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Research Paper 研究报告

Gene cloning, expression and characterization of an exo-chitinase with high β-glucanase activity from *Aeromonas veronii* B565

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Abstract: [**Objective**] We aimed to express and characterize biochemical properties of Chi92, a chitinase from *Aeromonas veronii* B565, and study its potential application as aquafeed supplement. [**Methods**] The chitinase gene *chi92* was cloned from *A. veronii* strain B565 and expressed in *Pichia pastoris* GS115. The recombinant chitinase (Chi92) was purified and characterized. Chi92 was supplemented in diets containing *P. pastoris* powder and fed to zebrafish for 14 days. By comparing with the control group, effect of Chi92 supplementation on growth, feed utilization, microvilli morphology, and disease resistance was investigated. [**Results**] The complete gene sequence encoded a polypeptide with 864 amino acids. Chi92 exhibited optimal activity at pH 6.0 and 40 °C, and was resistant to proteases and not substantially inhibited by metal ions. Chi92 had high chitinase activity (69.4 U/mL). The specific activity was 809.2 U/mg and 235.6 U/mg on colloidal chitin and *β*-1,3-1,4-glucan, respectively. Thin-layer chromatography and electrospray ionization–coupled mass spectrometry revealed that N-diacetylglucosamine was the dominant product of Chi92 when colloidal chitin was used as substrate. Moreover, Chi92 showed advantages over other chitinases for degradation of yeast cell wall. Supplementation of Chi92 in diet containing yeast product significantly improved the intestine microvilli length and density of zebrafish after two weeks of feeding. Marginally improved growth performance, feed utilization, as well as disease resistance were also observed in the Chi92 supplement group. [**Conclusion**] The pH stability, resistance against metal ions/chemical reagents/proteases, and high yeast cell wall degradation activity of Chi92 suggest its potential use as feed additive enzyme for warm water aquaculture.

Keywords: chitinase, Chi92, *Aeromonas veronii* B565, zebrafish

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Chitin is an insoluble polysaccharide composed of β-1,4 N-acetyl-D-glucosamine units[1]. Chitin is the second most abundant polysaccharide in nature after cellulose, with huge amounts (>10^{11} tons) produced in marine ecosystems each year[2−3]. Naturally occurring chitin is stabilized by both inter-chain and intra-chain hydrogen bonds[4], and this inherent stability limits its utilization.

Chitinases (EC 3.2.1.14) are the primary enzymes that catalyze the hydrolysis of chitin into soluble oligosaccharides, which are valuable natural products. Chitinases belong to glycoside hydrolase families 18 and 19 based on amino acid sequence similarity[5]. They are further classified into endo-chitinases, exo-chitinases, and N-acetyl-glucosaminidases according to differences of their sites of cleavage and hydrolysis products[6]. Endo-chitinases randomly cleave β-1,4-glycosidic bonds of chitin, whereas exo-chitinases cleave the chain from the non-reducing end to form diacetyl-chitobiose. N-acetyl-glucosaminidases hydrolyze diacetyl-chitobiose to yield N-acetyl-D-glucosamine or produce N-acetyl-D-glucosamine from the non-reducing end of N-acetyl-chitooligosaccharides[7]. Notably, certain chitinases have both exo- and endo-chitinase activities[8–9]. Chitinase have a very broad range of applications in agriculture[5,10]. However, the use of chitinase in aquafeed has rarely been investigated. Zhang et al. reported that dietary supplementation of a chitinase improved the growth and feed utilization of tilapia fed with diet containing shrimp bran[8], supporting its potential as aquafeed additive.

Yeast is widely used in industries such as wine, food, medicine, animal feed, and cosmetics[11]. Particularly, yeast products (primarily brewer's yeast and baker's yeast) are commonly used as feed ingredients in aquaculture because of high nutritional value[12]. Notably, the cell wall accounts for up to 30% of the dry weight of yeast[13]. The yeast cell wall is composed mainly of mannoproteins (–40% of dry weight), β-linked glucans (–60%), and chitin (–2%), with the presence of chitin in yeast cell wall probably accounting for its inherent insolubility[14]. The bottleneck of efficient utilization of yeast products is the resistance of yeast cell wall to enzyme-mediated hydrolysis[15].

Aeromonas veronii strain B565 is a gram-negative bacterium isolated from aquaculture pond sediment in China, and was found to have the ability to produce chitinase to control fungal or Myxozoa-related diseases[16]. The whole-genome sequence of *A. veronii* strain B565 (GenBank accession number CP002607) was reported in our earlier study[16]. Strain B565 encodes five chitinases, namely Chi92 (AEB48885.1), chitinase A (AEB48887.1), ChiB565 (AEB48892.1), and two other chitinases (AEB48889.1, AEB50059.1), with their sequence identities ranging from 9% to 81%. The five chitinase genes have homologs in *A. veronii* strain ATCC 7966, with identities of 88%, 78%, 90%, 78% and 76%, respectively, indicating an important role of these conserved chitinases in *Aeromonas* spp.[16]. However, the biochemical properties and potential applications of these chitinases have not been investigated. In this study, the *chi92* gene was cloned and expressed. This novel exo-chitinase showed relatively higher chitinase and β-glucanase activities which may efficiently hydrolyze yeast cell wall. Also, the effect of the enzyme as additive in aquafeed containing yeast product was studied in zebrafish.

1 Materials and methods

1.1 Bacterial strains, plasmids, media, and culture conditions

A. veronii strain B565 was originally isolated from aquaculture pond sediment in Tianjin, China. The strain was deposited in WDCM under the accession number CGMCC 3169. *Escherichia coli* Trans1-T1 (TransGen Biotech, Beijing, China) was used as the host strain for *chi92* cloning, and *Pichia pastoris* GS115 (maintained in our laboratory) was used as the host strain for expression. pEASY-T3 (TransGenBiotech) was used as the cloning vector, and pPIC9 (Invitrogen, Carlsbad, CA, USA) was used as the expression vector. Liquid or solid [1.5% (W/V) agar] Luria-Bertani medium (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) was used for cultivation of bacteria with ampicillin (100 μg/mL) or kanamycin (50 μg/mL) for the selection of transformants.

1.2 Preparation of substrate chitins

To prepare crude shrimp shell chitin, commercial white prawn *Litopenaeus vannamei* were purchased from a chain store in Beijing, China. Shells were peeled, washed, dried and ground into powder. Then, particles that passed through a 30-mesh sieve while retained in a 100-mesh sieve were selected, and used as crude shrimp shell chitin for enzyme activity assays. Purified shrimp shell chitin was prepared according to the method described by Feng et al.[17]. Commercial shrimp shell chitin was purchased from Sigma (V900332). Colloidal chitin was prepared as described by Zhang et al.[8].

