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Identification of a chitinase from the hepatopancreas of Chinese black sleeper (*Bostrychus sinensis*)

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Abstract

Chinese black sleeper (*Bostrychus sinensis*) is a fish that lives both in seawater and freshwater, feeds on crustaceans, aquatic insects and occasionally shellfish. The existence of digestive enzyme in viscera to act on chitinous exoskeleton of the prey is of interest. In this study, a chitinase was purified to homogeneity using ammonium sulfate precipitation, DEAE-Sephacel ion exchange, Sephacryl S-200 HR and Superdex 200 gel filtration columns. The purified protein presents a molecular mass of 58 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and results in a single band on native PAGE. According to peptide mass fingerprinting, two peptides containing a total of 20 amino acid residues, were 95% identical to a chitinase from yellow perch (*Perca flavescens*) and 100% identical to the chitinase from greater amberjack (*Seriola dumerili*). The purified chitinase showed optimum activity at pH 6.0, and was stable at acidic conditions and temperature below 55°C. The enzymatic activity was quite stable in the presence of NaCl, even at 1 mol/L . The chitinase was capable of degrading chitosan into low molecular mass chitooligosaccharides (COS) with sizes in a range of 200–700 Da, and the circular dichroism profile of the COS greatly differed from native chitosan. Full-length cDNA encoding the present chitinase was cloned and the transcript levels of chitinase in various tissues were determined by quantitative real-time PCR. The results showed that the transcript level of chitinase was highest in esophagus and hepatopancreas.

Key words: *Bostrychus sinensis*, chitinase, purification, characterization, molecular cloning, chitosan

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1 Introduction

Chinese black sleeper (*Bostrychus sinensis*) is a fish that inhabits inshore waters and enters fresh water, distributed throughout Southeast Asia and Indo-West Pacific coastal areas. In China, it is mainly distributed in the coastal area of the South China Sea, Taiwan Strait and East China Sea (Wang et al., 2011). This fish species feed on small crabs, shrimp, aquatic insects and occasionally shellfish. Due to its diet habit, the existence of such fish in aquaculture ponds of shrimp and crab can affect the production. On the other hand, from the biochemistry point of view, the action of digestive enzyme in viscera on the shell of prey is of interest.

Chitin is homopolymer consisting of β-1,4-linked N-acetyl-D-glucosamine (GluNAc) units in a linear form and is the second most abundant natural polysaccharide after cellulose (Madhuprakash et al., 2012). The insolubility of chitin in water is a major limitation to study its biological activities (Zakariassen et al., 2009). In comparison, N-acetylchitooligosaccharides $(GlcNAc)_n$ and GlcNAc, which are the hydrolysis products of polymeric chitin, are of increasing interest due to their solubility, various physiological functions and other potential applications (Halder et al., 2014; Xia et al., 2011). Chitin-hydrolyzing enzymes, also called chitinases, randomly hydrolyze the β-1,4-glycosidic bonds of chitin and are widely found in living organisms such as bacteria (Marcon et al., 2014; Liao et al., 2019), fungi (Hartl et al., 2012), plants (Kasprzewska, 2003) and animals (Strobel et al., 2013). Chitinases in fish digestive systems hydrolyze the chitin of crustaceans and aquatic insects to provide energy for the predator (Ikeda et al., 2013). Such chitinases have been purified and characterized from the stomach of several fish species, including greenling (*Hexagrammos otakii*) and common mackerel (*Scomber japonicus*) (Matsumiya et al., 2006), coelacanth (*Latimeria chalumnae*) (Matsumiya et al., 2008), silver croaker (*Pennahia argentatus*) (Ikeda et al., 2009, 2012), threeline grunt (*Parapristipoma trilineatum*) (Ikeda et al., 2013), marbled rockfish (*Sebastiscus marmoratus*) (Ikeda et al., 2014), and Japanese sardine (*Sardinops melanostictus*) (Kawashima et al., 2016). Tissue expression of chitinase in threeline as investigated by quantitative real-time PCR (qPCR) revealed that it only occurs in stomach (Ikeda et al., 2013). Except engagement in digestion, recent studies indicated that chitinase is involved in many other physiological processes. High expression level of chitinase genes in turbot mucosal tissues suggested its involvement in mucosal immunity against infection (Gao et al., 2017). In addition, chit-

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inase in the hepatopancreas of shrimp was proposed to be relevant to the molting, hatching, immunity and stress response (Fan et al., 2016; Zhou et al., 2017; Santos et al., 2019). However, little information concerning identification and characterization of chitinase from fish, which lives in both seawater and fresh water, is available.

In this study, a chitinase was purified and characterized from the hepatopancreas of Chinese black sleeper (*B. sinensis*) and its degradation on chitosan to produce chitooligosaccharides (COS) was evaluated. The full-length sequence of chitinase was cloned and the expression profile in different tissues was detected by qPCR. The possible physiological roles of this enzyme were discussed. Therefore, this study provided a theoretical reference for exploring the physiological functions of chitinase in fish.

2 Materials and methods

2.1 *Materials*

2.1.1 *Fish*

Male adult *B. sinensis* (n=10, body mass 70–80 g) which was farmed in aquaculture farms in the Dadeng Island (Fujian, China) was purchased alive from a local aquaculture market in Xiamen (Fujian, China) in March and April 2019. The fish was sacrificed and immediately placed on ice for subsequent experiments.

2.1.2 *Chemicals and reagents*

DEAE-Sephacel, Sephacryl S-200 HR media and Superdex 200 10/300 GL were purchased from GE Healthcare (Piscataway, USA). Chitosan (95% degree of deacetylation), phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), and 4-methylumbelliferyl-β-D-N,N′,N′-triacetylchitotrioside hydrate were from Sigma (USA). Protein markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were product of Thermo Fisher Scientific (USA). All other reagents were analytical grade.

2.2 *Determination of chitinase activity*

Chitinase activity was assayed using 4-methylumbelliferyl-β-D-N,N′,N′-triacetylchitotrioside as substrate during the purification and characterization of chitinase as described previously (Thompson et al., 2001). Briefly, the reaction was initiated by adding 50 μL of substrate (10 μmol/L) to a solution containing 900 μL of 50 mmol/L sodium phosphate buffer (pH 6.0) and 50 μL of appropriately diluted enzyme in 20 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L PMSF, 1 mmol/L EDTA and 0.02% NaN_3. After incubation at 37°C for 15 min, the reaction was terminated by adding 1.5 mL of 3 mol/L sodium carbonate. The fluorescence intensity of the released 4-methylumbelliferyl was measured using a fluorescence spectrophotometer (JASCO, FP-6200, Japan) at an excitation wavelength of 390 nm and an emission wavelength of 450 nm. Controls were done the same way with the reaction buffer substituting for the enzyme. One unit (U) of chitinase activity was defined as the amount of enzyme needed to release 1 μmol of 4-methylumbelliferyl per minute.

2.3 *Chitinase activity assay in different tissues*

Different tissues of *B. sinensis* including esophagus, stomach, intestine, kidney, hepatopancreas, muscle and blood were dissected and used immediately. The tissues were independently cut into small pieces using a scissor and homogenized with 4 volumes of buffer A. The homogenate was centrifuged at 12 000 r/min for 20 min and the supernatant was collected and used for enzymatic activity assays.

