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Distribution of Chitinolytic Enzymes in the Organs and cDNA Cloning of Chitinase Isozymes from the Liver of Golden Cuttlefish *Sepia esculenta*

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Abstract

The distribution of chitinolytic enzymes in eight organs of the golden cuttlefish *Sepia esculenta* was determined. Chitinase activity (activity of endo-type chitinolytic enzyme) was measured using *p*NP-$(GlcNAc)_n$ (n = 2, 3) as substrates, with high activity detected in the liver, posterior salivary gland, and stomach. *β*-*N*-acetylhexosaminidase (Hex) activity (activity of exo-type chitinolytic enzyme) was determined using *p*NP-(GlcNAc) as a substrate, and high activity was observed in six organs, including the liver, branchial heart, posterior salivary gland, and stomach. In addition, two chitin-binding proteins (CBP-A, CBP-B) were isolated from the liver using a chitin affinity column. Two full-length cDNAs (*SeChi*-1: 1484 bp; *SeChi*-2: 1748 bp) encoding chitinases were obtained from the liver of *S. esculenta*. *SeChi*-1 contained a 1377-bp open reading frame (ORF) encoding 459 amino acids, and *SeChi*-2 contained a 1656-bp ORF encoding 552 amino acids. Domain structures predicted from the deduced amino acid sequences of *SeChi*-1 and *SeChi*-2 (SeChi-1, SeChi-2) contained signal peptides, a GH Family 18 catalytic domain, one chitin binding domain (CBD) in SeChi-1, and two CBDs in SeChi-2. Proteome analysis revealed that 125 peptide residues of CBP-A were present in SeChi-1, and 116 peptide residues of CBP-B were present in SeChi-2. Organ expression analysis revealed that *SeChi*-1 and *SeChi*-2 were expressed only in the liver of *S. esculenta*. Phylogenetic analysis of SeChi-1, SeChi-2, and GH family 18 chitinases revealed that SeChi-2 belongs to a group of previously reported squid chitinases, while SeChi-1 does not belong to any previously reported group of mollusk chitinases.

Keywords

Chitinolytic Enzyme, Chitinase, Distribution, cDNA Cloning, Golden Cuttlefish *Sepia esculenta*

1. Introduction

Chitin, a β-1,4-linked polysaccharide of *N*-acetyl-D-glucosamine (GlcNAc), is an abundant reproducible biomass found widely in the exoskeletons of arthropods, the cell walls of fungi, and the cuticles of nematodes [1] [2] [3] [4]. Chitinolytic enzymes can be classified into two categories according to their degradation patterns: endo-type chitinolytic enzymes, called chitinase (EC 3.2.1.14), which degrade a random chitin polymer to produce chitin oligosaccharides $(GlcNAc)_n$, and exo-type chitinolytic enzymes, called β-*N*-acetylhexosaminidase (Hex) (EC 3.2.1.52), which degrade $(GlcNAc)_n$ from the nonreducing end of it to produce GlcNAc [5] [6]. Chitinases are found in various living organisms, including animals, plants, and microorganisms, and have important roles in biological processes, such as digestion, morphological changes during growth, and immunity [5] [7]. Chitinases are classified into glycoside hydrolase (GH) family 18 or 19, based on the homology of amino acid sequences [4] [8] and catalytic mechanisms in their active domains [9] [10]. GH family 18 chitinases are found widely in biology, including in microorganisms, animals, and plants [5]. Conversely, GH family 19 chitinases are found mainly in plants [11].

