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Characterization and primary structure of chitinase from the hepatopancreas of red snow crab *Chionoecetes japonicus*

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Introduction

Chitin, a β-(1,4) linked linear polymer of *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant biomass in nature after cellulose. The production volume of chitin is estimated to range from 100 million tons to 10 billion tons a year. It is widely distributed the exoskeletons of arthropods and the cell walls of fungi in nature. Chitin oligosaccharide $(GlcNAc)_n$ and GlcNAc, which is a degradation product of chitin have various physiological functions. For example, $(GlcNAc)_n$ has an antibacterial activity and antitumor effect depending on the length of sugar chain [1,2]. GlcNAc improves skin quality and alleviates osteoarthritis [3].

Chitinase is enzymes that randomly hydrolyze the β-(1,4) glycosidic bonds of chitin and produce $(GlcNAc)_n$. Chitinase is distributed widely organisms and play a very wide range of physiological roles, such as digestion, molting, self-defense and aggression. In crustacean, chitinase play a significant role in the molting process and digestion of chitin-containing foods.

Red snow crab *Chionoecetes japonicus* is an important fishery resource and its shell is the main source of chitin [4]. However, little has been reported on chitinase of *C. japonicus*. This study describes the characterization of the *C. japonicus* chitinase and the cloning of its corresponding cDNA.

Materials and methods

Materials and chemicals

Chionoecetes japonicas was caught in Ishikawa prefecture, Japan (Weight: 560 g, Carapace width: 12 cm). *p*-nitrophenyl-*N*-acetylchitooligosaccharides (*p*NP-$(GlcNAc)_n$, n = 2, 3) was purchased from Sigma (St. Louis, USA). Hydrophobic interaction chromatography resin (TOYOPEARL Butyl-650S) was purchased from Tosoh (Tokyo, Japan). ISOGEN II and ECOS™ Competent *Escherichia coli* DH5α were purchased Nippon Gene (Tokyo, Japan). GoTaq® Green Master Mix and pGEM-T Easy Vector were purchased Promega (Wisconsin, USA).

Enzyme preparation and activities

Hepatopancreas of *C. japonicas* was homogenized with five volume of phosphate buffer (pH 7.2) and centrifuged at 12,000 ×g for 20 min. Ammonium sulfate was added to the supernatant to give 80% saturation, and the preparation was left to stand for overnight. Precipitate was then collected by centrifuging at 12,000 ×g for 20 min and dialyzed in 20 mM sodium phosphate buffer (pH 7.2) containing 1 M ammonium sulfate. This solution was applied to TOYOPEARL Butyl-650S column (1.6 cm×16 cm) previously equilibrated with 20 mM sodium phosphate buffer (pH 7.2) containing 1 M ammonium sulfate and the non-adsorbed fractions were eluted with the same buffer. Adsorbed fractions were eluted with a gradient 20 mM phosphate buffer (pH 7.2) containing 1 M ammonium sulfate and 20 mM phosphate buffer (pH 7.2).

Chitinase activity was measured by the method of Ohtakara [5] using *p*NP-$(GlcNAc)_n$ (n = 2, 3) as a substrate. 3 µl of enzyme solution and 10 µl of 4 mM *p*NP-$(GlcNAc)_n$ were added to 10 µl of 0.2 M sodium phosphate-0.1 M citric acid buffer and incubating at 37°C for 20 min. After incubation, 65 µl of 0.2 M sodium carbonate was added, and the absorbency of the released *p*-nitrophenol was measured at 420 nm. Chitinase activity depending on pH was measured incubating at 37°C for 20 min, using a 0.2 M sodium phosphate-0.1 M citric acid buffer (pH 2.5 - 8.0) as a method of measuring enzyme activity.

cDNA cloning

Total RNA (tRNA) was extracted from 0.8 g of hepatopancreas of *C. japonicas* using ISOGEN II, isopropanol and ethanol according to the manufacturer's instructions. The extracted tRNA was stored at -20°C. The first-strand cDNA was synthesized

using tRNA and oligo dT primers with M-MLV Reverse Transcriptase (RNase H free) according to the manufacturer's instructions. Degenerate primers were designed from several arthropod GH family 18 chitinase conserved amino acid sequences. The first PCR was carried out with this cDNA from *C. japonicas* as a template and degenerate primers. Nested PCR was carried out with 10-fold dilution PCR product of the first PCR as the templates and degenerate primers. The Nested PCR product was transformed using the pGEM-T Easy Cloning Kit, and cDNA was sequenced using ECOS™ Competent *E. coli* DH5α according to the manufacturer's instructions. The similarity of the nucleotide sequence to other species of chitinase was determined using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

Chionoecetes japonicas chitinase (CjChi) was fractionated by TOYOPEARL Butyl-650S. The obtained chitinase activity showed pH dependent manner (Fig. 1). The optimal pH of CjChi against *p*NP-$(GlcNAc)_2$ was pH 5.5 and 42% of maximal activity was shown at pH 7.0. Reported crustacea chitinase, *Penaeus monodon* chitinase purified from hepatopancreas [6], *Marsupenaeus japonicus* chitinase purified from hepatopancreas [7], *Homarus americanus* chitinase purified from gastric juice [8], *Exopalaemon carinicauda* chitinase purified from hepatopancreas [9] had an optimal activity at pH 5.0, pH 6.2, pH 4.0 - 6.0 and pH 4.0, respectively, and were adapted to an acidic environment like CjChi. On the other hand, chitinase activity against *p*NP-$(GlcNAc)_3$ was not detected. Namely, CjChi has strong substrate selectivity not to *p*NP-$(GlcNAc)_3$ but to *p*NP-$(GlcNAc)_2$.

About 500 bp nucleotide sequence was obtained and determined as internal sequence of *CjChi*. The deduced amino acid sequence of *CjChi* was compared with those of other families 18 and 19 chitinases using BLAST software. BLAST analysis showed that CjChi had 73% identity with chitinase from *Portunus trituberculatus* of hepatopancreas and 68% identity with chitinase from *Eriocheir sinensis* of hepatopancreas. Chitinase from *E. sinensis* has been indicated that might play a role in the digestion of chitin-containing food [10]. Therefore, this analysis suggested that CjChi is likewise a chitinase involved in digestion.

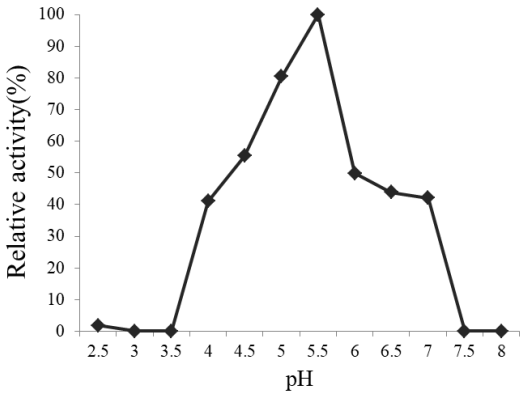

Fig. 1. Effects of pH on chitinolytic activity of CjChi when using *p*NP-$(GlcNAc)_2$.

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