2.4 *Purification of chitinase*

All the procedures were performed at 0–4°C. Approximately 90 g of fish hepatopancreas was homogenized with 4-times (w/v) 20 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L PMSF, 1 mmol/L EDTA and 0.02% NaN_3 (buffer A) using a homogenizer (Kinematica, PT-2100, Switzerland). The homogenate was centrifuged at 12 000 r/min for 20 min in a centrifuge (Avanti J-26S XP, Beckman Coulter, USA). Proteins in the supernatant were fractionated using increasing concentrations of solid ammonium sulfate (0–50% saturation). After centrifugation, the precipitate was collected and dissolved in a minimal volume of buffer A and dialyzed against the same buffer extensively at 4°C for 24 h. The dialysate was then applied to a DEAE-Sephacel column (2.5 cm × 10 cm), which had been previously equilibrated with buffer A. Then the column was washed with buffer A till the absorbance at 280 nm of the flow-through was below 0.05. Bound proteins were eluted with constant 0.1 mol/L NaCl, followed by elution with a linear gradient of NaCl from 0.1 mol/L to 0.6 mol/L in buffer A. Fractions with chitinase activity were pooled and concentrated by ultrafiltration using a YM-10 membrane (Millipore, USA) and applied to a Sephacryl S-200 HR gel filtration column (2.5 cm × 98 cm) pre-equilibrated with buffer A containing 0.15 mol/L NaCl at a flow rate of 0.8 mL/min. The pooled active fractions were concentrated once again by ultrafiltration and applied to the gel-filtration column of Superdex 200 10/300 GL (1.0 cm × 30 cm), which was connected to an AKTA system (UPC-10, GE Healthcare, USA). The column was pre-equilibrated with 50 mmol/L phosphate buffer (pH 7.0) containing 0.15 mol/L NaCl at a flow rate of 0.4 mL/min. Active fractions running through Superdex 200 10/300 GL were collected and used for electrophoresis and enzymatic characterization.

2.5 *SDS-PAGE and native-PAGE*

To estimate the molecular mass and purity of the target protein, aliquots of purified chitinase were subjected to SDS-PAGE under non-reducing (without β-mercaptoethanol) and reducing (with β-mercaptoethanol) conditions (Laemmli, 1970) using 10% polyacrylamide gels, which were subsequently silver stained as described previously (Hempelmann and Krafts, 2017).

To determine the purity of chitinase, Native-PAGE was also performed according to the method described (Nowakowski et al., 2014). Briefly, samples without heating and addition of SDS or reducing agents were used for electrophoresis at 4 °C. After electrophoresis, the gel was subjected to silver staining.

2.6 *Determination of protein concentration*

Protein concentration at each purification step was estimated by measuring the absorbance of sample solution at 280 nm using a Lambda 35 UV spectrophotometer (PerkinElmer, USA) or by the bicinchoninic acid (BCA) method using BSA as the standard (Smith et al., 1985).

2.7 *MALDI-TOF/TOF-MS/MS analysis*

To obtain primary structure information on the purified protein, samples were run on a 10% gel and subjected to silver staining. The target protein band was excised with destaining solution (30 mmol/L $K_3Fe(CN)_6$:100 mmol/L $Na_2S_2O_3$=1:1, v/v). This protein was subjected to trypsin digestion and the trypsin hydrolysates were injected into the mass spectrum. MALDI-TOF mass spectra were obtained using 4800 Plus MALDI-TOF/TOF-MS/MS

Analyzer (Applied Biosystems, Life Technologies, USA) by the Shanghai Applied Protein Technology Co. Ltd. The results were compared with the NCBI database.

2.8 *Effect of pH and temperature on the chitinase activity*

The effect of pH on the enzyme was determined at 37°C using the fluorogenic substrate 4-methylumbelliferyl-β-D-N,N′,N′-triacetylchitotrioside in 50 mmol/L of the following buffers: HCl-sodium acetate buffer (pH 2.0–3.0), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.0), Tris-HCl (pH 7.5–8.5) and Na_2CO_3-$NaHCO_3$ (pH 9.0–11.0). pH stability of the enzyme was evaluated by measuring the residual enzymatic activity at 37°C in 50 mmol/L sodium phosphate buffer (pH 6.0) after incubating the enzyme at 4°C for 30 min in buffers of varying pH from 2.0 to 11.0 with an interval of 1. The initial velocity of chitinase at 37°C was evaluated by measuring released 4-methylumbelliferyl at different reaction time points (0, 1 min, 3 min, 5 min, 7 min, 10 min, 15 min, 20 min, 30 min). To investigate its thermal stability, chitinase in 50 mmol/L sodium phosphate buffer (pH 6.0) was incubated at different temperatures (50°C, 55°C, 60°C, 65°C, 70°C) for 30 min and immediately cooled to room temperature by immersing the test tubes in ice water before the residual activity was measured at 37°C.

2.9 *Effect of NaCl on the chitinase activity*

To investigate the effect of NaCl on the enzyme, samples were pre-dialyzed with buffer A without NaCl. Then, NaCl was added into the reaction solution to a final concentration of 0–1 mol/L. The activity was measured by the method as described above. Control tests were performed in the absence of NaCl.

2.10 *Degradation of chitosan and ESI-MS analysis*

Two hundred milligrams of chitosan were dissolved in 20 mL of 0.1 mol/L acetic acid and digested with 0.2 mg of purified chitinase (120 U/mg) at 37°C for 24 h with a shaking speed of 150 r/min in an orbital shaker (Thermo Fisher Scientific). The hydrolysis reaction was terminated by inserting the mixture into a boiling water bath for 10 min. After centrifugation at 8 000 r/min for 15 min, the supernatant was collected and lyophilized using a lyophilizer (FD-1D-50, BIOCOOL, China).

Fifty micrograms of freeze-dried products were dissolved in 1 mL methanol and centrifuged at 10 000 r/min for 15 min. The supernatant was collected and filtered through a 0.22 μm nominal pore size organic membrane (Millipore). The filtrate was delivered directly to a Micromass LCT Premier XE electrospray ionization mass spectrometer (ESI/MS) (Waters, USA) using a syringe pump at a flow rate of 5 μL/min. MS was run in positive mode with a mass scan range of 200–1 000 Da, capillary temperature of 250°C, electrospray needle voltage of 3.5 kV, and analytical grade nitrogen sheath gas at 3.6 kg/in^2 (1 in = 2.54 cm). The molecular mass of the fragments was obtained (m/z, 200–1 000).

2.11 *Circular dichroism (CD)*

Structural change of chitosan during hydrolysis was examined by circular dichroism (CD) according to previous study with some modifications (Kittur et al., 2005). CD analyses were performed between 180 nm and 260 nm in a 0.5 nm path-length cuvette on a spectropolarimeter (Applied Photophysics, UK) using 5 mg/mL chitosan or COS in 0.1 mol/L perchloric acid. The baseline was obtained using 0.1 mol/L perchloric acid, and the CD spectra of each sample were calculated by averaging three repeats. The scanning speed was 100 nm/min with a response time of 1 s and a bandwidth of 1.0 nm.

2.12 *Cloning of chitinase cDNA*

Total RNA was prepared from *B. sinensis* hepatopancreas using RNAsimple Total RNA Kit (Tiangen, China). The first strand cDNA was synthesized with TIANScript RT Kit (Tiangen) according to the manufacturer's instruction. Based on the sequence of *Seriola dumerili* chitinase (GenBank accession No. XM_022767576.1) and *Perca flavescens* (GenBank accession No. XM_028582436.1), some primers (chitinase-F, chitinase-R) were designed (Table 1). Using these primers and cDNA synthesized, a fragment of the chitinase gene of approximately 1 400 bp was amplified by PCR. The PCR product was purified and cloned into pMD-18T vector for DNA sequencing.

