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Distribution of Chitinolytic Enzymes in the Organs and cDNA Cloning of Chitinase Isozymes from the Stomach of Two Species of Fish, Chub Mackerel (*Scomber japonicus*) and Silver Croaker (*Pennahia argentata*)

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Abstract

Chitinolytic activities were measured in two fish species having different feeding habits, chub mackerel (*Scomber japonicus*) and silver croaker (*Pennahia argentata*). Chitinase (an endo-type chitinolytic enzyme) activity was measured using pNP-$(GlcNAc)_n$ (n = 2, 3) as substrates; its level was significantly high in the stomachs of both species, as well as in the gills, intestine, pyloric appendage, testis, and liver of chub mackerel and in the spleen, kidney, pyloric appendage, ovaries, heart, and liver of silver croaker. *β-N*-Acetylhexosaminidase (an exo-type chitinolytic enzyme) activity was measured using pNP-(GlcNAc) as a substrate; it was detected at high levels in many parts apart from the digestive tracts of both species. The optimum pH for chitinase activity was 3.0 - 5.0 in the stomachs of both species, 4.0 in the liver of chub mackerel, and 4.0 and 8.0 in the kidney of silver croaker. Full-length cDNAs encoding two chitinase isozymes were obtained from the stomachs of the two fish species: *SjChi*-1 (1604 bp) and *SjChi*-2 (1512 bp) from chub mackerel and *PaChi*-1 (1630 bp) and *PaChi*-2 (1606 bp) from silver croaker. Expression analysis of these genes in the organs of the two species revealed strong expression of *SjChi*-1 in the stomach of chub mackerel and that of *PaChi*-1 and *PaChi*-2 in the stomach of silver croaker. The difference in the expression pattern of these genes is likely attributed to the difference in the feeding habits of the two fish species. Our results suggested the presence of novel chitinases in the two species.

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Keywords

Chitinolytic Enzyme, Chitinase, *β*-*N*-Acetylhexosaminidase, Distribution, Phylogenetic Tree Analysis

1. Introduction

Chitin is an amino polysaccharide containing *N*-acetyl-d-glucosamine (GlcNAc) units connected with *β*-1,4 linkages. It is a renewable biological resource that is abundantly present worldwide and is found in the exoskeletons of arthropods, cell walls of fungi, and the epidermis of nematodes [1]-[3]. The majority of naturally occurring chitin exists in the rigid, *α*-crystalline structure that is insoluble in common solvents, thus rendering it difficult for use [4]. However, degradation products of chitin exhibit various bioactivities, which have been attributed to the length and solubility of a polymer [5]; these include the promotion of bifidobacteria proliferation and immunostimulatory effect in chitooligosaccharides ($(GlcNAc)_n$) [6] and improvement of osteoarthritis in GlcNAc [7] [8].

Chitinolytic enzymes degrade chitin and are classified according to the degradation mechanisms as endo-type chitinolytic enzyme, which produce $(GlcNAc)_n$ by randomly degrading chitin internally, and exo-type chitinolytic enzyme, which produce GlcNAc by degrading chitin sequentially from the nonreducing ends [9]. The former is referred to as chitinase (EC 3.2.1.14) and belongs to the glycoside hydrolase GH family 18 or 19 based on the amino acid sequence homology within the catalytic domain [10]. The latter is referred to as *β*-*N*-acetylhexosaminidase (Hex) (EC 3.2.1.52) [11].

Although the majority of studies on chitinase have been conducted on microorganisms [12], chitinase is also found and has been investigated in many other biological species, including mammals [13]-[15], fish [16]-[19], mollusks [20] [21], insects [22]-[24], plants [22] [25], and fungi [1] [22]. Since the physiological role of chitinase varies in these organisms, the mechanism and efficiency of chitinase degradation and the substrate specificity have been reported to vary. For instance, acidic mammalian chitinase expressed in the stomach of mammals [26], and chitotriosidase produced by macrophages [14] [15] are thought to function in the digestion and absorption of food and defense against pathogens. In addition, chitinase has been detected at the onset of asthma and allergies, suggesting its involvement in diseases [13] [15]. In contrast, chitinase and Hex are also found in the digestive tracts of fish. The enzyme activities are generally associated with feeding habits, and their levels are high in fish that feed on organisms containing chitin [16] [27]. However, the distribution of chitinase and Hex in the body of fish is not known.

Our laboratory has been investigating the purification, characteristics, and cDNA cloning of chitinases by using various samples, including fish (osteichthyes [17] [28]-[30] and chondrichthyes [18]), mollusca [20] [21], and crustacea [31]. We purified chitinase isozymes that function at acidic pH and are involved in digestion from the stomachs of osteichthyes [28]-[30]. In addition, a subset of these chitinases was found to have superior degradation ability against crystalline *α*-chitin and broader substrate specificity compared to that of chitinases from other organisms [32]. Moreover, two genes encoding chitinase isozymes were found in the stomachs of these osteichthyes, and the deduced amino acid sequences revealed using phylogenetic analysis showed that these enzymes form two groups, acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2), which are unique to fish [17] [28]. However, which of these chitinase isozymes, AFCase-1 or AFCase-2, act dominantly in response to habitat and feeding habits is not known.

In this study, we determined the distribution of chitinase and Hex activities in chub mackerel, which lives in the ocean surface and feeds on chitin from zooplankton [32], and in silver croaker, which lives in the sandy, mud ocean floor and feeds on rigid chitin from shrimps and crabs [29] [30]: chitinase has been previously purified and characterized from the stomachs of both species. This study aimed to confirm the presence or absence of chitinase and Hex in the organs apart from the digestive tract. In addition, the optimum pH was measured in the stomach and liver of chub mackerel and the stomach and kidney of silver croaker, in which chitinase activities had been detected, in order to identify novel chitinases that are distinct from those found in the stomach and act under acidic conditions. cDNA cloning and organ expression analysis were also performed for the chitinase isozymes obtained from the stomachs of the two species, and the association between the expression of stomach chitinase isozymes and the feeding habits of fish was discussed.

2. Materials and Methods

2.1. Materials

Chub mackerel (mean body length: 40 cm) and silver croaker (mean body length: 25 cm) were freshly obtained on the day they were caught (June and August, respectively), and chitinolytic activity was measured and cDNA cloning were performed on the same day.

