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Purification and characterization of chitinase from the stomach of silver croaker *Pennahia argentatus*

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ABSTRACT

A chitinase was purified from the stomach of a fish, the silver croaker *Pennahia argentatus*, by ammonium sulfate fractionation and column chromatography using Chitopearl Basic BL-03, CM-Toyopearl 650S, and Butyl-Toyopearl 650S. The molecular mass and isoelectric point were estimated at 42 kDa and 6.7, respectively. The N-terminal amino acid sequence showed a high level of homology with family 18 chitinases. The optimum pH of silver croaker chitinase toward *p*-nitrophenyl *N*-acetylchitobioside (pNp-$(GlcNAc)_2$) and colloidal chitin were observed to be pH 2.5 and 4.0, respectively, while chitinase activity increased about 1.5- to 3-fold with the presence of NaCl. *N*-Acetylchitooligosaccharide ($(GlcNAc)_n$, $n = 2–6$) hydrolysis products and their anomer formation ratios were analyzed by HPLC using a TSK-GEL Amide-80 column. Since the silver croaker chitinase hydrolyzed $(GlcNAc)_{4–6}$ and produced $(GlcNAc)_{2–4}$, it was judged to be an endo-type chitinase. Meanwhile, an increase in β-anomers was recognized in the hydrolysis products, the same as with family 18 chitinases. This enzyme hydrolyzed $(GlcNAc)_5$ to produce $(GlcNAc)_2$ (79.2%) and $(GlcNAc)_3$ (20.8%). Chitinase activity towards various substrates in the order pNp-$(GlcNAc)_n$ ($n = 2–4$) was pNp-$(GlcNAc)_2$ >> pNp-$(GlcNAc)_4$ > pNp-$(GlcNAc)_3$. From these results, silver croaker chitinase was judged to be an enzyme that preferentially hydrolyzes the 2nd glycosidic link from the non-reducing end of $(GlcNAc)_n$. The chitinase also showed wide substrate specificity for degrading α-chitin of shrimp and crab shell and β-chitin of squid pen. This coincides well with the feeding habit of the silver croaker, which feeds mainly on these animals.

Chitin is a polysaccharide formed from β-1,4 links of *N*-acetyl-D-glucosamine (GlcNAc).[1] It is widely distributed in organisms, mainly in structural components such as arthropod exoskeletons, mollusk shells, and fungal cell walls. It is the second most abundant biomass next to cellulose [1,2]. Most of the chitin that exists in the natural world has an α- or β-chitin crystalline structure [3].

Chitinases (EC 3.2.1.14) are enzymes that randomly hydrolyze the β-1,4 glycosidic bonds of chitin and produce *N*-acetylchitooligosaccharides $(GlcNAc)_n$. Chitinases are widely distributed in nature and serve important biological functions in activities such as nutrient intake, morphological change, defense, and attack [4]. Chitinases found in the stomachs of fish [5–8] and the livers of squid [9,10] degrade chitinous substances ingested as food, whereas the chitinases present in insects and shellfish [11–13] serve to degrade chitinous substances in the exoskeleton during ecdysis. In plants, on the other hand, chitinases act as proteins for self-defense against fungal pathogens that contain chitinous substances [14–16]. Finally, chitinases are essential enzymes for the enzymatic production of $(GlcNAc)_n$ and GlcNAc, which are gradually proving to have a variety of physiological roles [17,18]. Consequently, research on chitinases in various organisms will not only clarify these physiological roles but will also be of use in the production of $(GlcNAc)_n$ and GlcNAc.

Chitinases are classified into two families of glycosyl hydrolases families 18 and 19, on the basis of the homology of their amino acid sequences [19] and their catalytic mechanisms [20,21]. Family 18 chitinases are widely distributed among microbes, animals, plants, and other organisms [19]. Family 19 chitinases, on the other hand, exist mainly in higher-order plants and are reported to have strong antibacterial properties [22].

Fish stomach chitinases have the physiological function of degrading chitinous substances ingested as food. They have been purified from the stomachs of several fish, and their properties have been clarified [7,8,23–25]. These reports suggest that the number of isozymes and substrate specificity of fish stomach chitinases differ depending on species of fish, in connection with their feeding habits [7]. The stomach of the greenling, for example, has been reported to contain chitinase isozymes with high

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[1] *Abbreviations used:* $(GlcNAc)_n$, *N*-acetylchitooligosaccharide; GlcNAc, *N*-acetyl-D-glucosamine; pNp-$(GlcNAc)_n$, *p*-nitrophenyl *N*-acetylchitooligosaccharides; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; IEF-PAGE, isoelectric focusing polyacrylamide electrophoresis.

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hydrolyzing activity toward only one of α-chitin or β-chitin, whereas that of the common mackerel has a chitinase with hydrolyzing activity toward both α-chitin and β-chitin [7].

In this study, therefore, the aim was to purify chitinase from the stomach of the silver croaker *Pennahia argentatus*, a hitherto unresearched demersal fish of the Percichthyidae family that feeds on shrimps, crab, and squid, and to characterize its various properties. By analyzing the N-terminal amino acid sequence of the purified chitinase and the anomer formation ratios of its $(GlcNAc)_n$ (n = 2–6) hydrolysis products, an attempt was made to identify the glycosyl hydrolases family of the silver croaker chitinase. Besides this, the aim was to clarify the enzymatic properties in order to study its potential for using silver croaker chitinase as a $(GlcNAc)_n$ producing enzyme. Silver croaker fish are caught in large numbers as a raw material for *kamaboko* and other fish paste products, but their organs are usually discarded. This study could also serve as basic research for the effective utilization of fishery processing waste.

Materials and methods

Materials

Silver croaker (*P. argentatus*) was purchased from the Tsukiji Market in Tokyo, Japan (number: 29, average body weight: 219 g, average stomach weight: 1.39 g). Glycol chitin, *p*-nitrophenyl *N*-acetylchitooligosaccharides (pNp-$(GlcNAc)_n$, n = 1–4), and *N*-acetylchitooligosaccharides ($(GlcNAc)_n$, n = 2–6) were purchased from Seikagaku Corp. (Tokyo). Crab shell chitin (α-chitin) was purchased from Tokyo Kasei (Tokyo). Shrimp shell chitin (α-chitin, Chitin EX) was purchased from Funakoshi (Tokyo). Silkworm cuticle chitin (α-chitin) was generously supplied by Dr. A. Haga. Squid pen chitin (β-chitin) was generously supplied by Kyowa Technos Co., Ltd. Microalgae chitin (β-chitin) was generously supplied by Dr. K.J. Kramer. Colloidal chitin was prepared by the method of Shimahara and Takiguchi [26]. Microbe *Streptomyces griseus* chitinase was purchased from Sigma-Aldrich (St. Louis, Mo, USA).