In marine animals, studies have reported the purification, properties, and cDNA cloning of chitinase isozymes mainly obtained from the fish stomach, which are involved in digestion [12]-[17]. Chitinases in the fish stomach are classified into two groups based on differences in their primary structure and the patterns of degradation of $(GlcNAc)_n$: acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2) [16] [17] [18] [19]. Conversely, studies reporting the cDNA cloning and expression of chitinases and chitinase-like proteins from bivalves and gastropods, which are mollusks, have noted that these play roles in shell formation [20] [21] [22], immunity [23] [24] [25] [26], and digestion [27]. Chitinase isozymes have been purified and studied from the liver of Decembrachiata (squid and cuttlefish), and are involved in digestion [28] [29] [30] [31]. Furthermore, two chitinase isozymes have been reported in the liver of Japanese common squid, and identified based on differences in molecular weight and N-terminal amino acid sequences [29] [30], and two chitinase isozymes have been reported in the liver of spear squid, and identified based on expressed sequence tag (EST) analysis [32]. However, the full-length genes have not yet been determined. Conversely, chitinases have been obtained from the posterior salivary gland of octopus [33] and cuttlefish [34], and found to act as poison. A chitotriosidase gene, which is involved in the induction of luminescent bacteria, has been found in the light organ of the Hawaiian bobtail squid *Euprym-*

nascolopes [35]. Thus, the roles of mollusk chitinases are not limited to digestion and range widely; thus, many isozymes exist to support these different roles.

Golden cuttlefish *Sepia esculenta*, used in the present study, belongs to Decembrachiata and is a type of mollusk that mainly ingests crustaceans, which contain chitinous substances, and fish [36]. We have previously reported the distribution of chitinase activity using glycolchitin as the substrate [37] and purification and properties of a chitinase obtained from the liver of *S. esculenta* [31]; however, no findings of enzyme proteins and genes corresponding with the chitinase isozymes have been reported. In this study, we first observed the distribution of chitinase activity using two kinds of chitinase specific substrates in the body of *S. esculenta,* and isolated two types of chitin-binding proteins (CBPs) from the liver that exhibited particularly high chitinase activity. Next, we cloned chitinase genes from the liver and obtained two types of full-length genes. Furthermore, the organ expression of the genes was analyzed, domain structures were compared, and phylogenetic analyses was performed based on the deduced amino acid sequences. The relationship between the two types of CBPs was elucidated and the different chitinase genes obtained were examined by proteome analysis. In summary, this study is the first to discuss the distribution of chitinolytic enzymes in *S. esculenta*, the presence of chitinase isozymes and features of their domain structure, and the positioning of chitinase isozymes in phylogenetic analysis.

2. Materials and Methods

2.1. Materials

Fresh *S. esculenta* was purchased from Tsukiji Fish Market (body weight: 183 g, liver weight: 9.5 g).

2.2. Measurement of Chitinolytic Enzyme Activity

Organs were removed from *S. esculenta* for subsequent analysis. Each organ was homogenized in five volumes of 20 mM phosphate buffer (pH 7.2), and then the homogenate was centrifuged at 7000 ×*g* for 20 min. The supernatant was used as the crude enzyme solution. Chitinase and Hex activities were measured using *p*-nitrophenyl $(GlcNAc)_n$, (*p*NP-$(GlcNAc)_n$) (n = 2, 3) (Seikagaku, Tokyo, Japan) and *p*NP-GlcNAc (Seikagaku) as substrates, respectively, according to the method described by Ohtakara [38], with slight modification. Briefly, 3.0 μL of crude enzyme solution and 2.5 μL of 4mM substrate solution were added to 10 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, and the absorbance of released *p*-nitrophenol was measured at 420 nm. One-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 organ.

2.3. Isolation of CBPs from the Liver of *S. esculenta*

Unless otherwise noted, all processes were carried out at 0˚C - 4˚C. Livers were collected from fresh *S. esculenta* and kept at −80˚C until use. The livers were homogenized with five volumes of 50 mM sodium acetate buffer (pH 5.5) and centrifuged at 7000 ×*g* for 20 min. Ammonium sulfate was added to the supernatant to give 70% saturation, and the preparation was left to stand for 24 h. The precipitate was then collected by centrifuging at 7000 ×*g* for 20 min, and dialyzed in 20 mM sodium phosphate buffer (pH 7.2). The dialyzed solution was centrifuged at 7000 ×*g* for 20 min and NaCl was added to bring the concentration to 1 M. This solution was applied to a chitin affinity column (Chitin EX column) (Funakoshi, Tokyo, Japan) (1.5 × 10 cm) previously equilibrated with 20 mM sodium phosphate buffer (pH 7.2) containing 1 M NaCl, and the non-adsorbed fractions were eluted with the same buffer. Adsorbed fractions were eluted with 0.1 M acetic acid. Finally, the adsorbed fractions were dialyzed with distilled water. Chitinase activity was measured using *p*NP-$(GlcNAc)_2$.