2.2. Measurement of Chitinolytic Enzyme Activity

The organs to be analyzed were removed from the chub mackerel and silver croaker. Next, 0.5 g of each organ was homogenized in three volumes of 20 mM phosphate buffer (pH 7.3), and then the homogenate was centrifuged at 9000 × g for 20 min. The supernatant was used as the crude enzyme solution. Chitinase and Hex activities were measured using *p*-nitrophenyl $(GlcNAc)_n$, (pNP-$(GlcNAc)_n$) (n = 2, 3) (Seikagaku, Tokyo, Japan) and pNP-GlcNAc (Seikagaku), respectively, as substrates according to the method described by Ohtakara [33], with slight modification. Briefly, 2.5 μL of crude enzyme solution and 2.5 μL of substrate solution were added to 6.5 μL of 0.2 M phosphate-0.1 M citrate buffer (pH 6.0), and then the solution was incubated at 37˚C for 20 min. After incubation, 65 μL of 0.2 M sodium carbonate solution was added to the solution, and the absorbance of released *p*-nitrophenol was measured at 420 nm. A unit of chitinolytic enzyme activity (U) was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute and was expressed as the activity per gram of the organs.

2.3. The Effect of pH of Chitinase and Hex Activity

The optimum pH was measured for the crude enzyme solutions obtained from the stomach and liver of chub mackerel and the stomach and kidney of silver croaker by using 0.2 M phosphate-0.1 M citrate buffer (pH = 2 - 8) and the mixture of 0.1 M glycine and 0.1 M NaCl-0.1 M NaOH (pH = 9), respectively, by using the same technique as was used for the measurement of chitinolytic activity.

2.4. cDNA Cloning of Chitinases Obtained from the Stomachs of Chub Mackerel and Silver Croaker

Total RNA was extracted from the stomachs of the chub mackerel and silver croaker by using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The extracted total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's instructions. Next, cDNA was synthesized using 1.0 μg of total RNA; a reverse transcriptase M-MLV (Takara Bio, Shiga, Japan); and an oligo dT primer (**Table 1**). The reaction conditions were 65˚C for 5 min, 42˚C for 60 min, and 70˚C for 10 min. Full-length cDNA from the chub mackerel and silver croaker stomach chitinases was amplified. The primers used are listed in **Table 1**, and the primer combinations are shown in **Figure 1**. Internal sequences were amplified using a solution containing the synthesized cDNA, Takara Ex Taq (Takara Bio), and degenerate primers designed using the conserved amino acid sequences of GH family 18 chitinases from several organisms. PCR parameters for the first PCR were as follows: initial denaturation at 95˚C for 30 s, followed by 35 cycles of 95˚C for 30 s, 55˚C for 1 min, and 72˚C for 2 min. Nested PCR was performed using the same PCR parameters except that the sample was 10-fold diluted for the first PCR products. Forward and reverse primers were designed from the chitinase gene sequences obtained by the internal sequence amplification, and the upstream (5′) and downstream (3′) regions were amplified using rapid amplification of cDNA ends (RACE) method. The 5′ and 3′RACE analyses were performed using kits provided by Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. Internal sequences and PCR products obtained by RACE were electrophoresed on 2% agarose gel, and DNA was extracted using Quantum Prep® Freeze'N Squeeze spin columns (Bio Rad, Hercules, CA) and ligated into the pGEM-T Easy Vector (Promega). Full-length chitinase genes obtained from the stomach of chub mackerel (*SjChi*-1 and *SjChi*-2) and the stomach of silver croaker (*PaChi*-1 and *PaChi*-2) were amplified using platinum® *pfx* DNA polymerase (Invitrogen), which has proofreading activity. The protocol was 35 cycles of 94˚C for 15 s, 55˚C for 30 s, and 68˚C for 2 min. The full-length genes obtained were extracted using the same method that was used for the internal sequence amplification and ligated into the pCR® Blunt II-TOPO® vector (Invitrogen). Base sequences were determined using the Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA).

Table 1. Primers used in this study.

	Primer name	Sequence (5'-3')	Length	Usage
	Oligo dT	CTGTGAATGCTGCGACTACGATTTTTTTTTTTTTTTTT	40mer	cDNA synthesis
	Chi-a (F)	TGYTAYTTYACNAAYTGG	19mer	Conserved region PCR
	Chi-b (F)	GAYATHGAYTGGGARTAYCC	19mer	
	Chi-c (R)	TTCCARTARTTCATNGCRTARTC	19mer	
	3'*RACE* (R)	CTGTGAATGCGACTACGAT	19mer	3'RACE PCR
	AAP (F)	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	36mer	5'RACE PCR
	AUAP (F)	GGCCACGCGTCGACTAGTACC	21mer	
	β-actin 1 (F)	AGCCAACAGAGGAGGCTCTTA	21mer	Tissue expression PCR
	β-actin 2 (R)	GATTCCTAAGAGTGAATG	18mer	
Chub mackerel	*SjChi*1-1(F)	ACCAACATTGACCCATGTCTCTGTG	25mer	3'RACE PCR
	*SjChi*1-2(F)	ATGGACAACAACATGATCAAGAC	23mer	
	*SjChi*2-1(F)	GGTGAGTGCAGTCCCCTGTTCA	22mer	
	*SjChi*1-3(R)	CTCCAATGGCCAACAGAGTCTTCA	24mer	5'RACE PCR
	*SjChi*1-4(R)	TGGAACTGTCCGTAGAGTTTTTC	23mer	
	*SjChi*1-5(R)	GTTGTTGTCCATTGATGCAAAGG	23mer	
	*SjChi*1-6(R)	GGTCAATGTTGGTGGGTAGGTACT	24mer	
	*SjChi*2-2(R)	AAGACGAATAGTCTAAGGGTT	21mer	
	*SjChi*2-3(R)	GAATCAATGGTGTCCTTTCCA	21mer	
	*SjChi*2-4(R)	ACAGCAGCAGACATCAGAAGACG	24mer	
	*SjChi*1-7(F)	TACAGAAGCAATATGGGCAAACTACTC	27mer	Full length amplification
	*SjChi*1-8(R)	AGTGGAATGGTCTTGTGTTTCATGATA	27mer	
	*SjChi*2-5(F)	CGGTAGCCATGGGGAAAGTACTG	23mer	
	*SjChi*2-6(R)	ACAACAGTTATCCAGTCAATTTAT	24mer	
	*SjChi*1-9(F)	CATCCACGTCATGTCCTACGA	21mer	Tissue expression PCR
	*SjChi*1-10(R)	CGTTGTTGTAGGCATATGGCA	21mer	
	*SjChi*2-8(F)	GTCATATGACTTCCATGGCTC	21mer	
	*SjChi*2-9(R)	CCCACTGATTTCCCTTGTAAG	21mer	
Silver croaker	*PaChi*1-1(F)	GGACTACTTCCACGTCATG	19mer	3'RACE PCR
	*PaChi*2-1(F)	TGTGGACTACGCCATGAACTAC	19mer	
	*PaChi*1-2(R)	CCACATTGAAGTAGATCAT	19mer	5'RACE PCR
	*PaChi*1-3(R)	CATGACGTGGAAGTAGTCC	19mer	
	*PaChi*1-4(R)	GCGAGGACGGTTGGTCTTCT	20mer	
	*PaChi*2-3(R)	GTAGTTCATGGCGTAGTCCACA	22mer	
	*PaChi*2-4(R)	GAAATAGATGAAGCCACCC	19mer	
	*PaChi*2-5(R)	GGCCGAGCTTGGGGATCTGAT	21mer	
	*PaChi*1-5(F)	TACAGAAGCACCATGGGCAAGCTAC	25mer	Full length amplification
	*PaChi*1-6(R)	GCTTTAAATGCGACGTTTATTTAA	24mer	
	*PaChi*2-6(F)	TACACGGTAGCCATGGGGAAA	21mer	
	*PaChi*2-7(R)	GCTTTCAAATCAACCACTCAAGACG	25mer	
	*PaChi*1-7(F)	GAGCACAATGTCGGAGAGAAC	21mer	Tissue expression PCR
	*PaChi*1-8(R)	ACCACTCTGCTTCAGCCACTG	21mer	
	*PaChi*2-8(F)	GACCCCATGACTGGTGAGTGC	21mer	
	*PaChi*2-9(R)	GTTACTCTTAGTCAGCCAGTC	21mer	