Purification of silver croaker chitinase

Unless otherwise noted, all processes were carried out at temperatures of 0–4 °C. Stomachs from fresh silver croakers were cut open, the contents removed, and the inner walls washed with chilled distilled water. The stomachs were homogenized with five volumes of 50 mM sodium acetate buffer (pH 5.5) and centrifuged at 13,000*g* for 20 min. Ammonium sulfate was added to the supernatant to give 70% saturation, and the preparation was left to stand for 2 h. Precipitate was then collected by centrifuging at 14,000*g* for 20 min, and dialyzed in 20 mM sodium phosphate buffer (pH 7.2). The dialyzed solution was centrifuged at 13,000*g* for 20 min, after which NaCl was added to bring the concentration to 1 M. This solution was applied to a Chitopearl Basic BL-03 column (1.6 cm × 30 cm) previously equilibrated with 20 mM sodium phosphate buffer (pH 7.2) containing 1 M NaCl, and the non-adsorbed fractions were eluted with the same buffer. Adsorbed fractions were eluted with 0.1 M acetic acid. The active fractions were collected and dialyzed with 20 mM sodium acetate buffer (pH 4.5), then applied to a CM-Toyopearl 650S column (1.6 cm × 30 cm) previously equilibrated with the same buffer. Enzymes were eluted with a linear gradient of NaCl from 0 to 0.7 M in the same buffer. The active fractions were collected and dialyzed with 20 mM sodium acetate buffer (pH 4.5), then ammonium sulfate was applied to the enzyme solution to bring the concentration to 0.8 M. This enzyme solution was applied to a Butyl-Toyopearl 650S column (1.6 cm × 30 cm) previously equilibrated with 20 mM sodium acetate buffer (pH 4.5) containing 0.8 M ammonium sulfate. Chitinases were eluted with a linear gradient of ammonium sulfate from 0.8 to 0 M in the same buffer, and the active fractions were collected and stored at −80 °C.

Assay of chitinase activity

Chitinase activity was measured using various substrates. First, pNp-$(GlcNAc)_2$ was used as a substrate to measure enzyme activity during chitinase purification. When using pNp-$(GlcNAc)_n$ (n = 1–4) as a substrate, enzyme activity was measured by the method of Ohtakara [27]. Namely, 25 μl of enzyme solution and 10 μl of 4 mM pNp-$(GlcNAc)_n$ were added to 25 μl of 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 4.5), and then incubated at 37 °C for 10 min. After incubation, 100 μl of 0.2 M sodium carbonate was added, and the absorbency of the released *p*-nitrophenol was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min at 37 °C. When using 0.5% colloidal chitin or, α- or β-chitin, enzyme activity was measured by the method of Ohtakara [27]. Namely, 250 μl of enzyme solution and 250 μl of each substrate (0.5%) were added to 500 μl of 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 4.5), and the mixture was incubated while being shaken at 37 °C for 2 h. After the incubation, the reaction was stopped by heating the mixture with boiling water for 3 min. The reactant solution was centrifuged, and 375 μl of the supernatant was sampled. To measure the amount of reducing sugar produced by the enzyme reaction, 500 μl of Schales's reagent was added to the reactant solution and the absorbency was measured at 420 nm. After this, the reactant solution was heated in boiling water for 15 min, and after cooling in flowing water its, absorbency was again measured at 420 nm. When glycol chitin was used as the substrate, enzyme activity was measured by the method of Imoto and Yagishita [28]. Namely, 100 μl of 0.1 M sodium acetate buffer (pH 4.0), 50 μl of enzyme solution, and 100 μl of 0.05% glycol chitin were incubated at 37 °C for 2 h, and the amount of reducing sugar produced as a result was measured by Schales's reagent. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of GlcNAc per min at 37 °C.

HPLC analysis of the hydrolysis products of $(GlcNAc)_n$ (n = 2–6) by chitinase

The hydrolysis products of $(GlcNAc)_n$ (n = 2–6) produced by silver croaker chitinase and their anomer formation ratios were analyzed by the method of Koga et al. [21]. Namely, 5 μl of enzyme solution and 25 μl of 0.22 mM $(GlcNAc)_n$ were added to 25 μl of 0.1 M sodium acetate buffer (pH 4.0) and incubated at 25 °C for 10 min. The reaction was stopped by cooling to 0 °C in an ice bath, and the reactant solution was analyzed by HPLC using a TSK-GEL Amide-80 column (4.6 mm × 250 mm). $(GlcNAc)_n$ was eluted with 70% acetonitrile at a flow rate of 0.8 ml/min, and absorbency was detected at 210 nm.

Characterization of purified chitinase

The optimum pH when using pNp-$(GlcNAc)_2$ and pNp-$(GlcNAc)_3$ as substrates was measured by incubating at 37 °C for 10 min, using a 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 2.0–8.0) as a method of measuring enzyme activity. The optimum pH when using colloidal chitin and glycol chitin as substrates was measured by incubating at 37 °C for 2 h, using a 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 2.0–8.0) as a method of measuring enzyme activity. For pH stability, the enzyme solution was incubated at pH 2.0–8.0 (0.2 M sodium phosphate–0.1 M citric acid buffer) at 60 °C for 10 min, and the remaining enzyme activity

was measured using pNp-$(GlcNAc)_2$ as a substrate, as a method of measuring enzyme activity. Optimum temperature was measured by incubating at 10–80 °C for 10 min, using pNp-$(GlcNAc)_2$ as a substrate, as a method of measuring enzyme activity. For temperature stability, the enzyme solution was incubated at pH 4.0 (0.2 M sodium phosphate–0.1 M citric acid buffer) at 10–90 °C for 10 min, and the remaining enzyme activity was measured using pNp-$(GlcNAc)_2$ as a substrate, as a method of measuring enzyme activity. For the effect of NaCl on chitinase activity, activity was measured by using pNp-$(GlcNAc)_2$, colloidal chitin, crab shell α-chitin, shrimp shell α-chitin, and squid pen β-chitin as substrates and NaCl was added to the reactant solution to a final concentration of 0–1 M as a method of measuring enzyme activity. For comparison, the effect of NaCl was similarly measured by using the chitinase of *S. griseus*.

