Advances in Bioscience and Biotechnology, 2018, 9, 36-51
http://www.scirp.org/journal/abb
ISSN Online: 2156-8502
ISSN Print: 2156-8456

Distribution of Chitinolytic Enzyme in the Organs and Molecular Cloning of a Novel Chitinase Gene from the Kidney of Marbled Rockfish *Sebastiscus marmoratus*

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How to cite this paper: Watanabe, M., Kakizaki, H., Tsukamoto, T., Fujiwara, M., Fukushima, H., Ueda, M. and Matsumiya, M. (2018) Distribution of Chitinolytic Enzyme in the Organs and Molecular Cloning of a Novel Chitinase Gene from the Kidney of Marbled Rockfish *Sebastiscus marmoratus*. *Advances in Bioscience and Biotechnology*, **9**, 36-51.
https://doi.org/10.4236/abb.2018.91004

Received: December 25, 2017
Accepted: January 27, 2018
Published: January 30, 2018

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Abstract

Actinopterygii express two types of chitinase (acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2)) that are active at acidic pHs and involved in digestion in the stomach. We proposed the existence of a new fish chitinase that has a non-digestive function. In this study, we used *Sebastiscus marmoratus*, for which characteristics and cDNA cloning of chitinase isozymes (SmChi-1, SmChi-2) in the stomach have been reported. Initially, we examined the distribution of chitinase and β-*N*-acetylhexosaminidase (Hex) in the body and then we tried to clone novel chitinase cDNA from the kidney. Chitinase and Hex activities were measured using *p*NP-(GlcNAc)$_n$, (n = 2, 3) and *p*NP-GlcNAc as substrates, respectively. Total RNA was extracted from the kidney. RT-PCR was performed to obtain chitinase cDNA fragments using reverse transcriptase with an oligo dT primer. The RACE method was used to obtain sequences of the upstream and downstream regions of cDNA. The full-length chitinase cDNA was determined using PrimeSTAR® Max DNA polymerase with proofreading activity. High chitinase activity was observed in the stomach, as previously reported. In addition, relatively high activity was observed in the liver, spleen, kidney, and heart. In contrast, Hex activity was detected in all organs. This result is consistent with the report that Hex is related to body-wide metabolism. Full-length cDNA (*SmChi*-3) of the novel chitinase was obtained from the kidney, which contained 1440 bp open reading frames. The domain structure of SmChi-3 was assumed to be similar to those of SmChi-1 and SmChi-2. SmChi-1 and SmChi-2 have a serine and glycine-rich linker region, which is characteristic of AMCase. In contrast, Sm-

Chi-3 contained no apparent sequence in the linker region. Phylogenetic analysis revealed the existence of a new chitinase group, which was named fish chitinase-3 (FCase-3) and differed from AFCase-1 and AFCase-2.

Keywords

Chitinolytic Enzyme, Chitinase, *Sebastiscus marmoratus*, Distribution, cDNA Cloning

1. Introduction

Chitin, a *β*-1,4-linked aminopolysaccharide of *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant component of biomass in the world, after cellulose, and is found in the exoskeletons of arthropods, the cell walls of fungi, and the cuticles of nematodes [1] [2] [3]. In recent years, byproducts degraded from chitin have been shown to have various biological activities. For example, chitin oligosaccharides $(GlcNAc)_n$, have been found to promote the growth of bifidobacteria, have immunostimulatory activity [4], and GlcNAc has been found to improve osteoarthritis [5] and dry skin [6].

Chitinolytic enzymes can be classified into the following two categories according to their degradation patterns: Endo-type chitinolytic enzymes, which degrade *β*-1,4-glycosidic bonds in a chitin polymer at random to produce chitin oligosaccharides $(GlcNAc)_n$ and are called chitinases (EC 3.2.1.14) [7] [8] [9]; and exo-type chitinolytic enzymes, which degrade a chitin polymer from the nonreducing end, one by one, to produce GlcNAc and are called *β*-*N*-acetylhexosaminidase (Hex) (EC 3.2.1.52) [8] [9] [10]. Chitinases have been found in various organisms including mammals [11] [12] [13], fish [14] [15] [16] [17], mollusks [18] [19], insects [20] [21], plants [22] [23] [24], and fungi [25] [26] [27] and play important physiological roles in digestion [11] [14] [15] [16] [17], defense [12] [13] [22], aggression [18] [19], and ecdysis [18], in each of these organisms. Chitinases are classified into glycoside hydrolase (GH) families 18 and 19 on the basis of homology of amino acid sequences [27] and catalytic mechanisms [28] [29]. Family 18 chitinases are widely found in animals, plants, microorganisms, etc. [20]. Family 19 chitinases, on the other hand, are found primarily in higher plants and are reported to have strong antibacterial properties [20] [23].

We have conducted basic research about chitinases in several aquatic organisms. In the process of our research, we purified chitinase isozymes from fish stomachs, which are active at acidic pHs and are involved in digestion, and we investigated their properties [14] [15] [17]. We also have shown, through phylogenetic analysis based on amino acid sequences, which fish stomach chitinases form two fish-specific groups: acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2) [30]. Furthermore, in fish, chitinase activity has been

observed not only in the digestive tract, but also in other organs [31]. Also, an acidic mammalian chitinase (AMCase) was found in mammals, which are vertebrates like fish, and this AMCase is involved in digestion and absorption of food [11]. In addition, Chitotriosidase, a chitinase produced by macrophages that plays a role in defense against pathogens, was found in mammals [12] [13]. Based on these findings, we predicted that additional chitinases existed in fish and that these new chitinases would have physiological roles other than digestion, like their counterparts in mammals.

In this study, we used marbled rockfish *Sebastiscus marmoratus*, as a model species. Our group had previously purified three chitinase isozymes, SmChiA, SmChiB, and SmChiC from the stomach of *S. marmoratus*. These chitinases showed the optimum pH in the acidic region (pH 2.0 - 4.5). SmChiA and SmChiB preferentially degrades the second glycosidic bond from the non-reducing end of $(GlcNAc)_n$ and SmChiC preferentially degrade the third glycosidic bond. *SmChi*-1 encoding SmChiA and SmChiB, and *SmChi*-2 encoding SmChiC were cloned. *SmChi*-1 and *SmChi*-2 were classified into AFCase-1 and AFCase-2, respectively [14]. In this study, we measured chitinase activity and Hex activity to search for the presence of new chitinases in *S. marmoratus* using crude enzyme solutions of organs. Also, we examined the effect of pH on chitinase activity using crude enzymes prepared from the kidney and stomach. Furthermore, we tried to obtain full-length genes of a new chitinase, different from *SmChi*-1 and *SmChi*-2, from the kidney of *S. marmoratus* using RT-PCR methods. Finally, we conducted phylogenetic analyses with data obtained in this study and data of family 18 chitinases that have been entered in a database to clarify phylogenetic relations among fish chitinases.