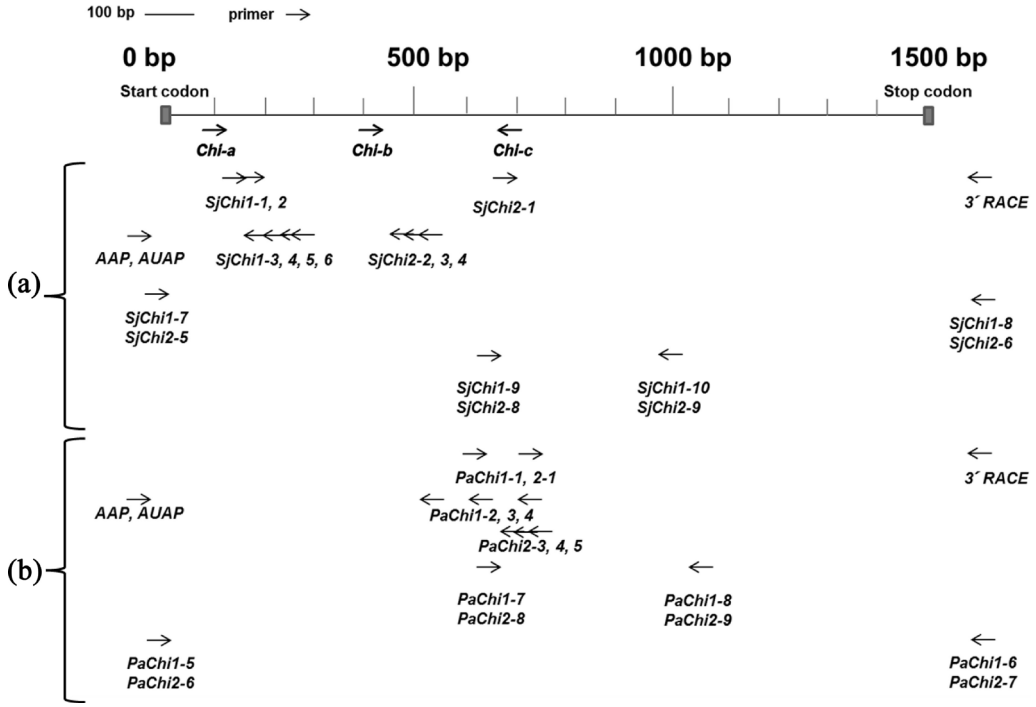


Figure 1. Primers position. (a) Chub mackerel; (b) Silver croaker.

2.5. Expression of *SjChi*-1, *SjChi*-2, *PaChi*-1, and *PaChi*-2 in the Organs

Total RNA was extracted from the organs of chub mackerel and silver croaker. cDNA was synthesized using 0.5 μg of total RNA obtained from each tissue and an oligo dT primer and amplified using PCR by using 1.0 μg of the synthesized cDNA; primers for *SjChi*-1, *SjChi*-2, *PaChi*-1, and *PaChi*-2; and fish *β*-actin amplification primers (**Table 1**) in the combinations shown in **Figure 1**. The PCR included 30 cycles of 94˚C for 30 s, 55˚C for 1 min, and 72˚C for 2 min.

2.6. Phylogenetic Analysis of the Chitinases

Phylogenetic tree analysis, based on the deduced amino acid sequences of the full-length genes of *SjChi*-1, *SjChi*-2, *PaChi*-1, and *PaChi*-2, was performed using the chitinase genes obtained from many organisms. The analysis was performed using ClustalW2 (EMBL-EBI) and Tree view programs.

3. Results and Discussion

3.1. Distribution of Chitinolytic Enzymes

Chitinolytic activity showed the highest levels of chitinase activity against pNP-$(GlcNAc)_2$ and pNP-$(GlcNAc)_3$ in the stomachs of chub mackerel and silver croaker. Chitinase activity was also detected in the gills, intestine, pyloric appendage, testes, and liver of chub mackerel (**Figure 2(a)**) and in the spleen, kidney, pyloric appendage, ovaries, heart, and liver of silver croaker (**Figure 2(b)**). The results are consistent with those of a previous study in which chitinase activity was found to be high in the stomach, a part of the digestive tract, in fish [16]. In addition, to our knowledge, this is the first study showing that chitinase activity is distributed beyond the digestive tract in the gills, testes, and liver of chub mackerel and in the spleen, kidney, ovaries, heart, and liver in silver croaker. In contrast, high levels of Hex activity were detected in all organs except the gills and heart in both species, which showed slightly lower activities (**Figure 2(c)**, **Figure 2(d)**). Thus, the two species likely digest dietary chitin down to GlcNAc owing to the high levels of chitinase activity in the stomachs, as well as Hex activity in the subsequent pyloric appendage, where food is digested after passing through the stomach, and in the intestine. In addition, in chub mackerel, chitinase activity was observed in the pyloric appendage, which is significantly developed as a part of the digestive system, suggesting that chitinase contributes to the digestion of

dietary chitin in the pyloric appendage as well. Moreover, the presence of chitinase and Hex activities in the organs apart from the digestive tract suggests that fish use chitinolytic enzymes for other physiological functions in addition to the digestion of dietary chitin.