1.3 Cloning of the chitinase 92 gene, *chi92*

Genomic DNA of strain B565 was extracted using DNA isolation kit reagents (Tiangen), and the DNA was used as template for amplification of *chi92* using the following primers *chi92*-F: 5′-GGAGAATTCGCGGCGCCCGGCAAGCC-3′, *chi92*-R: 5′-GGAGCGGCCGCTTTACAACTG GCGGCTCCCACATCCTG-3′; the underlined sequences represent *EcoR* I (*chi92*-F) and *Not* I (*chi92*-R) sites for cloning into pPIC9. The amplified PCR product was gel-excised, purified, and ligated into cloning vector pEASY-T3 and transformed into *E. coli* Trans1-T1 for blue/white selection. The positive clones were confirmed by sequencing. The recombinant plasmid Chi92-T3 was digested with *EcoR* I and *Not* I, and the liberated fragment was ligated into vector pPIC9 that had been digested with the same enzymes, yielding recombinant plasmid Chi92-pPIC9. Chi92-pPIC9 was transformed into *E. coli* Trans1-T1 with ampicillin selection, and positive clones were confirmed by DNA sequencing and cultured in Luria-Bertani medium containing 100 μg/mL ampicillin overnight at 37 °C. Chi92-pPIC9 was obtained from the cultures using the pure Midi Plasmid kit (Tiangen), digested with *Pme* I, and transformed into *P. pastoris* GS115 by electric shock using a MicroPulser (Bio-Rad, Hercules, CA, USA). Transformed yeast was cultured on minimal dextrose plates at 30 °C for 2 days for selection.

1.4 Selection of the positive transformants

In total, 200 colonies were selected randomly from the minimal dextrose plate and verified by PCR using the follow primers: 5′-AOX1: 5′-GGTTGAA TGAAACCTTTTTGCC-3′ and 3′-AOX1: 5′-CAACTAATTATTCGAAGGATCC-3′. The colonies containing a band of the expected size were incubated in 3 mL of buffered minimal glycerol

medium [BMGY; 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% (W/V) biotin, and 1% (V/V) glycerol] at 30 °C in a gyratory shaker at 200 r/min for 2 days. Cells were harvested by centrifugation at 4500 r/min at 10 °C for 5 min and cultured for 2 days in 1 mL of buffered minimal methanol medium [BMMY; the glycerol in BMGY was replaced by 1% (V/V) methanol]. The chitinase activity associated with each of the positive clones was assayed with the method described below.

1.5 Expression, purification, and identification of Chi92

The colony of the recombinant *P. pastoris* GS115 with the maximum chitinase activity was cultured in 200 mL BMGY at 30 °C with shaking at 200 r/min for 2 days. Cells were harvested by centrifugation at 4500 r/min at 10 °C for 5 min and cultured in 100 mL of BMMY at 30 °C with 200 r/min shaking for 2 days. After incubation, the culture medium was centrifuged at 12000 r/min for 10 min at 4 °C. The supernatant (crude enzyme) was harvested and purified by membrane filtration with a 5000 Da molecular weight cut off (Viwascienci, Hannover, Germany). The retentate (10 mL) was precipitated with acetone (20 mL) and proteins were resuspended in 20 mmol/L sodium phosphate-citrate (pH 6.0). The retentate loaded onto a HiTrap™ Desalting column (GE Healthcare, Uppsala, Sweden) for desalination and then onto a HiTrap™ Q Sepharose XL 5 mL FPLC column (GE Healthcare). 20 mmol/L sodium phosphate-citrate (pH 6.0) with 300 mmol/L NaCl was served as the eluent. SDS-PAGE was used to assess protein purity. The identity of the target recombinant protein was confirmed by liquid chromatography-coupled electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (Applied Biosystems 4000, Life Technologies, USA) at the Academy of Military Medical Sciences (Beijing, China).

1.6 Chitinase activity assay for Chi92

Chitinase activity was determined by the dinitrosalicylic acid method with some modifications[18]. The assay was carried out in 0.25 mL of 20 mmol/L sodium phosphate (pH 6.0) containing 1.0% (W/V) colloidal chitin and 0.25 mL Chi92 (86 μg/mL). The reaction mixture was incubated at 40 °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[18]. One unit of chitinase activity was defined as the amount of enzyme that released 1 μg reducing sugars per minute[10]. All reactions were performed in triplicate.

1.7 Substrate specificity and calculation of kinetic parameters for Chi92

Colloidal chitin, carboxymethylcellulose, *β*-1,3-1,4-glucan, powdered chitin, powdered chitosan, shrimp shell chitin (crude, purified, and commercial), glycol chitosan and porcine stomach mucin (M1778) were used as the substrate to determine the substrate specificity of Chi92. Chitinase activity was measured by the 3,5-dinitrosalicylic acid method described above. K_m and k_{cat} values for Chi92 when colloidal chitin was used as substrate were determined by the method described above except that colloidal chitin at concentrations of 0.1%–1.0 % (W/V) was used as the substrate. Initial velocity versus substrate concentration data were fitted to the Lineweaver-Burk transformation of the Michaelis-Menten equation, and the kinetic parameters of Chi92 were calculated accordingly.

1.8 Effects of pH on chitinase activity of Chi92

The optimal pH for Chi92 activity was determined in assays (500 μL) containing 250 μL Chi92 (86 μg/mL) and 250 μL 1.0% colloidal chitin in buffers of pH 2.0–12.0; reactions were incubated at 37 °C for 1 h. The buffers used were 100 mmol/L glycine-HCl (pH 2.0–3.0), 100 mmol/L sodium phosphate-citrate (pH 3.0–8.0), 100 mmol/L Tris-HCl (pH 8.0–9.0), and 100 mmol/L glycine-NaOH (pH 9.0–12.0). The pH stability of Chi92 activity was assessed by incubating Chi92 (86 μg/mL) in the above-mentioned buffers for 1 h before measuring the remaining activity in pH 6.0 buffer at 40 °C for 1 h.

1.9 Effects of temperature on chitinase activity of Chi92

The optimal temperature for Chi92 activity was determined in assays (500 μL) containing 250 μL Chi92 (86 μg/mL) and 250 μL 1.0% (W/V) colloidal chitin; reactions were incubated in 100 mmol/L sodium phosphate-citrate (pH 6.0) at different temperatures (20–80 °C) for 1 h. The temperature stability of Chi92 activity was assessed by incubating Chi92 (86 μg/mL) at the above-mentioned temperatures for 1 h in 20 mmol/L sodium phosphate-citrate (pH 6.0) before measuring the remaining activity in the same buffer at 40 °C for 1 h.

1.10 Effects of different metal ions and chemical reagents on chitinase activity of Chi92

The effects of various reagents on chitinase activity of Chi92 was determined by addition of Li^{+}, Na^{+}, K^{+}, Ag^{+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, Fe^{3+}, SDS, EDTA, or β-mercaptoethanol (1 mmol/L or 5 mmol/L) to the reaction mixture containing 250 μL Chi92 (86 μg/mL) and 250 μL 1.0% (W/V) colloidal chitin in 100 mmol/L sodium phosphate-citrate (pH 6.0), and incubation of the reaction mixture at 40 °C for 1 h. Reactions with no metal ion or chemical reagent were used as the control.