2. Materials and Methods

2.1. Material

The marbled rockfish *S. marmoratus* was purchased from a fresh fish shop of Kanagawa ($n = 3$; average standard length = 25 cm; average body weight = 275 g). *p*-Nitrophenyl Di-*N*-Acetyl-*β*-chitobioside (pNP-$(GlcNAc)_2$) and *p*-Nitrophenyl Tri-*N*-Acetyl-*β*-chitobioside (pNP-$(GlcNAc)_3$) were purchased from Seikagaku corporation (Tokyo, Japan). *p*-Nitrophenyl-*N*-Acetyl-*β*-D-glucosaminide (*p*NP-GlcNAc) was kindly supplied by Yaizu Suisankagaku Industry Co., Ltd. (Shizuoka, Japan).

2.2. Measurement of Chitinolytic Enzyme Activity

All organs (0.5 g) from *S. marmoratus* were homogenized in 3 volumes of 20 mM phosphate buffer (pH 7.3), and the homogenate was centrifuged at 9000× g for 20 min 4˚C. Chitinase assays were conducted using the method of Ohtakara [26], with slight modification. The assays were completed using pNP-$(GlcNAc)_n$, ($n = 1 - 3$) as the substrate. The crude enzyme solutions (12.5 µl) and 5 µl of 4 mM pNP-$(GlcNAc)_n$ were added to 12.5 µl of 0.2 M phosphate-0.1 M citrate

buffer (pH 6.0). The mixture was incubated at 37˚C for 20 min, and the reaction was stopped by adding 130 μl of 0.2 M sodium carbonate solution and the absorbance of the *p*-nitrophenol released was measured at 420 nm in a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that was liberated by 1 μmol of *p*NP per minute and was expressed as the activity per gram of the organs. The results are the mean of 3 replicates ± standard deviation.

2.3. Effect of pH on Chitinase Activity

The effect of pH on chitinase activity was determined from the crude enzyme solutions obtained from the kidney and stomach of *S. marmoratus*. Chitinase activity was measured in a buffer solution with a pH between 2.0 to 8.0 by the method of distribution of chitinolytic enzymes activity, with 4 mM *p*NP-$(GlcNAc)_2$ as the substrate.

2.4. Molecular Cloning of a Novel Chitinase Gene from the Kidney

Total RNA was extracted from the kidney of *S. marmoratus* using ISOGEN II (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Total RNA concentrations and purity were measured using the NanoVue spectrophotometer (GE Healthcare, Little Chalfont, UK). The first strand of cDNA was synthesized using 1.0 μg total RNA and oligo dT primers (**Table 1**) with PrimeScript™ II Reverse Transcriptase (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. For the reverse transcriptase-polymerase chain reaction (RT-PCR), three degenerate primers were designed from conserved sequences of the GH family 18 chitinases of vertebrates and internal sequence amplification was performed using GoTaq® Green Master Mix (Promega, Madison, USA). The primers used in this study are shown in **Table 1**. The first PCR was performed using cDNA as a template, and Chi3 F1 and Chi3 R as primers. The PCR parameters were 30 cycles of denaturation at 95˚C for 30 s, annealing at 50˚C for 30 s, and extension at 72˚C for 30 s. Nested PCR was performed using the products of the first PCR as templates, and Chi3 F2 and Chi3 R as primers, with the same PCR parameters as described above. cDNA fragments encoding the 3′region of *SmChi*-3 were amplified using *S. marmoratus* cDNA as the template and the primer pairs Chi3 3′F1 and 3′RACE, and Chi3 3′F2 and 3′RACE. The PCR parameters were 30 cycles of denaturation at 95˚C for 30 s, annealing at 50˚C for 30 s, and extension at 72˚C for 60 s. The 5′RACE was performed using the 5′-Full RACE Core Set (Takara Bio) according to the manufacturer's instructions. Full length *SmChi*-3 was performed by PCR using specific primers (Chi3 full F and Chi3 full R) and PrimeSTAR® Max DNA Polymerase (Takara Bio) with proofreading activity. The PCR products were resolved on a 2% agarose gel electrophoresis and purified using the Quantum Prep® Freeze 'N Squeeze spin columns (Bio Rad, Hercules, USA). We subcloned all of the PCR products into pGEM-T Easy Vector (Promega) according to the manufacturer's instructions.

Table 1. Primers used in this study.

Primer name	Sequence (5′-3′)	Length	Usage	Annealing temperature (˚C)
Oligo dT	CTGTGAATGCTGCGACTACGATTTTTTTTTTTTTTTTTTT	40 mer	cDNA synthesis	
Chi3 F1 (F)	TGYTAYTTYACNAAYTGG	19 mer	Conserved region PCR	50.0
Chi3 F2 (F)	GAYATHGAYTGGGARTAYCC	19 mer		
Chi3 R (R)	TTCCARTARTTCATNGCRTARTC	23 mer		
3′RACE (R)	CTGTGAATGCGACTACGAT	19 mer	3′RACE PCR	50.0
Chi3 3′F1 (F)	AAGAGGAAGTCCTCTGGAGG	20 mer		
Chi3 3′F2 (F)	GCTGAGGGAACAGCAACTGG	20 mer		
Chi3 5′R1 (R)	GCAGCGGAGACCATTAATC	19 mer	5′RACE PCR	50.0
Chi3 5′R2 (R)	CCAGTTGCTGTTCCCTCA	18 mer		
Chi3 5′F1 (F)	AGGAACCATTGATGCTGGTT	20 mer		
Chi3 5′F2 (F)	TGGCAAAGTACCTGGACTT	19 mer		
Chi3 5′F3 (F)	CGTGATGACGTATGACTTTCATG	23 mer		
Chi3 5′P	GTGATGTCCTGTCACGCT	18 mer		
Chi3 full F (F)	GACATGACAAGAGACCATCCAA	22 mer	Full length amplification	55.0
Chi3 full R (R)	ACAGAGCATTTCAAACAGAGGG	22 mer		
β-actin (F)	GATCATGTTCGAGACCTTCAACAC	24 mer	Organ expression	50.0
β-actin (R)	TCCAATCCAGACAGAGTATTTAGC	24 mer		
SmChi-1 (F)	ACTAGCGTGATCAAGTTCC	19 mer		
SmChi-1 (R)	ACAGCTCCAATCGCGGA	17 mer		
SmChi-2 (F)	CTCAGTCATTTAATTCCTGA	20 mer		
SmChi-2 (R)	CCTGGCCAAGCTTGGGAAT	19 mer		
SmChi-3 (F)	CAGTCTTCTGTCAACCTAC	19 mer		
SmChi-3 (R)	GGTACTTTGCCATCTCTGC	19 mer		

The full-length amplification products of *SmChi*-3 carried out A-tailing, and were subcloned into pGEM-T Easy vector. Inserts were sequenced using the Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Waltham, USA).