According to sequencing result of the above PCR product, specific oligonucleotide primers were designed for 5′-RACE (chitinase-5R1, chitinase-5R2, chitinase-5R3) and 3′-RACE (chitinase-3F1, chitinase-3F2, chitinase-3F3). All the primers were shown in Table 1. RACE and RACE-PCR were conducted using the SMARTer® RACE 5′/3′ Kit (TAKARA, USA). Nested-PCR was adopted to improve the specificity of RACE amplification and the program used for 5′-RACE and 3′-RACE was performed as follows: 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 45 s at 50°C, 60 s at 72°C, and a final extension of 7 min at 72°C. The PCR products were then purified, cloned and sequenced. Sequence and homology analyses were performed using DNAMAN software for multiple sequence alignments with proteins that are available from GenBank.

2.13 *Quantitative real-time PCR*

The total RNA was extracted from cells or tissues using an RNAsimple Total RNA Kit (Tiangen). The first-strand cDNA was

Table 1. Primers used for the cloning and qPCR assay

Primer	Sequence (5′-3′)
Chitinase-F	5′-ATGCGCATCCTAATTGTGATCTCAGGTCTC-3′
Chitinase-R	5′-CTAG/CGGCCG/AGTCACAGCACTTACAACTG/TTCTC-3′
Chitinase-5R1	5′-GCCAAACGAAATGCACGTCCGTAAGTAG-3′
Chitinase-5R2	5′-GTCCTGAATCATTTGAACAGTGGCTCCTT-3′
Chitinase-5R3	5′-GCATAGTTTCCCTCGCCACAGAACTGAC-3′
Chitinase-3F1	5′-TCAGTTCTGTGGCGAGGGAAACTATGC-3′
Chitinase-3F2	5′-GGAGCCACTGTTCAAATGATTCAGGACC-3′
Chitinase-3F3	5′-CTACTTACGGACGTGCATTTCGTTTGGC-3′
Chitinase-F (qPCR)	5′-GACCTTATTGGCAGTTGG-3′
Chitinase-R (qPCR)	5′-GCTTATCTTCTGGAGGACTT-3′
Actin-F (qPCR)	5′-GACAGGTCATCATCATTGGC-3′
Actin-R (qPCR)	5′-CAGACAGCACAGTGTTGGCATAC-3′

synthesized from the total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). TransStart® Top Green qPCR SuperMix (TransGen, China) was used to determine the transcript levels of chitinase according to the manufacturer's instructions in an ABI Prism 7300 System (Applied Biosystems). β-actin was used as a reference gene. Gene-specific primers used in this assay were listed in Table 1. Following initial denaturation at 95°C for 30 s, 45 cycles of PCR amplification were performed at 95°C for 5 s and 60°C for 31 s, with a dissociation curve at the end of the amplification reaction. The relative amount of the chitinase mRNA was normalized to that of β-actin.

2.14 *Homology modeling*

The 3-dimensional structure of *B. sinensis* chitinase was modeled using the SWISS-MODEL automated protein structure homology-modeling server (https://swissmodel.expasy.org/). The amino acid sequence of *B. sinensis* chitinase was highly identical to human chitotriosidase (53.35% identity). Hence, the crystal structure of human chitotriosidase (PDB ID: 5HBF) was chosen as the template to build the 3D structure on the web, and the generated PDB file was viewed using PyMOL software.

2.15 *Statistical analysis*

The results of enzyme activity and qPCR assay were obtained from three independent experiments. The data were expressed as the mean standard±deviation. GraphPad Prism 5 (GraphPad Software, USA) was utilized for analyzing results.

3 Results and discussion

3.1 *Chitinase activity in different tissues*

Chitinase was reported to be widely distributed in fish alimentary tracts and other tissues (Ikeda et al., 2017). However, the primary function of chitinase is still controversial and discrepant in different species. In this study, the chitinase activity was detected in various tissues of *B. sinensis*. As shown in Fig. 1, the highest chitinase activity was found in hepatopancreas, which is involved in metabolism and detoxification and contains much blood, followed by the digestive tract, including intestine, esophagus and stomach. These findings suggested that chitinases in fish show digestive roles in the digestive tract and physiological roles in non-digestive organs. It was speculated that chitinase is mainly associated with stomach (Ikeda et al., 2017), where it hydrolyzes exoskeleton of ingested shrimp and crab. Two chitinase isozymes from the stomachs of silver croaker (Ikeda et al., 2009, 2012), threeline grunt (Ikeda et al., 2013), and Japanese sardine (Kawashima et al., 2016) were purified by ammonium sulfate fractionation and column chromatography. Three chitinase isozymes from the stomachs of marbled rockfish (Ikeda et al., 2014) and greenling (Matsumiya et al., 2006) were also purified by the above methods. Among the three chitinases identified from Japanese flounder (*Paralichthys olivaceus*, fChi1, fChi2, fChi3), fChi1 and fChi2 were acidic chitinases and abundantly expressed in stomach, while fChi3 was expressed in spleen, pancreas, stomach, intestine, liver, kidney and gonads, proposing to participate in biodefence (Kurokawa et al., 2004). Chitinase secreted by hepatopancreas into intestinal tract may participate in the removal of intestinal fragment blockage (Lazado et al., 2012). Concerning the origin of chitinase in sharks, its activity was mostly found in the colon, demonstrating that chitinase is likely of microbial origin in this distal gut region (Jhaveri et al., 2015). Also, chitinase was identified in the pancreatic tissue of toad *Bufo japonicuski* (Oshima et al., 2002a) and the liver of golden cuttlefish (*Sepia esculenta*) (Nishino et al., 2014) and Japanese common squid (*Todarodes pacificus*) (Matsumiya et al., 2003). The exact function of chitinase involved in these species, however, has not been fully studied.

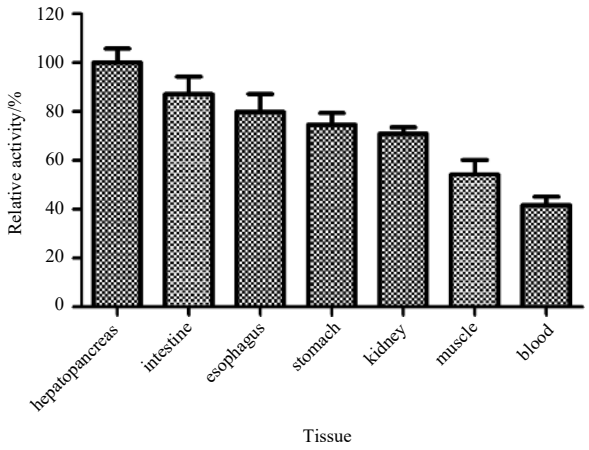

Fig. 1. Relative chitinase activity in different tissues. The highest activity in hepatopancreas was calculated as 100%.

