relative to total protein in the lysate supernatant was calculated with Quantity One (Bio-Rad Laboratories, Inc. USA).

1.5 Purification and identification of CHI-X

To purify crude CHI-X, the lysate supernatant was loaded onto an column containing 2 mL Ni-NTA agarose (Sample & Assay Technologies, Germany), which was processed with a linear 20–200 mmol/L imidazole gradient in 20 mmol/L Tris-HCl (pH 7.6), 500 mmol/L NaCl, 10% (V/V) glycerol. The eluent containing 200 mmol/L imidazole was collected and loaded into a dialysis bag with cut-off molecular weight as 15 kDa. The bag was then put into a 1 L beaker full of distilled water for dialysis. The purity and apparent molecular mass of CHI-X was determined by SDS-PAGE. The purity of CHI-X was evaluated by Quantity One software. The identity of purified CHI-X was verified by Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

1.6 Chitinase activity assay

The chitinase activity was measured following the method described by Zhang et al.[20]. Colloidal chitin was used as substrate in CHI-X activity assay. 0.25 mL purified CHI-X was mixed with 0.25 mL of 1% (W/V) substrate in McIlvaine buffer (0.2 mol/L Na_2HPO_4, 0.1 mol/L citric acid) at pH 4.0. The reaction mixture was incubated at 60 °C for 1 h. At the end of reaction, the amount of reducing sugars

released from the substrate was measured by the 3,5-dinitrosalicylic acid (DNS) assay[33]. One unit (U) of chitinase activity was defined as the amount of enzyme that released 1 μmol/L of reducing sugars from the substrate per hour. GlcNAc was used as the carbohydrate standard. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard. The specific activity of CHI-X was calculated as a ratio of the chitinase activity to the protein concentration of reaction system. All assays were performed in triplicate. The K_m and V_{max} values were determined using colloidal chitin as the substrate , and the substrate concentration was 10, 8, 6, 4, 3 or 2 g/L. Values for K_m and V_{max} were obtained from a Lineweaver-Burk plot.

1.7 Characterization of enzymatic property of CHI-X

The optimal pH for CHI-X activity was determined at 60 °C with pH varied between 2.0 and 12.0. The CHI-X activity at pH 4.0 was considered as 100%. The stability of CHI-X at different pH values was determined by incubating CHI-X at 60 °C for 1 h in solutions with pH between 2.0 and 12.0, followed by assay of CHI-X activity under standard conditions. The buffers used were 0.2 mol/L glycine-HCl (pH 2.0), 0.2 mol/L Na_2HPO_4, 0.1 mol/L citric acid for pH between 3.0 and 8.0; 0.2 mol/L Tris-HCl for pH between 8.0 and 9.0; and 0.2 mol/L glycine-NaOH for pH between 9.0 and 10.0. The CHI-X activity at 0 h was considered as 100%.

The optimal temperature for CHI-X activity was determined at pH 4.0 with the temperature varied from 0 °C to 80 °C. The activity of CHI-X at 60 °C was considered as 100%. The thermal stability of CHI-X was assessed by assay of CHI-X activity under standard conditions after incubating CHI-X at 60 °C, 70 °C or 80 °C for 0, 5, 20, 40, 60, 80, 100 and 120 min. The activity of CHI-X at 0 min was considered as 100%. The effects of metal ions and chemical reagents on CHI-X activity were determined by incubating CHI-X and the substrate with the respective metal ion or chemical reagent at 1 mmol/L and 5 mmol/L under standard conditions. The activity of CHI-X in the absence of any test ingredient was considered as 100%.

To evaluate the effects of proteases on CHI-X activity, CHI-X was firstly incubated with pepsin, trypsin, or chymotrypsin at 37 °C for 1 h at a ratio of 1: 10 (protease/CHI-X), and the enzymatic activity was assayed under standard conditions. The pHs during incubation differed for different proteases, with the value for trypsin, chymotrypsin and pepsin as 7.0, 7.0 and 2.0, respectively. The activity of CHI-X with no exposure to a protease was considered as 100%. The effects of grouper intestinal juice on the stability of CHI-X was assessed by assay of CHI-X activity under standard conditions after incubating CHI-X with grouper intestinal juice at a ratio of 1: 10 (grouper intestinal juice /CHI-X) at 37 °C, pH 7.0 for 0, 30, 60, 90 and 120 min.

The substrate specificity of CHI-X was determined by using colloidal chitin (according to Zhang et al. 2014)[20], chitin powder (Sigma-Aldrich, C7170, USA), β-(1,3)-(1,4)-glucan (Sigma-Aldrich, V900511, USA), sodium carboxymethylcellulose (Sigma-Aldrich, 419273, USA), or glycol chitosan (Sigma-Aldrich, G7753, USA) as the substrate under standard reaction conditions.

1.8 Hydrolysis products of colloidal chitin by CHI-X

CHI-X and colloidal chitin were incubated under standard conditions for 0 to 6 h. Instead of DNS assay, the solutions were boiled for 10 min after

reaction, cooled to room temperature, and centrifuged at 12000×g at room temperature for 5 min. The reaction products were analyzed by thin layer chromatography (TLC) as described previously[34]. The products after 6 h of reaction were also subjected to LC-ESI-MS analysis. Briefly, the supernatants were deionized through 50 w×8 cation resin and 1×8 anion resin (Dowex, USA) for analysis. The hydrolysis products were identified by an LC-ESI-MS 2010 System (Shimadzu Corporation, Japan) in Analysis and Test Center of Institute of Chemistry, Chinese Academy of Sciences. Chloroform was used as the nebulizing gas.

1.9 Combined effect of CHI-X and ChiB565

To test the combined effect of CHI-X and ChiB565, a chitinase from *Aeromonas veronii* B565 with both exo- and endo-chitinase activity[20], 30 U CHI-X and 30 U ChiB565, single or mixed, was incubated with 0.1 g of colloidal chitin at 37 °C for 1 h, and the amount of reducing sugars released from the substrate was measured with DNS assay[20].

1.10 Store stability of CHI-X

Purified and dialyzed CHI-X was lyophilized. The lyophilized powder was stored at –20 °C, 4 °C, 30 °C, or 37 °C for 2 h, 3 d and 28 d. The specific activities of the lyophilized powders were measured by the method described above, and the activity of the powder before storage was considered as 100%. Store stability of ChiB565 lyophilized powder was also assessed for comparison.