2.5. Phylogenetic Analysis of SmChi-3

Phylogenetic analysis of chitinase from several organisms was carried out using the ClustalW2 program (EMBL-EBI: The European Bioinformatics Institute, European Molecular Biology Laboratory, Hinxton, England) and the tree view program. A bacterial chitinase (GenBank: X03657) was used as the outgroup.

2.6. Organ-Specific Genes Expressions

Total RNA was prepared for all organs from *S. marmoratus*. First-strand cDNA was synthesized using each total RNA (0.5 μg) and an oligo dT primer, amplified using RT-PCR on the synthesized cDNA (1.0 μg). *SmChi*-1, *SmChi*-2, and *Sm*-

Chi-3, 250 bp gene fragments, were amplified using the first-strand cDNA as the template and the primer pairs *SmChi*-1 (F) and *SmChi*-1 (R) for *SmChi*-1, *SmChi*-2 (F) and *SmChi*-2 (R) for *SmChi*-2, and *SmChi*-3 (F) and *SmChi*-3 (R) for *SmChi*-3 (**Table 1**). To determine the amount of total RNA in each organ, *β*-actin mRNA fragments were amplified using specific primer pairs (**Table 1**). The PCR parameters were 30 cycles of denaturation at 95˚C for 30 s, annealing at 50˚C for 30 s, and extension at 72˚C for 20 s.

3. Results and Discussion

3.1. Distribution of Chitinolytic Enzyme

Measurement of activity of chitinolytic enzymes in organs of *S. marmoratus* (**Figure 1**) revealed that the chitinase activity assayed using *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$ as substrates agreed with results that were reported previously [32], in which glycol chitin was used as a substrate and had the highest value in the stomach. In addition, relatively high activity also was observed in organs other than the digestive tract, including the liver, which is involved in metabolism and detoxification and contains much blood; the spleen, which is involved in blood production and storage; the kidney, which is involved in metabolism and hormone secretion; and the heart, which is involved in blood circulation. These

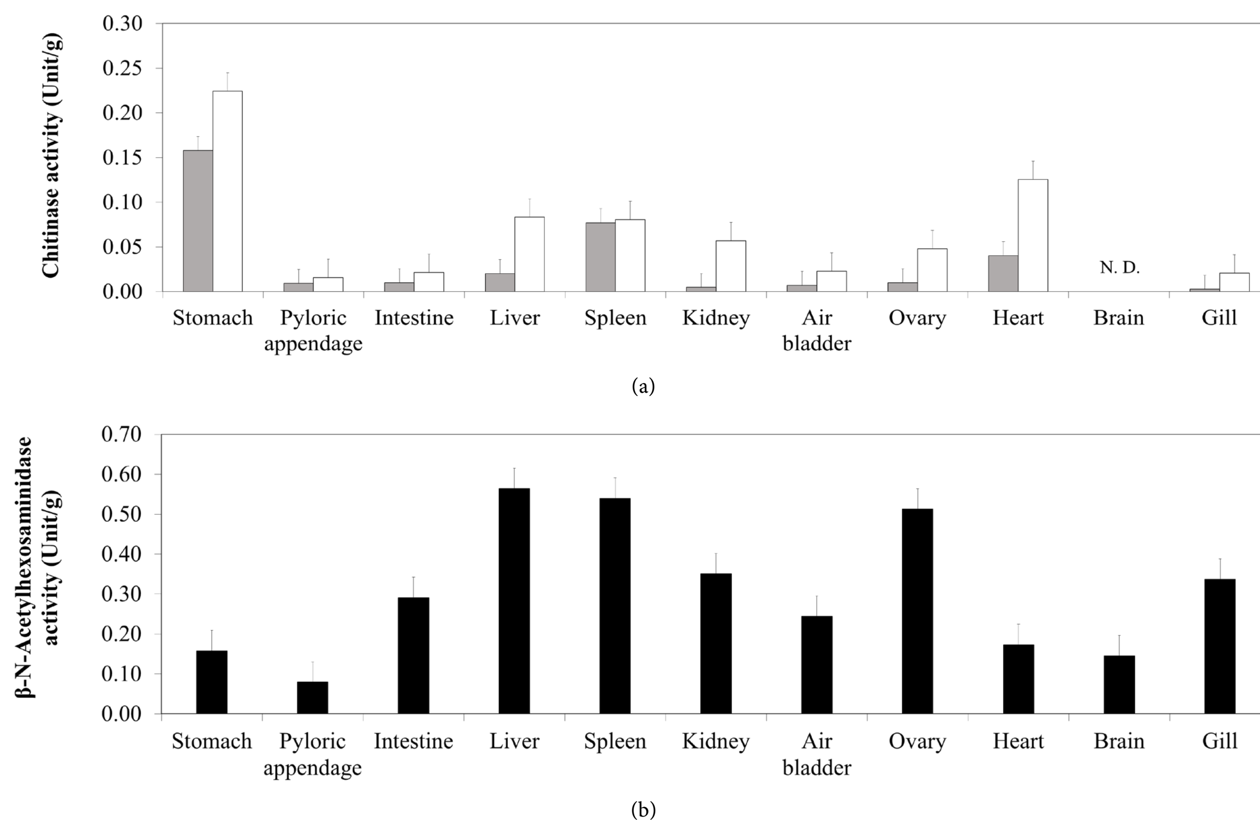

Figure 1. The distribution of the chitinolytic activities in the organs of *S. marmoratus*. (a): Chitinase activity, (b): *β*-*N*-Acetylhexosaminidase activity, ■: *p*NP-(GlcNAc), ■: *p*NP-$(GlcNAc)_2$, □: *p*NP-$(GlcNAc)_3$. Bars represent the standard deviation ($n = 3$).