3.2 *Purification of chitinase*

In this study, a chitinase was purified from the hepatopancreas of *B. sinensis* by ammonium sulfate fractionation and sequential column chromatography of DEAE-Sephacel, Sephacryl S-200 HR and Superdex 200 10/300 GL. Upon DEAE-Sephacel ion-exchange chromatography, the active fractions of chitinase were absorbed to the column and eluted at NaCl concentration of 0.2–0.4 mol/L (Fig. 2a). Active fractions were concentrated and then subjected to Sephacryl S-200 HR gel filtration and the chitinase was eluted as a single peak (Fig. 2b). After concentrating, chitinase was purified to homogeneity after further subjecting fractions to a gel-filtration column of Superdex 200 (Fig. 2c). Finally, 0.2 mg chitinase was obtained from 90.0 g hepatopancreas with a recovery of 1.5% and a 356.3-fold increase in specific activity (Table 2). The homogeneity of purified chitinase was checked by SDS-PAGE and native-PAGE. As shown in Fig. 2c, chitinase revealed a single band on SDS-PAGE with molecular mass of about 58 kDa under both non-reducing and reducing condition. The band in the native-PAGE was much smaller than in the denatured conditions, indicating that native chitinase was in a globular state which allowed it to migrate further. The molecular mass of *B. sinensis* chitinase was similar to a chitinase from silver croaker stomach (56 kDa) (Ikeda et al., 2009, 2012), and was between the isozyme of greenling stomach chitinase HoChiA (62 kDa) (Matsumiya et al., 2006) and a chitinase from stomach of the red scorpionfish (SsChi50, 50 kDa) (Laribi-Habchi et al., 2012). The molecular masses of these chitinases differed significantly from those of plant and seaweed (24.5–29 kDa) (Ikeda et al., 2017), indicating that the physiological functions of fish chitinases are disparate from those of plant chitinases. It is clear that some fish may actually assimilate N-acetyl-β-d-glucosaminidase in a nutritional context (German et al., 2015), proposing the purified enzyme participating in the dietary nutrition of fish.

3.3 *MALDI-TOF/TOF-MS/MS*

To identify the purified protein, the protein band was excised

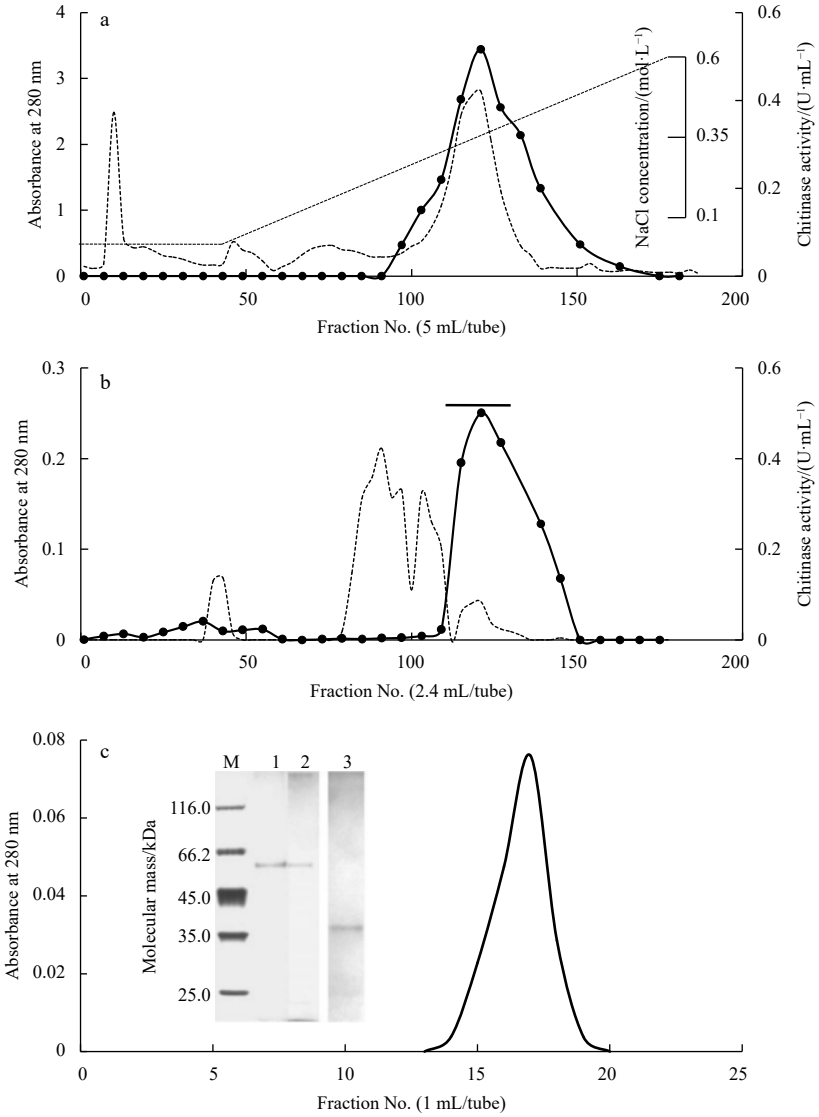

Fig. 2. Column chromatography purification of chitinase. a. DEAE-Sephacel; b. Sephacryl S-200 HR; c. Superdex 200 10/300 GL. ● Chitinase activity; ––– absorbance at 280 nm. Top line in b represented the active fractions pooled. SDS-PAGE and native-PAGE of purified chitinase were shown in the inset of c. M, molecular marker; Lane 1, under non-reducing condition; Lane 2, under reducing condition; Lane 3, native-PAGE of chitinase.

Table 2. Summary of the purification of chitinase from Chinese black sleeper (*B. sinensis*) hepatopancreas

Purification step	Total protein/mg	Total activity/U	Specific activity/(U·mg^{-1})	Purification (fold)	Yield/%
Crude enzyme	5 998.9	1 625.9	0.3	1.0	100.0
Ammonium sulfate	1 281.1	513.8	0.4	1.5	31.6
DEAE-Sephacel	248.3	180.3	0.7	1.8	11.1
Sephacryl S-200 HR	0.4	36.9	92.2	307.3	2.3
Superdex 200	0.2	24.6	106.9	356.3	1.5

Note: Crude enzyme was the first supernatant acquired from hepatopancreas homogenate after centrifugation.

from SDS-PAGE, digested with trypsin and the resulting peptide mixture was analyzed by MALDI-TOF/TOF-MS/MS. Peptide mass fingerprinting of the purified protein showed multiple peaks ranging from 800 Da to 5 000 Da, and peaks with signal-to-noise ratios higher than 50 were further analyzed by MS/MS (Fig. 3a). Subsequently, two fragments with 20 amino acid residues in total were obtained and the results were compared with the NCBI database. As shown in Fig. 3b, the 20 amino acid residues were 95% and 100% identical to chitinases from yellow perch (*P. flavescens*, GenBank accession No. XP_028438237.1) and greater amberjack (*S. dumerili*, GenBank accession No. XP_022623297.1), respectively, which strongly suggested that the purified protein is a chitinase.

3.4 *Effects of temperature and pH on the chitinase activity*

Fish stomach chitinases showed the optimum pH toward both small synthetic substrates and larger chitin molecules in the acidic region (pH 2.0–5.0), proposing their digestive function in