1.11 Stability of Chi92 activity in the presence of various proteases

The effect of different proteases on chitinase activity of Chi92 was determined by measuring the residual chitinase activity after incubation of Chi92 (86 μg/mL) in 100 mmol/L glycine-HCl containing 8.6 μg/mL pepsin (pH 2.0), 100 mmol/L Tris-HCl containing 8.6 μg/mL trypsin or α-chymotrypsin at pH 7.0 or 7.5, or 8.6 μg/mL tilapia intestinal juice (pH 7.0) for 30 or 60 min at 37 °C. Chi92 in the control reactions were subjected to the same procedures, except that no proteases were added during the incubation.

1.12 Thin layer chromatography (TLC) analysis of the hydrolysis products of colloidal chitin by Chi92

Hydrolysis products of Chi92 action were prepared as follows. Briefly, 500 μL Chi92 (86 μg/mL) and 500 μL 1.0% (W/V) colloidal chitin were incubated in 100 mmol/L sodium phosphate-citrate (pH 6.0) at 40 °C for 6 h and then boiled for 10 min. The control reaction mixture was directly boiled before incubation. Reactions were cooled to room temperature under flowing water, and each supernatant (hydrolysis products) was collected and centrifuged at 10000 r/min for 10 min. The products were analyzed by TLC on a silica gel plate (GF 254, Wanlanshitu, Beijing) as described by Lee et al.[19] with some modifications. A mixed solvent containing 1-butanol, 2-propanol, acetic acid, and H_2O (7:5:2:4)

was used as the mobile phase. After TLC, each plate was sprayed with a coloring reagent comprised of 4 mL aniline, 4 g diphenylamine, 200 mL acetone, and 30 mL of 85% phosphoric acid in a total volume of 4.23 mL. GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ (Sigma Chemical Company, St. Louis, MO, USA) were used as standards.

1.13 ESI-MS analysis of the hydrolysis products of colloidal chitin by Chi92

ESI-MS was used to identify the hydrolysis products of colloidal chitin by Chi92. Hydrolysis products were prepared as mentioned above. Free ions were removed by anion- and cation- exchange resins. Identification of the hydrolysis products was confirmed by ESI-MS/MS (LC-MS2010, Daojin, Kyoto, Japan) at the Institute of Chemistry, Chinese Academy of Sciences (Beijing, China).

1.14 Comparison of the specific activities among Chi92, ChiB565, and C6137

C6137 is a commercial exo-chitinase from *Streptomyces griseus* (Sigma-Aldrich). ChiB565 is another chitinase from *A. veronii* strain B565 expressed in our lab[8]. The specific activity of Chi92 was compared with the two chitinases. When colloidal chitin was used as substrate, the assay (500 μL) contained 250 μL Chi92/C6137 (86 μg/mL) and 250 μL 1.0% (W/V) colloidal chitin in 20 mmol/L sodium phosphate-citrate buffer (pH 6.0). The mixture was incubated at 40 °C for 1 h. For ChiB565 (86 μg/mL), the conditions were 20 mmol/L sodium phosphate-citrate (pH 5.0) at 50 °C. When crude, purified, and commercial shrimp shell chitin were used as the substrate, the assay (500 μL) contained 250 μL Chi92/C6137 (86 μg/mL) and 2.5 mg shrimp shell chitin power in 250 μL of 20 mmol/L sodium phosphate-citrate (pH 6.0). The mixture was incubated at 40 °C for 1 h. For ChiB565 (86 μg/mL), the conditions were 20 mmol/L sodium phosphate-citrate (pH 5.0) at 50 °C.

1.15 Comparison of the ability to degrade yeast cell wall among Chi92, ChiB565, and C6137

After incubation 30 min at 85 °C, heat-inactivated *P. pastoris* GS115 powder was prepared and used as the substrate to determine the ability of Chi92, ChiB565, and C6137 to degrade yeast cell wall. Each reaction contained 0.05 g yeast cell wall power and 7 U (measured by colloidal chitin as the substrate at 37 °C) of each chitinase (Chi92, ChiB565, or C6137) in a volume of 500 μL of sodium phosphate-citrate buffer (pH 6.0 for Chi92 and C6137, pH 5.0 for ChiB565). Reactions were incubated at 37 °C for 1.5 h or 3.0 h, after which the production of reducing sugars was measured by the 3,5-dinitrosalicylic acid method. Glucose yield by Chi92 was measured using a glucose detection kit (Liwen, Beijing, China). The hydrolysis products of heat-inactivated *P. pastoris* GS115 by Chi92 at 1.5 h or 3.0 h were collected and dried into powder. Microscopic morphology of the dried hydrolysis products and original GS115 powder were compared by scanning electron micrographs at the Institute of Agro-Products Processing Science and Technology, Chinese Academy of Agricultural Sciences (Beijing, China).

1.16 Effects of Chi92 as feed additive on growth of zebrafish

A 14-day feeding trial was conducted with zebrafish in a circulating aquaculture system at the Feed Research Institute, Chinese Academy of Agricultural Sciences. All experimental and animal

care procedures were approved by Feed Research Institute of Chinese Academy of Agricultural Sciences Animal Care Committee, under the auspices of the China Council for Animal Care (Assurance # 2014-HFM01). The rearing conditions were as follows: water temperature 25 °C, pH 7.0, dissolved oxygen>5.0 mg/L, NH_4^+-N< 0.5 mg/L, and NO_2-N< 0.05 mg/L. Three experimental diets were prepared: control, 95% (W/W) of the basal diet and 5% (W/W) heat-inactivated *P. pastoris* powder; diet Chi92 (0.23 U/g), the control diet supplemented with 0.23 U/g of Chi92, and diet Chi92 (2.3 U/g), the control diet supplemented with 2.3 U/g of Chi92. Each diet was randomly assigned to five replicate tanks with 12 fish in each. The fish were fed to apparent satiation twice a day at 9:00 and 15:30. The fish were batch weighed at the beginning (IBW) and end (FBW) of feeding period, and weight gain (WG), feed conversion ratio (FCR), and survival rate were determined.

WG=100×(FBW- IBW)/IBW

FCR=IT_d (g)/(FBW-IBW) (g) where IT_d is the total dry diet consumed during the experimental period.

1.17 Microvilli density and length

After 14-day feeding trial, three fish from each group were sacrificed with overdose of MS-222. The gut was rinsed three times with PBS buffer (130 mmol/L NaCl, 10 mmol/L NaH_2PO_4, pH 7.4) and immediately placed in 2.5% glutaraldehyde solution for fixation. The microscopic morphology of microvilli was analyzed through scanning electron microscopy. Microvilli density and length were calculated by Image J2x, USA.

1.18 Gut alkaline phosphatase activity assay

After 14-day feeding trial, eight fish from each tank were immersed in 10^8 CFU/mL *A. hydrophila* NJ-1 for 24 h[20]. Then, the whole gut was sampled from fish for determination of the alkaline phosphatase (AKP) activity following the method described earlier[20]. In brief, the gut samples of fish were homogenized by PBS, followed by centrifugation at 4 °C. The supernatant was incubated in a *p*-nitrophenylphosphate liquid substrate system (Sigma) for 30 min, and then absorbance was measured at 405 nm. The concentration of total protein was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, America). The alkaline phosphatase activity was calculated as the ratio of enzyme activity and protein content.

1.19 Statistics

Data from the animal experiment were analyzed with one-way ANOVA, and Duncan's multiple range test was used to compare the means between any two groups. Differences with a *P* value lower than 0.05 were considered as significant. All the statistical analysis was conducted on SPSS 17.0.