findings suggested a possibility that chitinases in fish have physiological roles in several, non-digestive organs, as well as having a role in the digestion of ingested chitinous substances in the stomach. In addition, while the ratio of the degradation ability for *p*NP-$(GlcNAc)_2$ to that for *p*NP-$(GlcNAc)_3$ in the stomach was approximately 1:1.5, the ratio in the kidney was 1:12.5, which considerably differed from that in the stomach. This finding suggested the presence of chitinases in the kidney that degrade *p*NP-$(GlcNAc)_3$ better than *p*NP-$(GlcNAc)_2$, such as SmChiC in the stomach of *S. marmoratus* [14]. Hex activity measured at the same time had high values in the spleen, kidney, and heart. In fish, Hex activity in digestive organs has been thought to play a role in the degradation of $(GlcNAc)_n$ into GlcNAc, produced via the effect of chitinases in the stomach [31]. It has been reported that fish contain chitinous substances in their scales [33], have chitin synthases [34], and have a mechanism of carbohydrate metabolism [35]. From these findings, we thought that after ingested chitin was degraded to $(GlcNAc)_n$, by chitinases in the stomach, *S. marmoratus* would degrade $(GlcNAc)_n$ into GlcNAc with chitinases and Hex, which would allow for the metabolism and use of $(GlcNAc)_n$ that had been absorbed in the intestines and transferred to the bloodstream.

3.2. Effect of pH on Chitinase Activity

Chitinase activity of crude enzymes obtained from the stomach and kidney of *S. marmoratus* was measured at each pH using *p*NP-$(GlcNAc)_2$ as a substrate (**Figure 2**). The stomach chitinase activity was greatest at pH 2.5, but declined to 55% of the maximum at pH 5.0, and to 15% at pH 7.0. In nature, the optimum pH for chitinase activity has been shown to be 2.5, which was similar to that for chitinases obtained from the stomachs of fish including *S. marmoratus* [14] [15] [17] and to a property of AMCase [11]. On the other hand, the activity of the crude enzyme of the kidney peaked at pH 5.0 and 40% of the maximum at pH 7.0. This was similar to a property of chitotriosidase, which for which the optimum pH is 5.0 [36]. The optimum pH for chitinase contained in the kidney was

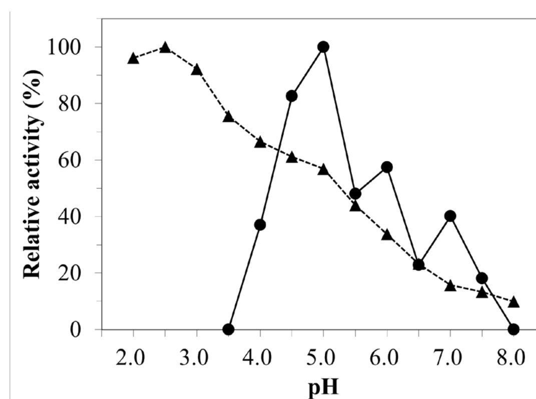

Figure 2. Effect of pH on chitinase activities in the kidney (●) and stomach (▲) of *S. marmoratus*. Substrate: *p*NP-$(GlcNAc)_2$.

slightly more neutral than the optimum pH of the stomach chitinase and this finding suggested that the chitinase activity detected in the kidney was attributable to the effect of a new chitinase that was different from the stomach chitinase. Chitinases have been obtained from fish organs other than those of the digestive system; for example, previous studies have reported the activity of chitinase in the blood serum of *Salmo gairdneni* [37] and a 75 kDa chitinase obtained from the blood serum of freshwater, which has an optimum pH of 7.0 and considerable degradation capacity for $4MU(GlcNAc)_2$ rather than $4MU(GlcNAc)_3$, which was used as a substrate [38]. Although the kidneys of *S. marmoratus* are also non-digestive organs, the ability of the kidney chitinase of *S. marmoratus* to degrade substrates and the optimum pH differed from those of the chitinase of the plasma of *Oreochromis niloticus*. These results demonstrated the possibility that the chitinase of the kidney of *S. marmoratus* is a new chitinase isozyme different from chitinases contained in the blood serum of fish.

3.3. Molecular Cloning of *SmChi*-3

As a result of amplification of internal sequences of a gene of a new chitinase obtained from the kidney of *S. marmoratus*, 350 bp gene fragments were obtained. Analysis of base sequences of the gene fragments, conducted by the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI Blast), showed 94% homology with *fChi*3 of *Paralichthys olivaceus*. After this analysis, unknown regions of the upstream and downstream regions of the chitinase obtained from the kidneys of *S. marmoratus* were amplified using the RACE method. As a result, initiation codons and termination codons were found in the upstream regions and the downstream regions, respectively. Full-length genes of the chitinase obtained from the kidney of *S. marmoratus* were amplified using a sense primer and an antisense primer (designed with upstream and downstream untranslated regions (UTRs) in mind, respectively) and an enzyme with proofreading activity. As a result, a 1618 bp full-length gene of the chitinase obtained from the kidney of *S. marmoratus* (*SmChi*-3) was obtained. This gene contained a 1440 bp open reading frame (ORF) encoding 480 amino acids (**Figure 3**). The base sequence of *SmChi*-3 and some pieces of information were registered with the database of DDBJ and an accession number (LC077733) was issued.

Molecular weight of *SmChi*-3 was estimated at 51 kDa based on its deduced amino acid sequence (SmChi-3) using the Compute pI/Mw tool of the Expert Protein Analysis System (ExPASy, Lausanne, Switzerland). This value is most similar to that of SmChi-1 (AB686658; 49 kDa), SmChi-2 (AB686659; 52 kDa), and chitotriosidase (51 kDa) obtained from a mammal (*Rattus norvegicus*), which were estimated using the same method. This value was considerably lower than the value of 75 kDa for the chitinase obtained from blood serum of *O. niloticus* [38]. **Figure 4** shows SmChi-3, two chitinases obtained from the stomach of *S. marmoratus* (SmChi-1 and SmChi-2) [14], and the deduced amino acid