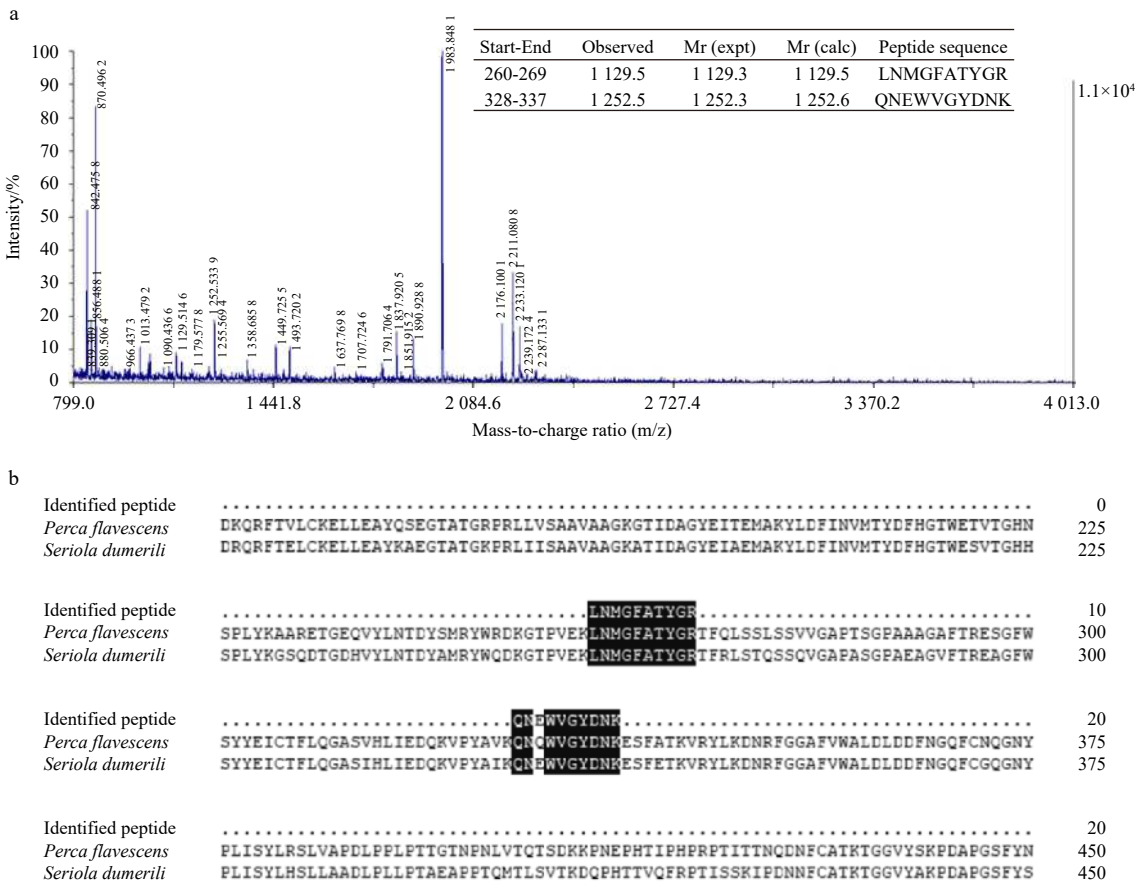

Fig. 3. Peptide mass fingerprint of purified chitinase. a. MALDI-TOF/TOF-MS/MS analysis of the purified chitinase following trypsin digestion. Three peptide fragments of chitinase were shown in the insert. b. Alignment of tryptic peptide fragments of purified chitinase with chitinases from yellow perch (*Perca flavescens*, GenBank accession No. XP_028438237.1) and greater amberjack (*Seriola dumerili*, GenBank accession No. XP_022623297.1). Mr (expt) and Mr (calc) represent experimental molecular weight and calculated molecular weight, respectively. Result of partial sequence alignment including the identified peptides was shown. Identical amino acid residues show a dark background.

the stomach (Ikeda et al., 2017). The optimum pH of stomach chitinases from threeline grunt (PtChiA) and silver croaker (PaChiA) toward pNp-$(GlcNAc)_2$ was 2.5 (Ikeda et al., 2013, 2009). This characteristic of fish stomach chitinases is suitable for the digestion of chitinous substances ingested as food in the presence of gastric acid. A previous study showed that the optimum activity of chitinase isolated from the stomach and intestine of snakehead fish was achieved at pH 6.0 (Baehaki et al., 2018). In this study, the optimum pH of the *B. sinensis* chitinase was 6.0. The enzyme showed activity throughout a wide range of pH from 4.0 to 8.0. It exhibited about 50% activity at pH 4.0, while only 20% activity at pH 9.0. However, at pH 3.0, its enzymatic activity sharply decreased to 5% (Fig. 4a). The optimum pH of chitinase from the liver of golden cuttlefish (SeChi) was 3.5 (Nishino et al., 2014), while that of toad pancreatic chitinase was 6.0 (Oshima et al., 2002a), which is concordant with the value for *B. sinensis* chitinase.

pH stability study showed that the enzyme was stable in pH range from 3.0 to 11.0, while incubation at pH 2.0 for 30 min resulted in a significant decrease of activity to only 5% (Fig. 4b). Several chitinases from other fish species, such as threeline grunt and marbled rockfish, have been reported to be stable and showed activity under acidic pH conditions (Ikeda et al., 2013, 2014). The present chitinase, however, was less stable than chitinases from other fish species under acidic conditions.

When 4-methylumbelliferyl-β-D-N,N′,N′-triacetylchitotrioside was used as substrate, the initial velocity of the enzyme at 37°C was 3.52 nmol/(L·min) (Fig. 4c). The enzyme expressed its highest activity to the substrate in 15 min. For thermal stability, the enzyme is relatively stable below 55°C (Fig. 4d). However, the enzyme activity decreased to 50% after incubation at 60°C for 30 min and was almost completely inactivated after heating at 70°C merely for 10 min (Fig. 4d). The optimum temperature of snakehead fish chitinases was recorded as 70°C (Baehaki et al., 2018). When heated for 10 min at 70°C, chitinases from threeline grunt (PtChiA and PtChiB) showed stability of more than 50% (Ikeda et al., 2013). These results indicated that *B. sinensis* chitinase is a thermolabile enzyme.

3.5 *Effect of NaCl on chitinase activity*

To determine the effect of NaCl on chitinase activity, NaCl was added into the reaction solution to a final concentration of 0 to 1 mol/L. Chitinase activity was not influenced by NaCl at 1 mol/L, as relative activity was 105.7% (data not shown). By comparison, the relative activity of silver croaker PaChiB decreased to 50% in the presence of 1 mol/L NaCl, while the activity of PaChiA increased 3-fold under the same condition (Ikeda et al., 2012). Regarding to this property, it is clear that the chitinase from *B. sinensis* exhibited parallel activity in the presence or absence of NaCl, in accordance with its living habit, migration between

freshwater and seawater.

3.6 *Characterization of chitinase degraded chitooligosaccharides (COS)*

COS have attracted much attention as they possess versatile functional properties, including antioxidant, antimicrobial and immunity-enhancing activity (Yang et al., 2017). In this study, the degradation of chitosan by the purified chitinase produced COS with molecular masses mostly of 200–700 Da, main peaks being at 269 Da, 360 Da, 433 Da, 515 Da, 597 Da (Fig. 5), which meets

