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Substrate Specificity of Chitinases from Two Species of Fish, Greenling, Hexagrammos otakii, and Common Mackerel, Scomber japonicus, and the Insect, Tobacco Hornworm, Manduca sexta

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Substrate Specificity of Chitinases from Two Species of Fish, Greenling, *Hexagrammos otakii*, and Common Mackerel, *Scomber japonicus*, and the Insect, Tobacco Hornworm, *Manduca sexta*

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Three chitinase isozymes, HoChiA, HoChiB, and HoChiC, were purified from the stomach of the greenling, *Hexagrammos otakii*, by ammonium sulfate fractionation, followed by column chromatography on Chitopearl Basic BL-03 and CM-Toyopearl 650S. The molecular masses and pIs of HoChiA, HoChiB, and HoChiC are 62 kDa and pH 5.7, 51 kDa and pH 7.6, and 47 kDa and pH 8.8, respectively. Substrate specificities of these chitinases were compared with those of another fish stomach chitinase from the common mackerel, *Scomber japonicus* (SjChi), as well as two from the tobacco hornworm, *Manduca sexta* (MsChi535 and MsChi386). The efficiency parameters, k_{cat}/K_m, toward glycolchitin for HoChiA and SjChi were larger than those for HoChiB and HoChiC. The relative activities of HoChiA and SjChi toward various forms of chitin were as follows: shrimp shell or crab shell α-chitin > β-chitin ≫ silkworm cuticle α-chitin. On the other hand, the relative activities of HoChiB and HoChiC were β-chitin ≫ silkworm α-chitin > shrimp and crab α-chitin. MsChi535 preferred silkworm α-chitin to shrimp and crab α-chitins, and no activity was observed toward β-chitin. MsChi386, which lacked the C-terminal linker region and the chitin-binding domain, did not hydrolyze silkworm α-chitin. These results demonstrate that fish and insect chitinases possess unique substrate specificities that are correlated with their physiological roles in the digestion of food or cuticle.

Key words: chitinase; greenling (*Hexagrammos otakii*); tobacco hornworm (*Manduca sexta*); substrate specificity; crystalline chitin

Chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant biomasses in the world, second only to cellulose, and is now regarded as a renewable resource.[1,2] It is a major structural component of arthropod exoskeleton and fungal cell walls. Most of the chitin present in nature has either an α- or a β-crystalline structure, with the α-form predominant.[3]

Chitinases (EC 3.2.1.14) are enzymes that hydrolyze β-1,4-*N*-acetylglucosaminide linkages in the chitin polymer, which are widely distributed in organisms and have been isolated and characterized from a variety of sources.[4–6] Chitinases play important physiological roles in nutrition, morphogenesis, defense, and aggression.[7] Chitinases from fish[8–10] and mollusk[11] digest chitin present in prey. In insects[12] and crustaceans,[13,14] chitinases also catalyze exoskeletal molting, whereas plant and seaweed chitinases act as self-defense proteins against fungi.[5] Since the physiological roles of chitinases vary among different organisms, the substrate specificity might also be different. Furthermore, chitinases are used to produce *N*-acetylchitooligosaccharides $(GlcNAc)_n$ and GlcNAc, which have biological activities.[15] Study of the substrate specificity of chitinases is important not only to reveal physiological roles but also to degrade chitin to generate novel products with industrial applications.

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Abbreviations: HoChi, greenling stomach chitinase; SjChi, common mackerel stomach chitinase; MsChi, tobacco hornworm chitinase; EDTA, ethlenediamine tetraacetic acid

Chitinases have been isolated from several fish[16–19] and insect species,[20,21] but substrate specificity, especially toward crystalline forms of chitin, has not been investigated. In this study, we purified and characterized three chitinase isoforms from the stomach of the greenling fish, *Hexagrammos otakii*, and compared their substrate specificity with those of chitinases from the common mackerel, *Scomber japonica*, as well as an insect chitinase from the tobacco hornworm, *Manduca sexta*. This study is to our knowledge the first report of substrate specificity of animal chitinases towards several crystalline forms of chitin.

Materials and Methods

Chemicals. Glycolchitin and *p*-nitrophenyl *N*-acetylchitooligosaccharides (pNp-$(GlcNAc)_n$, n = 1 to 3) were purchased from Seikagaku Kogyo (Tokyo, Japan). Shrimp shell chitin (α-chitin, chitin EX) was from Funacoshi (Tokyo, Japan), and crab shell chitin (α-chitin) was from Tokyo Kasei (Tokyo, Japan). Silkworm cuticle chitin (α-chitin) was prepared by the method of Zhang *et al.*[22] Squid pen chitin (β-chitin) was a generous gift from Kyowa Tecnos (Chiba, Japan). All other reagents were of the highest grade commercially available.