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GACATGACAAGAGACCATCCAAACGCAACCACC
ATGAGCAGGTTAATTCTAATTACAGGTCTCTGTCTTTCCTTTGGCAGTTTGGTGTCATCCTCCAGACTGATGTGT   75
 M  S  R  L  I  L  I  T  G  L  C  L  S  F  G  S  L  V  S  S  S  R  L  M  C
TATTTCACTAACTGGTCCCAATACCGACCTGGCAATGGGAAGTTTATGCCTCTAAATATTGATCCAAACCTGTGT   150
 Y  F  T  N  W  S  Q  Y  R  P  G  N  G  K  F  M  P  L  N  I  D  P  N  L  C
ACCCACCTGATCTATGCCTTTGCTGGTATTAACGAGGCAAATGAGTTGGTCACCATAGAATGGAATGATGATGTA   225
 T  H  L  I  Y  A  F  A  G  I  N  E  A  N  E  L  V  T  I  E  W  N  D  D  V
CTCTATAAATCCTTTAATGGACTCAAACAGAGGAATCCGAATCTTAAAACACTATTGGCTGTCGGAGGCTGGAAC   300
 L  Y  K  S  F  N  G  L  K  Q  R  N  P  N  L  K  T  L  L  A  V  G  G  W  N
TTTGGAACACAAAAGTTCACAACAATGGTGTCAACACAAGCCAACCGAAATGCATTTATCCAGTCTTCTGTCAAC   375
 F  G  T  Q  K  F  T  T  M  V  S  T  Q  A  N  R  N  A  F  I  Q  S  S  V  N
CTACTGAGAAAATATAGTTTTGATGGACTTGATTTGGACTGGGAATACCCCAGTTCAAGAGGAAGTCCTCTGGAG   450
 L  L  R  K  Y  S  F  D  G  L  D  L  D  W  E  Y  P  S  S  R  G  S  P  L  E
GACAAGCAGAGATTCACGGTGTTATGCAAGGAACTCCTAGAGGCCTATCAGGCTGAGGGAACAGCAACTGGCCGG   525
 D  K  Q  R  F  T  V  L  C  K  E  L  L  E  A  Y  Q  A  E  G  T  A  T  G  R
CCCAGATTAATGGTCTCCGCTGCTGTGGCGGCTGGGAAAGGAACCATTGATGCTGGTTATGAAATTGCAGAGATG   600
 P  R  L  M  V  S  A  A  V  A  A  G  K  G  T  I  D  A  G  Y  E  I  A  E  M
GCAAAGTACCTGGACTTTATTAACGTGATGACGTATGACTTTCATGGCACCTGGGAGAGCGTGACAGGACATCAC   675
 A  K  Y  L  D  F  I  N  V  M  T  Y  D  F  H  G  T  W  E  S  V  T  G  H  H
AGCCCCTTATACAACGGATCCCATGACACCGGGGACCATGTCACCTTAAACACTGACTTTGCCATGAGATATTGG   750
 S  P  L  Y  N  G  S  H  D  T  G  D  H  V  T  L  N  T  D  F  A  M  R  Y  W
CGGGACAAGGGAACACCTGTAGAAAAGCTAAATATGGGCTTTGCTACATACGGGAGAGCATTCCGACTGTCTACT   825
 R  D  K  G  T  P  V  E  K  L  N  M  G  F  A  T  Y  G  R  A  F  R  L  S  T
CAATCCTCTGAGGTTGGAGCACCAACCAGTGGTGCCGCTGCTGCTGGTGTTTTTACAAGGGAGGCCGGCTTCCGG   900
 Q  S  S  E  V  G  A  P  T  S  G  A  A  A  A  G  V  F  T  R  E  A  G  F  R
TCCTATTATGAGATTTGCACTTTTCTTCAAGGGGCCAGTGTCCACCTGATTGAGGATCAGAAAGTTCCATATGCG   975
 S  Y  Y  E  I  C  T  F  L  Q  G  A  S  V  H  L  I  E  D  Q  K  V  P  Y  A
ATCAAACTAAATGAGTGGGTTGGATATGACAACAAAGAAAGTTTTGAGACTAAGGTCCGTTACCTAAAGGAGAAC   1050
 I  K  L  N  E  W  V  G  Y  D  N  K  E  S  F  E  T  K  V  R  Y  L  K  E  N
AGATTTGGAGGAGCTTTCGTCTGGACTCTGGATCTGGATGACTTTAAGGGACAGTACTGCGGACAAGGGAACTAC   1125
 R  F  G  G  A  F  V  W  T  L  D  L  D  D  F  K  G  Q  Y  C  G  Q  G  N  Y
CCCCTCATCAGCTATCTCCGCTCTCTTGTAGCCCCAGATCTTCCTGCCCTTCCCACTACAGACACCACCCCAGAT   1200
 P  L  I  S  Y  L  R  S  L  V  A  P  D  L  P  A  L  P  T  T  D  T  T  P  D
CAAGTGACTCCATCTACAAACATAGATCAACCTGACACCACATCTCGCCCCAGACCAGCAATCACTACTTCATCC   1275
 Q  V  T  P  S  T  N  I  D  Q  P  D  T  T  S  R  P  R  P  A  I  T  T  S  S
AATATCCCGGACAACTTCTGTGCTACAAAGGCTGGTGGCATTTATGCCAAACCTGATGCCCCAGGTTCCTTTTAC   1350
 N  I  P  D  N  F  C  A  T  K  A  G  G  I  Y  A  K  P  D  A  P  G  S  F  Y
AGTTGTGCCAACGGCATCACCTGGGTCCTAAACTGCCCAGCTAATTTGATCTTTCAGGATAGCTGCAAATGCTGT   1425
 S  C  A  N  G  I  T  W  V  L  N  C  P  A  N  L  I  F  Q  D  S  C  K  C  C
AACTGGCCTAAATTGTAGTTATCAACAGTGAGCAAAAGTAATTCTTGTTGATTTACAATTTTAAGTTAAAGGGGA   1443
 N  W  P  K  L  *
CCTATTATGCTTATTTTCAGGTGCATACTTCTTGTATTTGGGCTTTTCTTTTCTCATACCGGCTGTGCTGCAGTA
CCTCTTTCA
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Figure 3. Deduced amino acid sequences and bases for *SmChi*-3 from the kidney of *S. marmoratus*. DDBJ accession No. LC077733.

sequences of genes, corresponding to Chi-3, of four varieties of fish and their predicted domain structures for comparison. SmChi-1, SmChi-2, and SmChi-3 consisted of N-terminal signal peptides, a catalytic domain, a chitinase insertion domain, a linker region, and a chitin binding domain, and the catalytic domain contained a sequence peculiar to the active site of GH family 18 chitinase, DXXDXDXE [39]. While SmChi-1 and SmChi-2, like AMCase [13], have repeated sequences of turn/coil-forming amino acids serine and glycine in the linker region, SmChi-3 does not have such characteristic repeated sequences. The same was true for fChi3 of *P. olivaceus* [16], CHIT3 of *Scophthalmus maximus* [40], and Chi-3 of *Danio rerio* [41]. Furthermore, comparison of Chi-3 of *S. marmoratus*, *Hexagrammos otakii*, *Sardinops melanostictus*, *P. olivaceus* [16], and *Thunnus orientalis* showed homology of 45% or more probability among the five varieties of fish. These results suggested the possibility that a *Chi*-3 gene ubiquitously exists in fish.