2 Results and Analysis

2.1 Isolation and identification of chitin-degrading bacteria

We attempted to isolate bacteria with chitin-degrading ability from the intestinal samples of juvenile large yellow croaker fed with trash fish, which comprised anchovies and some chitin-rich crustaceans. Finally, a strain with chitin-degradation function was isolated from the intestinal content of fish fed with trash fish. The strain was confirmed as *Citrobacter freundii* by 16S rRNA sequence alignment, with over 99% identity with *Citrobacter freundii* strain P10159 (CP012544.1). The strain was deposited in the China General Microbiological Culture Collection Center (Beijing, China) (Accession No: CGMCC7536).

2.2 Sequence analysis of CHI-X

A 428 bp fragment of chitinase gene was amplified from genomic DNA of the *Citrobacter freundii* strain. Flanking sequences of the fragment were amplified by FPNI-PCR. The retrieved full sequence of the gene was 1482 bp. The ORF of *chi-X* encodes a protein with 493 residues. ProtParam analysis showed that the molecular mass of and pI of CHI-X were 54650.9 and 5.03, respectively. The enzyme was estimated to be stable and hydrophilic, as indicated by the instability index and grand average of hydropathicity score (21.070 and –0.365, respectively). BLAST analysis showed that the deduced CHI-X sequence was most similar to the hypothetical protein of *Citrobacter freundii* (WP_019078334.1), with 100% sequence identity at amino acid level and 99% sequence identity at nucleotide level. The nucleotide sequence of *chi-X* was deposited into the GenBank database under the accession number KC290945.

No N-terminal signal peptide was found by SignalP 4.1 in the deduced CHI-X sequence. Smart analysis indicated that CHI-X has a glycoside hydrolase family-18 catalytic domain (Glyco_18) formed by residues between 51 and 460. A multiple sequence alignment indicated that CHI-X contains

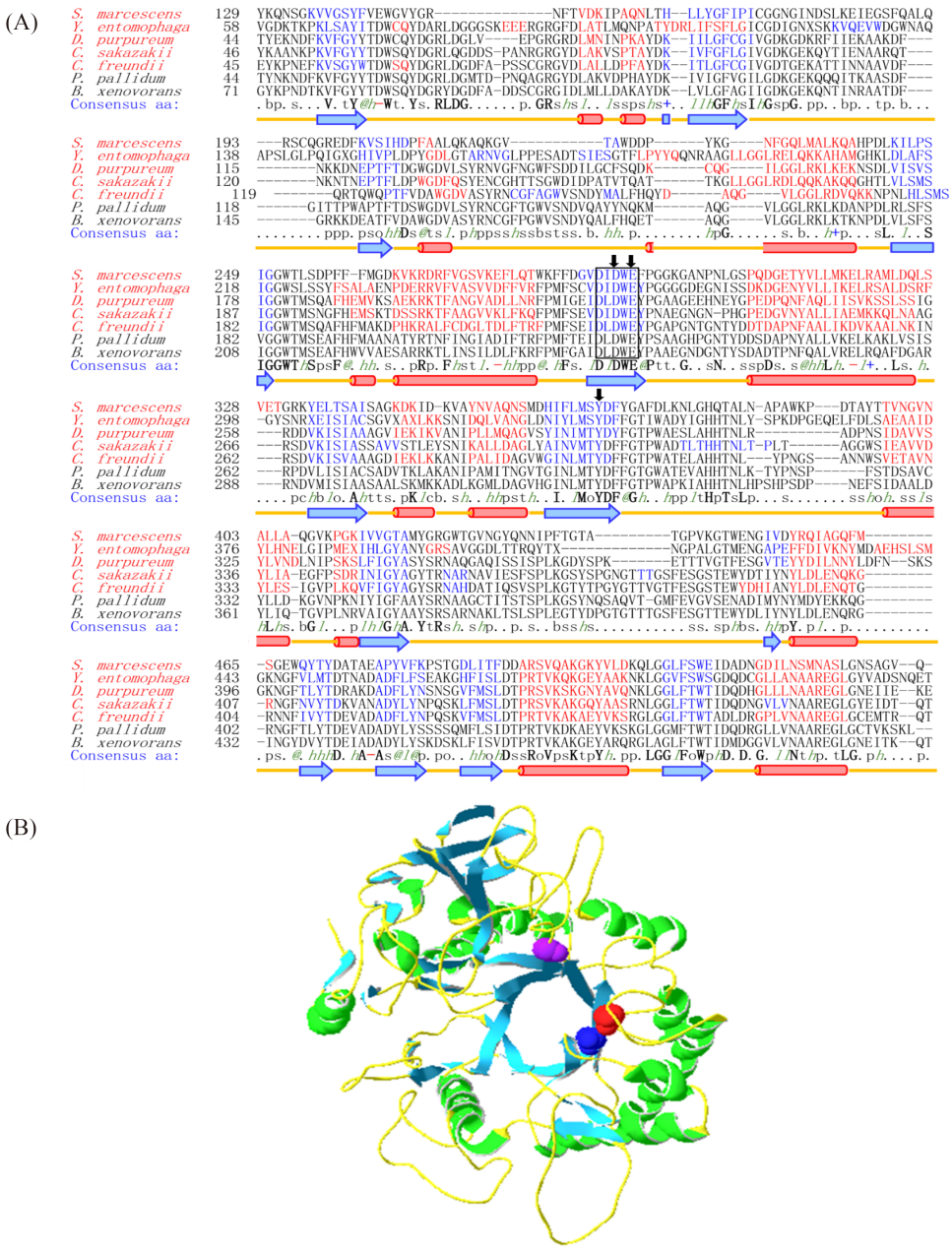

Figure 1. Structure of CHI-X. A: comparison of CHI-X from *C. freundii* with other similar sequences from six species indicated at the beginning of the alignment. Representative sequences have magenta names and they are colored according to predicted secondary structures (red: alpha-helix, blue: beta-strand). Consensus predicted secondary structure symbols: alpha-helix, cylinder; beta-strand, arrows. Conserved amino acids are in bold and uppercase letters. Consensus amino acid symbols: aliphatic (I, V, L), l; aromatic (Y, H, W, F), @; hydrophobic (W, F, Y, M, L, I, V, A, C, T, H), h; alcohol (S, T), o; polar residues (D, E, H, K, N, Q, R, S, T), p; tiny (A, G, C, S), t; small (A, G, C, S, V, N, D, T, P), s; bulky residues (E, F, I, K, L, M, Q, R, W, Y), b; positively charged (K, R, H), +; negatively charged (D, E), –; charged (D, E, K, R, H), c. The conserved sequence motif DXDXE of GH18 chitinases is boxed. Arrows point to predicted active sites (Asp224, Glu226 and Tyr299) of CHI-X. B: the predicted three-dimensional structure of the protein CHI-X based on the template 4 dwsD (1.80 A). In the picture, the purple, red and blue balls represent Tyr299, Glu226 and Asp224, respectively.