Enzymes. Greenling (*Hexagrammos otakii*) stomach chitinases were purified in this study (see below). Chitinase SjChi was purified from the stomachs of the common mackerel, *Scomber japonicus*, by the method of Matsumiya *et al.*[18] Tobacco hornworm (*Manduca sexta*) full length recombinant chitinase (MsChi535, 81 kDa) and a truncated form lacking both the C-terminal chitin binding domain and the linker region (MsChi386, 48 kDa) were prepared by the method of Arakane *et al.*[23]

Purification of greenling chitinases. Fresh greenling (no. of samples, 2; average body weight, 935 g; average stomach weight, 13 g) was purchased from the Tokyo Central Wholesale Market. The stomach was cut open, food items were removed, and the mucosal surface was washed free of any adhering particles with cold distilled water. The stomach (26 g) was homogenized in 5 volumes of 50 mM sodium phosphate buffer (pH 6.2), and the homogenate was centrifuged at 12,000 *g* for 30 min. Ammonium sulfate was added to the supernatant to give 70% saturation. The precipitate was collected after centrifugation at 9,000 *g* for 20 min and dialyzed against 20 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl. The dialysate was centrifuged at 12,000 *g* for 30 min and the supernatant was applied to a Chitopearl Basic BL-03 column (1.6 × 21 cm) that was equilibrated with 20 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl. The enzyme was eluted with 0.1 M acetic acid. The active fractions were dialyzed against 20 mM sodium acetate (pH 4.5) and applied to a CM-Toyopearl 650S column (1.6 × 20 cm) that was equilibrated with the same buffer. The chitinase isozymes were eluted with a linear gradient of NaCl from 0 to 0.6 M in the same buffer. The active fractions were collected and stored at −80 °C.

Assay of chitinase activity. Chitinase activity was assayed using various substrates. When glycolchitin was used as the substrate, the reducing sugar produced was measured by the method of Imoto and Yagishita.[24] One milliliter of 0.1 M sodium acetate buffer solution (pH 4.0), 0.5 ml of enzyme solution, and 1 ml of 0.05% (w/v) glycolchitin solution were incubated for 30 min at 37 °C. For MsChi535 and MsChi386, 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 8.0) was used. Two ml of Schales' reagent was added to the solution to stop the reaction. The solution was then heated in a boiling water bath for 15 min. After cooling under running water, absorbance was measured at 420 nm. The value recorded was converted into the amount of GlcNAc produced using the standard curve prepared using authentic GlcNAc. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of GlcNAc per min at 37 °C.

When colloidal chitin was used as the substrate, enzyme activity was assayed according to the method of Ohtakara[25] by measuring the amount of reducing sugar produced by an enzyme reaction. A reaction mixture containing 2 ml of 0.1 M sodium acetate buffer (pH 4.0), 1 ml of enzyme solution, and 1 ml of 0.5% colloidal chitin was incubated for 1 h at 37 °C with shaking. For assays using MsChi535 and MsChi386, 0.2 M sodium phosphate–0.1 M citric acid buffer (pH 8.0) was used. The reaction was stopped by boiling for 3 min. After centrifugation, 1.5 ml of the supernatant was mixed with 2 ml of Schales' reagent. Reducing sugars produced were measured by the same protocol that was utilized when glycol chitin was used as substrate. When α-chitin or β-chitin was used as the substrate, powdered substrates that passed thorough a 100-mesh screen were used. Activity was measured by the method described above. In the case of enzyme solutions that did not contain reducing sugars, we determined sample blank reactions in the manner described above using buffer solutions without enzyme for activity measurement toward these insoluble substrates. In the case of enzyme solutions that contain reducing sugars, we also determined sample blank reactions using distilled water instead of substrates.

When pNp-$(GlcNAc)_n$ (n = 1 to 3) was used as the substrate, enzyme activity was assayed by the method of Ohtakara.[25] The enzyme solution (0.5 ml) and 0.2 ml of 4 mM pNp-$(GlcNAc)_n$ were added to 0.5 ml of 0.2 M sodium phosphate-0.1 M citric acid buffer (pH 4.5). For MsChi535 and MsChi386, the same buffer, but adjusted to pH 8.0, was used. The mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding 2 ml of 0.2 M sodium carbonate, and the *p*-nitrophenol released