2.4. Amino Acid Sequence of the CBPs Isolated from the Liver of *S. esculenta*

The chitinase-active fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with AE-1360 Ez Stain Silver (ATTO, Tokyo, Japan). A gel slice was cut into small pieces and destained with destaining solution (15 m$MK_3[Fe(CN)_6]$, 50 mM $Na_2S_2O_3$). Destained gel pieces were trypsinized as described in the manual for the In-Gel Tryptic Digestion Kit (Thermo Scientific, Waltham, MA). The peptide mixtures obtained were subjected to a nanoscale liquid chromatography-electrospray (Thermo Scientific) equipped with a captive spray ionization source (Michrom Bioresources, Auburn, CA) and an Advance UHPLC System (Michrom Bioresources).

2.5. cDNA Cloning of Chitinases from the Liver of *S. esculenta*

Total RNA was extracted from the *S. esculenta* liver using ISOGEN II (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Next, cDNA was synthesized using 1.0 μg of total RNA, a PrimeScript reverse transcriptase (Takara Bio, Shiga, Japan), and an oligo dT primer (Table 1). The reaction conditions were 90˚C for 3 min, 42˚C for 60 min, and 70˚C for 10 min. The primers used are listed in Table 1, and the primer combinations are shown in Figure 1. Internal sequences were amplified in a solution containing the synthesized cDNA, Go Taq Green Master Mix (Promega, Madison, USA), 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 follow: initial denaturation at 95˚C for 2 min, 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

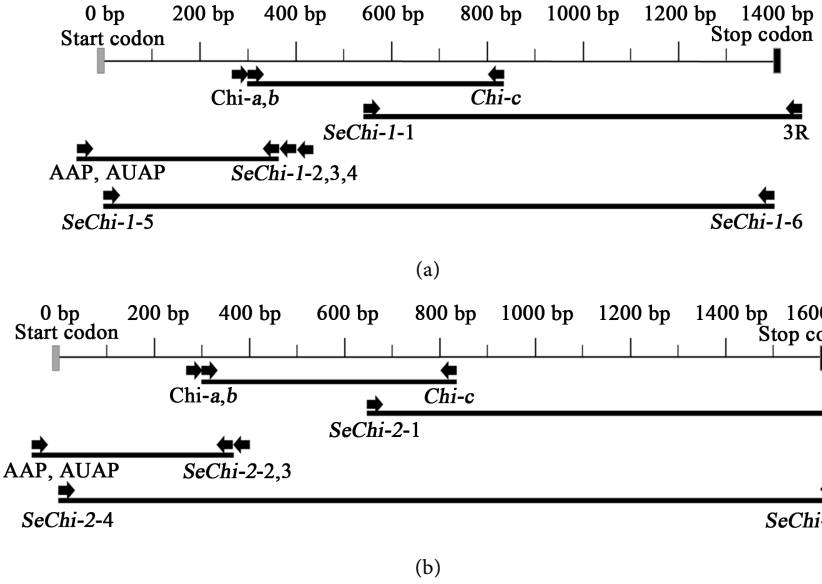

Figure 1. Schematic representation of the cDNA structure of (a) *SeChi*-1, (b) *SeChi*-2 and location of the primers. Arrowheads indicate the primers, and lines between the arrowheads indicate the amplified cDNA fragments.

Table 1. Primers used for PCR, RACE, and organ expression.