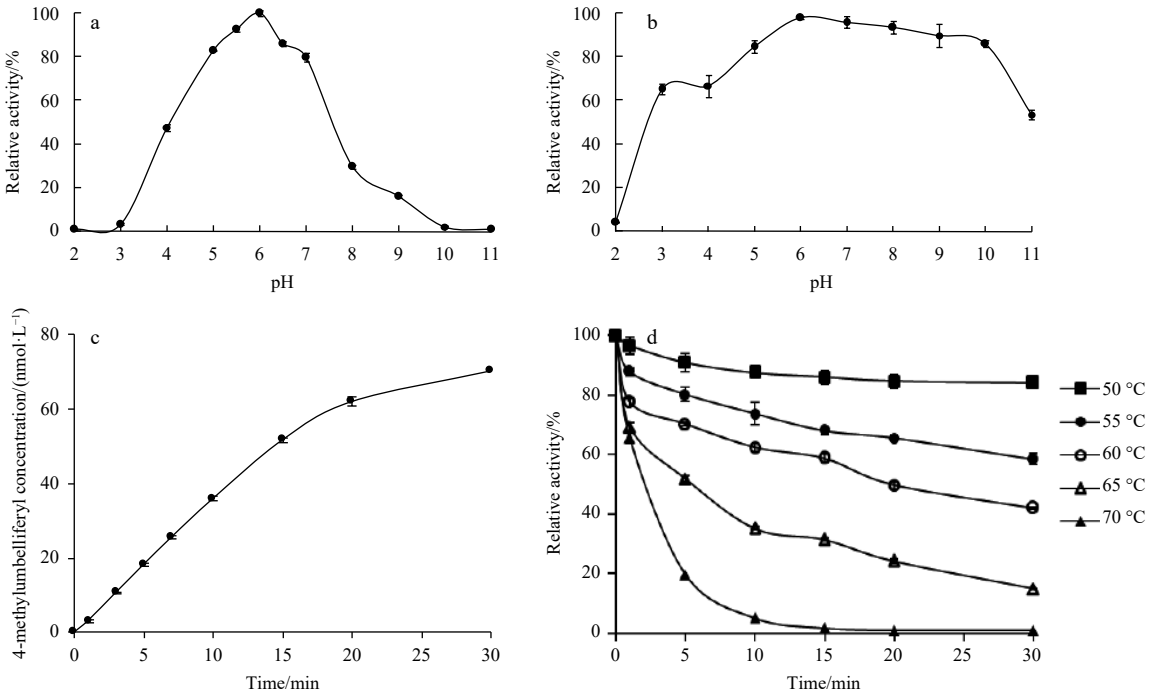

Fig. 4. Effect of pH and temperature on chitinase. a. Optimum pH; b. pH stability; c. initial velocity; d. thermal stability. The data were expressed as the mean standard±deviation.

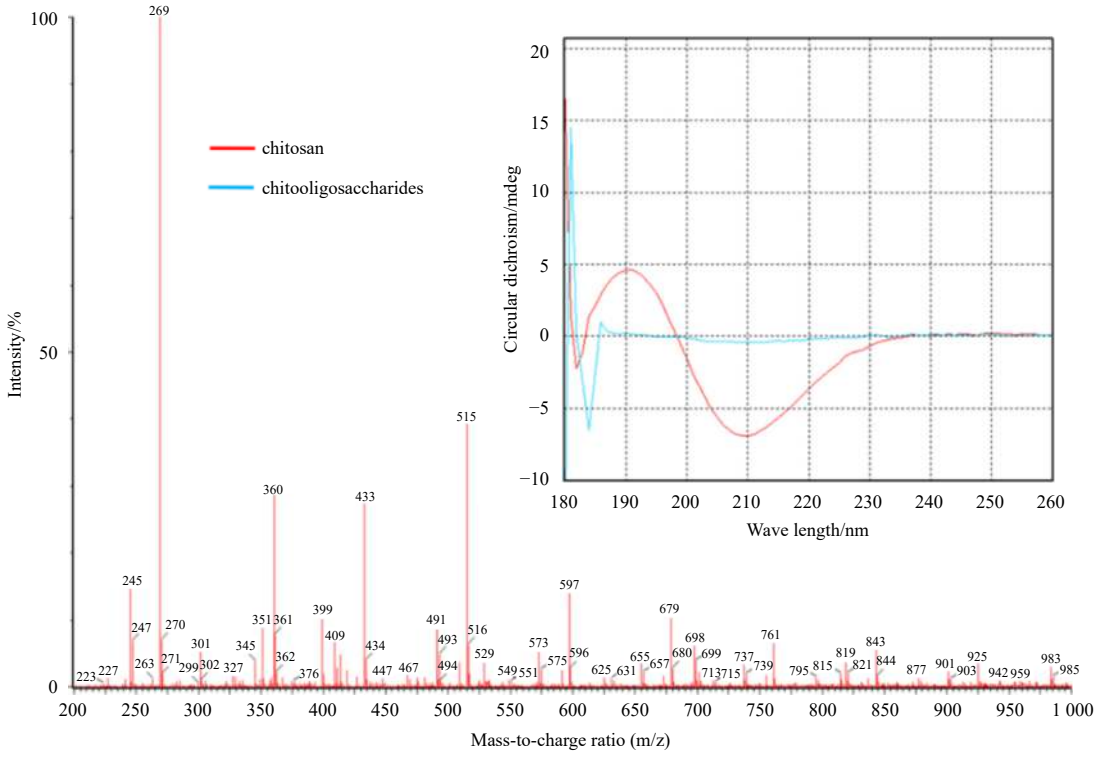

Fig. 5. ESI-MS and CD analysis of chitinase degraded COS. The concentration of COS is 50 μg/mL. CD analysis of chitosan and COS is shown in the inset.

the requirement to be a COS (≤10 kDa). It was assured that with the aid of chitinase, chitosan was decomposed into lower molecular mass oligosaccharides. Beneficial effects of COS on rainbow trout and koi growth performance, immunity, or blood profiles have been reported (Lin et al., 2012). However, the biochemical and physiological functions of COS to *B. sinensis* remain to be explored.

In the CD spectra, the peak near 210 nm corresponds to the GlcNAc residue (Fig. 5). In the presence of chitinase, as depolymerization progresses, a radical decrease in the peak height was observed, indicating extensive decrease of GlcNAc. It was proposed that the peak near 211 nm was due to the η→π * transition corresponding to the GlcNAc residue, which was related to the degree of acetylation, independent of chain conformation, length and ionic strength (Kumar et al., 2007). In observation, a decrease in the absorbance peak at 210 nm appeared in COS, that is, the degree of acetylation was reduced. Together with degradation of chitosan into low molecular mass COS as revealed by ESI/MS analysis, this study present results supported the fact that in the presence of chitinase, chitosan was depolymerized into low molecular mass products.

3.7 *Cloning of chitinase gene*

The full-length sequence of *B. sinensis* chitinase (2 337 bp) contains a 5′-untranslated region (UTR) of 33 bp, a 3′-UTR of 882 bp, and an open reading frame of 1 422 bp. The predicted protein contains 473 amino acid residues with a signal peptide of 20 amino acids located at the N-terminus, a GH18 catalytic domains and a chitin-binding domain (Fig. 6a). The mature deduced chitinase presents a theoretical isoelectric point of 5.94 and a predicted molecular mass of 52.6 kDa, which was smaller than the purified native chitinase on SDS-PAGE (58 kDa). The molecular mass difference suggests the possibility of post-translational modification of the protein at potential N-glycosylation sites of Asn29 and Asn392. BLAST analysis revealed that the deduced amino acids of *B. sinensis* chitinase showed 72.0%, 72.7% and 72.2% identity to chitinases from *P. flavescens* (GenBank accession No. XP_028438237.1), *S. dumerili* (GenBank accession No. XP_022623297.1) and *Oreochromis niloticus* (GenBank accession No. XP_019205294.1). The active site and GH18 catalytic domain were well conserved in different fish species (Fig. 6b). In the active site, the Asp (D) and Glu (E) in the DG-D-D-E motif is considered to be essential for chitinase activity of the GH18 chitinases (Meekrathok et al., 2017; Oshima et al., 2002b). Moreover, in the chitin-binding domain six cysteine residues composing three sets of disulfide bonds were perfectly conserved in the fish species (Fig. 6b), which is reported to be significant for exerting chitinolysis (Rogozhin et al., 2018).