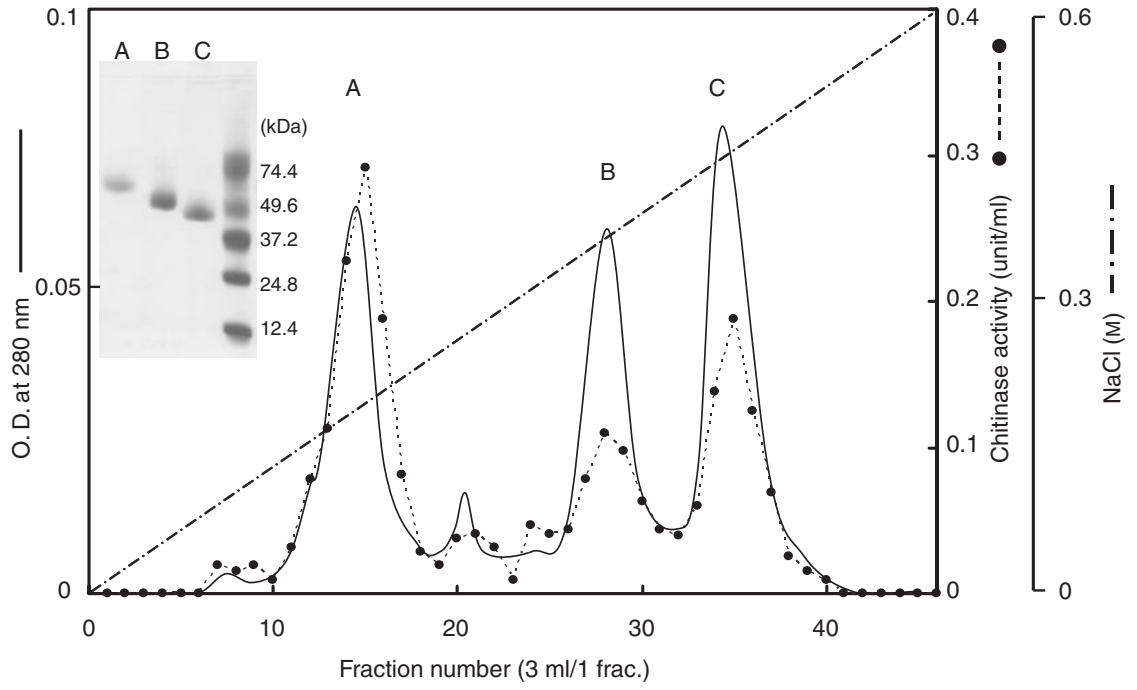

Fig. 1. CM-Toyopearl 650S Column Chromatography of Greenling Stomach Extract.

An enzyme solution obtained from Chitopearl Basic BL-03 column chromatography was applied to a CM-Toyopearl 650S column (1.6 × 20 cm) previously equilibrated with 20 mM sodium acetate buffer (pH 4.5). The enzymes were eluted with a linear gradient of NaCl from 0 to 0.6 M in the same buffer. Inset: SDS–PAGE patterns of chitinase activity peaks, A, B, and C. The amounts of applied proteins were 0.4, 0.8, and 0.6 µg respectively. Marker proteins used were cytochrome c hexamer (74.4 kDa), cytochrome c tetramer (49.6 kDa), cytochrome c trimer (37.2 kDa), cytochrome c dimer (24.8 kDa), and cytochrome c monomer (12.4 kDa).

was measured spectrophotometrically 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.

Protein measurement. Protein concentration was measured by the method of Bradford[26] using bovine serum albumin as the standard protein.

Gel electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in 12.5% polyacrylamide gel according to the manufacturer's instructions (Phast Gel, Amersham Biosciences, Piscataway, NJ). The sample was heated for 5 min in 1 mM EDTA, 2.5% SDS, and 5% 2-mercaptoethanol prior to loading. Isoelectric focusing was performed in a pH range of 3.5–9.5 on a flatbed analytical electrofocusing apparatus in a thin layer polyacrylamide gel (Amersham Biosciences). The proteins in the gels were stained with Coomassie brilliant blue R-250.

Amino acid sequence analysis. The N-terminal amino acid sequences of HoChiA, -B and -C, and SjChi were analyzed using a protein sequencer (PE Applied Biosystems 447/120A, Foster City, CA).