the conserved DXDXE motif (Figure 1-A) which is typical for chitinases in the glycoside hydrolase family 18[35]. The structure of CHI-X was predicted with SWISS-MODEL using ChiA from *Serratia marcescens* (PDB code:1EDQ_A; 1.55–Å resolution; predicted Glyco_18 domain: residues from 135 to 521) and Chi2 from *Yersinia entomophaga* (PDB code: 4DWS_D; 1.80–Å resolution; predicted Glyco_18 domain: residues from 64 to 500) as the templates, and two nearly identical models were obtained[8,36]. The sequences of CHI-X and ChiA are quite different (21.77% identity). The critical catalytic residues in ChiA include Glu315 (the proton donor), the adjacent Asp313, and Tyr390 at the opposite position of Glu315[36], while the corresponding CHI-X residues are Asp224, Glu226, and Tyr299. Notably, Glu226 was also predicted as a residue in the active site of CHI-X by Pfam. The sequence identity of CHI-X and Chi2 was 37.26%. The predicted three-dimensional structure of CHI-X with Chi2 used as the template is illustrated in Figure 1-B. The positions of Asp224, Glu226, and Tyr299 were highlighted.

2.3 Expression and purification of CHI-X

After fifteen hours incubation at 20 °C, cells harboring the CHI-X expression plasmid yielded a prominent SDS-PAGE band at 54 kDa, which corresponded well with the theoretical mass (54650 Da) (Figure 2-A). The maximum yield of CHI-X was 52.81% of total protein in the lysate supernatant. CHI-X was purified from the cell lysate by Ni^{2+}-affinity chromatography. The yield was 15.6 mg CHI-X/L culture, with the purity as high as 98.7% (Figure 2-B). LC-MS/MS analysis of the purified enzyme showed that 31% of the predicted CHI-X sequence was covered by identified peptides, indicating that the isolated protein was CHI-X.

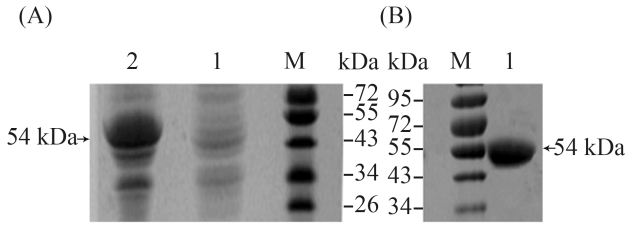

Figure 2. SDS-PAGE gel of proteins. A: SDS-PAGE gel of pET28a (+) and recombinant plasmid expressed from *E. coli* BL21 (DE3). M: protein molecular-mass markers; lane 1: cell extract of *E. coli* BL21 (DE3) harboring an empty pET28a (+); lane 2: cell extract of *E. coli* BL21 (DE3) harboring recombinant plasmid. B: SDS-PAGE gel of purified CHI-X. M: protein molecular-mass markers; lane 1: purified CHI-X liquid.

2.4 Chitinase activity of CHI-X

The chitinase activity of CHI-X was 137.7 U/mg toward colloidal chitin. The K_m and V_{max} values of CHI-X toward colloidal chitin at pH 4.0 and 60 °C are 6.24 mg/mL and 2.49 μmol/min, respectively.

2.5 Effect of pH and temperature on the activity of CHI-X

CHI-X exhibited similar activity profiles at different pH values for the two substrates. In both cases, the optimal pH of CHI-X was 4.0. CHI-X maintained more than 60% of its activity in the pH range of 3.0 to 8.0 for colloidal chitin at 60 °C (Figure 3-A). The pH stability of CHI-X was measured by the maintenance of activity after incubation at different pH, and higher maintenance of activity over a broad pH range was observed when colloidal chitin was used as the substrate, with over 90% of the activity maintained after incubation at pH from 3.0 to 11.0 (Figure 3-B).

The optimal temperature for CHI-X on colloidal chitin is 60 °C. 24.5%–39.8% of the activity was observed between 30–40 °C, which was the temperature range for tropical marine fish (Figure 3-C). Similarly, thermal stability of CHI-X was measured by the maintenance of activity after incubation at different temperatures. 66.4% of the activity was maintained after incubation of CHI-X at 60 °C for 2 h in colloidal chitin assay. CHI-X rapidly lost its activity at temperatures higher than 60 °C (Figure 3-D).