3.4. Phylogenetic Analysis of SmChi-3

Phylogenetic analysis of SmChi-3 was conducted on the basis of homology of deduced amino acid sequences of family 18 chitinases of other vertebrates and of

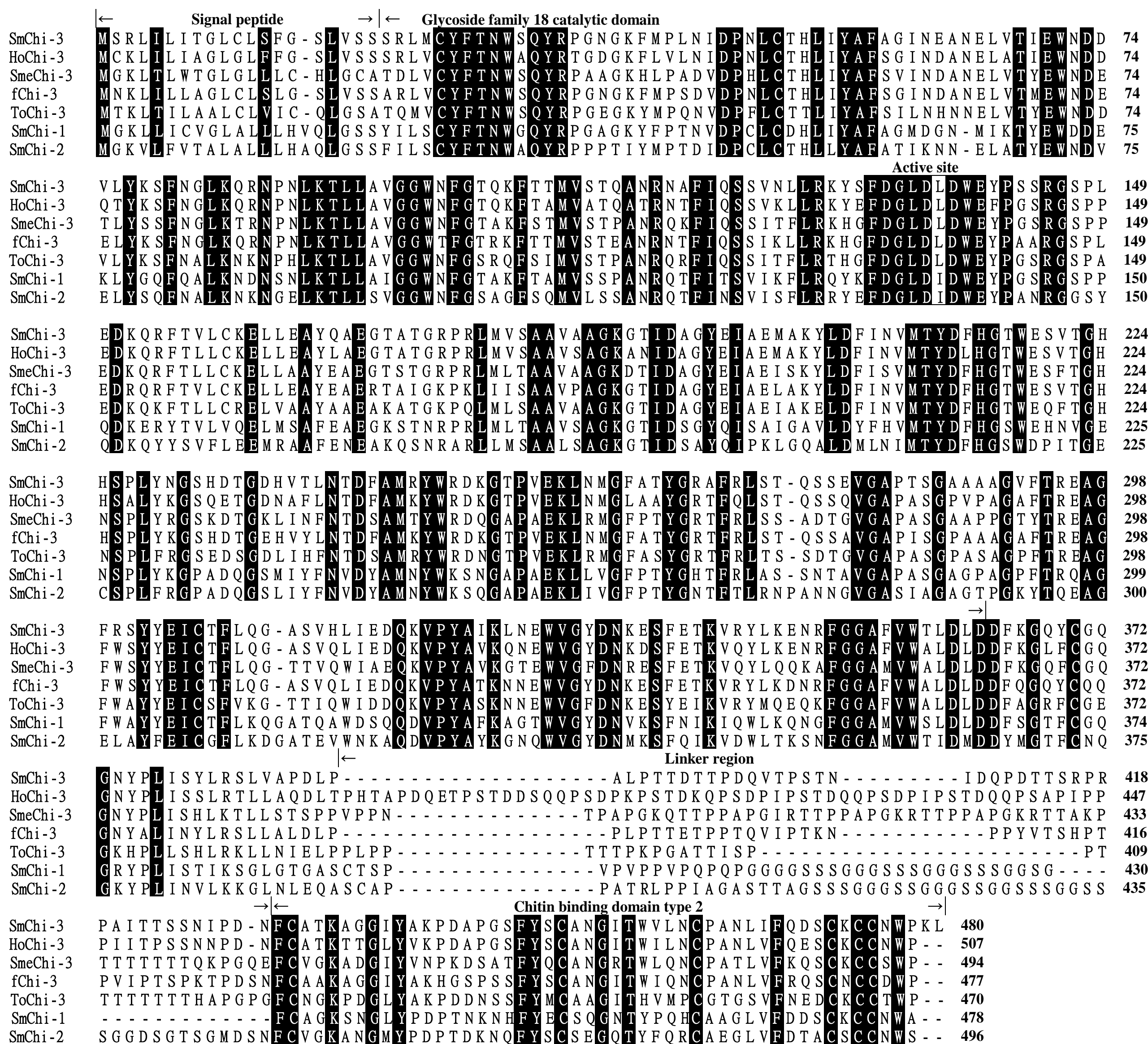
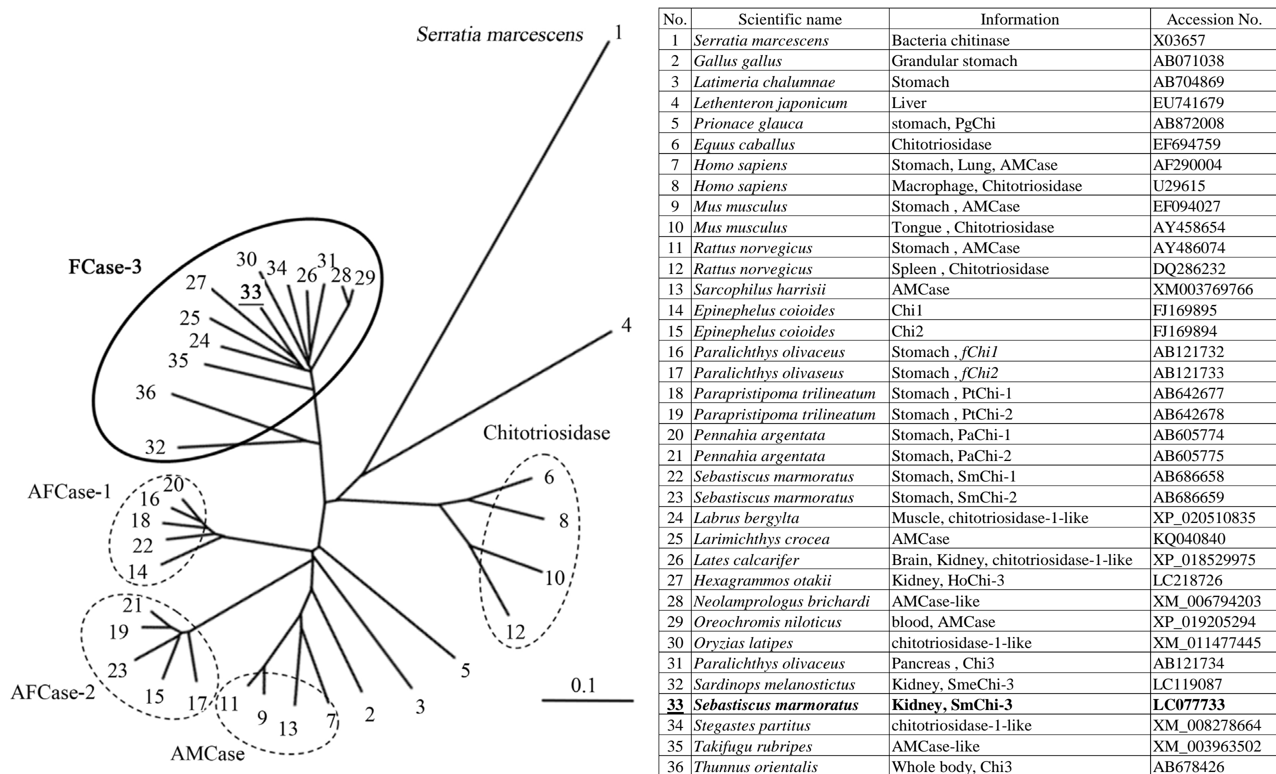


