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Purification, characterization, and molecular cloning of chitinases from the stomach of the threeline grunt *Parapristipoma trilineatum*

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ABSTRACT

Two chitinase isozymes, PtChiA and PtChiB, were purified from the stomach of the threeline grunt, *Parapristipoma trilineatum*. The molecular masses of PtChiA and PtChiB were estimated to be 50 and 60 kDa by SDS-PAGE, respectively. Both chitinases were stable at pH 3.0–6.0 (acidic) and showed the optimum pH toward both short and long substrates in the acidic region (pH 2.5–5.0). PtChiA and PtChiB preferentially degraded the second and third glycosidic bonds from the non-reducing end of *N*-acetylchitooligosaccharides, respectively. PtChiA and PtChiB exhibited wide substrate specificities toward crystalline chitin. Moreover, 2 cDNAs encoding PtChiA and PtChiB, *PtChi-1* and *PtChi-2*, respectively, were cloned. The deduced amino acid sequences of both chitinase cDNAs comprised N-terminal signal peptides, glycoside hydrolase 18 catalytic domains, linker regions, and C-terminal chitin-binding domains. Phylogenetic tree analysis of vertebrate chitinases revealed that fish stomach chitinases including *PtChi-1* and *PtChi-2* form unique chitinase groups, acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2), which differ from the acidic mammalian chitinase (AMCase) group. The present results suggest that fish have a chitin-degrading enzymatic system in which 2 different chitinases, AFCase-1 and AFCase-2, with different degradation patterns are expressed in the stomach.

1. Introduction

Chitin, a β-1,4-linked polysaccharide of *N*-acetyl-D-glucosamine (GlcNAc), is widely found in the exoskeletons of arthropods such as crustaceans and insects as well as the cell walls of fungi; it is the second most abundant biomass in nature after cellulose [1,2]. Since crab and shrimp shell chitin has a rigid α-crystal structure [3] and is insoluble in water and organic solvents, these have not been found to be useful to most processes [4]. Therefore, chitin is mainly used as chitosan and glucosamine after deacetylation [2,5]. On the other hand, chitooligosaccharides $(GlcNAc)_n$ and GlcNAc, which are the degradation products of chitin, have various physiological functions. For example, $(GlcNAc)_n$ has immunostimulatory activity [4,6], and GlcNAc improves skin quality and alleviates osteoarthritis [7,8].

Chitinases (EC 3.2.1.14) are enzymes that randomly hydrolyze the β-1,4 glycosidic bonds of chitin and are necessary for the enzymatic production of physiologically active $(GlcNAc)_n$ and GlcNAc. They are widely distributed in various living organisms and have been isolated and characterized from various sources [9–12]. Chitinases play roles in various biological processes including ecdysis in crustaceans, defense in plants, digestion and aggression in mollusks, and digestion and defense in vertebrates [13,14].

On the basis of the homology of the amino acid sequence of their active domain [15] and catalytic mechanisms [16,17], chitinases are classified into 2 glycoside hydrolase (GH) families: 18 and 19. GH family 18 chitinases are widely distributed among organisms including microbes, animals, and plants [15]. On the other hand, GH family 19 chitinases are mainly found in higher-order plants [18]. Only GH family 18 chitinases have been detected in vertebrates [19,20].

Fish chitinases are reported to have high activity in the stomach [21,22]. Such fish chitinases have been purified from the stomachs of several fish species, and their properties have been characterized [11,23–26]. Moreover, fish stomach chitinases work well in the acidic pH range [11,23,25,26], and the numbers of their isozymes as well as substrate specificities differ by fish species [23,25,26]. Furthermore, chitinase isozymes that have high degradation activity against crab and shrimp shell α-chitin have been purified from the stomachs of greenling (*Hexagrammos otakii*), common mackerel (*Scomber japonicus*) [23], and silver croaker (*Pennahia argentatus*) [26]; they have the potential to be useful in the enzymatic production of $(GlcNAc)_n$ and GlcNAc.