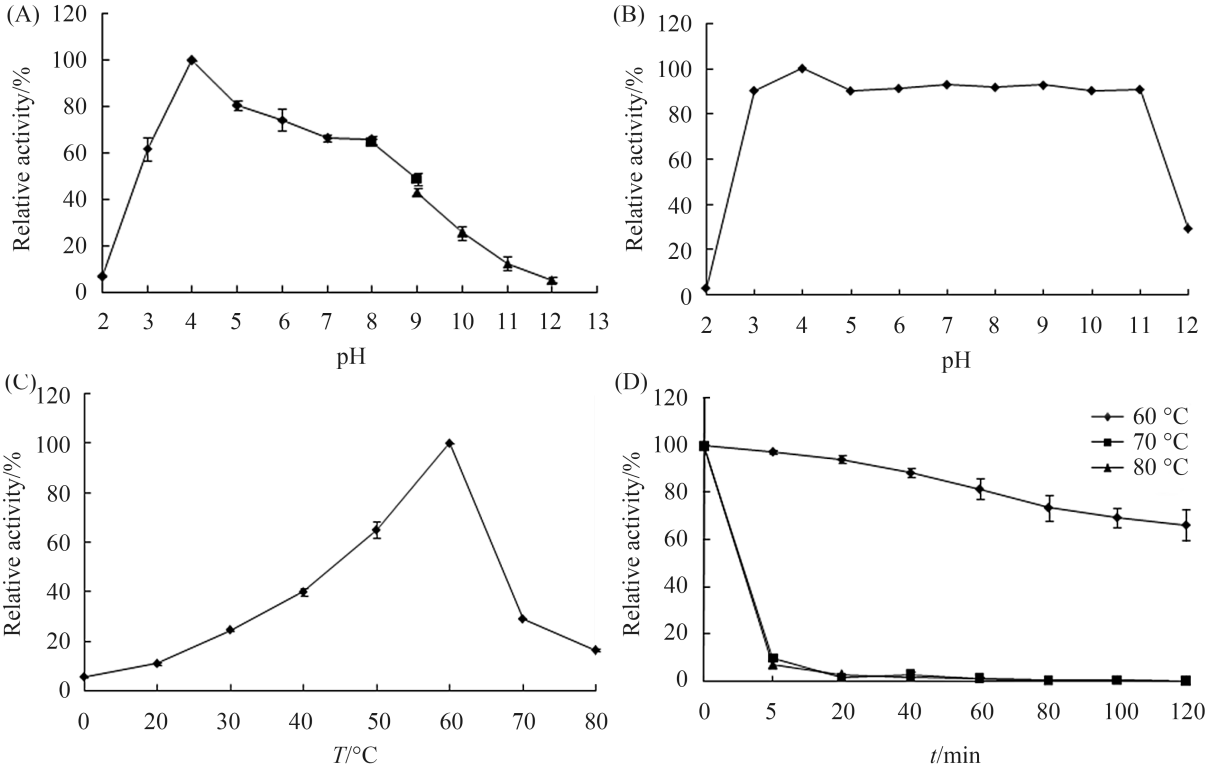

Figure 3. The enzymatic properties of CHI-X with colloidal chitin. A: Effect of pH on CHI-X activity at 60 °C. B: Effect of pH on the stability of CHI-X at 60 °C. C: Effect of temperature on CHI-X activity at pH 4.0. D: Effect of temperature on the stability of CHI-X.

2.6 Effects of metal ions, proteases and grouper intestinal juice on the activity of CHI-X

The effects of various metal ions and chemicals on CHI-X activity were shown in Table 2. Mn^{2+} significantly activated CHI-X activity while Ag^{+} and SDS greatly inhibited CHI-X. CHI-X retained more than 90% of its activity after incubation with proteases with colloidal chitin as the substrate (Figure 4). The effect of grouper intestinal juice on the activity of CHI-X was measured by the maintenance of activity after incubation with grouper intestinal juice. 70.4% of the activity was maintained after incubation of CHI-X with grouper intestinal juice at 37 °C for 2 h in colloidal chitin assay (Figure 5).

2.7 Substrate specificity of CHI-X

As indicated above, CHI-X may hydrolyze colloidal chitin. However, CHI-X exhibited no activity toward chitin powder, glycol chitosan, β-(1,3)-(1,4)-glucan, and sodium carboxymethylcellulose, indicating a narrow substrate spectrum.

Table 2. Effects of metal ions and chemicals on CHI-X activity using colloidal chitin substrates

Chemical	Relative activity/%		Chemicals	Relative activity/%	
	1 mmol/L	5 mmol/L		1 mmol/L	5 mmol/Laa
None	100.00	100.00	Co^{2+}	95.14±8.20	129.90±7.40
K^{+}	131.64±2.70	111.77±7.30	Ca^{2+}	80.81±9.30	119.93±9.70
Na^{+}	84.50±5.80	119.39±6.80	Ni^{2+}	134.29±3.90	91.73±8.10
Li^{+}	137.14±3.40	104.78±6.60	Mn^{2+}	123.00±9.90	174.57±7.30
Ag^{+}	3.58±2.20	4.59±2.70	Cr^{3+}	97.77±0.80	74.47±6.20
Pb^{2+}	79.12±4.10	43.86±3.00	Fe^{3+}	117.70±3.80	54.86±6.20
Mg^{2+}	80.42±5.70	112.61±8.40	EDTA	87.29±3.80	91.01±7.70
Zn^{2+}	116.83±2.60	97.64±2.70	SDS	81.83±1.60	3.59±1.90
Cu^{2+}	120.75±2.80	105.15±5.10			

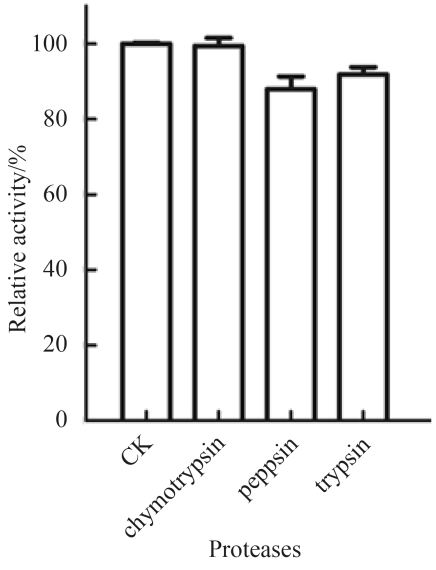

Figure 4. The effects of different proteases on catalytic activity of CHI-X on colloidal chitin. Values are expressed in mean±SEM.

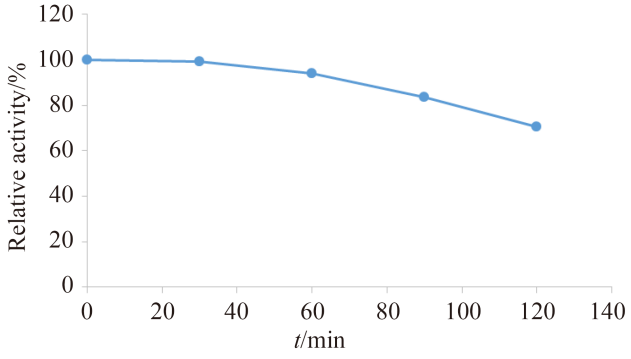

Figure 5. The effect of grouper intestinal juice on the stability of CHI-X on colloidal chitin.