1.20 NCBI accession numbers

The nucleotide sequence of the chitinase gene (*chi92*) was deposited in GenBank under the accession number CP002607.1.The amino acid sequence of Chi92 was assigned GenBank NCBI Reference Sequence YP_004391502.1.

2 Results

2.1 Cloning of chi92 and sequence analysis

The *chi92* gene encoded a protein of 864 residues. Chi92 has high amino acid sequence identity to the glycoside hydrolase (98%) from *A. veronii* Hm21 (NCBI Reference Sequence WP_021229704.1), the glycoside hydrolase (98%) from *A. veronii* AER39 (NCBI Reference Sequence: WP_005335247.1), a

chitinase (GenBank number CAD24484.1) (81%) from *Aeromonas caviae* WS7b[21], and a chitinase (GenBank number AAG09437.1) (81%) from *Aeromonas hydrophila* JP101[22]. A 23 amino acid residue signal peptide was found by SignalP 4.1. The enzyme contains a glycoside hydrolase family 18 catalytic domain (residues Val158-Asp543), and two carbohydrate binding domains (residues Pro770-Trp809, Trp819-Trp855), as predicted by Pfam (http://pfam.sanger.ac.uk/search). Multiple sequence alignment indicated that Chi92 contained the consensus substrate binding sequence SXGG (Ser270-Val271-Gly272-Gly273) and catalytic sequence DXXDXDXE (Asp307-Gly308-Val309-Asp310-Ile311-Asp312-Trp313-Glu314) of family 18 chitinases[23] (Figure 1).

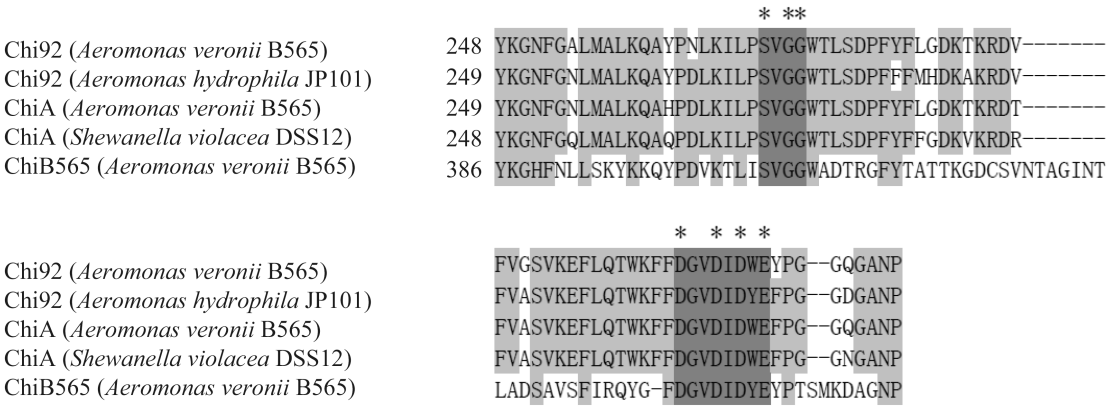

Figure 1. Amino acid sequence alignment of Chi92 with other chitinases by CLUSTAL. Enzymes include Chi92 from *A. veronii* B565, Chi92 from *A. hydrophila* JP101, ChiA from *A. veronii* B565, ChiA from *Shewanella violacea* DSS12, and ChiB565 from *A. veronii* B565.

2.2 Expression, purification, and characterization of recombinant Chi92

The successful expression and purification of recombinant Chi92 were confirmed by SDS-PAGE. The yield of Chi92 from *P. pastoris* was 86 mg/L. The band migrated to a position near the theoretical molecular mass of the recombinant Chi92 (-92 kDa; Figure 2). LC-ESI-MS/MS was used to ensure the identity of the purified protein. Twenty-eight percent of the recombinant Chi92 sequence was confirmed (data not shown). The biochemical parameters of purified recombinant Chi92 were shown in Table 1.

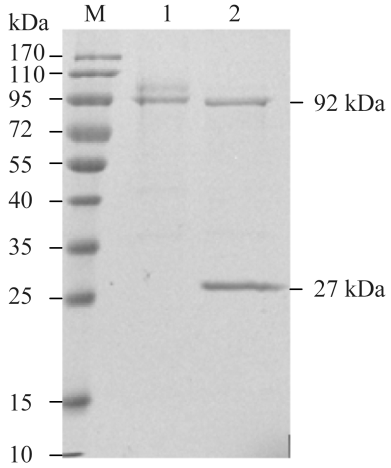

Figure 2. SDS-PAGE analysis of purified recombinant Chi92. M, molecular mass standards; lane 1, purified recombinant Chi92; lane 2, Chi92 that had been deglycosylated with Endo H. The band in lane 2 at about 92 kDa represents purified Chi92, and the band at –27 kDa represents Endo H.

2.3 Effects of temperature, pH, metal ions, chemical reagents, and proteases on Chi92 activity

The optimal pH for Chi92 was 6, and over 80% of the maximal activity was retained at the pH range

of 6.0–9.0 (Figure 3-A). Chi92 activity was generally stable over a broad pH range. After incubation at pH ranging from 4.0 to 9.0, Chi92 retained over 58% of its maximum activity (Figure 3-B). The optimum temperature for Chi92 activity was 40 °C, and it retained more than 48% of its maximum activity when assayed at temperatures ranging from 20 °C to 40 °C (Figure 3-C). Chi92 showed good thermal stability, retaining 70% and 93% of its maximal activity after preincubation for 2 h at 40 °C or 25 °C, respectively (Figure 3-D).

The effects of metal ions and chemical reagents on chitinase activity were assessed under standard assay conditions, i.e., 40 °C, pH 6.0, and incubation for 1 h. Mn^{2+} (5 mmol/L) increased the activity by –50%, whereas other ions such as Cr^{3+}, Cu^{2+}, and Fe^{3+} (1 mmol/L or 5 mmol/L) inhibited the enzymatic activity to different extents (Table 2). *β*-mercaptoethanol (1 mmol/L) increased the activity of Chi92 by 30%. The chelating agent EDTA had almost no effect at 1 mmol/L but inhibited activity by –90% at 5 mmol/L (Table 2). Chi92 activity exhibited almost complete resistance to proteolysis after 60 min preincubation with trypsin, or pepsin. The enzyme retained –40% of maximal activity after preincubation with α-chymotrypsin or tilapia intestinal juice (Figure 4).