Primer	Sequence (5'-3')	Purpose
Oligo dT	CTGTGAATGCGACTACGATTTTTTTTTTTTTTTTT	cDNA synthesis
*Chi-a**	GGNGGNTGGAAYATGGG	Primary PCR
*Chi-b**	TNGCNGCNTTYGARTGGAAYGA	Primary PCR
*Chi-c**	ANCANRANCCNTTRGTYACCCA	Primary PCR
SeChi-1-1	GCACCAAAGAAAAAAGTTGAT	3'RACE
SeChi-2-1	GGACATAACAGCCCTCTG	3'RACE
3R	CTGTGAATGCGACTACGAT	3'RACE
SeChi-1-2	GCCCATGTTCCAGCCACC	5'RACE
SeChi-1-3	TTCGTCGTTCCACTCAAAAGC	5'RACE
SeChi-1-4	CCCATTCAGTTTGGCAAAAGC	5'RACE
SeChi-2-2	CGCTACCATGGCAGTGAAAGG	5'RACE
SeChi-2-3	CTTCATCCATGGTTCAGATTC	5'RACE
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	5'RACE
AUAP	GGCCACGCGTCGACTAGTAC	5'RACE
SeChi-1-5	ACACATTACAGCAAA	Full-length ORF
SeChi-1-6	TAATAAATACCAAGATTAT	Full-length ORF
SeChi-2-4	CGAGTTCTGGTGGACAAA	Full-length ORF
SeChi-2-5	GGCTGAAAAATAAAATGT	Full-length ORF
β-actin-a	GGTATGTGCAAAGCTGGTTTT	Organ expression
β-actin-b	GTGGGTGACACCATCACCAGA	Organ expression
SeChi-1-a	GAAACTTTGATGGTTTGGACAT	Organ expression
SeChi-1-b	TGTGTCCATACAAAGCAATTCC	Organ expression
SeChi-2-a	GAGAAATACCCACTGCTGAAGA	Organ expression
SeChi-2-b	AGAACAGTTAGGAATAGCGGAT	Organ expression

*Degenerate primers; 5'AAP: 5'RACE abridged anchor primer; AUAP: abridged universal anchor primer.

gene sequences obtained by the internal sequence amplification, and the upstream (5') and downstream (3') regions were amplified using the rapid amplification of cDNA ends (RACE) method. PCR parameters for the 3' RACE analyses were as follow: initial denaturation at 95˚C for 2 min, followed by 35 cycles of 95˚C for 30 s, 55˚C for 1 min, and 72˚C for 2 min. The 5' 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 in 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 liver of *S. esculenta* were amplified using platinum®*Pfx* DNA polymerase (Invitrogen), which has proofreading activity. The reaction conditions were: 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 described for the internal sequence amplification. Base sequences were determined using the Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA).

2.6. Organ Expression of *SeChi*-1 and *SeChi*-2

Total RNA was extracted from *S. esculenta* organs. cDNA was synthesized using 0.5 μg of total RNA obtained from each tissue and an oligo dT primer, and amplified using PCR with 1.0 μg of the synthesized cDNA, primers for *SeChi*-1, *SeChi*-2, and Decembrachiata β-actin amplification primers (**Table 1**). The reaction conditions were: 30 cycles of 95˚C for 30 s, 55˚C for 1 min, and 72˚C for 2 min.

2.7. Phylogenetic Analysis of Chitinases

Phylogenetic analysis, based on the deduced amino acid sequences of the full-length *SeChi*-1 and *SeChi*-2 genes, was performed using chitinase genes obtained from multiple organisms. The analysis was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/) and Tree view.

3. Results and Discussion

3.1. Distribution of Chitinolytic Activities

Chitinolytic activity measurement using *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$ as substrates showed that out of eight measured organs of *S. esculenta*, the liver and stomach, which are involved in digestion, and the posterior salivary gland, which contains chitinase genes that were also found to be present in other cephalopods [33] [34], exhibited high activity (**Figure 2(a)**). When using glycolchitin as substrate, chitinase activity was detected only liver, stomach, and caecum [37]. Moreover, Hex activity, which is characteristic of exo-type chitinolytic enzymes, was high in the following six organs: the liver, heart, branchial heart, posterior salivary gland, stomach, and caecum (**Figure 2(b)**).