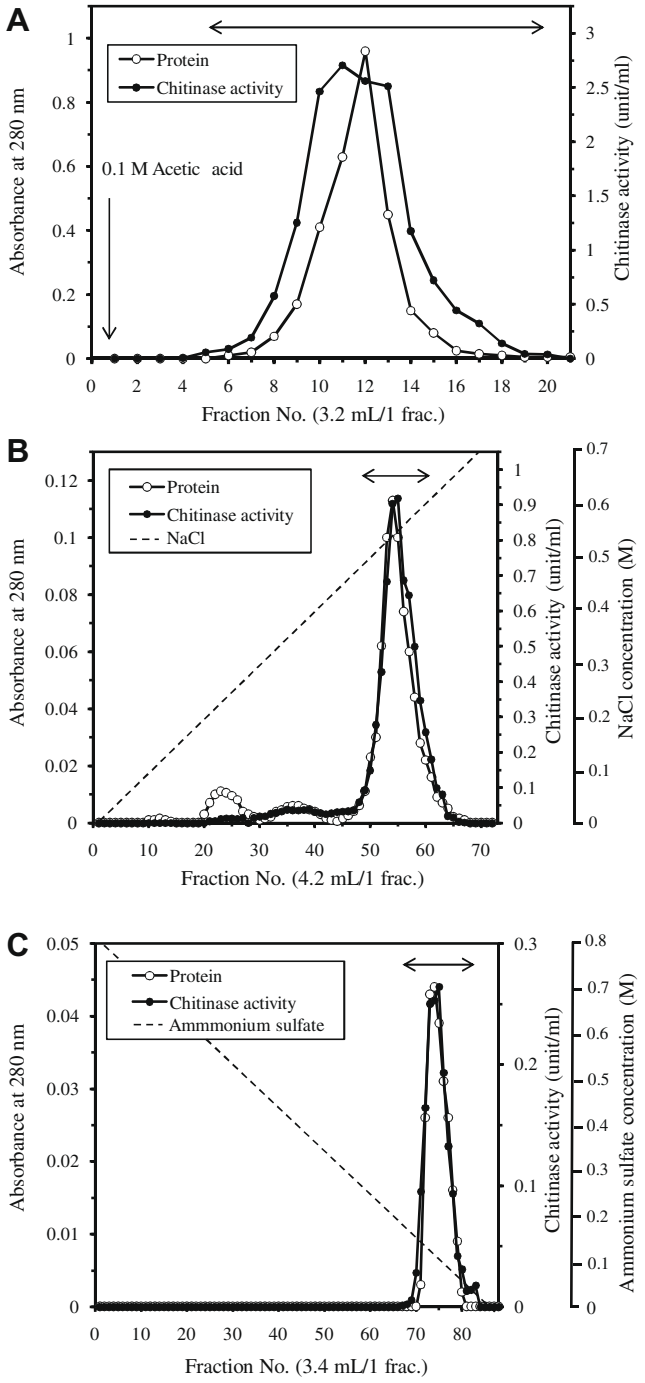

Fig. 1. Purification of silver croaker chitinase. (A) Sample solution was applied to a Chitopearl Basic BL-03 column previously equilibrated with 20 mM sodium phosphate buffer solution (pH 7.2) containing 1 M NaCl, and the non-absorbed fractions were eluted with the same buffer. Absorbed fractions were eluted with 0.1 M acetic acid. (B) The active fractions were collected and applied to a CM-Toyopearl 650S column previously equilibrated with 20 mM sodium acetate buffer (pH 4.5). Enzymes were eluted with a linear gradient of NaCl from 0 to 0.7 M in the same buffer. (C) The active fractions were collected and applied to a Butyl-Toyopearl 650S column previously equilibrated with 20 mM sodium acetate buffer (pH 4.5) containing 0.8 M ammonium sulfate. Chitinase was eluted with a linear gradient of ammonium sulfate from 0.8 to 0 M in the same buffer.

Protein measurement

Protein concentration was measured by the method of Bradford, using bovine serum album as the standard protein [29].

Gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) was carried out in 12.5% polyacrylamide gel (e-PAGEL, ATTO, Tokyo) following Laemmli [30]. The samples were mixed with Ez Apply (ATTO, Tokyo) and heated for 5 min. Gel proteins were stained with Coomassie Brilliant Blue R-250. Isoelectric focusing polyacrylamide electrophoresis (IEF-PAGE) was conducted using pI 3.0–9.0 thin-layer polyacrylamide gel (Phast Gel, GE Healthcare Bioscience, Uppsala, Sweden).

N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was analyzed by using a protein sequencer (PE Applied Biosystems 447/120A, Foster City, CA).

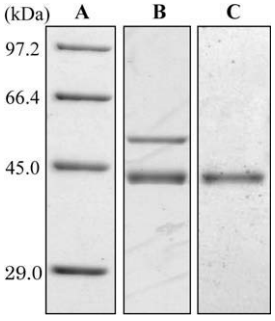

Fig. 2. SDS-PAGE of silver croaker chitinase. (A) Marker proteins used were phosphorylase b (97.2 kDa), serum albumin (66.4 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (29.0 kDa). (B) Chitinase active fractions obtained by using CM-Toyopearl 650S column chromatography. (C) Chitinase active fractions obtained by using Butyl-Toyopearl 650S column chromatography. The amount of protein applied in C was 0.4 μg.

Table 1
Molecular masses and isoelectric points of chitinase purified from silver croaker and other fish stomach.

Chitinase	Molecular mass (kDa)	Isoelectric point
Pennahia argentatus	42	6.7
Hexagrammos otakii (HoChiA)	62	5.7
Hexagrammos otakii (HoChiB)	51	7.6
Hexagrammos otakii (HoChiC)	47	8.8
Pagrus major	46	8.3
Anguilla japonica	50	6.2
Scomber japonicus	38	7.6

The other fish stomach chitinases and their reference nos. are: *Hexagrammos otakii* [7], *Pagrus major* [23], *Anguilla japonica* [24], *Scomber japonicus* [25].

Results

Purification of silver croaker chitinase

As stated in Materials and methods above, enzyme solution was added to a Chitopearl Basic BL-03 column after ammonium sulfate fractionation. Chitinases adsorbed to a chitin affinity column were eluted with 0.1 M acetic acid (Fig. 1A). Next, these chitinase active fractions were further purified using a CM-Toyopearl 650S ion exchange column (Fig. 1B). When the adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.7 M in the same buffer, the chitinase was eluted at around 0.55 M of NaCl. The purity of the chitinase was studied by using SDS–PAGE. As shown in Fig. 2B, the chitinase active fractions obtained from CM-Toyopearl 650S column chromatography contained two types of protein (molecular mass 50 and 42 kDa) eluted at the same NaCl concentration. Thus, these active fractions were further applied to Butyl-Toyopearl 650S hydrophobic column chromatography, and the adsorbed proteins were eluted with a linear gradient of ammonium sulfate from 0.8 to 0 M in the same buffer (Fig. 1C). The peak at which chitinase activity and elution of proteins coincided was obtained at an ammonium sulfate concentration of around 0.1 M. On SDS–PAGE, the purified chitinase showed a single band, and the molecular mass was estimated to be 42 kDa (Fig. 2C). The molecular mass of 42 kDa for this enzyme lay between the 46 kDa for red sea bream [23] and the 38 kDa for common mackerel [25] (Table 1). The isoelectric point of this enzyme was estimated by IEF-PAGE to be 6.7. It has been reported that the isoelectric point of fish stomach chitinases is 5.7–8.8 (Table 1), and the pI 6.7 of this enzyme was within that range. Table 2 shows the total activity, total proteins, specific activity, and other data obtained during the purification of this chitinase. The specific activity of CM-Toyopearl 650S eluted fractions containing the two types of protein was 6.14 U/mg, but the specific activity of chitinase purified using the Butyl-Toyopearl 650S decreased to 3.33 U/mg. These results suggested that the CM-Toyopearl 650S active fractions contained both the purified enzyme (42 kDa) and a chitinase (50 kDa) with higher specific activity toward pNp-$(GlcNAc)_2$ than the purified enzymes (42 kDa), and that the specific activity decreased as a result of separating the enzyme (50 kDa) using Butyl-Toyopearl 650S column chromatography. Meanwhile, the stability of the 50 kDa enzymes was markedly low, and these enzymes were inactivated by Butyl-Toyopearl 650S column chromatography. The recovery of the final purified chitinase was 3.3%, and the purification was 49.0 times (Table 2).