Table 1. Biochemical parameters of Chi92

Parameters	Substrates		
	Colloidal chitin	*β*-1,3-1,4-glucan	Commercial shrimp shell chitin
K_m/ (mg/mL)	3.967	1.938	1.349
V_{max}/[μmol/(mg·min)]	4798.464	596.659	757.576
k_{cat}/(s^{-1})	7374.440	916.965	1164.268

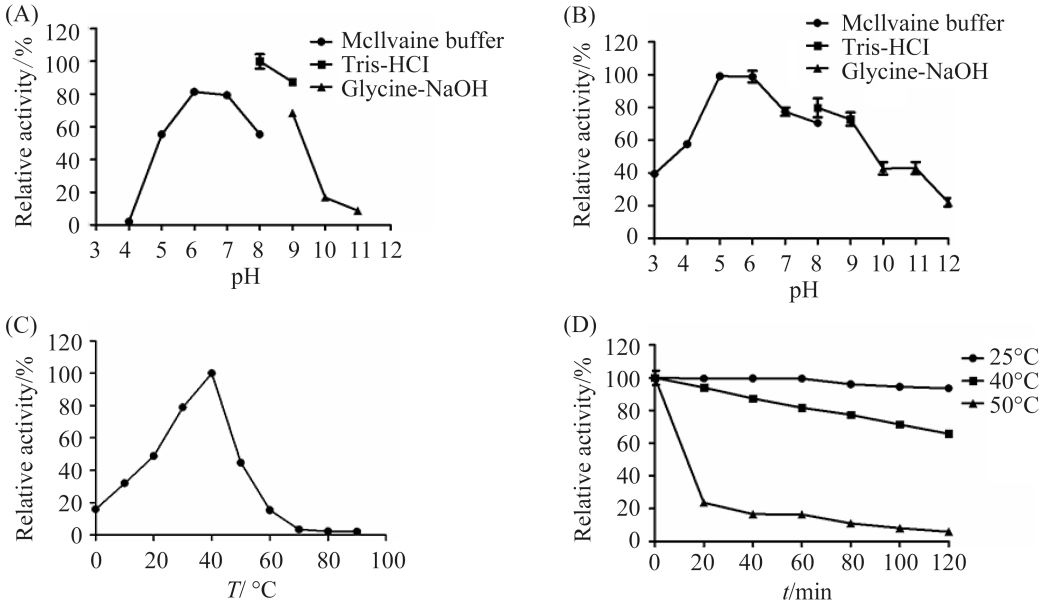

Figure 3. Effects of pH and temperature on purified recombinant Chi92. A: Effect of pH on the enzymatic activity of Chi92; B: pH stability of Chi92; C: Effect of temperature on enzymatic activity of Chi92; D: Thermostability of Chi92. Data were presented as percentage of the maximum activity in each experiment, which was set as 100%. The data represent mean±S.E. of triplicate samples. Experiments were repeated for three times.

Table 2. Effect of metal ions and chemical reagents on the enzymatic activity of recombinant Chi92

Chemicals	Relative activity/%[a]	
	1 mmol/L	5 mmol/L
None	100.0±2.0	100.0±5.5
Cr^{3+}	49.3±3.6	36.9±1.2
Co^{2+}	88.7±3.2	116.3±2.4
Li^{+}	68.5±3.1	115.9±0.7
Ca^{2+}	95.9±3.8	112.7±0.9
Zn^{2+}	93.3±0.3	87.3±2.0
K^{+}	97.1±0.7	96.0±3.0
Na^{+}	98.6±0.6	103.1±3.9
Ni^{+}	81.5±1.4	80.5±1.9
Cu^{2+}	33.9±1.2	35.7±6.8
Mg^{2+}	82.7±0.7	102.0±5.1
Fe^{3+}	23.0±0.6	20.0±6.1
Mn^{2+}	108.1±4.0	151.2±4.6
Pb^{2+}	69.4±2.6	65.3±4.4
Ag^{+}	91.0±2.0	115.2 ±4.3
EDTA	100.1±1.0	11.8±1.2
SDS	73.7±1.5	87.3±3.5
Mercaptoethanol	129.3±0.9	104.0±6.1

The data represent the mean±S.E. of triplicate samples. All experiments were repeated three times.

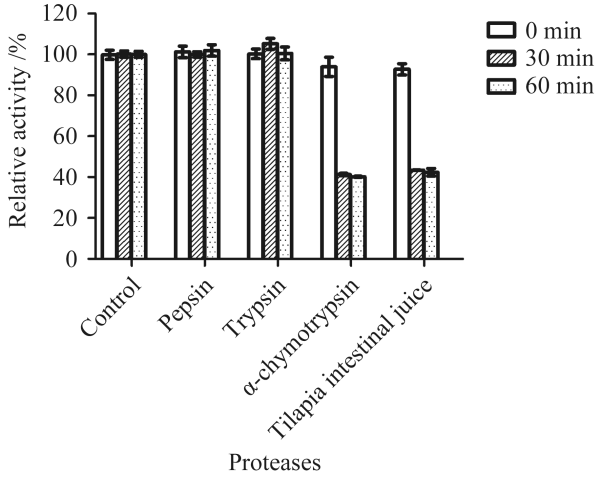

Figure 4. Effect of proteases on the activity of purified recombinant Chi92. The data represent the mean±S.E. of triplicate samples. Experiments were repeated for three times.

2.4 Hydrolysis products of colloidal chitin by Chi92

The hydrolysis products of 1.0% colloidal chitin by Chi92 were analyzed by TLC (Figure 5-A) and ESI-MS (Figure 5-B). The theoretical molecular mass of GlcNAc is 221.21 Da, and that of $(GlcNAc)_2$ is 424.4 Da. As chloroform was used as an electrospray reagent for ESI-MS, the theoretical molecular mass of GlcNAc and $(GlcNAc)_2$ including Cl^{-} from chloroform is 256.21 Da and 459.4 Da, respectively, which is consistent with results in Figure 5-B. Therefore, the major hydrolysis products as assessed with ESI-MS were GlcNAc and $GlcNAc_2$, with the level of $(GlcNAc)_2$ around 4-fold higher than that of GlcNAc. In accordance with the ESI-MS result, $(GlcNAc)_2$ was also assayed as the major product by TLC. However, GlcNAc was not detected, because the level of GlcNAc was below the detection limit of TLC.

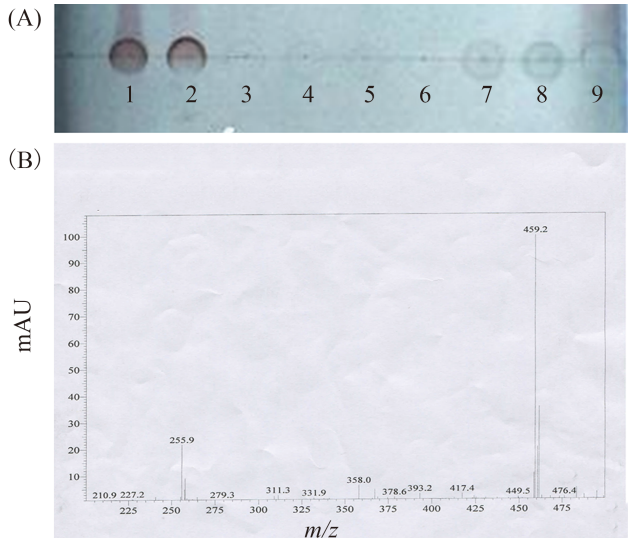

Figure 5. TLC (A) and ESI-MS (B) analysis of the hydrolysis products of colloidal chitin by Chi92. Lanes in A: 1–6, $GlcNAc_6$ to GlcNAc standards, respectively; 7, hydrolysis products of colloidal chitin by Chi92; 8, control; 9, mixture of $GlcNAc_6$ to GlcNAc standards.