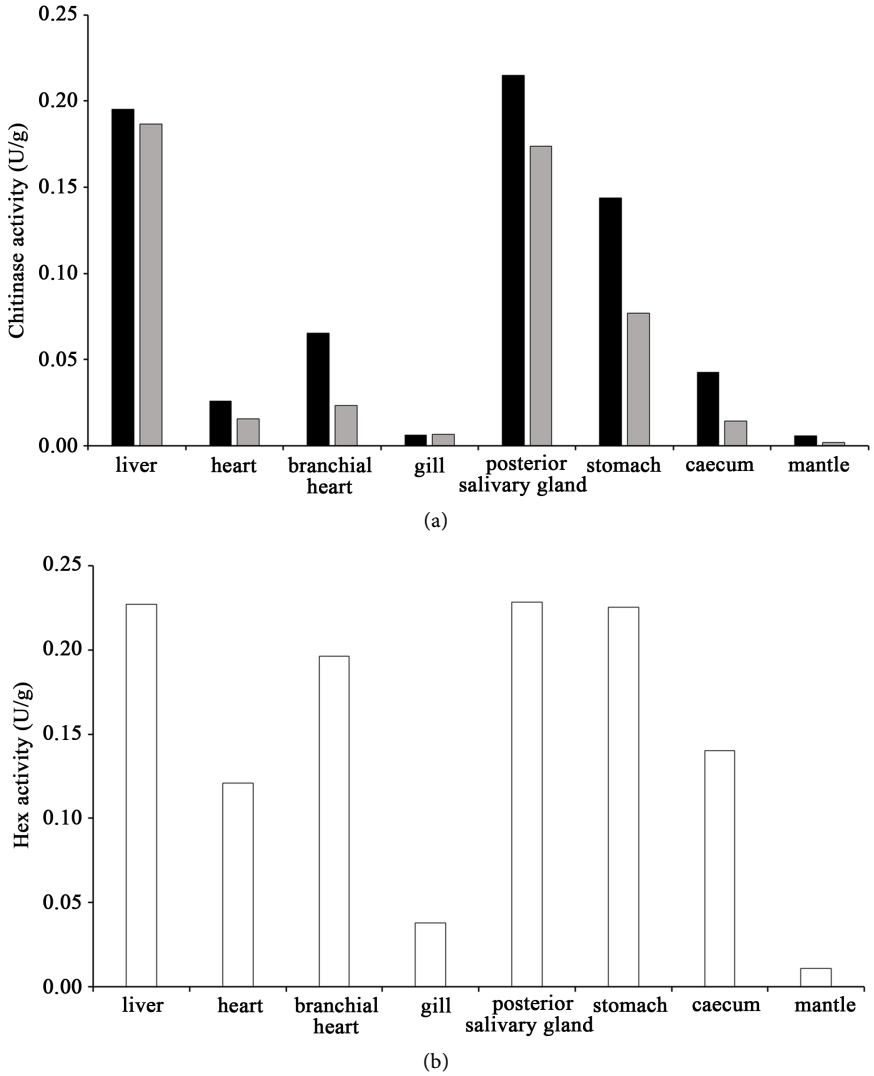

Figure 2. The distribution of the chitinolytic activities in the organs: (a) Chitinase activity; (b) Hex activity. (▪) *p*NP-$(GlcNAc)_2$; (▫) *p*NP-$(GlcNAc)_3$; (□) *p*NP-(GlcNAc).

In digestive organs, chitinases and Hex are involved in the degradation of chitinous substances following their intake as feed. This is consistent with the feeding habit of *S. esculenta*; that is, *S. esculenta* ingests organisms containing chitinous substances, such as shrimps and crabs [36], suggesting that *S. esculenta* degrades chitin from feed into GlcNAc using both endo- and exo-type enzymes. In addition, it is possible that chitinases in the posterior salivary gland act as a poison, as observed in other cephalopods [33] [34]. Furthermore, because blood chitinases in mollusks have important roles in immunity [23], chitinases in the heart and branchial heart, which are not involved in digestion, are involved in defense against organisms containing chitinous substances, such as parasitic crustaceans and nematodes.