Relative high identity was found in the amino acid sequence of *B. sinensis* chitinase to human chitotriosidase (53.35% identity). Therefore, based on the crystal structure of human chitotriosidase (PDB ID: 5HBF), the three-dimensional structure of *B. sinensis* chitinase was predicted (Fig. 6c). Structurally, the GH18 catalytic domain and chitin-binding domain were spa-

a

```
1       GAGTACGCGGGGGAACACTCAGGGATAGGCACAATGAGGAAGCTACTTTTAATTGCAGCCCTCGCTATTGTTTGC
1                                                 M  R  K  L  L  L  I  A  A  L  A  I  V  C
76      AATAGTTTTGTCTCAGCCACCAGATTGGTGTGCTATTACACCAACTGGTCACAGTACAGGCCCGAGGGTGGAAAG
15       N  S  F  V  S  A  T  R  L  V  C  Y  Y  T  N  W  S  Q  Y  R  P  E  G  G  K
151     TTTGTACCAACCAATATTGATCCAAACCTATGCACCCACTTGATCTATGCCTTTGCTGCCATCAATGATGCAAAT
40       F  V  P  T  N  I  D  P  N  L  C  T  H  L  I  Y  A  F  A  A  I  N  D  A  N
226     GAGTTGGTAACTGTGGAGTGGAATGATGATGTACTGTACAAGTCATTTAATGGGCTTAAACAAAGGAATCCAAAC
65       E  L  V  T  V  E  W  N  D  D  V  L  Y  K  S  F  N  G  L  K  Q  R  N  P  N
301     CTTAAGACCTTATTGGCAGTTGGAGGCTGGAACTTTGGATCACAAAAATTTACTACGATGGTCTCAACACAAGCC
90       L  K  T  L  L  A  V  G  G  W  N  F  G  S  Q  K  F  T  T  M  V  S  T  Q  A
376     AACAGAAATATGTTCATCAAATCCTCCATCAAATTTCTAAGAGATAACAGCTTTGATGGGCTTGACCTGGACTGG
115      N  R  N  M  F  I  K  S  S  I  K  F  L  R  D  N  S  F  D  G  L  D  L  D  W
451     GAATACCCAGCTGCAAGAGGAAGTCCTCCAGAAGATAAGCAGAGATTCACACTGCTGTGTAAGGAACTTCTTGAG
140      E  Y  P  A  A  R  G  S  P  P  E  D  K  Q  R  F  T  L  L  C  K  E  L  L  E
526     GCCTATGAAGCTGAGGGAACAGTAACGGGCCGTCCCAGATTATTGCTGTCTGCTGCTGTGTCTGCTGGAAAAGCA
165      A  Y  E  A  E  G  T  V  T  G  R  P  R  L  L  L  S  A  A  V  S  A  G  K  A
601     ACTATTAATGCTGGTTATGAAGTAGCAGAAATTGCTAAATACCTGGATTTCATTAATGTGATGACGTATGACTTC
190      T  I  N  A  G  Y  E  V  A  E  I  A  K  Y  L  D  F  I  N  V  M  T  Y  D  F
676     CATGGTTCTTGGGAGACGCTCACCGGACATAACAGCCCTTTGTACAAGGGATCTCATGACACTGGGGACAATGTT
215      H  G  S  W  E  T  L  T  G  H  N  S  P  L  Y  K  G  S  H  D  T  G  D  N  V
751     TATTTAAATACTGACTTTGCTGTGACTTACTGGCAAGTACAGGGAACTCCTGTGGAGAAGTTAAATATGGGTTTT
240      Y  L  N  T  D  F  A  V  T  Y  W  Q  V  Q  G  T  P  V  E  K  L  N  M  G  F
826     GCTACTTACGGACGTGCATTTCGTTTGGCCACTCAGTCCAGCCAGGTTGGAGCGCCAGCTAGCGGCCCGGCTAAT
265      A  T  Y  G  R  A  F  R  L  A  T  Q  S  S  Q  V  G  A  P  A  S  G  P  A  N
901     CCTGGCAAATATACACGAGAGTCTGGCTTCTGGTCTTATTATGAGGTTTGTTCTTTTCTTCAAGGAGCCACTGTT
290      P  G  K  Y  T  R  E  S  G  F  W  S  Y  Y  E  V  C  S  F  L  Q  G  A  T  V
976     CAAATGATTCAGGACCAGAAGGTTCCCTATGCCACAAAGCAAAATGAGTGGGTTGGTTATGATAATAAGAACAGT
315      Q  M  I  Q  D  Q  K  V  P  Y  A  T  K  Q  N  E  W  V  G  Y  D  N  K  N  S
1 051   TTTCAAACTAAAGTTCAATACCTAAAAGAGAAAATGCTTGGCGGAGCCTTTGTTTGGGCTTTAGATCTGGATGAT
340      F  Q  T  K  V  Q  Y  L  K  E  K  M  L  G  G  A  F  V  W  A  L  D  L  D  D
1 126   TTCTCTGGTCAGTTCTGTGGCGAGGGAAACTATGCCCTCATCAGCTACCTACACACTCTCTTGACTTCAGAAACA
365      F  S  G  Q  F  C  G  E  G  N  Y  A  L  I  S  Y  L  H  T  L  L  T  S  E  T
1 201   AACCCAAATCCAACAGATTCATCAACAGAAAAACCTTCTCCCTCTACCAAACCTCCCATTAAACTTATATTTCAA
490      N  P  N  P  T  D  S  S  T  E  K  P  S  P  S  T  K  P  P  I  K  L  I  F  Q
1 276   ACACCATCCACTGTGGCCGCTGACAACTTCTGTGCCACAAAGAGCAGCGGTTTGTACTCTAAACCTGATGCCCTG
415      T  P  S  T  V  A  A  D  N  F  C  A  T  K  S  S  G  L  Y  S  K  P  D  A  L
1351    ACATCTTTCTACCACTGTGCCAATGGGATAACATGGATCCTAGAGTGTCCAGCTAATCTGGTGTTTAAAGACAGC
440      T  S  F  Y  H  C  A  N  G  I  T  W  I  L  E  C  P  A  N  L  V  F  K  D  S
1 426   TGCAAGTGCTGCGATTATGCTGCTTTGTAACGCACACTTGATCAATCAAAATATTGAGATAAATTGTTGTATAAA
465      C  K  C  C  D  Y  A  A  L  *
1 501   ATTTGTTTGTCTCACAATGGTACTAAATACTATAAAAAATCCTGACTTATACTTTTTCAGTTAGCTTGCTAATAG
1 576   ACTAACAAACAAACAAAACAAGCAAAAGTACATCAGCAAAATCATGTCATTGGCACGGGTAATAAAATAAAGTCG