2.5 Comparison the specific activities of Chi92, ChiB565, and C6137

As shown in Table 3, colloidal chitin was the best substrate for Chi92, followed by *β*-1,3-1,4-glucan, commercial shrimp shell chitin, carboxymethylcellulose, crab shell chitin, chitosan powder, crude shrimp shell chitin, and glycol chitosan. Chi92 had high specific activity for colloidal chitin (809.2 U/mg) and *β*-1,3-1,4-glucan (235.6 U/mg), which were substantially higher than those measured for ChiB565 (553.8 and 94.2 U/mg, respectively) and C6137 (620.3 and 115.6 U/mg, respectively). The specific activity of Chi92 was 293.8 U/mg for commercial shrimp shell chitin, which was much higher than that of ChiB565 (152.6 U/mg) but substantially lower than that of C6137 (454.6 U/mg). Notably, Chi92 showed low specific activity on porcine stomach mucin (149.2 U/mg), which was substantially lower than that of ChiB565 (325.1 U/mg).

Table 3. The relative substrate specificities for Chi92, Chi565 and C6137 among the various substrates tested

Substrates	Specific activity/(U/mg)		
	Chi92	ChiB565[a]	C6137[a]
Colloidal chitin	809.2±3.2	553.8±4.2*	620.3±3.5*
β-1,3-1,4-glucan	235.6±2.0	94.2±2.9*	115.6±3.7*
Commercial shrimp shell chitin	293.8±0.1	152.6±3.8	454.6±1.7
Mucin from porcine stomach	149.2±1.3	325.1±3.1	ND[b]
Carboxymethylcellulose	139.7±0.1	ND[b]	–[c]
Crab shell chitin	38.9±1.5	108.6±0.3*	ND[b]
Crude shrimp shell chitin	28.5±0.3	400.8±0.1*	ND[b]
Purified shrimp shell chitin	12.9±0.4	16.5±0.2	ND[b]
Glycol chitosan	9.7±1.4	ND[b]	–[c]

[a]C6137, the commercial exo-chitinase from Sigma-Aldrich; ChiB565, another chitinase from *A. veronii* strain B565[8]. [b]ND, not detected. [c]–, not determined. *Data determined in our previous paper[8]. The data represent the mean±S.E. of triplicate samples. All experiments were repeated three times.

2.6 Comparison of the abilities of Chi92, ChiB565, and C6137 to degrade yeast cell wall

Yeast cell wall was treated with 7 U of Chi92, ChiB565, or C6137. After 1.5 h or 3.0 h treatment, the total amount of reducing sugars increased significantly in the suspensions containing Chi92 or C6137 (Figure 6), and the production of reducing sugars by Chi92 was significantly higher than that of C6137. Notably, considerable amount of the reducing sugars released by Chi92 was glucose, with its level significantly higher after 3 h treatment of Chi92 than that of 1.5 h (Figure 7), supporting the *β*-glucanase activity of Chi92. As shown in Figure 8, the *P. pastoris* GS115 cell wall hydrolysed by Chi92 became porous. Also, the number and depth of holes increased significantly with hydrolysis time.

2.7 Effects of dietary Chi92 on growth performance of zebrafish

As shown in Table 4, dietary Chi92 supplement has no significant effect on weight gain and feed utilization of zebrafish after feeding for 14 days. Nevertheless, both the weight gain and feed

conversion ratio of the Chi92 groups tend to be improved compared with the control, suggesting marginal positive effect of the enzyme supplementation on growth performance and feed utilization of the fish.

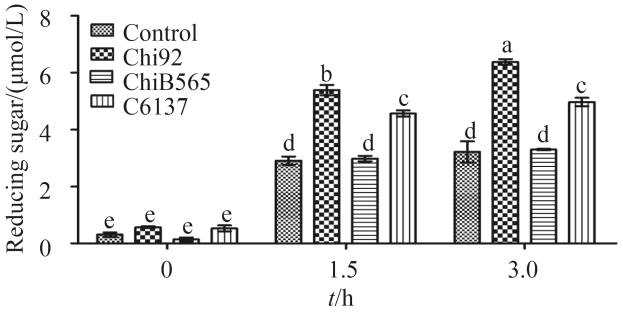

Figure 6. The ability of purified recombinant Chi92, ChiB565, or C6137 to degrade yeast cell wall. The amount of reducing sugars released increased significantly in the suspensions containing Chi92 or C6137. The production of reducing sugars by Chi92 was significantly higher than that of C6137. The data represent the mean±S.E. of triplicate samples. Experiments were repeated for three times. Data sharing the same superscript letters have no significant difference (P>0.05).

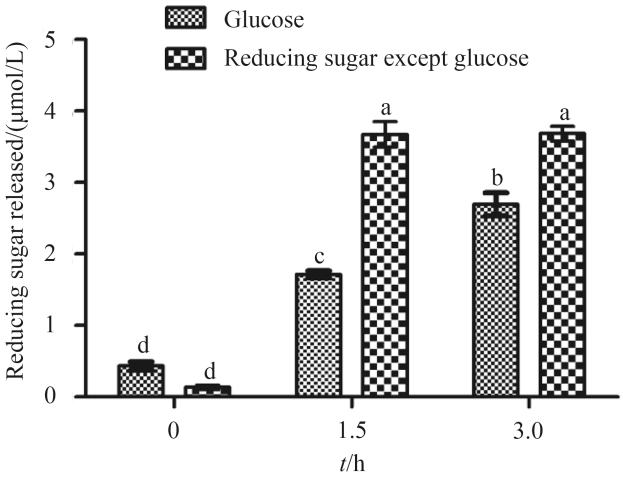

Figure 7. The hydrolysis products of yeast cell wall by Chi92. The data represent the mean±S.E. of triplicate samples. Experiments were repeated for three times. Data sharing the same superscript letters have no significant difference (P>0.05).

2.8 Microvilli morphology

Dietary Chi92 at 2.30 U/g significantly improved (P<0.05) microvilli density and length. However, dietary Chi92 at 0.23 U/g only showed positive effects (P<0.05) on microvilli density (Figure 9).

2.9 Gut alkaline phosphatase activity for assessment of the protection against *A. hydrophila* NJ-1 infection

The gut alkaline phosphatase activity was positively correlated with the numbers of *A. hydrophila* cells in the intestine after challenge. Therefore, lower gut AKP activity after challenge indicates improved resistance of the host against pathogen (Liu et al. unpublished data). As shown in Figure 10, there were no significant differences in the activity of gut alkaline phosphatase (AKP) between treatments after *A. hydrophila* NJ-1 infection. However, groups fed diets containing Chi92 at 0.23 U/g and 2.30 U/g tended to have lower gut alkaline phosphatase activity, suggesting higher level of resistance against *A. hydrophila* infection.