N-terminal amino acid sequence of silver croaker chitinase

The N-terminal amino acid sequence of the silver croaker chitinase was analyzed up to the 25th residue, and compared with those of other families 18 and 19 chitinases (Fig. 3). The N-terminal amino acid sequence of this enzyme was consistent with those of chi 1 of olive flounder stomach chitinase [31]. Also, consistency was seen in 13 out of 23 residues in the N-terminal amino acid sequence of fish stomach chitinases. The N-terminal amino acid sequence of this enzyme showed high homology with those of family 18 chitinases (Fig. 3).

Effect of pH and temperature on silver croaker chitinase activity

The optimum pH of silver croaker chitinase toward pNp-$(GlcNAc)_2$ and pNp-$(GlcNAc)_3$ were observed to be pH 2.5 and 4.5, respectively, and no more than 20% of maximum activity was shown at pH 8.0 (Fig. 4A). On the other hand, maximum activity toward colloidal chitin, an insoluble long substrate, was observed to be 4.0, and even at pH 8.0 only about 50% of maximum activity was shown (Fig. 4B). The optimum pH of red sea bream [23] and eel stomach [24] chitinases toward colloidal chitin has been reported in the acidic region of pH 5.5 and 4.4, respectively, and the value for silver croaker chitinase was close to these. Meanwhile, maxi-

Table 2
Purification of silver croaker chitinase.

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	128	49.1	726	0.068	100	1
Ammonium sulfate fractionation	15.0	24.9	47.2	0.528	50.7	7.8
Chitopearl Basic BL-03	51.2	15.2	3.59	4.23	31.0	62.2
CM-Toyopearl 650S	50.4	5.97	0.972	6.14	12.2	90.3
Butyl-Toyopearl 650S	61.2	1.60	0.481	3.33	3.3	49.0

One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min at 37 °C.
Protein concentration was measured by the method of Bradford, using bovine serum album as the standard protein.

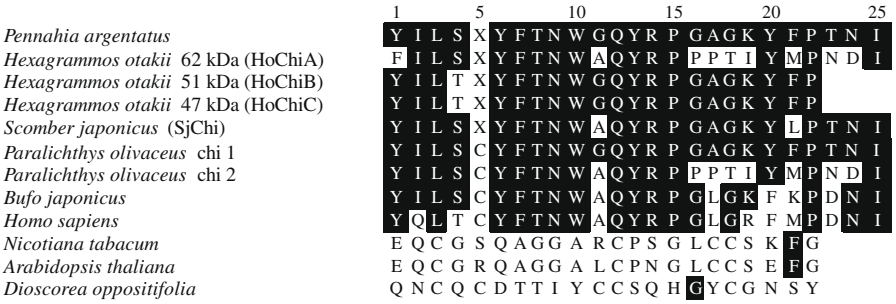

Fig. 3. Comparison of the N-terminal amino acid sequence of silver croaker stomach chitinase with those of other families 18 and 19 chitinases. Identical residues of silver croaker stomach chitinase are in white with a black background. X is an unidentified amino acid. The family 18 chitinases and their reference nos. and/or GenBank Accession Nos. are: *Hexagrammos otakii* [7], *Scomber japonicas* [25], *Paralichthys olivaceus* fChi 1 (GenBank Accession No. AB121732), *Paralichthys olivaceus* fChi 2 (GenBank Accession No. AB121733), *Bufo japonicas* [32], and *Homo sapiens* (GenBank Accession No. AAG60019.1). The family 19 chitinases and their GenBank Accession Nos. are: *Nicotiana tabacum* (GenBank Accession No. AAB23374), *Arabidopsis thaliana* (GenBank Accession No. BAA82818), and *Dioscorea oppositifolia* (GenBank Accession No. BAC56863).

mum activity of this enzyme toward glycol chitin, a water-soluble long substrate, was detected at pH 8.0, and more than 70% of maximum activity was observed at pH 4.0–7.5 (Fig. 4B). From these results, it became clear that silver croaker chitinase is an enzyme that shows activity toward short substrates only in the acidic region, but shows activity toward long substrates at a wide range of pH from acidic to neutral. Also, as shown in Fig. 4C, silver croaker chitinase was recognized to be stable in the acidic regions of pH 3.0–5.0 when incubating at 60 °C for 10 min. From these results, it became clear that the enzyme is stable in the acidic region, and that moreover the chitinase shows activity toward both short and long substrates in the acidic region.