Figure 4. Alignment of amino acid sequences of SmChi-3 (*S. marmoratus* Chi-3), HoChi-3 (*H. otakii* Chi-3: DDBJ accession No. LC218726), SmeChi-3 (*S. melanostictus* Chi-3: DDBJ accession No. LC119087), fChi-3 (*P. olivaceus* Chi-3: DDBJ accession No. AB121734), ToChi-3 (*T. orientalis* Chi-3: DDBJ accession No. AB678426), SmChi-1 (*S. marmoratus* Chi-1: DDBJ accession No. AB686658), and SmChi-2 (*S. marmoratus* Chi-2: DDBJ accession No. AB686659).

chitinase from *Serratia marcescens*, which was used as an outgroup (Figure 5). As a result, like in previous work [14] [15] [31], chitinases expressed in the stomachs and lungs of mammals form an AMCase cluster, chitinases expressed in the tongue and spleen and derived from macrophages form Chitotriosidase clusters, and chitinases from the stomach of ray-finned fish form AFCase-1 and AFCase-2 clusters. However, SmChi-3 belonged neither to AFCase-1 cluster nor AFCase-2 cluster. *SmChi-*3 of *S. marmoratus*, the full-length gene sequence of which was obtained in this study, formed a new cluster that is obviously different from other chitinases, with the following chitinases registered in gene database under the various designations: *SmeChi-*3 of *S. melanostictus*, *HoChi-*3 of



No.	Scientific name	Information	Accession No.
1	*Serratia marcescens*	Bacteria chitinase	X03657
2	*Gallus gallus*	Grandular stomach	AB071038
3	*Latimeria chalumnae*	Stomach	AB704869
4	*Lethenteron japonicum*	Liver	EU741679
5	*Prionace glauca*	stomach, PgChi	AB872008
6	*Equus caballus*	Chitotriosidase	EF694759
7	*Homo sapiens*	Stomach, Lung, AMCase	AF290004
8	*Homo sapiens*	Macrophage, Chitotriosidase	U29615
9	*Mus musculus*	Stomach , AMCase	EF094027
10	*Mus musculus*	Tongue , Chitotriosidase	AY458654
11	*Rattus norvegicus*	Stomach , AMCase	AY486074
12	*Rattus norvegicus*	Spleen , Chitotriosidase	DQ286232
13	*Sarcophilus harrisii*	AMCase	XM003769766
14	*Epinephelus coioides*	Chi1	FJ169895
15	*Epinephelus coioides*	Chi2	FJ169894
16	*Paralichthys olivaceus*	Stomach , *fChi1*	AB121732
17	*Paralichthys olivaseus*	Stomach , *fChi2*	AB121733
18	*Parapristipoma trilineatum*	Stomach , PtChi-1	AB642677
19	*Parapristipoma trilineatum*	Stomach , PtChi-2	AB642678
20	*Pennahia argentata*	Stomach, PaChi-1	AB605774
21	*Pennahia argentata*	Stomach, PaChi-2	AB605775
22	*Sebastiscus marmoratus*	Stomach, SmChi-1	AB686658
23	*Sebastiscus marmoratus*	Stomach, SmChi-2	AB686659
24	*Labrus bergylta*	Muscle, chitotriosidase-1-like	XP_020510835
25	*Larimichthys crocea*	AMCase	KQ040840
26	*Lates calcarifer*	Brain, Kidney, chitotriosidase-1-like	XP_018529975
27	*Hexagrammos otakii*	Kidney, HoChi-3	LC218726
28	*Neolamprologus brichardi*	AMCase-like	XM_006794203
29	*Oreochromis niloticus*	blood, AMCase	XP_019205294
30	*Oryzias latipes*	chitotriosidase-1-like	XM_011477445
31	*Paralichthys olivaceus*	Pancreas , Chi3	AB121734
32	*Sardinops melanostictus*	Kidney, SmeChi-3	LC119087
33	***Sebastiscus marmoratus***	**Kidney, SmChi-3**	**LC077733**
34	*Stegastes partitus*	chitotriosidase-1-like	XM_008278664
35	*Takifugu rubripes*	AMCase-like	XM_003963502
36	*Thunnus orientalis*	Whole body, Chi3	AB678426

Figure 5. Phylogenetic tree for chitinase amino acid sequences developed using the neighbor joining method in the ClustalW program. The right table lists the scientific name, information, and accession No. of chitinase used in the phylogenetic tree analysis. The numbers in the phylogenetic tree and table correspond to each other. A chitinase from bacteria was used as an outgroup. The scale bar indicates the substitution rate per residue.

H. otakii, AMCase of *Larimichthys crocea*, AMCase-like of *Neolamprologus brichardi*, chitotriosidase-1-like of *Oryzias latipes*, *fChi*3 of *P. olivaceus*, chitotriosidase-1-like of *Stegastes partitus*, AMCase-like of *Takifugu rubripes*, Chi3 of *T. orientalis*, chitotriosidase-1-like of *Labrus bergylta* (muscle) (XP_020510835), with AMCase of *O. niloticus* (blood) (XP_019205294), and chitotriosidase-1-like of *Lates calcarifer* (brain, kidney) (XP_018529975). One of the functions of chitinases in fish organs other than the stomach is said to be defense-related [16] [38] [40] [41] [42]. Examples are chitinases in blood plasma [38] [42] and chitinases on the surface of mucous membranes, which prevent attachment to and entry of pathogens [40]. Because these chitinase genes of various fish, which were grouped into this new cluster, are widely distributed in the body, including in the liver, kidneys, brain, muscle, and blood, it was suggested that the chitinase genes belonging to this cluster are present in fish regardless of fish variety and have various physiological roles besides digestion and defense. We named this new cluster of fish chitinase genes Fish Chitinase-3 (FCase-3). Furthermore, because Chi-3 of *S. melanostictus* and that of *T. orientalis* branch off in this phylogenetic analysis, the FCase-3 cluster may be divided further.