2.8 Cleavage patterns of CHI-X toward colloidal chitin and the additive activity with ChiB565

TLC showed that $GlcNAc_2$ started to be detected after 15 min, and maintained as the only detectable product through 6 h of reaction (Figure 6), indicating that CHI-X is an exo-chitinase. The result of LC-ESI-MS analysis is shown in Figure 7. The peak at *m/z* = 35 represents chloroform, the electrospray reagent for ESI-MS. The theoretical molecular mass of GlcNAc with Cl^{-} from chloroform is 256.21 Da, and that of $(GlcNAc)_2$ is 459.4 Da, which correspond to the major mass spectrometric peaks at *m/z* = 256.0 and 459.2, respectively (Figure 7). Therefore, the major hydrolysis products of CHI-X after 6 h of incubation were GlcNAc and $(GlcNAc)_2$. Collectively, the TLC and LC-ESI-MS results indicated that CHI-X is an exo-chitinase possessing both chitobiosidase and β-*N*-acetylglucosaminidase activities[8]. Moreover, an additive effect was observed between CHI-X and ChiB565, a wide substrate spectrum enzyme with both exo- and endo-chitinase activity, on colloidal chitin) (Table 3).

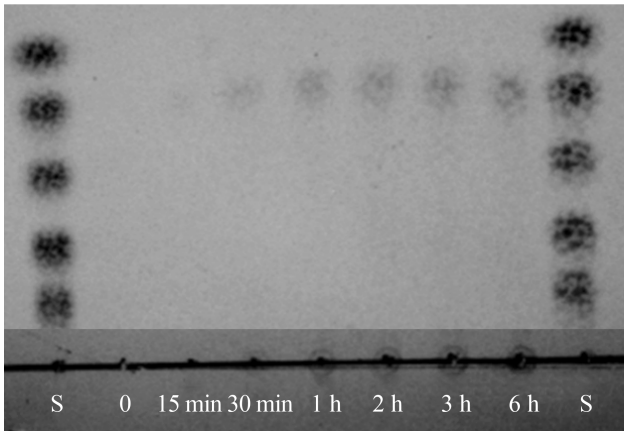

Figure 6. TLC of hydrolysis products of colloidal chitin by CHI-X. The reaction mixture (1 mL) containing 2.5 mg of colloidal chitin in PBS buffer was incubated with 66 U CHI-X at 60 °C for 0, 15, 30 min, 1, 2, 3 or 6 h. 10 μL of reaction mixture or 8 μL of standard chitin oligosaccharides (containing 1 mg/mL of each oligosaccharide) were chromatographed on a silica gel plate (Dieselgel 60: Merck Co., Berlin, Germany) for TLC analysis. Lane S, standard chitin oligosaccharides from GlcNAc (top) to GlcNAc5 (bottom).

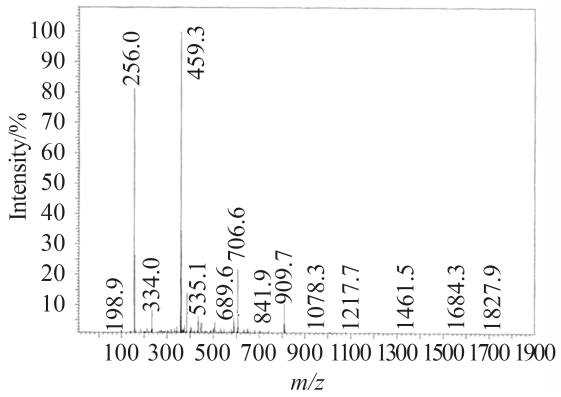

Figure 7. ESI-MS analysis of hydrolysis products of colloidal chitin by CHI-X. The reaction mixture (1 mL) containing 5 mg of colloidal chitin in PBS buffer was incubated with 44 U CHI-X at 60 °C for 6 h.

2.9 Stability of CHI-X during storage

The stability during storage is an important parameter for enzyme additives. Lyophilized CHI-X retained more than 50% of the original activity after storage at –20, 4, 30, 37 °C for 28 days, which is similar to ChiB565 (Figure 8).

Table 3. The additive effects of CHI-X and ChiB565 in degrading colloidal chitin [$]

Treatment	Enzymes	Substrates	The production of reducing sugar (μmol)
1	CHI-X 30U	Colloidal chitin 0.1 g	11.01±0.23[a]
2	ChiB565 30U	Colloidal chitin 0.1 g	15.03±0.41[b]
3	CHI-X 30U and ChiB565 30U	Colloidal chitin 0.1 g	26.06±0.25[c]

[$]All reactions were incubated at 37 °C, pH 7.0 for 1 hour. Different superscripts indicated statistically significant differences (P<0.05).

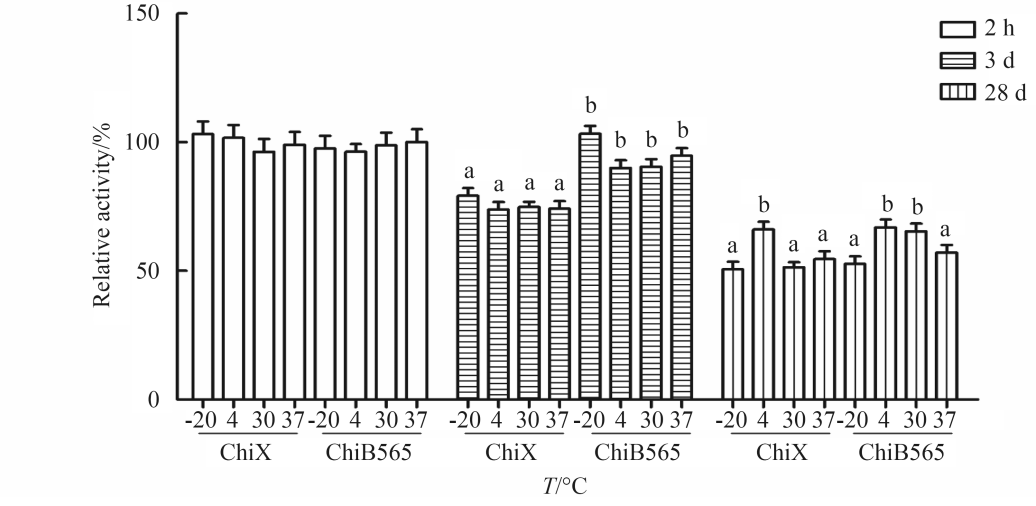

Figure 8. The storage stability of chitinases CHI-X and ChiB565 determined with colloidal chitin. Values are expressed in mean±SEM; different letters mean significantly difference (P<0.05).