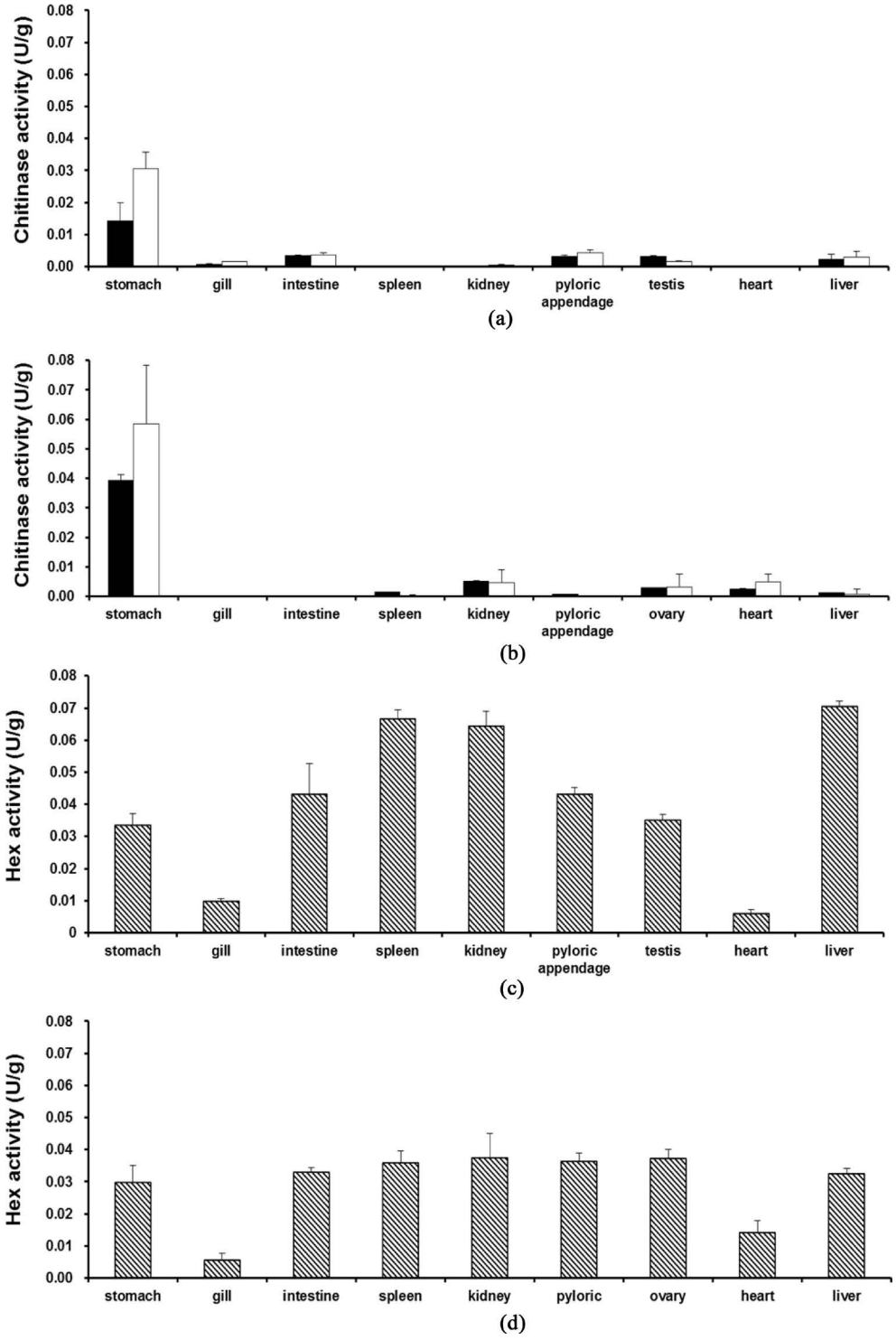


Figure 2. The distribution of the chitinolytic activities in the organs. (a) Chub mackerel; (b) silver croaker; (c) chub mackerel; (d) silver croaker. Results show the average of three individuals. Bars represent the standard deviation. (■) pNP-$(GlcNAc)_2$, (□) pNP-$(GlcNAc)_3$, (▨) pNP-(GlcNAc).

3.2. The Effect of pH on Chitinase Activities

The effect of pH on chitinase activities was determined in the stomach and liver of chub mackerel and the stomach and kidney of silver croaker in order to compare the optimum pH for chitinase activity in the digestive tract and the other organs. Maximum activity was observed at pH of 3.0 against pNP-$(GlcNAc)_2$ and at pH 5.0 against pNP-$(GlcNAc)_3$ in the stomach of chub mackerel, whereas, at pH 7.0, the activity against the two substrates remained less than 35% of the maximum activity. In contrast, the optimum pH was found to be 4.0 against both substrates in the liver of chub mackerel, and approximately 70% of the maximum activity was retained even at pH 7.0. More than 80% of the maximum activity was observed at pH 3.0 to 6.0 against the two substrates in the stomach of silver croaker, whereas the activity reduced to less than 15% of the maximum activity at pH 8.0. Maximum activity was observed at pH 4.0 and 8.0 against both substrates in the kidney (**Figure 3**). For Hex activity, the optimum pH was found to be around 4.0 to 5.0 in both organs of the two species (data not shown).