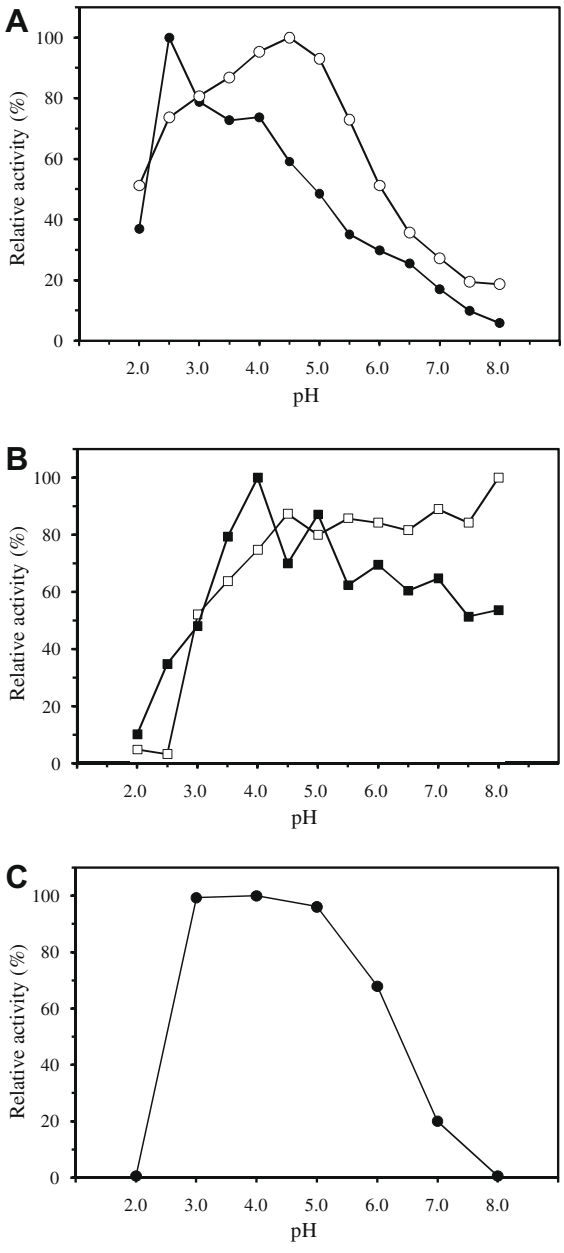

Fig. 4. Effect of pH on silver croaker chitinase activity. (A) The optimum pH when using pNp-$(GlcNAc)_2$ (●) and pNp-$(GlcNAc)_3$ (○) as substrates was measured by incubating at 37 °C for 10 min, using a 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 2.0–8.0). (B) The optimum pH when using colloidal chitin (■) and glycol chitin (□) as substrates was measured by incubating at 37 °C for 2 h, using a 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 2.0–8.0). (C) For pH stability, enzyme solution was incubated at pH 2.0–8.0 (0. 2 M sodium phosphate–0.1 M citric acid buffer) at 60 °C for 10 min, and remaining activity was measured using pNp-$(GlcNAc)_2$ as a substrate.

As shown in Fig. 5A, the optimum temperature of silver croaker chitinase toward pNp-$(GlcNAc)_2$ with a reaction time of 10 min was observed to be 60 °C. The optimum temperature of both greenling stomach chitinase isozymes (HoChiA) [7] and common mackerel stomach chitinase (SjChi) [25] has been reported as 60 °C, and that of silver croaker chitinase was consistent with the values for this chitinase. Silver croaker chitinase was stable up to incubating at 40 °C for 10 min, but incubating at 60 °C for 10 min caused the activity to fall to 70%, and activity more or less ceased when incubating at 80 °C for 10 min (Fig. 5-B).

Effect of NaCl on silver croaker chitinase activity

When using pNp-$(GlcNAc)_2$ as a substrate, chitinase activity was activated by the presence of NaCl, and 298% of the relative activity was observed with the presence of 0.9 M NaCl (Fig. 6A). On the other hand, the chitinase was also activated, dependent on concentration, when using colloidal chitin as a substrate, showing 208% activity with the presence of 0.8 M NaCl. However, the rate of activation decreased with the addition of 0.9 M NaCl or more, showing 125% with the addition of 1 M (Fig. 6B). In the *S. griseus* chitinase studied for comparison, no activation was observed in activity measurement of either substrate with the addition of NaCl, and activity was inhibited with the presence of 0.8 M NaCl (Fig. 6A and B).

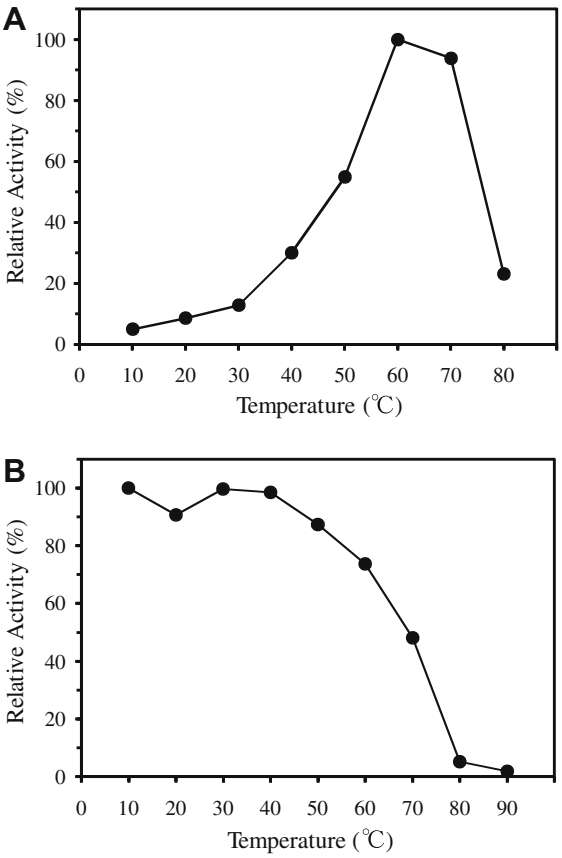

Fig. 5. Effect of temperature on silver croaker chitinase activity. (A) Optimum temperature was measured by incubating at 10–80 °C for 10 min, using pNp-$(GlcNAc)_2$ as a substrate. (B) For temperature stability, enzyme solution was incubated at pH 4.0 (0.2 M sodium phosphate–0.1 M citric acid buffer) at 10–90 °C for 10 min, and remaining activity was measured using pNp-$(GlcNAc)_2$ as a substrate.

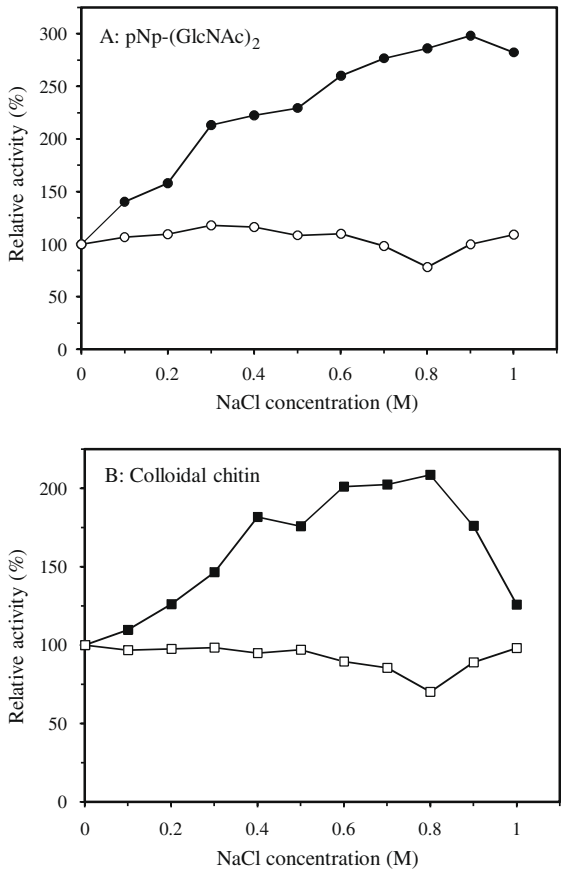

Fig. 6. Effect of NaCl on silver croaker (●, ■) and *Streptomyces griseus* (○, □) chitinase activities toward pNp-$(GlcNAc)_2$ and colloidal chitin. Chitinase activity was measured by using pNp-$(GlcNAc)_2$ (A) and colloidal chitin (B) as substrates, and adding NaCl to the reactant solution so that the final concentration was 0 to 1 M. For comparison, the effect of NaCl was similarly measured using the chitinase of *Streptomyces griseus*.