3.2. Isolation of CBPs from the Liver of *S. esculenta*

Using a chitin affinity column, CBPs were separated from the enzyme solution

obtained from *S. esculenta* liver via 0% - 70% ammonium sulfate fractionation (Figure 3). SDS-PAGE was performed using the fraction with the highest chitinolytic activity; CBPs with molecular weights of 52 and 62 kDa (CBP-A, CBP-B) were detected (Figure 4).

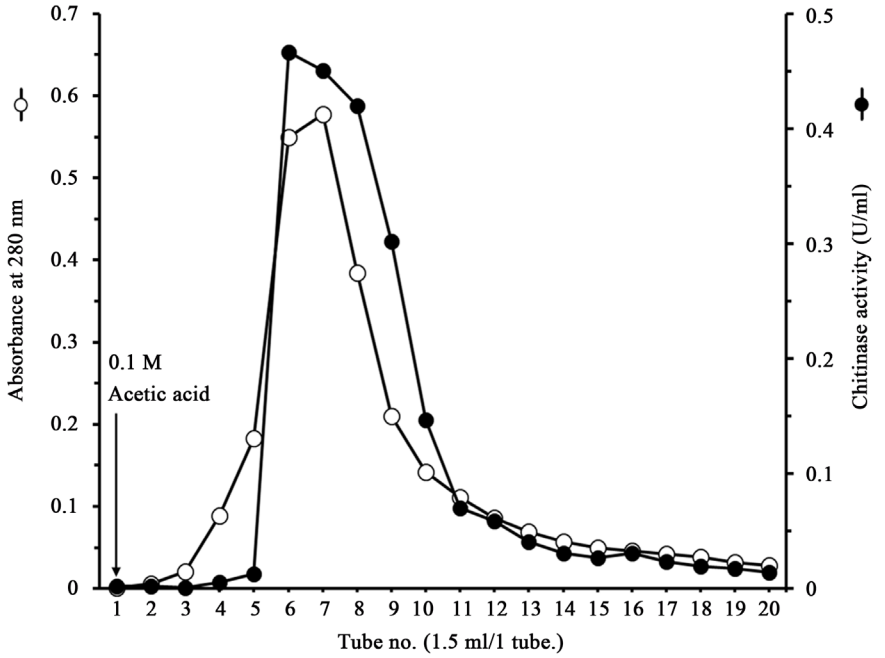

Figure 3. Separation of chitin binding proteins (CBPs) from the liver of *S. esculenta* by using chitin affinity column chromatography. Sample solution was applied to a Chitin EX column previously equilibrated with 20 mM sodium phosphate buffer solution (pH 7.2) containing 1 M NaCl, and the non-adsorbed fraction was eluted with the same buffer. The adsorbed fraction was eluted with 0.1 M acetic acid.

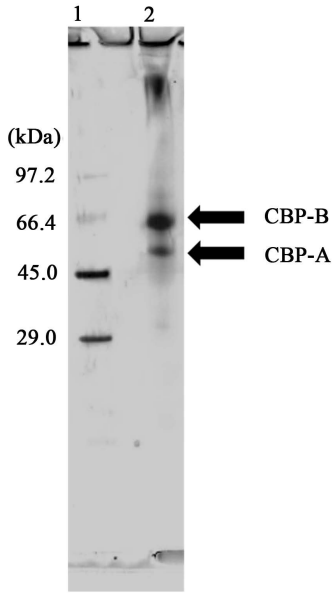

Figure 4. SDS-PAGE of CBPs. (1: Marker; 2: Chitinase active fraction obtained by Chitin EX column chromatography.)