The acidic optimum pH for chitinase activity observed in the stomachs of the two species, liver of chub mackerel, and kidney of silver croaker was similar to that observed for chitinase isozymes purified from the stomachs of marbled rockfish [17], threeline grunt [28], and silver croaker [29] [30]. However, the presence of a chitinase with novel characteristics that retains its activity at around neutral pH was suggested in the liver of chub mackerel. Some chitinases have double optimum pH values toward long substrate, glycol chitin and/or colloidal chitin, but those chitinase have single optimal pH value toward short substrate used this study, pNP-$(GlcNAc)_2$ and pNP-$(GlcNAc)_3$ [29]-[31]. The optimum pH of 8.0 for chitinase activity observed in the kidney of

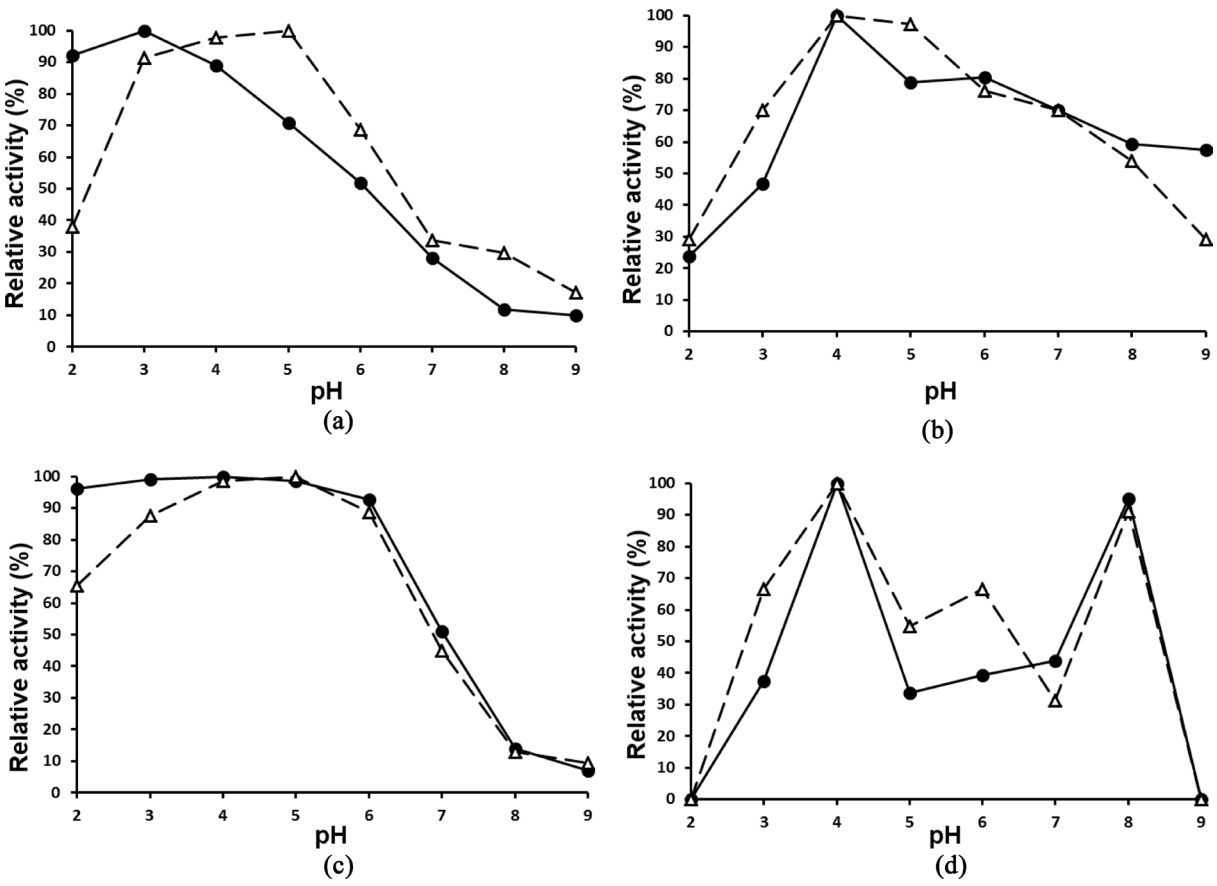


Figure 3. Effect of pH on chitinase activity. The optimum pH when pNP-$(GlcNAc)_2$ and pNP-$(GlcNAc)_3$ were used as the substrate was measured by incubating the enzyme-substrate complex at 37˚C for 20 min in 0.2 M sodium phosphate-0.1 M citric acid buffer (pH 2.0 - 8.0) and 0.1 M glycine + 0.1 M NaCl-0.1 M NaOH buffer (pH 9.0). (●) pNP-$(GlcNAc)_2$, (△) pNP-$(GlcNAc)_3$. (a) Stomach, chub mackerel; (b) liver, chub mackerel; (c) stomach, silver croaker; (d) kidney, silver croaker.

silver croaker was similar to that of 7.0 and 9.0 for chitinase activity in the serum of Nile tilapia [34]. This suggests the presence of a chitinase isozyme in the kidney of silver croaker that acts at pH 8.0, in addition to the chitinase isozyme similar to those expressed in the stomach that act under acidic conditions.

3.3. cDNA Cloning of Chitinases Obtained from Chub Mackerel and Silver Croaker

Two genes of approximately 350 bp were obtained after amplification of the internal sequence of chitinase genes obtained from the stomachs of chub mackerel and silver croaker. Sequence analysis by NCBI Blast revealed high degrees of homology with threeline grunt stomach chitinase genes (*PtChi*-1 and *PtChi*-2) [28] and marbled rockfish stomach chitinase genes (*SmChi*-1 and *SmChi*-2) [17]. Thus, the upstream and downstream regions of chitinase genes were amplified using RACE method. The start and stop codons were found in the upstream and downstream regions, respectively, of all four genes obtained. Full-length cDNAs were then amplified using Platinum®*Pfx* DNA polymerase. Thus, full-length cDNAs of two genes, *SjChi*-1 (1604 bp) and *SjChi*-2 (1512 bp), were obtained from the stomach of chub mackerel, which contained 1422 bp and 1467 bp open reading frames (ORFs), respectively (**Figure 4**). In addition, full-length cDNAs of two genes, *PaChi*-1 (1630 bp) and *PaChi*-2 (1606 bp), were obtained from the stomach of silver croaker, which contained 1446 bp and 1470 bp ORFs, respectively (**Figure 5**). The deduced amino acid sequence revealed that the genes consisted of a signal peptide, GH 18 catalytic domain, linker region, and chitin-binding domain, and the GH 18 catalytic domain contained a sequence unique to the active site of GH family 18 chitinase of vertebrates. Moreover, sections of the amino acid sequence for *SjChi*-1, *PaChi*-1, and *PaChi*-2 were found to match the N-terminus amino acid sequences for SjChi [32], PaChiA [29], and PaChiB [30], respectively, that were previously purified at our laboratory, suggesting that the three genes encoded SjChi, PaChiA, and PaChiB, respectively. DDBJ accession numbers have been obtained for the four full-length genetic sequences: AB686657 for *SjChi*-1, AB689022 for *SjChi*-2, AB605774 for *PaChi*-1, and AB605775 for *PaChi*-2.