3.5. Organ-Specific Genes Expressions

Expression of *SmChi*-1, *SmChi*-2, *SmChi*-3, and β-actin, which is used as a house-

keeping gene, in organs of *S. marmoratus* were analyzed by a semi-quantitative RT-PCR method and the result is shown in **Figure 6**. *SmChi*-1, classified into the AFCase-1 cluster, was expressed strongly in the stomach and heart and also expressed slightly in the pyloric appendage and ovary. *SmChi*-2, classified into the AFCase-2 cluster, was expressed strongly in the stomach and also expressed slightly in the heart. In contrast, *SmChi*-3, classified into the FCase-3 cluster, was newly identified in this study and was expressed in the liver and kidney, where *SmChi*-1 and *SmChi*-2 were not expressed. *fChi*1 of *P. olivaceus* [16], *PaChi*-1 of *P. argentata* [31], and *SmeChi*-1 of *S. melanostictus* [15], which were classified into the AFCase-1 cluster in the phylogenetic analysis, were reported to be expressed strongly in the stomach and slightly in the gonad. *fChi*2 of *P. olivaceus* [16], *PaChi*-2 of *P. argentata* [31], and *SmeChi*-2 of *S. melanostictus* [15], which were classified into the AFCase-2 cluster were reported to be expressed strongly in the stomach. Distribution of mRNA of *SmChi*-1 and *SmChi*-2 was similar to that of fish classified into AFCase-1 and AFCase-2, respectively [15] [16] [31]. These findings indicated the possibility that AFCase-1 and AFCase-2 in fish have the same roles. In other words, *SmChi*-1 and *SmChi*-2 play roles in digestion and degradation of ingested chitin and chitin-containing organisms (which enter the stomach and pyloric appendage after initial entry into the digestive tract through the mouth), defense against chitin-containing external organisms in the gonad (ovary), and degradation of chitin oligosaccharides $(GlcNAc)_n$ transferred to blood in the heart. On the other hand, because *SmChi*-3 was expressed only in the liver and kidney, *SmChi*-3 may be a new chitinase that plays physiological roles not only in digestion, but also in metabolism and carbohydrate storage.

4. Conclusion

In the body of *S. marmoratus*, relatively high chitinase activity was observed not only in the stomach, but also in the liver, spleen, kidney, and heart. Hex activity, on the other hand, was detected widely throughout the body; especially high Hex activity was observed in the spleen, kidney, and heart. Optimum pH for a chitinase contained in the crude enzyme of the stomach of *S. marmoratus* was

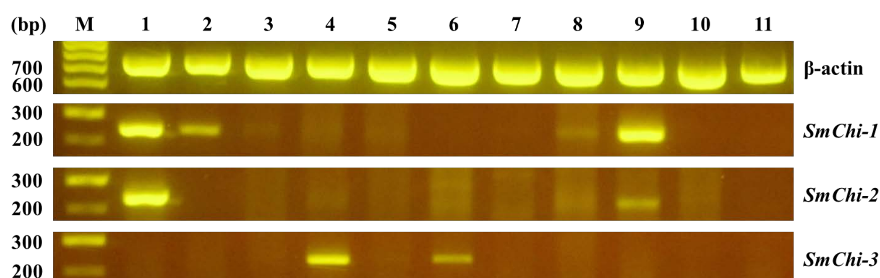


Figure 6. Expressions of *SmChi*-1, *SmChi*-2, *SmChi*-3, and *β*-actin mRNA in organs of *S. marmoratus* by RT-PCR. Lane M: markers. Lane 1: stomach. Lane 2: pyloric appendage. Lane 3: intestine. Lane 4: liver. Lane 5: spleen. Lane 6: kidney. Lane 7: air bladder. Lane 8: ovary. Lane 9: heart. Lane 10: brain. Lane 11: gill. *β*-actin was used as a control.

measured using *p*NP-$(GlcNAc)_2$ as a substrate and was determined to be at pH 2.5. Its optimal pH was similar to that of previously reported fish stomach chitinases. On the other hand, the ratio of the degradation ability for *p*NP-$(GlcNAc)_2$ to that for *p*NP-$(GlcNAc)_3$ of a chitinase contained in the kidney and its optimum pH was different from those of previously reported chitinases obtained from the stomach of *S. marmoratus* and the blood plasma of *O. niloticus*. Molecular weight estimated from a deduced amino acid sequence of the new chitinase gene *SmChi*-3 (ORF, 1440 bp) obtained from the kidney of *S. marmoratus* in this study was 51 kDa, which was most similar to the molecular weight of chitotriosidase obtained from *R. norvegicus* (50 kDa), but was remarkably lower than a chitinase obtained from the blood plasma of *O. niloticus* (75 kDa). These findings suggested that SmChi-3 was a new chitinase that was distinct from stomach chitinases and blood plasma chitinases. Although SmChi-3 had a domain structure analogous to that previously reported for SmChi-1 and SmChi-2, SmChi-3 did not have SG repeated sequences in the linker region as observed in SmChi-1 and SmChi-2. Phylogenetic analysis revealed that SmChi-3 formed a peculiar cluster Fish Chitinase-3 (FCase-3), which is different from clusters of previously reported chitinases obtained from ray-finned fish (AFCase-1 and AFCase-2). Furthermore, because Chi-3 of *T. orientalis* and that of pilchard branch off in this phylogenetic analysis, the FCase-3 cluster may be divided further. Expression analysis of *SmChi*-1, *SmChi*-2, and *SmChi*-3 in organs made by a semi-quantitative RT-PCR method showed that expression of *SmChi*-3 in the liver and kidney, where *SmChi*-1 and *SmChi*-2 were not expressed. Furthermore, the results of chitinase activity measurement in the body of *S. marmoratus* and expression analysis of each gene suggested a possibility that chitinase activity in the kidney and liver was attributable to *SmChi*-3, but chitinase activity in the spleen was attributable to another chitinase different from *SmChi*-1, *SmChi*-2, and *SmChi*-3.

Acknowledgements

This work was supported in part by College of Bioresource science, Nihon University Grant (2017).

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