Results

Purification of greenling stomach chitinases

As described in "Materials and Methods" after ammonium sulfate fractionation, the greenling stomach ammonium sulfate fraction was applied to a Chitopearl Basic BL-03 column. The bound proteins with affinity to chitin were eluted with 0.1 M acetic acid. The chitinases were further purified by cation exchange chromatography using a CM-Toyopearl 650S column. Chitinases were eluted at 0.19 M NaCl (HoChiA), 0.37 M NaCl (HoChiB), and 0.48 M NaCl (HoChiC) (Fig. 1). The purity of the chitinases was examined by SDS–PAGE. As shown in Fig. 1, the final enzyme preparations of HoChiA, HoChiB, and HoChiC exhibited a single protein band after SDS–PAGE with apparent molecular masses of 62, 51, and 47 kDa, respectively. The recoveries of HoChiA, HoChiB, and HoChiC were 13.7, 5.9, and 8.4% and their specific activities were 3.04, 1.25, and 2.37 U/mg, respectively (Table 1). As shown in Table 2, fish stomach chitinase isozymes have not been purified previously. The molecular mass of HoChi-A, 62 kDa, was larger than that of any of the other fish stomach chitinases.[16–19] The isoelectric points of HoChiA, HoChiB, and HoChiC were pH 5.7, 7.6, and 8.8, respectively.

Table 1. Purification of Chitinases from the Stomach of Greenling

Purification step		Total activity (units/min)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Recovery (%)
Crude extract		23.16	526.6	0.0440	1	100
Ammonium sulfate fractionation		18.04	332.6	0.0542	1.2	77.9
Chitopearl Basic BL-03		9.15	4.084	2.24	34.2	39.5
CM-Toyopearl 650	HoChiA	3.16	1.041	3.04	69.1	13.7
	HoChiB	1.36	1.084	1.25	28.4	5.9
	HoChiC	1.96	0.825	2.37	53.9	8.4

Table 2. Molecular Masses and Isoelectric Points of Greenling Stomach and Other Fish Stomach Chitinases

Chitinase	Molecular mass (kDa)	Isoelectric point	Reference
HoChiA	62	5.7	This study
HoChiB	51	7.6	This study
HoChiC	47	8.8	This study
SjChi	38	7.6	18
Pagrus major	46	8.3	16
Anguilla japonica	50	6.2	17
Latimeria chalumnae	46	9.1	19

N-Terminal amino acid sequences of greenling and common mackerel chitinases

The N-terminal amino acid sequences of HoChiA, HoChiB, and HoChiC, and SjChi were determined and compared to those of other family 18 chitinases (Fig. 2). Based on the amino acid sequence alignment with other chitinases, several of the unknown amino acids in these N-terminal sequences (positions 5, 28, and 30) are likely to be cysteine residues. The N-terminal residues of HoChiB and HoChiC were identical. Of the 30 N-terminal residues in the fish stomach chitinases compared, 17 were identical. The N-terminal amino acid sequences of these fish chitinases showed high homology to other family 18 chitinases as well (Fig. 2).

Optimum pH and temperature

Chitinase activity was measured using glycolchitin at 37 °C for 30 min from pH 1 to 10. The buffers used were 0.1 M sodium acetate–0.1 M HCl (pH 1–3), 0.2 M sodium phosphate–0.1 M citric acid (pH 3–8), and 0.1 M ammonium chloride–0.1 M ammonia (pH 8–10). A dual optimum pH toward glycolchitin has been reported for some fish stomach chitinases.[18,19,27] As shown in Fig. 3, HoChiA had the highest activity around pH 2 and exhibited a second optimum at pH 8 with 35% of the maximal activity at pH 2. The pH profile of HoChiA was similar to that of SjChi.[18] Conversely, HoChiB and HoChiC had > 50% activity from pH 1 to 9, and the highest activity was at pH 8. The effect of temperature on enzyme activity was determined using glycolchitin in 0.1 M sodium acetate buffer, pH 4.0 for 30 min at