Moreover, when the effect of NaCl was measured using crystalline chitin substrates (i.e. crab shell α-chitin, shrimp shell α-chitin, and squid pen β-chitin), silver croaker chitinase was activated to about 145–190% on both substrates with the addition of 0.5 or 0.8 M NaCl (Fig. 7). Compared to this, the activity of *S. griseus* chitinase toward these substrates was inhibited with the presence of NaCl, showing 34–76% activity. As shown in the foregoing, it became clear that the short and long substrates activity of silver croaker chitinase was activated with the presence of NaCl.

Substrate specificity of silver croaker chitinase toward pNp-$(GlcNAc)_n$ (n = 1–4)

Although silver croaker chitinase released pNp from pNp-$(GlcNAc)_n$ (n = 2–4), it did not exhibit activity toward pNp-(GlcNAc) (Table 3), suggesting that this enzyme does not have exo-type chitinolytic activity. The enzyme showed high activity (0.974 U/mg) toward pNp-$(GlcNAc)_2$, but its activity toward pNp-$(GlcNAc)_n$ (n = 3, 4) decreased markedly.

HPLC analysis of cleavage patterns and anomeric forms of products in the hydrolytic reaction of $(GlcNAc)_n$ (n = 2–6) by silver croaker chitinase

Fig. 8 shows the results of analysis by HPLC of products in the hydrolytic reaction of $(GlcNAc)_n$ (n = 2–6) by silver croaker chitinase. The enzyme showed no hydrolytic activity toward $(GlcNAc)_{2,3}$, but it hydrolyzed $(GlcNAc)_{4-6}$ and produced $(GlcNAc)_{2-4}$

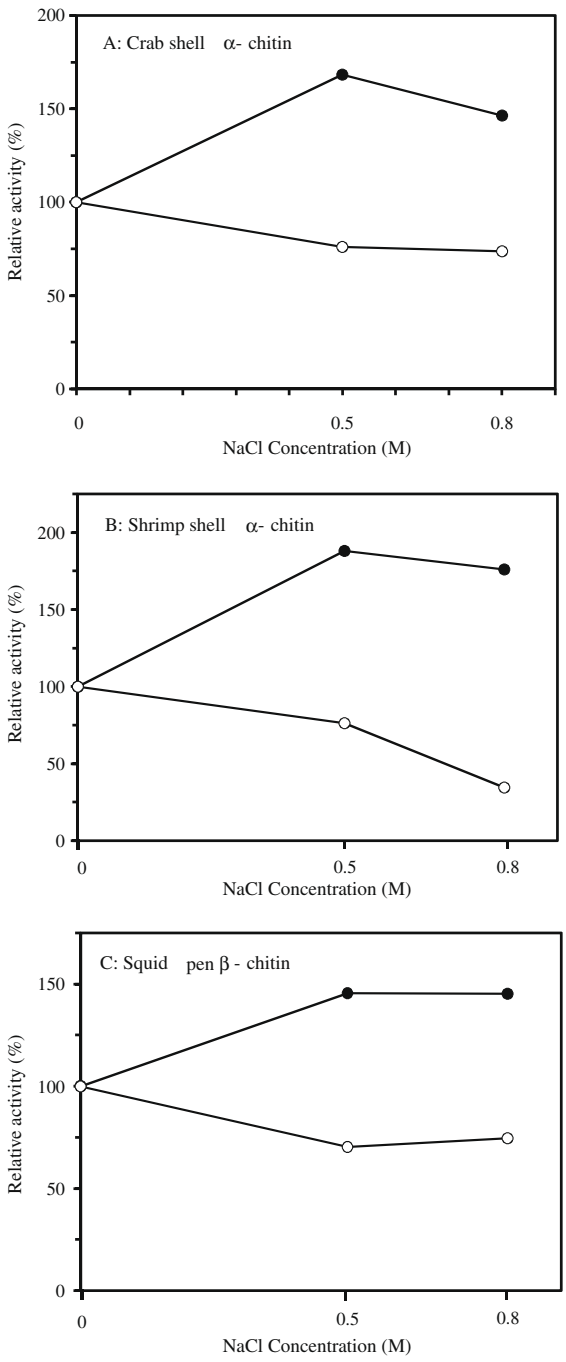

Fig. 7. Effect of NaCl on silver croaker (●) and *Streptomyces griseus* (○) chitinase activities toward crystalline chitins. Chitinase activity was measured by using crab shell α-chitin (A), shrimp shell α-chitin (B), and squid pen chitin β-chitin (C) as insoluble crystalline substrates, and adding NaCl to the reactant solution so that the final concentration was 0 to 1 M. For comparison, the effect of NaCl was similarly measured using the chitinase of *Streptomyces griseus*.

Table 3
Substrate specificity of silver croaker chitinase toward pNp-$(GlcNAc)_n$ (n = 1–4).

Substrate	Specific activity (U/mg)
pNp-(GlcNAc) (G-P)	ND
pNp-$(GlcNAc)_2$ (G-G-P)	0.974
pNp-$(GlcNAc)_3$ (G-G-G-P)	0.109
pNp-$(GlcNAc)_4$ (G-G-G-G-P)	0.296

ND, not detected; G, GlcNAc; P, pNp.

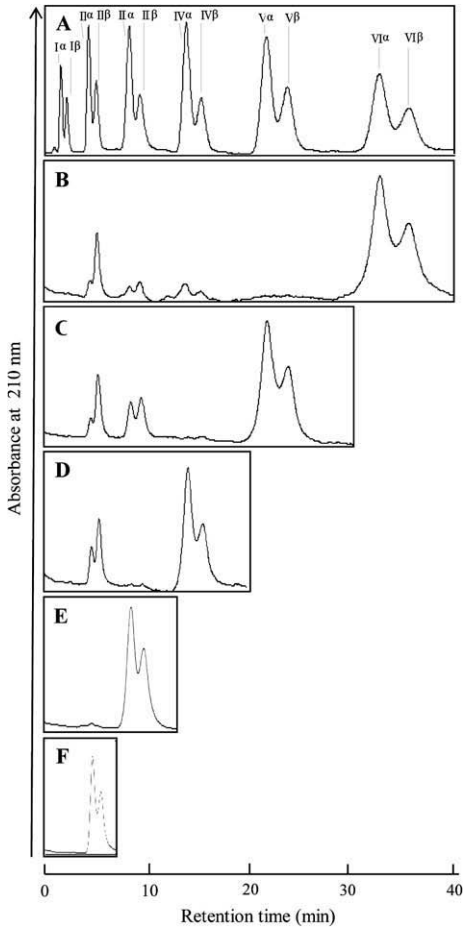

Fig. 8. HPLC analysis of the hydrolysis products of $(GlcNAc)_n$ (n = 2–6) by silver croaker chitinase. (A), $(GlcNAc)_n$ (n = 1–6) as standard; (B), $(GlcNAc)_6$; (C), $(GlcNAc)_5$; (D): $(GlcNAc)_4$; (E): $(GlcNAc)_3$; (F): $(GlcNAc)_2$. Five microliters of enzyme solution and 25 μl of 0.22 mM $(GlcNAc)_n$ were added to 25 μl of 0.1 M sodium acetate buffer solution (pH 4.0), and the mixture was incubated at 25 °C for 10 min. The reaction was stopped by cooling to 0 °C in an ice bath, and the reactant solution was analyzed by HPLC using a TSK-GEL Amide-80 column (4.6 mm × 250 mm). $(GlcNAc)_n$ was eluted using a 70% acetonitrile solution at a flow rate of 0.8 ml/min, and absorbency was detected at 210 nm [21].