1 651   TTCTTCTTGCTTTGTTTAAGAGCCCAGCCTAGTATAAATGTCATAATTAGATATGTCTACGTTGAGGATTTTTAA
1 726   TCTTCAGTGAGCGTCACAAATAAATACTGGTTAAATTGTATTATTATCGTCAGTGTCTTTTCAGGTGAGGTCACT
1 801   TGCAGAGCTTTGTGTGCATGTGAAACACTTGACATCCACCCTTTCATAGTAACTAATAATTAGTATTATTAATAA
1 876   TTAGAGTTAATGGTTCTACATGCTGCACAGAAGGAATGCTTAATTACAAATGTGCTGAGGCGTGCCTCCGAGCAC
1 951   ACCCAGACCAGAACTGTGGCAGGTGTGGTGGCTTTTTTTTGTGTGTTGCCTCAAGTTACAGGTGCAGGTACATGT
2 026   TGATGATAAACAGAGAAAAAAAATAAACATACACACACAAAATAAATAAACACATAAACAAATAAATAAATAAAT
2 101   AGGTGAACTAGTGACAGTACAAATACAGTTAACAATTGTGGCAACATGGAGTAGCTGGATCAAACTTAGCACCTG
2 176   CAGAAGCTGGACTGACAGAGCTTTGAATGCTCCTGGCTGTTTTACAATAATAACGCAAATTTAGATAAACCACTG
2 251   AATTAATTTGTACCTAATTAAACGTTATTTTTCTTGTCTGTAAATAAAGCAATAAAAACAAAAAAAAAAGAAAAA
2 326   AAAAAAAAAAAA
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Fig. 6.

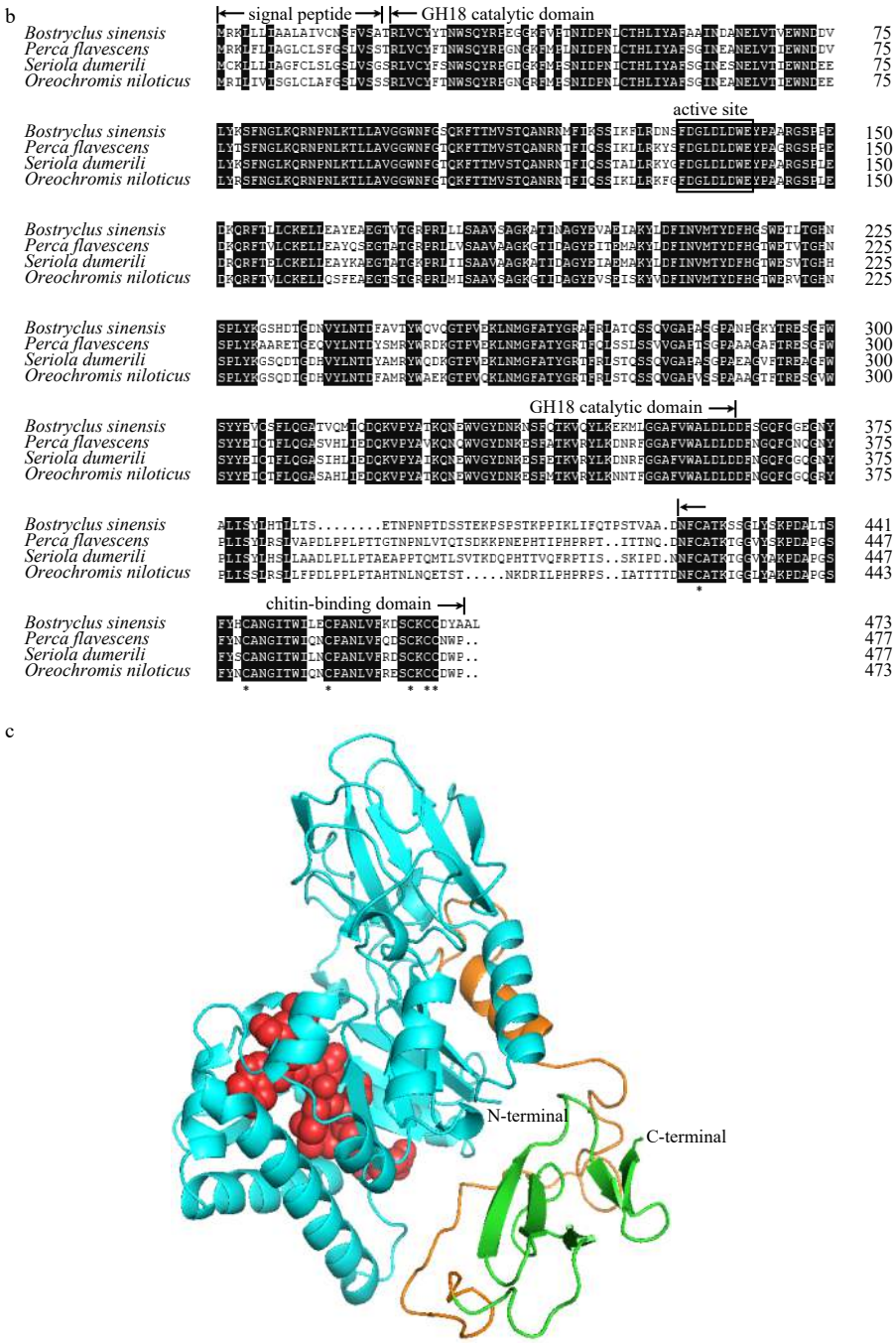

Fig. 6. Chitinase gene cloning and three-dimensional structure. a. Nucleotide and deduced amino acid sequences of *Bostryclus sinensis* chitinase. The start codon and stop codon are boldfaced. The putative signal peptides are shown in gray background. The GH18 catalytic domain and chitin-binding domain are shown with solid line and dot line, respectively. b. Alignment of *B. sinensis* chitinase (GenBank accession No. MN905598) with counterparts from *Perca flavescens* (GenBank accession No. XP_028438237.1), *Seriola dumerili* (GenBank accession No. XP_022623297.1) and *Oreochromis niloticus* (GenBank accession No. XP_019205294.1). Signal peptide, GH18 catalytic domain and chitin-binding domain are indicated with arrows. Active site is included in box. The conserved cysteine residues were indicated with asterisk. c. Three-dimensional structure diagram of *B. sinensis* chitinase. GH18 catalytic domain, linker and chitin-binding domain were shown in cyan, orange and green colors, respectively. Active sites were shown with red sphere.

tially separated, and the active site was located in the inner space of GH18 catalytic domain.

3.8 *Expression analysis of chitinase in different tissues*

Northern blot analysis revealed the toad chitinase mRNA to be expressed in the pancreas, but not in other organs (Oshima et al., 2002a), while threeline grunt chitinase was expressed solely in stomach (Ikeda et al., 2013). To investigate the tissue-specific expression profile of chitinase, qPCR analysis was used to determine the transcript levels of chitinase in *B. sinensis* different tis-

sues. The results showed that the chitinase gene was broadly expressed in all tissues selected. As shown in Fig. 7, chitinase mRNA was highly expressed in esophagus and hepatopancreas, followed by intestine, while mRNA levels were barely detected in stomach, kidney, muscle and blood. Hence, the transcript levels of chitinase were correlated with the enzyme activities in various tissues.

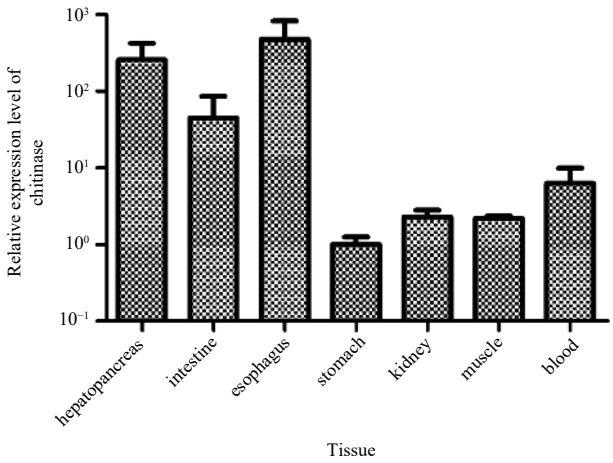

Fig. 7. Determination of chitinase transcript level in different tissue of *Bostryclus sinensis* by qPCR. Lane headings indicated the specific tissue. β-actin was used as the reference gene for internal control. Columns represented the mean of triplicate assays.

4 Conclusions

A chitinase was purified to homogeneity from the hepatopancreas of *B. sinensis*. The enzymatic properties of chitinase were investigated and its degradation on chitosan to produce low molecular mass COS were explored. Its full-length sequence was cloned and the expression profile in different tissues was detected by qPCR, which provided a theoretical basis for studying the physiological function of chitinase in different tissues of *B. sinensis*. This study represented the leading report on the investigation of chitinase in *B. sinensis*.

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