CBP-B was considered to be SeChi, which is a chitinase purified from the liver of *S. esculenta* [31], because the molecular weight of CBP-B (62 kDa) was consistent with that of SeChi. Two types of chitinase isozymes with molecular weights of 38 [28] and 42 kDa [30] have previously been purified from the liver of Japanese common squid. This suggests that the CBP-A newly detected in this study is an isozyme of chitinase in the liver of *S. esculenta*.

3.3. cDNA Cloning of Chitinases from the Liver of *S. esculenta*

The liver of *S. esculenta* was used as a sample, and internal sequences of chitinase cDNA were amplified by PCR using degenerate primers designed from conserved amino acid sequences of GH family 18 chitinases. As a result, amplified fragments approximately 550 bp in size were found, and two types of base sequences were obtained by base sequencing. NCBI Blast analysis revealed that these base sequences share homology with chitotriosidase of Hawaiian bobtail squid *E. scolopes* [35]. The upstream and downstream sequences of the aforementioned base sequences were amplified by the RACE method. As a result, initiation and termination codons were identified in the upstream and downstream regions. Then, full-length cDNAs were amplified using Platinum® *Pfx* DNA polymerase.

Two full-length cDNAs, *SeChi*-1 (1484 bp) and *SeChi*-2 (1748 bp), were obtained and found to contain 1377-bp (459 amino acids) and 1656-bp (552 amino acids) open reading frames (ORFs), respectively. The molecular weights of SeChi-1 and SeChi-2 were 51.2 and 61.0 kDa, respectively, based on the deduced amino acid sequences of *SeChi*-1 and *SeChi*-2, (**Figure 5** and **Figure 6**). The molecular weights were very similar to those of CBP-A (52 kDa) and CBP-B (62 kDa), respectively, which were determined by SDS-PAGE. Isoelectric points calculated from the amino acid sequences of SeChi-1 and SeChi-2 were 8.87 and