Table 4
Reaction pattern and cleavage patterns of $(GlcNAc)_n$ (n = 2–6) by silver croaker chitinase.

Substrate	Reaction pattern	Initial velocity (nM/s)	Cleavage patterns
$GlcNAc_2$ (II)	ND	ND	ND
$GlcNAc_3$ (III)	ND	ND	ND
$GlcNAc_4$ (IV)	IV → II + II	3.03	○ ○ ○ ○ ↑ 100%
$GlcNAc_5$ (V)	V → II + III	6.70	○ ○ ○ ○ ○ ⬆ ↑ 79.2% 20.8%
$GlcNAc_6$ (VI)	VI→II+IV	—	○ ○ ○ ○ ○ ○ ⬆ ↑ 76.8%
	III+III		○ ○ ○ ○ ○ ○ ↑ 23.2%

Initial velocities were measured in the reaction of 0.1 mM *N*-acetylchitooligosaccharides with 100 nM silver croaker chitinase in 0.1 M sodium acetate buffer (pH 4.0) at 25 °C. ○ represent GlcNAc. The left side in the cleavage patterns is the non-reducing end. The thick arrows show main cleavage sites. ND, not detected.

Table 5
Kinetic analysis of silver croaker and other fish stomach chitinases toward glycol chitin.

Chitinase	K_m (mg/ml)	k_{cat} (1/S)	k_{cat}/K_m (ml/mg/s)
Pennahia argentatus	0.521	2.33	4.47
Hexagrammos otakii 62 kDa (HoChiA)	0.205	3.15	15.4
Hexagrammos otakii 51 kDa (HoChiB)	0.471	1.06	2.25
Hexagrammos otakii 47 kDa (HoChiC)	0.494	1.85	3.74
Scomber japonicus	0.182	2.21	12.1

The other fish stomach chitinases and their reference nos. are: *Hexagrammos otakii* [7], *Scomber japonicus* [25].

with increased β-anomers, in the same way as family 18 chitinases. Also, GlcNAc was not detected in the hydrolysis products. As shown in Table 4, the enzyme hydrolyzed $(GlcNAc)_4$ to produce two molecules of $(GlcNA)_2$, and it hydrolyzed $(GlcNAc)_5$ to produce $(GlcNAc)_2$ (79.2%) and $(GlcNAc)_3$ (20.8%). Furthermore, it hydrolyzed $(GlcNAc)_6$ to produce two molecules of $(GlcNAc)_3$ (23.2%) and $(GlcNAc)_2$ + $(GlcNAc)_4$ (76.8%, n = 2 >> 4).

Kinetic analysis of silver croaker chitinase

Silver croaker chitinase activity was measured by using glycol chitin (0.2–2 mg/ml) and $(GlcNAc)_4$ (0.15–1 mg/ml), and kinetic parameters were obtained by Lineweaver–Burk double reciprocal plot. The result toward glycol chitin is shown in Table 5. It became clear that the enzyme had a K_m value of 0.521 (mg/ml), higher than other types of fish, and its affinity toward the substrate was rather low. Conversely, the k_{cat} value of 2.33 (1/S) was similar to the 2.21 (1/S) of common mackerel stomach chitinase [7]. The k_{cat}/K_m value of the enzyme was 4.47 (ml/mg/s), about a third of common mackerel stomach chitinase and greenling stomach chitinase isozyme HoChiA but 1.2–2 times higher than the greenling stomach chitinase isozymes HoChiB and HoChiC [7]. The K_m value, k_{cat} value, and k_{cat}/K_m value toward $(GlcNAc)_4$ were 0.189 (mg/ml), 0.0192 (1/S), and 0.102 (ml/mg/s), respectively. The substrate inhibition reported in the red sea bream [33] was not observed.

Substrate specificity of silver croaker chitinase toward insoluble substrates

The substrate specificity of silver croaker chitinase toward insoluble substrates was measured by using crystalline α-chitin (crab shell chitin, shrimp shell chitin, and silkworm cuticle chitin), crystalline β-chitin (squid pen chitin and microalgae chitin), and non-crystalline colloidal chitin (Fig. 9). Of these insoluble substrates, the enzyme degraded the non-crystalline colloidal chitin the most efficiently (0.496 U/mg). This was followed by β-chitin (squid pen chitin and microalgae chitin). The activities of the enzyme toward α-chitin, which has the most robust crystalline structure, were 0.149 U/mg toward shrimp shell α-chitin and 0.116 U/mg toward silkworm cuticle α-chitin, but activity toward crab shell α-chitin was low at 0.038 U/mg. From these results, it became clear that the substrate specificity of the enzyme toward insoluble substrates was in the order non-crystalline chitin > β-chitin > α-chitin. Among the α-chitins, shrimp shell and silkworm cuticle α-chitin were degraded more efficiently than crab shell α-chitin.

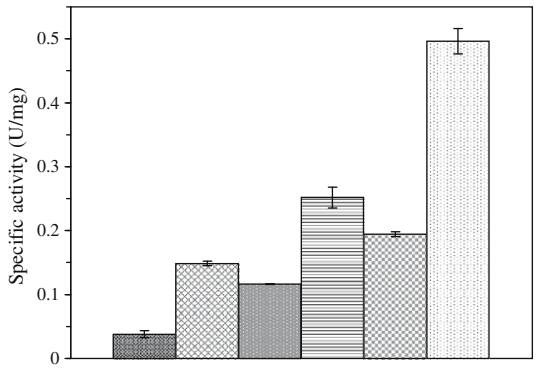

Fig. 9. Substrate specificity of silver croaker chitinase toward insoluble substrates. ▩: crab shell α-chitin; ▨: shrimp shell chitin α-chitin; ▩: silkworm cuticle α-chitin; ▤: squid pen β-chitin; ▨: microalgae β-chitin; ▨: colloidal chitin. Vertical bars are standard deviations.