3.4. Phylogenetic Analysis of Chitinases from Different Species

Phylogenetic tree analysis was performed based on sequence homology among the amino acid sequences of *SjChi*-1, *SjChi*-2, *PaChi*-2, and *PaChi*-2 as well as those of chitinases from other biological species, where *SjChi*-1 and *SjChi*-2 as well as *PaChi*-1 and *PaChi*-2 were found to be classified into AFCase-1 and AFCase-2, respectively, that include fish stomach chitinases (**Figure 6**). These results strongly support the hypothesis that chitinases from fish form distinct groups (AFCase-1 and AFC-2) [17] [28].

3.5. Expression of *SjChi*-1, *SjChi*-2, *PaChi*-1, and *PaChi*-2 in the Organs

The expression of *SjChi*-1, *SjChi*-2, *PaChi*-1, and *PaChi*-2 was examined in the organs of chub mackerel and silver croaker. *SjChi*-1 was strongly expressed in the stomach of chub mackerel, whereas the expression of *SjChi*-2 was insignificant. This suggests that chub mackerel, which lives in the ocean surface, mainly uses *SjChi*-1 for the degradation of α-chitin, which is a structural component of zooplankton exoskeletons. In addition, the expression of *SjChi*-1 was observed in the pyloric appendage, in which chitinase activity was detected, suggesting that *SjChi*-1 is an enzyme involved in the digestion of chitin in the pyloric appendage. In contrast, both *PaChi*-1 and *PaChi*-2chitinases were found to be strongly expressed in the stomach of silver croaker (**Figure 7**). We previously reported that chitinase isozymes PaChiA and PaChiB, purified from the stomach of silver croaker, possess degradation ability against a broad range of insoluble polymer substrates [29] [30]. Silver croaker, which lives in the sandy, mud ocean floor, likely expresses both chitinase isozymes for the digestion of chitin from diverse biological species, including α-chitin from crustacean such as shrimps and crabs as well as β-chitin from cephalopods such as squid and polychaetes such as ragworm. The weak expression of *PaChi*-1 in the ovaries suggests that the chitinase isozyme *PaChiA* also has a possibility to plays a role in the defense against the entry of nematodes that have chitin in their epidermis.

In the present study, chitinase activity was observed in the organ where the expression of chitinase genes of the two fish species was not detected, suggesting the presence of novel chitinases that are distinct from those encoded by chitinases belonging to AFCase-1 and AFCase-2. This finding, together with the results shown in **Figure 3**, implies that novel chitinase isozymes likely act under acidic and neutral conditions, and that the genes encoding these chitinases are likely involved in the defense mechanisms, as has been reported for chitinase 3 in

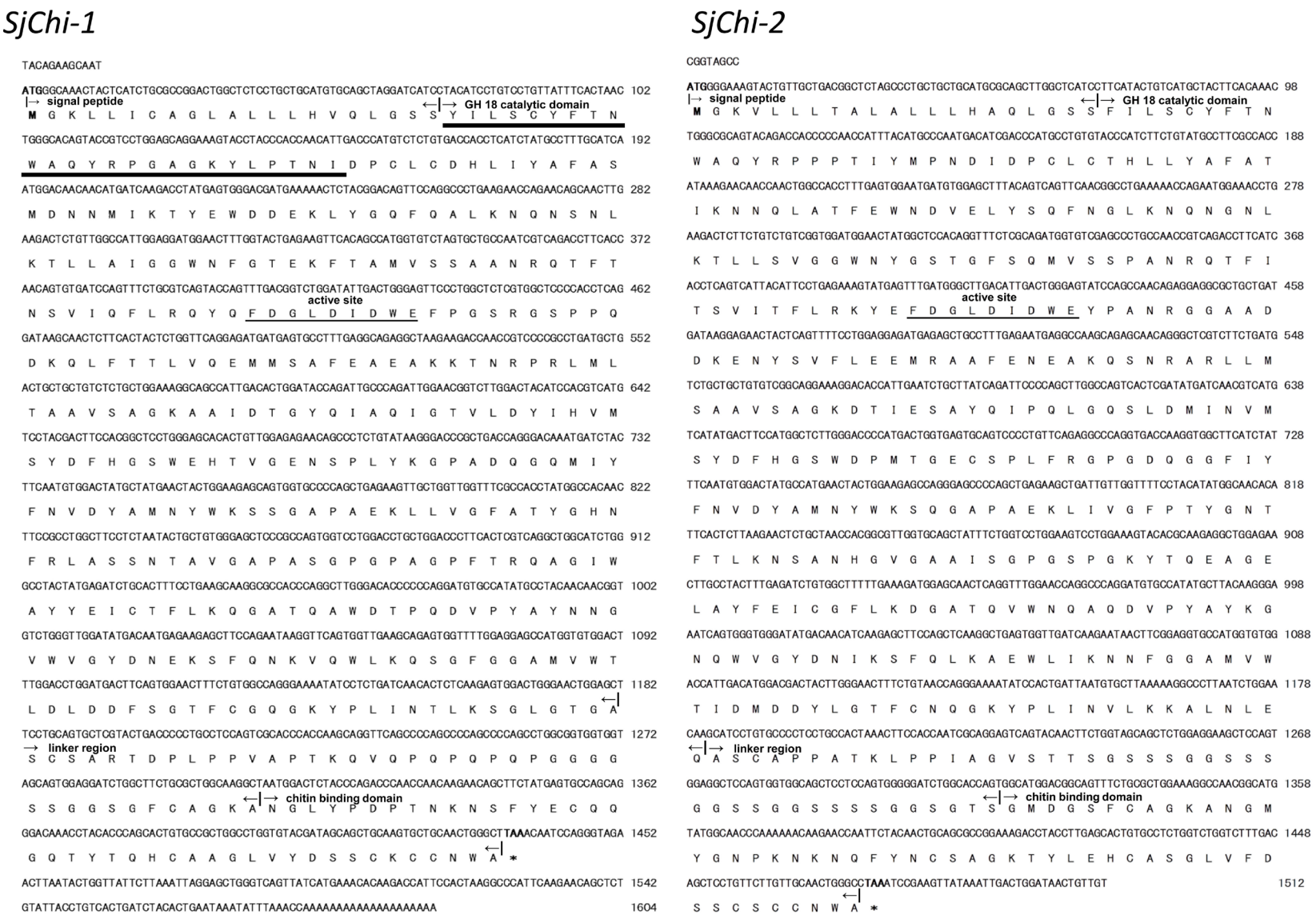


Figure 4. Deduced amino acid sequences and bases for *SjChi*-1 and *SjChi*-2. DDBJ accession Nos.: *SjChi*-1, AB686657; *SjChi*-2, AB689022. Underlined text indicates the N-terminal amino acid sequences of purified SjChi.