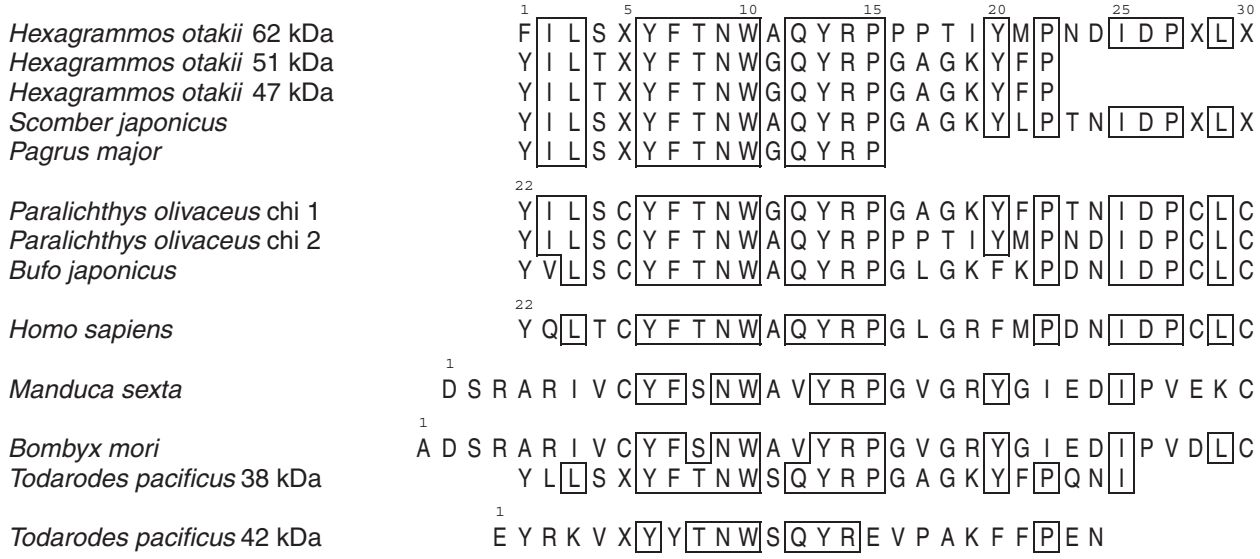

Fig. 2. Comparison of the *N*-Terminal Amino Acid Sequences of Greenling and Common Mackerel Chitinases with Those of Other Family 18 Chitinases.

Identical residues of fish stomach chitinases are enclosed in the box. X is an unidentified amino acid (probably a cysteine). The other chitinases and their reference nos. are: *Pagrus major*,;[27] *Paralichthys olivaceus* chi 1 and chi 2,;[28] *Bufo japonicus*,;[29] *Homo sapiens*,;[30] *Manduca sexta* MsChi535, MsChi386,;[31] *Bombyx mori*,;[21] *Todarodes pacificus* 38 kDa and 42 kDa.[32]

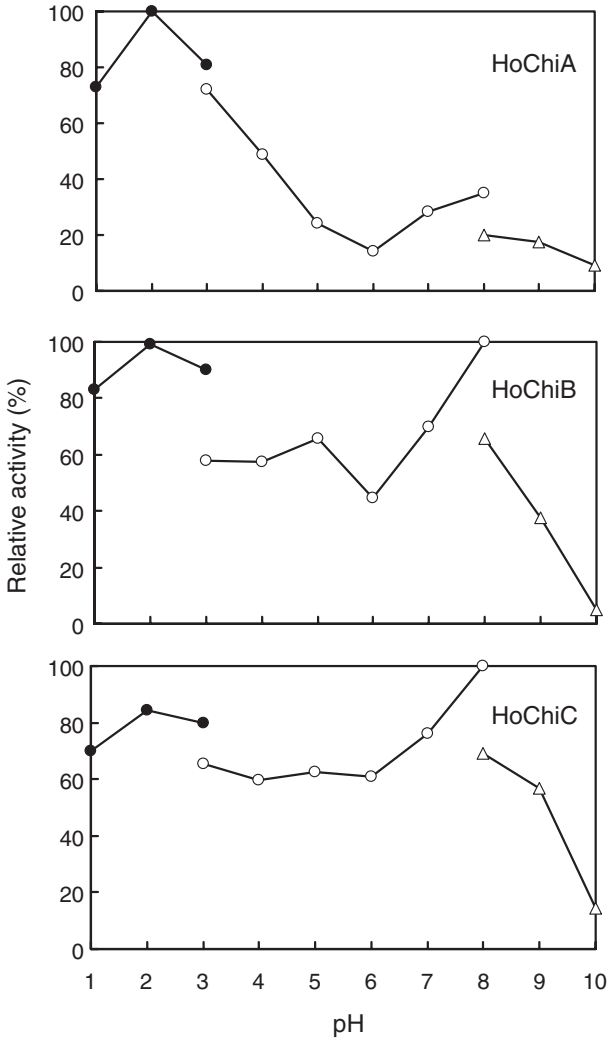

Fig. 3. Optimum pH Values for Catalytic Activity of Greenling Chitinases.

The chitinases were incubated with 0.05% glycol chitin at 37 °C for 30 min at various pH values. The buffers used were: 0.1 M sodium acetate–0.1 M HCl (pH 1–3, ●), 0.2 M sodium phosphate–0.1 M citric acid (pH 3–8, ○), 0.1 M ammonium chloride–0.1 M ammonia (pH 8–10, △).