3 Discussion

In this study, we cloned and expressed a novel exo-chitinase gene (*chi92*) from *A. veronii* strain B565. We compared the characteristics of Chi92 with reported exo-chitinases or chitinases with both exo- and endo-chitinolytic activities. To our knowledge, Chi92 has the highest specific activity (809.2 U/mg, colloidal chitin as substrate) among known exo-chitinases. Remarkably, Chi92 showed relatively high specific activity on β-1,3-1,4-glucan, suggesting that it is a bifunctional enzyme with both chitinase and glucanase activity. Consistently, the reducing sugars released from yeast cell wall by Chi92

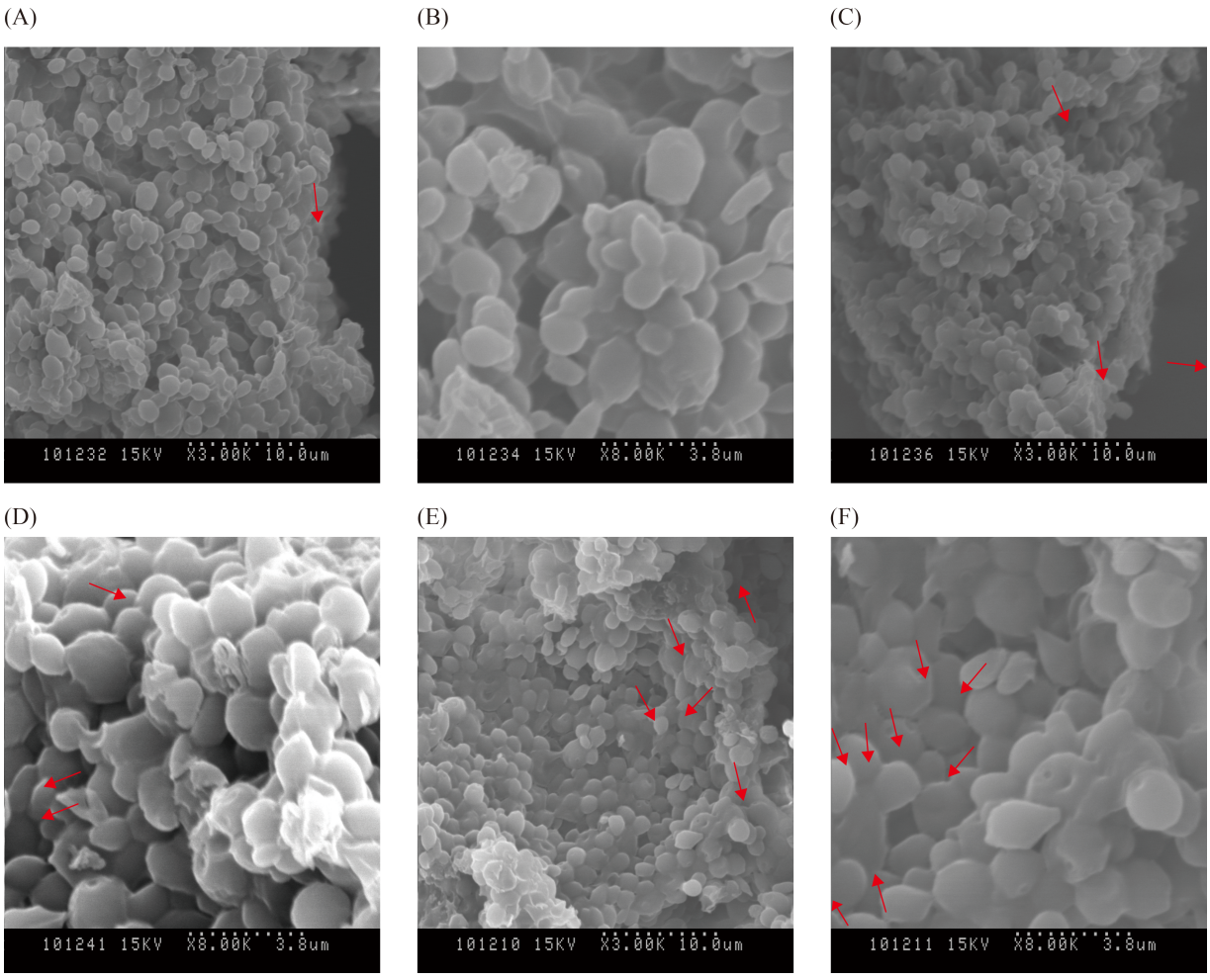

Figure 8. Scanning electron micrographs of the hydrolysis products of yeast cell wall by Chi92. A, B: Heat-inactivated *P. pastoris* GS115 powder; C, D: Heat-inactivated *P. pastoris* GS115 powder hydrolysed by Chi92 after 1.5 h; E, F: Heat-inactivated *P. pastoris* GS115 powder hydrolysed by Chi92 after 3 h. The red arrow points to the holes at the cell wall.

Table 4. The weight gain, feed utilization, survival rate of zebrafish fed different diets for 14 days

Group	Initial body weight/g	Final body weight/g	Weight gain/%	Feed conversion rate	Survival rate/%
Control	0.119±0.000	0.150±0.002	26.49±1.73	1.97±0.12	100
Chi92 (0.23U/g)	0.119±0.001	0.156±0.004	28.40±1.34	1.78±0.13	100
Chi92 (2.30U/g)	0.119±0.001	0.155±0.003	27.10±0.87	1.75±0.15	100

The data represent the mean±S.E. from five independent fish tanks.

comprised considerable amount of glucose, which were most likely the hydrolysis product of the β-linked glucans in yeast cell wall by the glucanase activity of Chi92. Compared with ChiB565 or C6137, Chi92 had a significantly greater ability to hydrolyze yeast cell wall, which is probably due to the higher specific activity of Chi92 as both chitinase and glucanase. Also, the chitinase and glucanase activity were more balanced in Chi92, as indicated by higher ratio of specific activity on β-glucan over that on

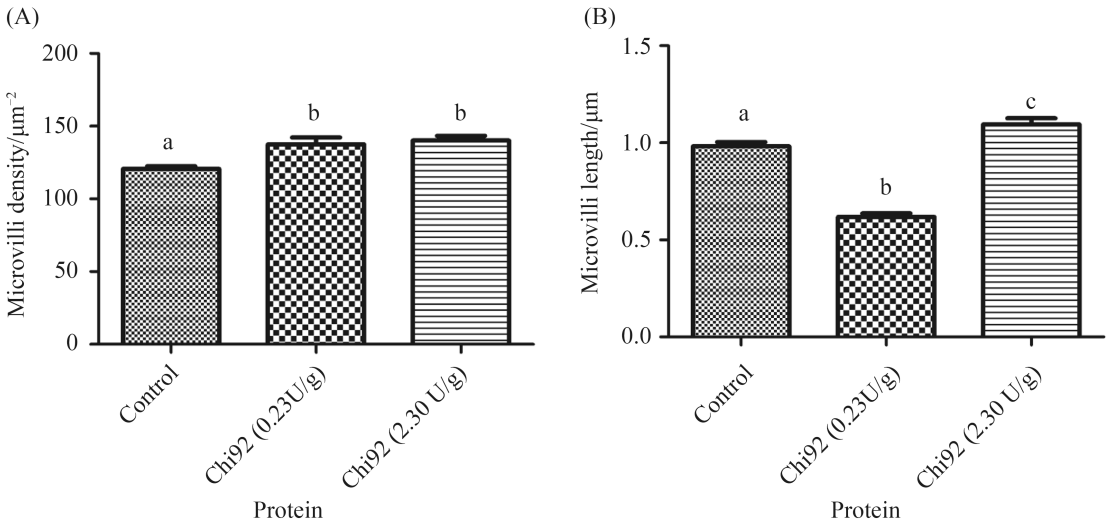

Figure 9. The microvilli density (A) and microvilli length (B) of the fish intestine after 14-day feeding trial. The data represent the mean±S.E. of triplicate samples. Data sharing the same superscript letter have no significant difference (P>0.05).