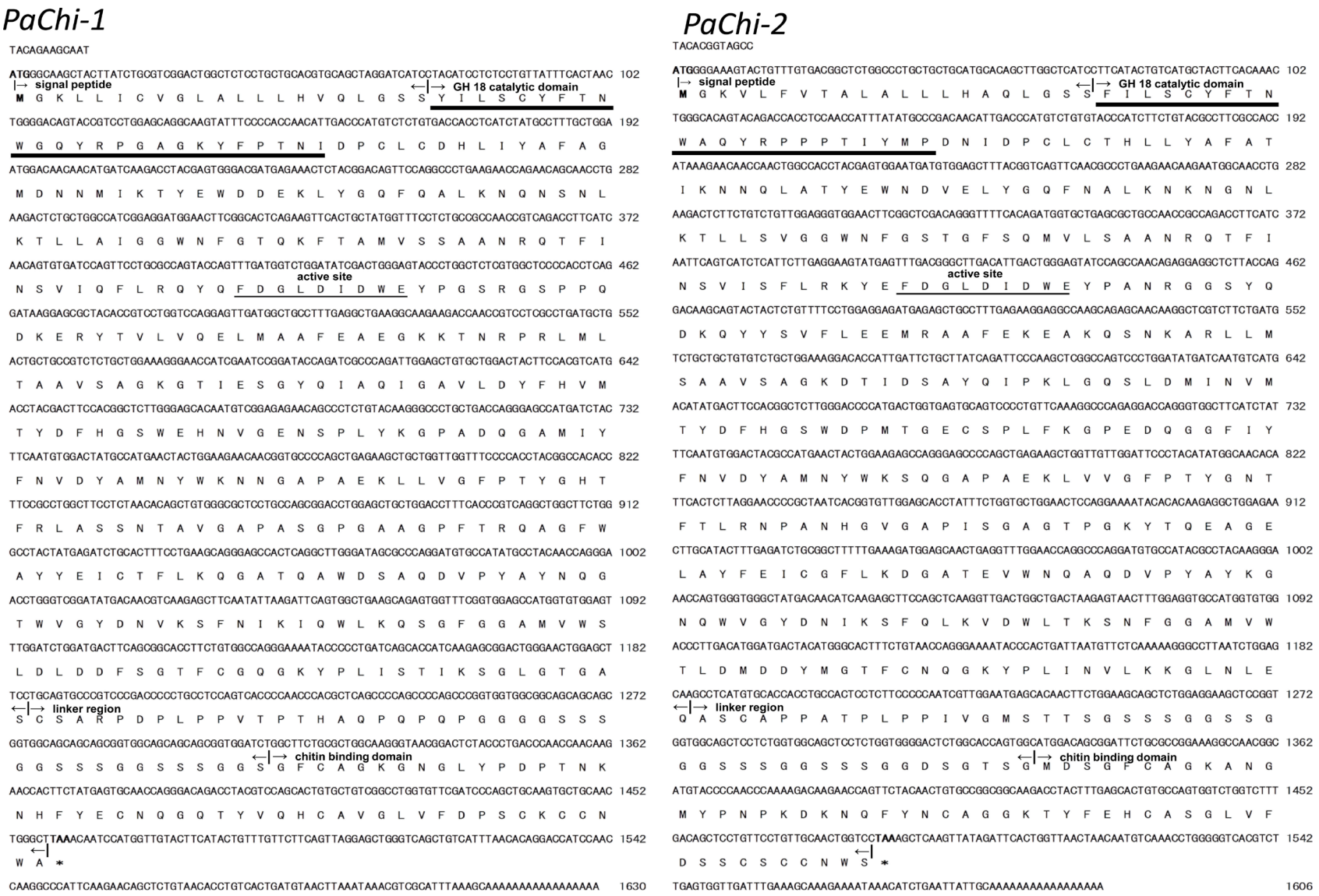
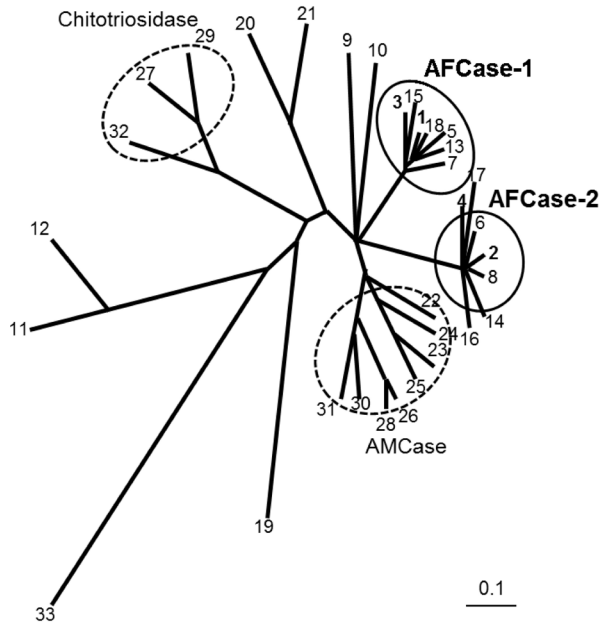


Figure 5. Deduced amino acid sequences and bases for *PaChi*-1 and *PaChi*-2. DDBJ accession Nos.: *PaChi*-1, AB605774; *PaChi*-2, AB605775. Underlined text indicates the N-terminal amino acid sequences of purified PaChiA and PaChiB.