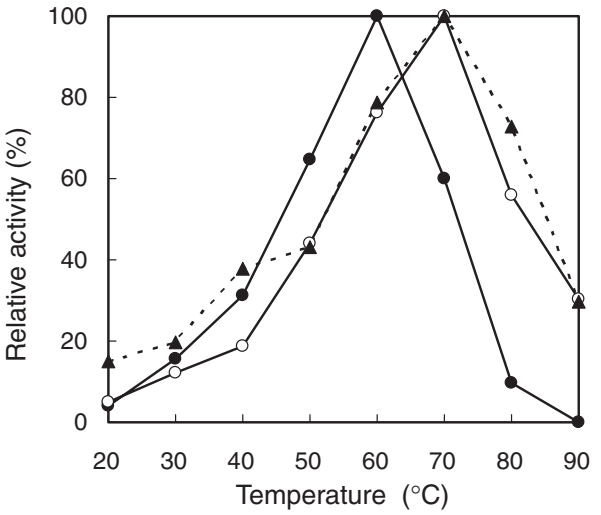

Fig. 4. Optimum Temperature for Catalytic Activity of Greenling Chitinases.

The chitinases were incubated with 0.05% glycolchitin in 0.1 M sodium acetate buffer (pH 4.0) for 30 min at various temperatures. HoChiA, ●; HoChiB, ○; HoChiC, ▲.

Table 3. Kinetic Parameters of Chitinases toward Glycolchitin

Chitinase	K_m (mg/ml)	k_{cat} (1/s)	k_{cat}/K_m (ml/mg per s)
HoChiA	0.205	3.15	15.4
HoChiB	0.471	1.06	2.25
HoChiC	0.494	1.85	3.74
SjChi	0.182	2.21	12.1

temperatures ranging from 20 to 90 °C. As shown in Fig. 4, HoChiA had the highest activity at 60 °C and was completely inactivated at 90 °C. On the other hand, both HoChiB and HoChiC exhibited the highest activity at 70 °C, and 30% activity remained even at 90 °C. The optimum temperatures for all three greenling chitinase isozymes were higher than that of *Pagrus major* chitinase, which was 50 °C.[16]

Kinetic analyses

MsChi535 and MsChi386 had no activity toward a commercial preparation of glycolchitin, a soluble long substrate. This result was contrary to that obtained by Koga *et al.*[20] who demonstrated glycolchitin hydrolysis by tobacco hornworm chitinase using a laboratory-made preparation of glycolchitin that, apparently, was less chemically modified and more susceptible to enzymatic hydrolysis than the commercial preparation. In contrast, the fish enzymes, HoChiA, HoChiB, HoChiC, and SjChi, did hydrolyze commercial glycolchitin at 37 °C in 0.1 M sodium acetate buffer, pH 4.0. Kinetic parameters were obtained from Lineweaver-Burk double reciprocal plots. As shown in Table 3, the K_m values of HoChiA and SjChi were smaller than those of HoChiB and HoChiC, suggesting that the former chitinases have higher affinity for glycolchitin than the latter two enzymes. The reaction velocity (k_{cat}) and substrate affinity ($1/K_m$) for HoChiA and SjChi were about 2–3 times higher than those for HoChiB and HoChiC. Consequently, the catalytic efficiency parameter, k_{cat}/K_m, for HoChiA and SjChi was approximately 5 times larger than those of HoChiB and HoChiC.

Substrate specificity of chitinases toward insoluble substrates

Chitinase activities were measured using non-crystalline chitin, colloidal chitin, and two crystalline chitins, α-chitin from shrimp shell, crab shell, and silkworm cuticle, and β-chitin from squid pen. The results are summarized in Table 4. Whereas all chitinases hydrolyzed colloidal chitin, SjChi showed the highest activity (83.6 units/μmol enzyme). HoChiA and SjChi showed higher activity toward shrimp shell and crab shell α-

chitin and squid pen β-chitin than toward silkworm cuticle α-chitin. On the other hand, the insect chitinase, MsChi535, showed higher activity towards silkworm cuticle α-chitin than towards shrimp shell and crab shell α-chitins. No activity toward squid pen β-chitin was observed with insect chitinase. The activities of HoChiB and HoChiC for the various substrates were in the following order: squid pen β-chitin $\gg$ silkworm cuticle α-chitin $>$ shrimp shell and crab shell α-chitin, suggesting that these chitinases prefer substrates with a β-crystalline chitin structure. Unlike the full length enzyme MsChi535, the truncated MsChi386, which lacks both the C-terminal chitin-binding domain and linker region, did not hydrolyze silkworm α-chitin. Apparently, the chitin-binding domain facilitates hydrolysis of the insoluble polymeric substrate, a hypothesis also in agreement with observations reported previously.[23)]