Abbreviations: $(GlcNAc)_n$, *N*-acetylchitooligosaccharides; GlcNAc, *N*-acetyl-D-glucosamine; pNp-$(GlcNAc)_n$, *p*-nitrophenyl *N*-acetylchitooligosaccharides; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; 5′ AAP, 5′ RACE abridged anchor primer; AUAP, abridged universal amplification primer.

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Studies of vertebrate chitinase genes indicate the presence of chitotriosidase in mammalian macrophages [27–29] and acidic mammalian chitinase (AMCase) in mammalian stomachs [30–32]. In addition, chitinase genes have been isolated from the glandular stomach of chickens [31] and the stomach of the toad, *Bufo japonicus* [33].

A study on fish chitinase genes detected 2 chitinase genes, *fChi1* and *fChi2*, in the stomach of Japanese flounder (*Paralichthys olivaceus*), and 1 chitinase gene, *fChi3*, in multiple organs including the pancreas; these 3 genes were cloned [34]. In addition, phylogenetic tree analysis in that study revealed that *fChi1* and *fChi2* belong to an acidic chitinase group together with chitinases from human and chicken stomachs [34].

Although the properties and functions of fish stomach chitinases have gradually been elucidated, there is little information concerning the genes encoding them. We previously reported the purification and characterization of 2 chitinase isozymes, PaChiA and PaChiB, from the stomach of *P. argentatus* [25,26], which belongs to the order Perciformes. In this study, we cloned the cDNAs encoding chitinases from the stomach of the threeline grunt, *Parapristipoma trilineatum*, which feeds on crustaceans and also belongs to the order Perciformes, and determine the primary structures of the obtained cDNA. Tissue expression and phylogenetic tree analyses of the chitinase genes were also performed.

Furthermore, we also aimed to purify 2 chitinase isozymes from the stomach of *P. trilineatum* and characterize their properties. In particular, we investigated the enzymatic reaction characteristics in detail to evaluate the differences of the functions of 2 chitinase isozymes present in the stomach of *P. trilineatum*.

2. Materials and methods

2.1. Chemicals

Glycol chitin, *p*-nitrophenyl *N*-acetylchitooligosaccharides [pNp-$(GlcNAc)_n$, $n = 1–4$] and *N*-acetylchitooligosaccharides [$(GlcNAc)_n$, $n = 2–6$] were purchased from Seikagaku (Tokyo). Crab shell chitin (α-chitin) was from Tokyo Chemical Industry (Tokyo). Shrimp shell chitin (α-chitin, Chitin EX) was from Funakoshi (Tokyo). Silkworm cuticle chitin (α-chitin) was a generous gift from Dr. Atsunobu Haga, and squid pen chitin (β-chitin) was kindly provided by Kyowa Technos (Chiba, Japan). Chitin nanofiber was a generous gift from Dr. Shinsuke Ifuku. Colloidal chitin was prepared according to the method of Shimahara and Takiguchi [35].

2.2. Enzymes

Chitinase isozymes (PtChiA and PtChiB) were purified from the stomach (stomach weight: 25 g) of *P. trilineatum* by ammonium sulfate fractionation (0–70% saturation) and column chromatography using Chitopearl Basic BL-01 and CM-Toyopearl 650S according to the method of Ikeda et al. [25,26].

2.3. Chitinase activity assay

Chitinase activity was assayed using various substrates. When pNp-$(GlcNAc)_n$ ($n = 1–4$) was used as substrate, enzyme activity was assayed according to the method of Ohtakara [36]. Briefly, 12.5 μL enzyme solution and 5 μL 4 mM pNp-$(GlcNAc)_n$ were added to 12.5 μL 0.2 M phosphate–0.1 M citrate buffer (pH 4.5) and incubated for 20 min at 37 °C. After incubation, 130 μL 0.2 M sodium carbonate solution was added, and the absorbance of the *p*-nitrophenol released was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol *p*-nitrophenol per min at 37 °C.