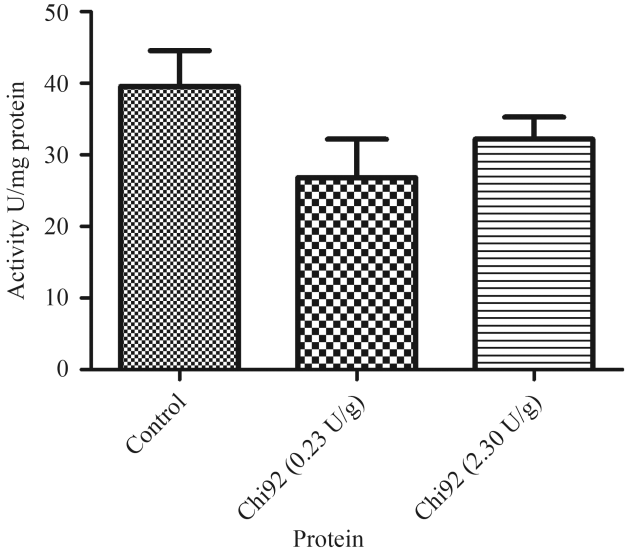

Figure 10. The activity of gut alkaline phosphatase of zebrafish after challenge with *A. hydrophila* NJ-1. The data represent the mean±S.E. of eight samples. Experiments were repeated for three times.

colloidal chitin. This property may contribute to the potential synergistic effect between the chitinase and glucanase activity of the enzyme on yeast cell wall, giving rise to a greater overall activity. Further investigation is warranted to confirm this hypothesis.

Chi92 activity had a relatively low optimal temperature (40 °C), and –50% of its maximal activity was retained when assayed at temperatures between 20 °C and 35 °C, a range generally encountered in warm water aquaculture. The stability of Chi92 at acidic pH is a useful characteristic as feed additive, as the enzyme has to pass through the acidic gastric environment of the host. Also, Chi92 was nearly completely resistant to proteases trypsin and pepsin, and partially resistant to α-chymotrypsin or tilapia intestinal juices, which means it can resist degradation by intestinal proteases *in vivo*. Moreover, Chi92 showed relatively low specific activity on mucin. As highly glycosylated large glycoproteins at mucosal surfaces[24–25], mucins are important epithelial products of the intestine, and are essential for a proper epithelial barrier function. Therefore, the low activity of Chi92 on mucin suggests that it may exert less negative influence on the intestinal protective barrier of fish. Collectively, the temperature range, pH stability, ion and protease resistance, as well as low specific activity on mucin of Chi92 support it as a candidate aquafeed additive enzyme.

Yeasts, as single cell proteins, are frequently used in aquafeed for substitution of fishmeal[26]. Also, yeast products contain some immunostimulatory ingredients, i.e., the complex carbohydrate components and nucleic acids[27]. The key factor determining the quality of the yeast products was the hydrolysis of the yeast cell wall. As Chi92 exhibited high activity to hydrolyze yeast cell wall, we expected it would be an effective dietary supplement in combination with yeasts. Hydrolysis of yeast cell wall by Chi92 may help the release of proteins, amino acids, nucleic acids as well as carbohydrates, which may stimulate the growth and immune response of fish. Also, hydrolysis product of Chi92 on yeast cell wall chitin *in vivo* should be mainly $(GlcNAc)_2$, according to the *in vitro* results. Chito-oligosaccharides (COS) have been reported to improve the growth, immune response and resistance against pathogens in various fish species[28-30]. Results of the feeding experiment with Chi92 as dietary supplement showed that Chi92 may improve the microscopic microvillus morphology in terms of length and density. Generally, improved microvilli length and density indicate more mature epithelium and enhanced absorptive function due to increased absorptive area of the villus[31], which may contribute to better nutrient digestibility and growth performance of fish. However, the improved microvillus morphology didn't translate into significantly improved growth and feed utilization of fish in this study, and only marginally improved values were achieved, suggesting that microvillus morphology is not dominating in the combination of factors determining the growth and feed utilization of fish. The gut alkaline phosphatase activity post challenge may negatively reflect the resistance level of the host against pathogen (Liu et al. unpublished data). Resistance against the infection by *A. hydrophila* was marginally improved by Chi92 supplementation, as indicated by the trend of lower post-challenge gut AKP activity in groups of fish fed Chi92 diets compared with the control. The marginal scale of improvement of both growth performance and disease resistance by Chi92 supplement may be attributed to various reasons including dosage of the enzyme, duration of feeding, as well as the low addition level of yeast products in feed, which deserves further investigation.

To our knowledge, there have been no reports on the usage of microbial chitinase as aquafeed additive for degradation of yeast products in the feed. Although no significant improvement in the growth and disease resistance of zebrafish was observed in this study, supplementation of Chi92 has the potential to increase the feed utilization efficiency and health status of fish fed diets with higher level of yeast products.

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一种具有高β-葡聚糖酶活性的几丁质外切酶基因的克隆、表达和性质鉴定

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摘要：【目的】表达并鉴定来源于维氏气单胞菌的几丁质酶Chi92并研究其作为水产饲用酶的有效性。【方法】自*A. veronii* B565中克隆*chi92*基因并在*Pichia pastoris* GS115中进行表达，对表达成功的Chi92进行分离纯化和生化鉴定。最后将Chi92添加到含有毕赤酵母粉的饲料中饲喂斑马鱼2周，研究Chi92添加对斑马鱼生长、饲料利用率、肠道微绒毛形态和抗病性能的影响。【结果】*chi92*基因编码具有864个氨基酸残基的多肽。Chi92在pH 6.0和40 °C时表现最佳酶活。Chi92对蛋白酶有抗性，同时酶活不受金属离子显著影响。Chi92具备高几丁质酶活 (69.4 U/mL)。以胶体几丁质和*β*-1,3-1,4-葡聚糖作为底物时，比活力分别为809.2 U/mg和235.6 U/mg。薄层层析和电喷雾电离质谱联用技术均表明N-乙酰葡糖胺二聚体是Chi92酶解胶体几丁质的主要产物。Chi92在对酵母细胞壁的降解方面比其他几丁质酶性能更加优良。经过2周饲喂，添加有Chi92的饲料显著提高了斑马鱼肠道微绒毛的高度和密度，同时斑马鱼的生长，饲料利用率，以及抗病性能均得到了一定提高。【结论】Chi92具有pH稳定性、抗逆性和高酵母细胞壁降解功能，能较好地作为饲用酶用于温水水产养殖。

关键词：几丁质酶，Chi92，维氏气单胞菌，斑马鱼

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