Table 4. Comparison of properties of CHI-X with other exo-chitinases reported

Names	Sources	Molecular mass/kDa	Optimal pH	pH stability	Optimal temperature/ °C	Thermal stability/ °C	Metallic ions and chemicals	Substrate specificity	References
CHI-X	*Citrobacter freundii*	54.0	4.0	3.0–11.0	60	⩽60	Mn^{2+}↑, Ag^{+}↓	colloidal chitin shrimp-shell chitin	Present study
Chi36	*Bacillus thuringiensis* HD-1	36.0	6.5		65			4-MU $(GlcNAc)_2$	Arora et al. 2003
Chitinase	*Penicillium ochrochloron* MTCC 517	64.0	7.0		40		Hg^{2+}, Zn^{2+}, K^{+}, NH_4^{+}↓	colloidal chitin>N, N′-diacetylchitobiose> p-nitrophenyl N-acetyl-β-D-glucosaminide> glycol chitin> glycol chitosan> chitosan	Patil et al. 2013
Chitinase	*Trichoderma saturnisporum*	24.0	4.0		60	50	Mn^{2+}, Zn^{2+}↑	4-MU $(GlcNAc)_2$	Sharma et al. 2012
Chitinase	*Aeromonas* sp. GJ-18	34.0	6.0	5.0–8.0	30–50	50	Zn^{2+}↑	colloidal chitin	Jeong et al. 2012
*Hs*ChiA1p	*Halobacterium salinarum* CECT 395	66.5	7.3	6.0–8.5	40	25–45	Mg^{2+}, Ca^{2+}, K^{+}↑ Mn^{2+}↓	*p*-NP-(GlcNAc)3, *p*-NP-(GlcNAc), crystalline chitin, colloidal chitin	García-Fraga et al. 2013
ChiA	*Bacillus licheniformis*	66.0	6.0		60	⩽60		colloidal chitin	Songsiriritthigul et al. 2010
A. fumigatus chitinase	*Aspergillus fumigatus* YJ-407	46.0	5.0	4.0–8.0	60	<45	Hg^{2+}, Pb^{2+}, Ag^{+}, Fe^{2+}, Mn^{2+}, Zn^{2+}↓	glycol chitin> partially deacetylated chitosan> colloidal chitin	Xia et al. 2001
ChiB565	*Aeromonas veronii* B565	110.0	5.0	4.5–9.0	50	<50	Cu^{2+}, Mn^{2+}↑, SDS↓	colloidal chitin> shrimp-shell chitin> powdered chitin> β-1, 3-1, 4-glucan	Zhang et al. 2013

↑ signifies that metal ions or chemicals increase the enzymatic activity; ↓ signifies that metal ions or chemicals decrease the enzymatic activity.

3 Discussion

In this study, we attempted to isolate chitin-degrading bacteria from the intestinal samples of trash-fish fed croakers, and the isolated strain with chitin-degradation activity consisted with our expectation. The cleavage patterns of chitinases varied among different enzymes. Chitinase from *Aspergillus fumigatus* YJ-407 has both endo- and exo-hydrolytic activities[37]; a chitinase from *Streptomyces olivaceoviridis* generates chitobiose from colloidal chitin[38]; ChiB and Chisb, from *Clostridium parapurtificum* and *Bacillus* sp. DAU101 respectively, both produce GlcNAc and GlcNAc2[34,39]. ChiB565 hydrolyzed colloidal chitin to $(GlcNAc)_4$, $(GlcNAc)_3$, and $(GlcNAc)_2$, indicating both exo- and endo-chitinase activity[20]. In our study, GlcNAc and $(GlcNAc)_2$ units were identified as the major CHI-X hydrolysis product, suggesting that CHI-X possesses exo-chitinase activity. Consistent with the activity result, the predicted CHI-X active site appears to be similar to that of ChiA, which is

also an exo-chitinase. Additive effect was observed between CHI-X and ChiB565, probably due to a simultaneous attacking of different regions of the substrate by the two chitinases.

Chitinases from different microorganisms have varied enzymatic properties. The enzymatic properties of CHI-X and those of other exo-chitinases are summarized in Table 4. In comparison with most of the other chitinases listed in Table 4, CHI-X has higher optimal temperature (60 °C) and lower optimal pH value (4.0). Also, CHI-X is characteristic with stability over a broader pH range (3.0–11.0). For ion effects, CHI-X activity is enhanced by Mn^{2+}, which is similar to ChiB565 and the chitinase from *Trichodermas aturnisporum*[20,40]. In contrast, the chitinases from *Halobacterium salinarum* CECT 395 and *Aspergillus fumigatus*YJ-407 are both inhibited by Mn^{2+} [36,41]. On the other hand, CHI-X is strongly inhibited by Ag^{+}, similar with the chitinase from *A. fumigates*. CHI-X showed narrow substrate profile, as it only hydrolyzes colloidal chitin. In contrast, some other exo-chitinases exhibited wide substrate spectrum, such as the chitinase from *Penicillium ochrochloron* MTCC 517[42], *Hs*ChiA1p of *Halobacterium salinarum* CECT 395[41], and ChiB565 of *Aeromonas veronii* B565[20] (Table 4).

The thermal stability of CHI-X is not desirable as a potential feed additive, because feeds are usually processed by pelleting, which involves treatment at 80 °C for a few seconds[43]. In future, the specific amino acids can be substituted to improve thermal stability of CHI-X by referring to other high thermal stability isoenzymes. The modified CHI-X would hopefully maintain activity after the pelleting treatment.