Substrate specificity of chitinases for pNp-N-acetylchitooligosaccharides

Chitinase activities were measured using several soluble short substrates, pNp-$(GlcNAc)_n$ ($n = 1$–3). The results are summarized in Table 5. None of the chitinases showed any activity toward pNp-GlcNAc, suggesting that these chitinases have no exo-type chitinolytic activity. HoChiA, SjChi, and MsChi535 released pNp from pNp-$(GlcNAc)_n$ ($n = 2$, 3 with a preference for the second glycosidic bond). On the other hand, HoChiB and HoChiC exhibited strong activity toward pNp-$(GlcNAc)_2$ but only very weak activity toward pNp-$(GlcNAc)_3$. These results suggest that HoChiB and HoChiC have a preference for the glycosidic bond which is second from the reducing end. Even though MsChi386 and MsChi535 were about equally active toward pNp-$(GlcNAc)_3$, MsChi386 had about only half of the activity of MsChi535 toward pNp-$(GlcNAc)_2$.

Discussion

To correlate the substrate specificities of several chitinases from different organisms with their physiological roles, we purified three chitinase isozymes, HoChiA, HoChiB, and HoChiC, from the stomach of the greenling fish. Greenlings inhabit the bottom of the sea and feed on both crustaceans such as shrimp and crab, which have α-chitin in their exoskeletons,[33)] and Polychaeta, which have β-chitin.[33)] The HoChiB and HoChiC enzymes are similar, if not identical in many of their properties, including N-terminal amino acid sequence, optimal pH, optimal temperature, and K_m and k_{cat}/K_m values. We suspect that HoChiC might be a C-terminal truncated form of HoChiB. The relative activity at 20 °C (Fig. 4) of HoChiC is 3 times higher than that of HoChiB, suggesting that HoChiC is more active than HoChiB at the temperature of their habitat, below 30 °C,[34)] which is typical for marine fishes. The enzymatic properties of HoChiA were different from those of HoChiB and HoChiC, but similar to SjChi from the common mackerel, which inhabits the surface area of the sea and feeds primarily on crustaceans. As shown in Table 4, SjChi and HoChiA hydrolyzed both shrimp and crab shell α-chitin and squid β-chitin, whereas HoChiB and HoChiC hydrolyzed β-chitin well but exhibited little or no activity toward shrimp and crab shell α-chitin. These results are what might be expected when considering their respective diets. Greenlings feed on polychaeta as well as crustaceans such as shrimp and crab, suggesting that HoChiA digests both α- and β-forms of chitin, whereas HoChiB and HoChiC probably play a role in digesting only β-chitin. Regarding the insect chitinases and chitin substrates, MsChi535 showed a high activity only toward silkworm cuticle α-chitin, suggesting that this enzyme is designed to hydrolyze cuticular chitin during molting. MsChi535 and MsChi386 had no activity toward commercial glycol chitin. We have observed that we have to limit the glycolation of chitin before complete modification during preparation of glycol chitin for the product to be useful as a substrate for insect chitinases. Apparently, insect chitinase can hydrolyze the polymer of *N*-acetyl-D-glucosamine, but not those of glycolated *N*-acetyl-D-glucosamine. Therefore, the substrate specificity of insect chitinase might be narrower than that of the fish chitinases. All of these results indicate that chitinases from different sources possess unique substrate specificities that are consistent with their intended physiological roles.

Previously, we reported that MsChi386, which lacks both the C-terminal chitin-binding domain and the linker region, showed activity toward several non-crystalline forms of chitin.[23)] In this study, we also demonstrated the activity of MsChi386 not only toward non-crystalline chitin, colloidal chitin, and pNp-$(GlcNAc)_n$ ($n = 2$, 3), but also towards crystalline chitins, including shrimp shell and crab shell α-chitin. However, we also observed that MsChi386 was unable to hydrolyze silkworm cuticle α-chitin, the physiological target of insect chitinases, suggesting that the C-terminal chitin-binding domain is required for hydrolysis of silkworm cuticle α-chitin. Although MsChi386 showed little or no activity toward the preparations of shrimp and crab shell α-chitin used in the present study, MsChi386 might weakly hydrolyze the amorphous part of other preparations of shrimp and crab α-chitin produced during powdering process[35)] to an extent similar to the activity towards colloidal chitin (Table 4).