When 0.5% colloidal chitin, α- or β-chitin, or chitin nanofiber was used, enzyme activity was assayed according to the method of Ohtakara [36]. Briefly, 250 μL enzyme solution and 250 μL substrate solution (0.5%) were added to 500 μL 0.2 M phosphate–0.1 M citrate buffer (pH 4.5), and the mixture was incubated for 2 h at 37 °C with shaking. After incubation, the reaction was stopped by 3 min boiling. The reaction solution was centrifuged at 14,000 × *g* for 5 min, and 375 μL supernatant was sampled. To measure the amount of reducing sugar produced by the enzymatic reaction, 500 μL Schales' reagent was added to the collected solution and the absorbance was measured at 420 nm. The solution was then boiled for 15 min and cooled in running water. Then, the absorbance was re-measured at 420 nm. When glycol chitin was used as substrate, enzyme activity was assayed according to the method of Imoto and Yagishita [37]. In brief, 100 μL 0.1 M sodium acetate buffer (pH 4.0), 50 μL enzyme solution, and 100 μL 0.05% glycol chitin were incubated for 2 h at 37 °C, and the amount of reducing sugar produced was measured using Schales' reagent. The standard curve was prepared using authentic GlcNAc, and the absorbance was then converted into the amount of GlcNAc. One unit of enzyme activity was defined as the amount of enzyme required to degrade substrates at 37 °C and produce reducing sugars corresponding to 1 μmol GlcNAc per min.

2.4. HPLC analysis of the hydrolysis products of $(GlcNAc)_n$ ($n = 2–6$) by PtChiA and PtChiB

The hydrolysis products of $(GlcNAc)_n$ ($n = 2–6$) produced by PtChiA and PtChiB and their anomer formation ratios were analyzed according to the method of Koga et al. [17]. Briefly, 5 μL enzyme solution and 25 μL 0.22 mM $(GlcNAc)_n$ were added to 25 μL 0.1 M sodium acetate buffer (pH 4.0), and the mixture was incubated for 10 min at 25 °C. The reaction was stopped by cooling to 0 °C in an ice bath. The reaction solution was analyzed at 25 °C by HPLC using a TSK-GEL Amide-80 column (Ø4.6 mm × 250 mm, Tosoh, Tokyo). $(GlcNAc)_n$ was eluted with 70% acetonitrile solution at a flow rate of 0.8 mL/min, and the absorbance was measured at 210 nm.

2.5. Effect of pH and temperature chitinase activity

When pNp-$(GlcNAc)_2$ for PtChiA and pNp-$(GlcNAc)_3$ for PtChiB were used as a substrate, the optimum pH was determined by assaying enzyme activity. Specifically, the solution was incubated for 20 min at 37 °C in 0.2 M phosphate–0.1 M citrate buffer (pH 2.0–8.0). When chitin nanofiber was used as a substrate, the optimum pH was measured by assaying enzyme activity. The solution was incubated for 2 h at 37 °C in 0.2 M phosphate–0.1 M citrate buffer (pH 2.0–8.0). To measure the pH stability of the enzyme, the enzyme solution was heated for 10 min at 60 °C at pH 2.0–8.0 (0.2 M phosphate–0.1 M citrate buffer), and the residual enzyme activity was then measured by assaying enzyme activity using pNp-$(GlcNAc)_3$ as a substrate.

The optimum temperature was determined by assaying enzyme activity using pNp-$(GlcNAc)_2$ for PtChiA and pNp-$(GlcNAc)_3$ for PtChiB as a substrate and incubating the reaction solution for 10 min at 10–90 °C. For temperature stability, the enzyme solution was incubated at pH 4.0 (0.2 M phosphate −0.1 M citrate buffer) at 10–90 °C for 10 min, and the residual enzyme activity was measured using pNp-$(GlcNAc)_2$ for PtChiA and pNp-$(GlcNAc)_3$ for PtChiB as a substrate, as a method of measuring enzyme activity.

2.6. Protein measurement

Protein concentrations were measured according to the method of Bradford using bovine serum albumin as a standard [38].