In present study, a novel chitinase named CHI-X was obtained from a *Citrobacter* strian isolated in the intestines of large yellow croaker juveniles. The enzyme hydrolyzes colloidal chitin, with stability over a broad pH spectra (3.0–11.0). The enzyme may be adapted to an acidic environment, a temperature range appropriate for warm-water marine fish culture (30–40 °C), and is resistant to proteases. These properties suggest that CHI-X would adapt well to the environment of marine fish stomachs/intestines and can be developed as a potential feed additive enzyme for warm water marine fish.

参考文献

[1] Souza CP, Almeida BC, Colwell RR, Rivera ING. The importance of chitin in the marine environment. *Marine Biotechnology*, 2011, 13(5): 823–830.

[2] Sagheer FAA, Al-Sughayer MA, Muslim S, Elsabee MZ. Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydrate Polymers*, 2009, 77(2): 410–419.

[3] Hoell IA, Vaaje-Kolstad G, Eijsink VGH. Structure and function of enzymes acting on chitin and chitosan. *Biotechnology and Genetic Engineering*, 2010, 27(1): 331–366.

[4] Shen KT, Chen MH, Chan HY, Jeng JH, Wang YJ. Inhibitory effects of chitooligosaccharides on tumor growth and metastasis. *Food and Chemical Toxicology*, 2009, 47(8): 1864–1871.

[5] Tsai GI, Wu ZY, Su WH. Antibacterial activity of a chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. *Journal of Food Protection*, 2000, 63(6): 747–752.

[6] Omumasaba CA, Yoshida N, Ogawa K. Purification and characterization of a chitinase from *Trichoderma viride*. *The Journal of General and Applied Microbiology*, 2001, 47(2): 53–61.

[7] Annamalai N, Giji S, Arumugam M, Balasubramanian T. Purification and characterization of chitinase from *Micrococcus* sp. AG84 isolated from marine environment. *African Journal of Microbiology Research*, 2010, 4(24): 2822–2827.

[8] Busby JN, Panjikar S, Landsberg MJ, Hurst MRH, Lott JS. The BC component of ABC toxins is an RHS-repeat-containing

protein encapsulation device. *Nature*, 2013, 501(7468): 547–550.

[9] Bhushan B. Production and characterization of a thermostable chitinase from a new alkalophilic *Bacillus* sp. BG-11. *Journal of Applied Microbiology*, 2000, 88(5): 800–808.

[10] Ikeda M, Miyauchi K, Matsumiya M. Purification and characterization of a 56 kDa chitinase isozyme (PaChiB) from the stomach of the silver croaker, *Pennahia argentatus*. *Bioscience, Biotechnology and Biochemistry*, 2012, 76(5): 971–979.

[11] Molinari LM, Pedroso RB, Scoaris DO, Ueda-Nakamura T, Nakamura CV, Filho BPD. Identification and partial characterisation of a chitinase from Nile tilapia, *Oreochromis niloticus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 2007, 146(1): 81–87.

[12] Gutowska MA, Drazen JC, Robison BH. Digestive chitinolytic activity in marine fishes of Monterey Bay, California. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 2004, 139(3): 351–358.

[13] Matsumiya M, Arakane Y, Haga A, Muthukrishnan S, Kramer KJ. Substrate specificity of chitinases from two species of fish, greenling, *Hexagrammos otakii*, and common mackerel, *Scomber japonicus*, and the insect, tobacco hornworm, *Manduca sexta*. *Bioscience, Biotechnology and Biochemistry*, 2006, 70(4): 971–979.

[14] Ikeda M, Miyauchi K, Mochizuki A, Matsumiya M. Purification and characterization of chitinase from the stomach of silver croaker *Pennahia argentatus*. *Protein Expression and Purification*, 2009, 65(2): 214–222.

[15] Laribi-Habchi H, Dziril M, Badis A, Mouhoub S, Mameri N. Purification and characterization of a highly thermostable chitinase from the stomach of the red scorpionfish *Scorpaena scrofa* with bioinsecticidal activity toward cowpea weevil *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Bioscience, Biotechnology, and Biochemistry*, 2012, 76(9): 1733–1740.

[16] Goodrich TD, Morita RY. Bacterial chitinase in the stomachs of marine fishes from Yaquina Bay, Oregon, USA. *Marine Biology*, 1977, 41(4): 355–360.

[17] Lindsay GJH, Gooday GW. Chitinolytic enzymes and the bacterial microflora in the digestive tract of cod, *Gadus morhua*. *Journal of Fish Biology*, 1985, 26(3): 255–265.

[18] Gokul B, Lee JH, Song KB, Rhee SK, Kim CH, Panda T. Characterization and applications of chitinases from *Trichoderma harzianum*-a review. *Bioprocess Engineering*, 2000, 23(6): 691–694.

[19] Danulat E. Role of bacteria with regard to chitin degradation in the digestive tract of the cod, *Gadus morhua*. *Marine Biology*, 1986, 90(3): 335–343.

[20] Zhang YT, Zhou ZG, Liu YC, Cao YA, He SX, Huo FM, Qin CB, Yao B, Ringø E. High-yield production of a chitinase from *Aeromonas veronii* B565 as a potential feed supplement for warm-water aquaculture. *Applied Microbiology and Biotechnology*, 2014, 98(4): 1651–1662.

[21] Jian JC, Wu ZH. Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Richardson). *Aquaculture*, 2003, 218(1/4): 1–9.

[22] Cheung KC, Leung HM, Wong MH. Metal concentrations of common freshwater and marine fish from the Pearl River Delta, South China. *Archives of Environmental Contamination and Toxicology*, 2008, 54(4): 705–715.

[23] Ray AK, Ghosh K, Ringø E. Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture Nutrition*, 2012, 18(5): 465–492.

[24] He SX, Zhou ZG, Liu YC, Shi PJ, Yao B, Ringø E, Yoon I. Effects of dietary *Saccharomyces cerevisiae* fermentation product (DVAQUA®) on growth performance, intestinal autochthonous bacterial community and non-specific immunity of hybrid tilapia (*Oreochromis niloticus* ♀×*O. aureus* ♂) cultured in cages. *Aquaculture*, 2009, 294(1/2): 99–107.

[25] Patel RK, Jain M. NGS QC Toolkit: A tool kit for quality control of next generation sequencing data. *PLoS One*, 2012, 7(2): e30619.

[26] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 2010, 7(5): 335–336.

[27] Delong EF. Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States America*, 1992, 89(12): 5685–5689.