It has been reported that *Bacillus* sp. PI-7S chitinase prefers β-chitin to shrimp shell α-chitin,[36)] and that *Bacillus* sp. chitinase prefers beetle larval cuticular α-chitin to shrimp shell α-chitin.[22)] The substrate specificities of HoChiB and HoChiC toward crystalline forms of chitin resemble those of the microbial chitinases. Since the k_{cat}/K_m values of HoChiA and SjChi toward the long substrate, glycolchitin, were markedly higher than those

Table 4. Substrate Specificity of Chitinases for Insoluble Substrates

Substrate	Specific activity (units/μmol enzyme)					
	HoChiA	HoChiB	HoChiC	SjChi	MsChi535	MsChi386
Colloidal chitin	19.5 ± 1.6	5.1 ± 0.6	9.4 ± 0.5	83.6 ± 3.6	33.3 ± 2.2	6.7 ± 1.0
Shrimp shell chitin (α-Chitin)	63.2 ± 2.5	3.1 ± 0.4	4.6 ± 0.4	49.8 ± 4.2	3.1 ± 1.2	7.4 ± 1.4
Crab shell chitin (α-Chitin)	62.6 ± 1.3	A trace	3.3 ± 0.4	63.8 ± 4.1	7.7 ± 1.5	9.3 ± 1.7
Silkworm cuticle chitin (α-Chitin)	3.3 ± 0.5	23.4 ± 1.1	29.5 ± 2.7	A trace	32.6 ± 2.0	ND
Squid pen chitin (β-Chitin)	40.5 ± 2.7	122 ± 2.2	191 ± 3.0	32.7 ± 3.9	ND	ND

ND, not detected. Mean value ± S.D. ($n = 3$)

Table 5. Substrate Specificities of Chitinases for pNp-*N*-Acetylchitooligosaccharedes

Substrate		Specific activity (units/μmol enzyme)					
		HoChiA	HoChiB	HoChiC	SjChi	MsChi535	MsChi386
pNp-(GlcNAc)	(G-P)	ND	ND	ND	ND	ND	ND
pNp-$(GlcNAc)_2$	(G-G-P)	93.6 ± 0.2	108 ± 1.5	244 ± 2.3	105 ± 0.7	58.9 ± 0.5	29.8 ± 0.2
pNp-$(GlcNAc)_3$	(G-G-G-P)	80.6 ± 1.1	1.5 ± 0.1	6.6 ± 0.1	32.3 ± 0.4	8.1 ± 0.2	7.9 ± 0.1

ND, not detected. Mean value ± S.D. ($n = 3$)

of HoChiB and HoChiC (Table 3), and since both of the former enzymes preferentialy cleave the third glycosidic link from the non-reducing end of pNp-$(GlcNAc)_n$ (Table 5), these properties might be necessary for digestion of shrimp shell and crab shell α-chitin. HoChiA and SjChi appear to be very proficient at digesting α-chitin from shrimp and crab shells, which are the most abundant natural sources of biologically active substances such as chitooligosaccharides and GlcNAc.

The N-terminal amino acid sequences of all of the fish chitinases are homologous to N-terminal sequences of members of family 18 animal chitinases, which includes representatives of many species including insects, mollusks, amphibians, and humans (Fig. 2). Several amino acid residues are completely conserved (positions 6Y, 9N, 10W, 13Y, and 14R) in greenling and common mackerel chitinases as well as in all the other members of family 18 chitinases shown in Fig. 2. Thus, it is very likely that HoChiA, HoChiB, HoChiC, and SjChi all belong to family 18 glycosyl hydrolases, as does *Pagrus major* chitinase.[27] This hypothesis is strongly supported by our cloning data for the cDNAs from HoChiA, HoChiB, HoChiC, and SjChi encoding the region between the N-terminus and conserved region II of family 18 chitinases (unpublished data). These cDNA fragments have both conserved regions I and II of family 18 chitinases and the catalytically critical residues, DWE (-Asp-Trp-Glu-) within conserved region II.[37]

Nearly all of the plant chitinases have molecular masses of 25–35 kDa.[38,39] Some of the plant chitinases have very acidic pI values. For example, yam chitinases H1 and A have pI values of 3.6 and 3, respectively, and yam chitinase E has a pI of 3.8.[39] On the other hand, the molecular masses and pI values of fish stomach chitinases range from 38–62 kDa and 5.7–9.1 (Table 2), indicating that the physicochemical properties of fish chitinases are quite different from those of plant chitinases. Fish stomach chitinases have been purified by chitin affinity column chromatography,[18,19] demonstrating that these chitinases have chitin-binding ability. Recently, Kurokawa *et al.*[28] reported that *Paralichthys olivaceus* chitinases have type 2 chitin-binding domains.

Some species of fish secrete hydrochloric acid into their stomachs, creating acidic conditions for digestion and a pH range of 2–4.[40] Chitinases from the common mackerel,[18] coelacanth,[19] and greenling (Fig. 3) exhibited high activity at around pH 2. Therefore, for digestion of chitinous food, some of the fish stomach chitinases have the following favorable characteristics: neutral to basic pI values, molecular masses of 38–62 kDa, and domains with chitin-binding ability, as well as excellent activity in an acidic environment.

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