No.	Scientific name	Information	Accession No.
1	***Pennahia argentata***	**Stomach *PaChi1***	**AB605774**
2	***Pennahia argentata***	**Stomach *PaChi2***	**AB605775**
3	***Scomber japonicus***	**Stomach *SjChi1***	**AB686657**
4	***Scomber japonicus***	**Stomach *SjChi2***	**AB689022**
5	*Sebastiscus marmoratus*	Stomach *SmChi1*	AB686658
6	*Sebastiscus marmoratus*	Stomach *SmChi2*	AB686659
7	*Parapristipoma trilineatum*	Stomach *PtChi-1*	AB642677
8	*Parapristipoma trilineatum*	Stomach *PtChi-2*	AB642678
9	*Latimeria chalumnae*	Stomach *LcChi*	AB704869
10	*Prionace glauca*	Stomach *PgChi*	AB872008
11	*Portunus trituberculatus*	Hepatopancreas *PotChi1*	AB874469
12	*Portunus trituberculatus*	Hepatopancreas *PotChi2*	AB890123
13	*Paralichthys olivaceus*	Stomach fChi1	AB121732
14	*Paralichthys olivaceus*	Stomach fChi2	AB121733
15	*Epinephelus coioides*	Chi1	FJ169895
16	*Epinephelus coioides*	Chi2	FJ169894
17	*Pagrus major*	Hepatopancreas Chi2	AB678430
18	*Larimichthys crocea*	Muscle AMCase-like	XP_010745222
19	*Lethenteron japonicum*	Liver	EU741679
20	*Xenopus silurana tropicalis*	Whole body	BC090382
21	*Bufo japonicas*	Pancreatic	AJ345054
22	*Alligator mississippiensis*	AMCase-like	XP_006278821
23	*Gallus gallus*	Grandular stomach	AB071038
24	*Caprimulgus carolinensis*	AMCase	XP_010171779
25	*Cuculus canorus*	AMCase	XP_009560368
26	*Mus musculus*	Stomach AMCase	EF094027
27	*Mus musculus*	Tongue Chitotriosidase	AY458654
28	*Rattus norvegicus*	Stomach AMCase	AY486074
29	*Rattus norvegicus*	Spleen Chitotriosidase	DQ286232
30	*Macaca fascicularis*	AMCase	FJ685619
31	*Homo sapiens*	Stomach Lung	AF290004
32	*Homo sapiens*	Chitotriosidase	U29615
33	*Serratia marcescens*	Bacteria chitinase	X03657

Figure 6. Phylogenetic tree for chitinase amino acid sequences developed using the neighbor joining method in the ClustalW program. Right table lists the scientific name, information and accession No. of chitinase used in phylogenetic tree analysis. The numbers in phylogenetic tree and table correspond each other. A chitinase from *Serratia marcescens* was used as an outgroup. The scale bar indicates the substitution rate per residue.

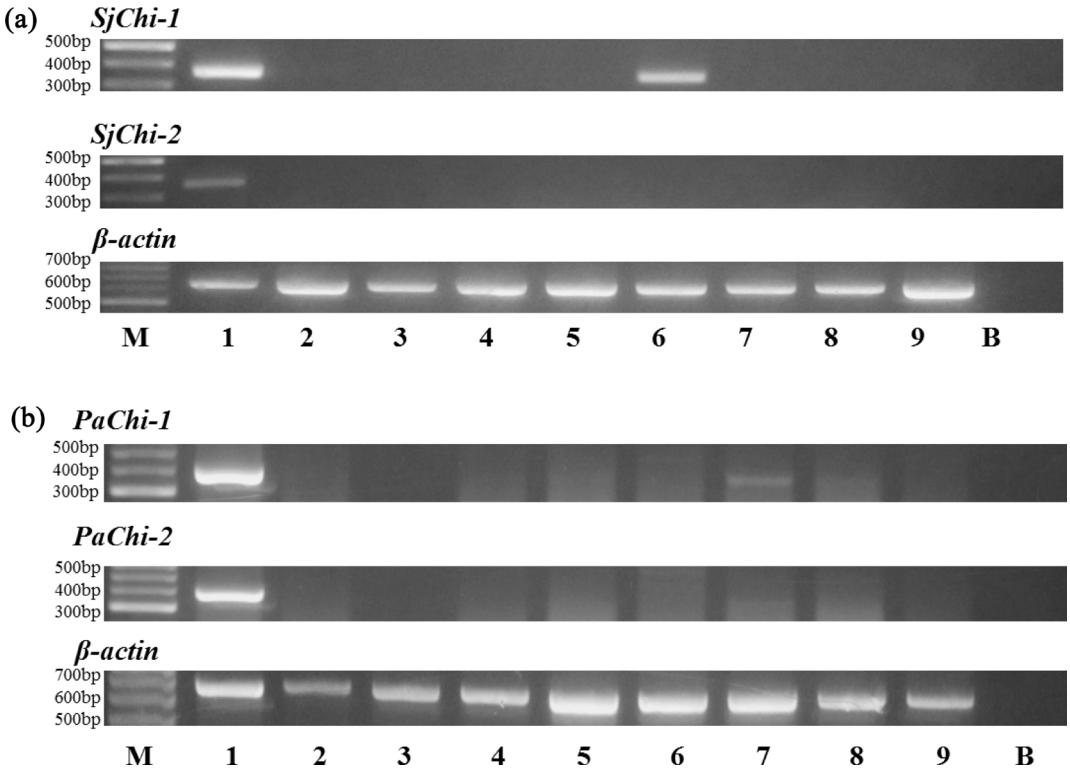


Figure 7. Chitinase and *β*-actin expressions in various tissues (M, marker; 1, stomach; 2, gill; 3, intestine; 4, spleen; 5, kidney; 6, pyloric appendage; 7, ovaries; 8, heart; 9, liver; B, blank). (a) Chub mackerel; (b) silver croaker.

flounders [35] and chitotriosidase in the lungs of mammals [14]. cDNA cloning and expression analysis of novel chitinase genes are currently in progress.

4. Conclusion

The distribution patterns of chitinolytic enzymes in chub mackerel and silver croaker revealed, to our knowledge, for the first time that chitinase activities are broadly distributed in the organs of the two species, in addition to the digestive tract, including the stomach. Measurement of optimum pH for chitinase activity in chub mackerel and silver croaker revealed the presence of a chitinase that shows maximum activity at pH 4.0 and 70% of the maximum activity at pH 7.0 in the liver of chub mackerel, and a chitinase that shows maximum activity at pH 4.0 and 8.0 in the kidney of silver croaker. This suggests the presence of novel chitinases in the two fish species that act at around neutral pH, in addition to enzymes that are analogous to the previously reported fish stomach chitinases that have the optimum pH in the range of 3.0 to 5.0. Full-length cDNAs encoding the two chitinase genes for each species, *SjChi*1 and *SjChi*2, as well as *PaChi*1 and *PaChi*2, were obtained from the stomachs of the two species; these genes belong to AFCase-1 and AFCase-2, respectively. Expression analysis of these chitinase genes in the organs of the two fish species showed significant differences in the expression pattern in the stomachs, which was likely attributed to the difference in the feeding habits between the two species. In addition, the expression of these genes was observed only in some of the organs in which chitinase activity was detected, suggesting the presence of novel chitinases that are distinct from those encoded by genes belonging to AFCase-1 and AFCase-2.

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