Discussion

Fish stomach chitinases have the physiological roles of digesting chitinous substances ingested as food. These chitinases have been purified from the stomachs of several different fish, and their properties have been clarified [7,8,23–25]. In this study, chitinase was purified from the stomach of a previously unresearched species, the silver croaker *P. argentatus*, and its characteristics were investigated. Especially, substrate specificity was investigated in particular detail in order to determine the potential of this enzyme for use as a $(GlcNAc)_n$ producing enzyme.

To attempt to identify the glycosyl hydrolases family of silver croaker chitinase, the N-terminal amino acid sequence of purified chitinase was analyzed, together with the anomer formation ratios $(GlcNAc)_n$ (n = 2–6) of hydrolysis products formed from the chitinase. Although the N-terminal amino acid sequence of this enzyme showed a high level of homology with those of family 18 chitinases, it differed markedly from those of family 19 chitinases (Fig. 3). In substrate degradation, family 18 chitinases are reported to hydrolyze the substrate by a retaining mechanism, producing β-anomers. Conversely, family 19 chitinases hydrolyze the substrate by an inverting mechanism, producing α-anomers [20,21]. The enzyme hydrolyzed $(GlcNAc)_{4-6}$ and produced $(GlcNAc)_{2-4}$ with increased β-anomers, in the same way as family 18 chitinases (Fig. 8). These results strongly suggested that silver croaker chitinase should be classified as a family 18 glycosyl hydrolases.

Common mackerel stomach chitinase shows activity and stability in acidic regions [25]. This characteristic is thought to suit the role of this enzyme in digesting chitinous substances ingested as food under conditions in which gastric acid exists [34]. The enzyme was stable in acidic regions of pH 3.0–5.0, and it showed activity toward short substrates in acidic regions only, but toward long substrates in a wide range of pH regions from acidic to neutral (Fig. 4). From these results, it became clear that silver croaker chitinase has the activity to hydrolyzes both short and long substrates in an acidic regions. Furthermore, silver croaker chitinase activity toward pNp-$(GlcNAc)_2$, colloidal chitin, crab shell α-chitin, shrimp shell α-chitin, and squid pen β-chitin was markedly activated by the presence of NaCl (Figs. 6 and 7). On the other hand, *S. griseus* chitinase measured under the same conditions was not activated by the presence of NaCl, and α- and β-crystalline chitin degrading activity was inhibited. Activation of the degradation of short and long substrates in the presence of NaCl was therefore seen as characteristic of silver croaker chitinase. This characteristic was thought to correspond well to the role of this enzyme in the digestion of chitinous substances ingested as food together with seawater (ca. 0.5 M NaCl).

In $(GlcNAc)_n$ degradation by chitinases, red sea bream stomach chitinase has been reported to be a typical endo-type chitinase that hydrolyzes $(GlcNAc)_{4-6}$ and produces $(GlcNAc)_{2-4}$ but does not produce GlcNAc [33]. Conversely, it has been reported that an insect, silkworm *Bombyx mori*, chitinases hydrolyze $(GlcNAc)_{3-6}$ to produce $(GlcNAc)_{1-4}$ [13,35]. Insect chitinases are thought to have random-type chitin degradation activity, since they produce the monosaccharide GlcNAc. In contrast, the silver croaker chitinase hydrolyzed $(GlcNAc)_{4-6}$ to produce $(GlcNAc)_{2-4}$, whereas GlcNAc was not detected in its hydrolysis products (Fig. 8). These results proved that $(GlcNAc)_n$ degradation by the enzyme is more or less the same as that by red sea bream stomach chitinase [33].

Silver croaker chitinase degraded $(GlcNAc)_4$ to produce bimolecular $(GlcNA)_2$, and degraded $(GlcNAc)_5$ to produce $(GlcNAc)_2$ (79.2%) and $(GlcNAc)_3$ (20.8%). The enzyme also degraded $(GlcNAc)_6$ to produce bimolecular $(GlcNAc)_3$ (23.2%) and $(GlcNAc)_2 + (GlcNAc)_4$ (76.8%, $n = 2 >> 4$) (Fig. 8, Table 4), successfully producing $(GlcNAc)_2$ on all substrates. Moreover, when pNp-$(GlcNAc)_n$ (n = 1–4) was used as a substrate, the enzyme showed no pNp-(GlcNAc) activity but activity toward pNp-$(GlcNAc)_{2-4}$ (Table 3). From these results, it was thought that this enzyme is an endo-type chitinase, and that it hydrolyzes the 2nd to 4th glycosidic links from the non-reducing end of $(GlcNAc)_n$. The enzyme also showed no $(GlcNAc)_3$ hydrolytic activity but degraded $(GlcNAc)_4$ and upwards. Therefore, in the hydrolysis of $(GlcNAc)_n$, at least two molecules of GlcNAc are likely to be required at the reducing end of the hydrolyzing glycosidic linkage.

The number of chitinase isozymes present in fish stomach chitinases and the substrate specificity of each of these isozymes differ according to fish species and feeding habit [7]. The greenling stomach contains HoChiA (62 kDa), which degrades both crab shell or shrimp shell α-chitin and squid pen β-chitin, and HoChiB (51 kDa) and HoChiC (47 kDa), which specifically degrade squid pen β-chitin only. The common mackerel stomach contains only SjChi (38 kDa), which has about the same substrate specificity as HoChiA. However, both HoChiA and SjChi have markedly low silkworm cuticle α-chitin degrading activity. On the other hand, tobacco hornworm (*Manduca sexta*) chitinase efficiently degrades only silkworm cuticle α-chitin and shows markedly poor, or no, hydrolysis of crab shell, shrimp shell α-chitin, and squid pen β-chitin [7]. As these reports show, fish stomach and insect chitinases display differing substrate specificities toward crystalline chitin in accordance with their respective physiological roles.

The silver croaker chitinase obtained in this study had low activity toward crab shell α-chitin, but it efficiently degraded shrimp shell and silkworm cuticle α-chitin, as well as squid pen and microalgae β-chitin (Fig. 9). This enzyme therefore shows wider substrate specificity than HoChiA, B, C, and SjChi. These traits coincide well with the feeding habits of the silver croaker, which occupies a demersal habitat and feeds on shrimps, crab, and squid.

Fish stomach chitinases have been reported to have a chitin binding domains [31] and are adsorbed to affinity columns using chitin as a carrier [7,25]. Silver croaker chitinase was also adsorbed to the Chitopearl Basic BL-03 chitin affinity column and was eluted from the column with 0.1 M acetic acid (see Fig. 1A). Silver croaker is caught in large numbers as a raw material for *kamaboko* and other fish paste products, but its organs are usually discarded. It was thought that this study, which used affinity column chromatography to separate chitinase from the discarded stomachs of silver croaker, could be important basic research for the effective utilization of silver croaker organs and other fishery processing wastes as sources of chitinase.

Acknowledgments

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