[28] Wang Z, Ye SF, Li JJ, Zheng B, Bao MZ, Ning GG. Fusion primer and nested integrated PCR (*FPNI-PCR*): a new high-efficiency strategy for rapid chromosome walking or flanking. *BMC Biotechnology*, 2011, 211: 109.

[29] Petersen TN, Brunak S, Von Heijne G, Nielsen H. SignalP 4. 0: discriminating signal peptides from transmembrane regions. *Nature Methods*, 2012, 8(10): 785–786.

[30] Guex N, Peitsch MC. SWISS-MODEL and the Swiss-Pdb Viewer: an environment for comparative protein modeling. *Electrophoresis*, 1997, 18(15): 2714–2723.

[31] Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Research*, 2003, 31(13): 3381–3385.

[32] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*, 2006, 22(2): 195–201.

[33] Tasun K, Chose P, Ghen K. Sugar determination by DNS method. *Biotechnology and Bioengineering*, 1970, 12: 921–922.

[34] Lee YS, Park IH, Yoo JS, Chung SY, Lee YC, Cho YS, Ahn SC, Kim CM, Choi YL. Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101. *Bioresource Technology*, 2007, 98(14): 2734–2741.

[35] Synstad B, Gåseidnes S, Van Aalten DMF, Vriend G, Nielsen JE, Eijsink VGH. Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase. *European Journal of Biochemistry*, 2004, 271(2): 253–262.

[36] Papanikolau Y, Tavlas G, Vorgias CE, Petratos K. *De novo* purification scheme and crystallization conditions yield high-resolution structures of chitinase A and its complex with the inhibitor allosamidin. *Acta Crystallographica Section D*, 2003, 59(2): 400–403.

[37] Xia GQ, Jin CS, Zhou J, Yang SJ, Zhang SZ, Jin C. A novel chitinase having a unique mode of action from *Aspergillus fumigatus* YJ-407. *European Journal of Biochemistry*, 2001, 268(14): 4079–4085.

[38] Blaak H, Schnellmann J, Walter S, Henrissat B, Schrempf H. Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. *European Journal of Biochemistry*, 1993, 214(3): 659–669.

[39] Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. Cloning, sequencing, and expression of the gene encoding *Clostridium paraputrificum* chitinase ChiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain. *Journal of Bacteriology*, 1997, 179(23): 7306–7314.

[40] Sharma V, Shanmugam V. Purification and characterization of an extracellular 24 kDa chitobiosidase from the mycoparasitic fungus *Trichoderma saturnisporum*. *Journal of Basic Microbiology*, 2012, 52(3): 324–331.

[41] García-Fraga B, Da Silva AF, López-Seijas J, Sieiro C. Functional expression and characterization of a chitinase from the marine archaeon *Halobacterium salinarum*CECT 395 in *Escherichia coli*. *Applied Microbiology and Biotechnology*, 2014, 98(5): 2133–2143.

[42] Patil NS, Waghmare SR, Jadhav JP. Purification and characterization of an extracellular antifungal chitinase from *Penicillium ochrochloron* MTCC 517 and its application in protoplast formation. *Process Biochemistry*, 2013, 48(1): 176–183.

[43] Vahjen W, Simon O. Biochemical characteristics of non starch polysaccharide hydrolyzing enzyme preparations designed as feed additives for poultry and piglet nutrition. *Archives of Animal Nutrition*, 1999, 52(1): 1–14.

源自大黄鱼肠道柠檬酸杆菌的外切几丁质酶的特性分析

许洁[1,2#]，杨雅麟[2#]，刘阳[2]，冉超[2]，李娟[2]，何夙旭[2]，徐俐[2]，艾春香[1*]，周志刚[2*]

[1] 厦门大学海洋与地球学院，福建 厦门 361005

[2] 中国农业科学院饲料研究所，农业部饲料生物技术重点实验室，北京 100081

摘要：【目的】从饲喂富含几丁质饲料的大黄鱼肠道分离具有几丁质分解功能的菌株并分离鉴定新的几丁质酶。【方法】利用胶体几丁质平板分离饲喂杂鱼的大黄鱼肠道中的几丁质分解菌。对几丁质酶基因*chi-X*进行了克隆并在大肠杆菌中表达。对CHI-X的酶学性质进行了分析。【结果】从饲喂杂鱼的大黄鱼肠道内容物中分离出1株具有几丁质分解功能的费氏柠檬酸杆菌，其中的几丁质酶基因编码1个含493个氨基酸残基的蛋白，其中包含一个糖苷水解酶18家族催化域。CHI-X对胶体几丁质具有分解功能。最适pH和温度分别是4.0和60 °C。CHI-X具有很强的pH稳定性，在pH 3.0–11.0的范围培育1 h仍保留90% 左右的活性。Mn^{2+}, Li^{+}和K^{+}可促进CHI-X酶活，Ag^{+}对CHI-X有抑制作用。CHI-X对蛋白酶和石斑鱼肠道内容物有较强的抗逆性。CHI-X可分解胶体几丁质为N-乙酰葡萄糖胺和N-乙酰葡萄糖胺二聚体，表明它是一个几丁质外切酶。最后，CHI-X和另一个几丁质酶Chi565表现出酶活性的加和效应。【结论】分离自肠道菌的CHI-X能很好适应海水鱼类的肠道环境，可以作为温水海水养殖鱼类的饲料添加剂使用。

关键词：几丁质酶，费氏柠檬酸杆菌，大黄鱼

(本文责编：张晓丽)

基金项目：海洋公益性行业科研专项经费项目(201305022-6)；国家科技支撑计划课题(2012BAD25B02, 2013BAD10B01-2-3)；北京市现代农业产业技术体系(SCGWZJ20121104-4)；中国农业科学院基本科研业务费预算增量项目(2013ZL043)；中央级公益性科研院所基本科研业务费专项(2013ywf-sl-4)

[*]**通信作者。**艾春香，Fax：+86-592-2186397，E-mail：chunxai@xmu.edu.cn；周志刚，Tel：+86-10-82106073，Fax：+ 86-10-82106054，E-mail：zhouzhigang03@caas.cn

[#]**共同第一作者。**

收稿日期：2015-09-10；**修回日期：**2015-12-05；**网络出版日期：**2015-12-11