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                                                                AAATAGTGAATTTCCAAGGAGATACACATTACAGCAAA   -1
ATGTCCATGAAGTGTTTCTTTTCTCTGTTGCTGTTTTTATTTATTGCAAGTAGAATAGAAGCTTCACGCCGGTGGTGCTTCTATACAAACTGGGCTCAGTATCGAAAAGGTGGTGCTAGA  120
M  S  M  K  C  F  F  S  L  L  L  F  L  F  I  A  S  R  I  E  A  S  R  R  W  C  F  Y  T  N  W  A  Q  Y  R  K  G  G  A  R
TTTCTTCCCAAAGATATTGATGCAAGATTTTGCACCCACATCTCTTATGCTTTTGCTACATTGAAGAATGGTGAATTAGCAGCTTTTGAATGGAATGATGATGACACACCTTATGCAGAA  240
F  L  P  K  D  I  D  A  R  F  C  T  H  I  S  Y  A  F  A  T  L  K  N  G  E  L  A  A  F  E  W  N  D  D  D  T  P  Y  A  E
GGAATGTATAAACAAGTGAATAATGTGAAGAAACAAAATCCTGGTTTGAAGACTCTTCTCGCAATTGGTGGTTGGAATATGGGTTCAAATTTATTTTCTGATATGGTTGCTACAAAACAA  360
G  M  Y  K  Q  V  N  N  V  K  K  Q  N  P  G  L  K  T  L  L  A  I  G  G  W  N  M  G  S  N  L  F  S  D  M  V  A  T  K  Q
ACCCGCCAAAAGTTCATTACATCAACTATATCATTCTTGAGCTCAAGAAACTTTGATGGTTTGGACATTTGCTGGGAATATCCAACAAAGAGAGGCAGCCCACCCCAAGACAAAGAAAGG  480
T  R  Q  K  F  I  T  S  T  I  S  F  L  S  S  R  N  F  D  G  L  D  I  C  W  E  Y  P  T  K  R  G  S  P  P  Q  D  K  E  R
TTTGGACTTCTTCTGAAGGAATTAAGAACTGCATTTGATGAGAATGCCAAAAAAGGTTTGTCAAAACTTATCCTTGGAATAGTTGTGGGTACAGATGAAAATTTGATTGAAAATGCTTAT  600
F  G  L  L  L  K  E  L  R  T  A  F  D  E  N  A  K  K  G  L  S  K  L  I  L  G  I  V  V  G  T  D  E  N  L  I  E  N  A  Y
GACATTGATGCTATTAAATCATCAGTAGATGCTGTGTCCCTTTTGTCCTATGATTTCTACAGTGCAATGTCAACTGACTCTGCTGTTCATACCAGTGCTCTTTATGCAAGTAATATAACT  720
D  I  D  A  I  K  S  S  V  D  A  V  S  L  L  S  Y  D  F  Y  S  A  M  S  T  D  S  A  V  H  T  S  A  L  Y  A  S  N  I  T
AAAGGTTCAGATGGTAAAAAGAATGTTGAGTATGTTGCTAAATCTTGGGTCAAGAATGGAATTCCAAAGAACCTGATTAATATAGGAATTGCTTTGTATGGACACAGTTATCGTTTAAAG  840
K  G  S  D  G  K  K  N  V  E  Y  V  A  K  S  W  V  K  N  G  I  P  K  N  L  I  N  I  G  I  A  L  Y  G  H  S  Y  R  L  K
GATACAAATGCAAAGGGAGAAGGTGCCTTAATCAGCGGACCAGGTGCTGCTGGACGTTACACTAATACTCCAGGTTTTCTAGCCTATTATGAAGTCTGTGAGATGATAAATAATGGTGGC  960
D  T  N  A  K  G  E  G  A  L  I  S  G  P  G  A  A  G  R  Y  T  N  T  P  G  F  L  A  Y  Y  E  V  C  E  M  I  N  N  G  G
ATTGTTACATTCATAAAAGGGAGAGGTGTTCCTTACCTGGTGCTGGGCAATCAATGGGTTGCTTTTGAAAATGAAGAGAGTGTTACACTGAAAACAAAATTTGCTCTCAATGAAGGTTAT  1080
I  V  T  F  I  K  G  R  G  V  P  Y  L  V  L  G  N  Q  W  V  A  F  E  N  E  E  S  V  T  L  K  T  K  F  A  L  N  E  G  Y
GGTGGTGTGATGATCTGGTCATTTGATAATGATGACTTTTCTGGGATGTGTCAAGGTGGAAAAATTTATCCCCTTTTTAAAGCCTTCTATAATGCAATGCAAATGCCTCAAACAACACCA  1200
G  G  V  M  I  W  S  F  D  N  D  D  F  S  G  M  C  Q  G  G  K  I  Y  P  L  F  K  A  F  Y  N  A  M  Q  M  P  Q  T  T  P
GATCCTAATTGGCCAAAGAAGTTCTGCTTAAAACATGGCAATGGATTTTTTGGATTAGACTGTAAACGGTTTATGATATGCACCAATGGAAATGGTTTTGTAAGTCAGTGTACACAAGGT  1320
D  P  N  W  P  K  K  F  C  L  K  H  G  N  G  F  F  G  L  D  C  K  R  F  M  I  C  T  N  G  N  G  F  V  S  Q  C  T  Q  G
CAACTCTGGGATAAAAAGCTAAATACATGTGTAAATGCAAAACTGACTACATGTACATAAACTGTTGTTATTAGTTGTTGTATTAGTGTTGTTATACAGTTGTTCATAAATAAATGTTCA  1440
Q  L  W  D  K  K  L  N  T  C  V  N  A  K  L  T  T  C  T  *
TAAATTTCAACAAAAAAAAAAAAAAAAA                                                                                          1560
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Figure 5. cDNA and deduced amino acid sequence of *SeChi*-**1**. DDBJ accession nos. AB986212. Underlined sequence show matching with the peptide fragments of the separated and trypsinized CBP-A (coverage: 27.23%, 125 residues). Calculated molecular weight: 51228.59. Isoelectric point: 8.87.

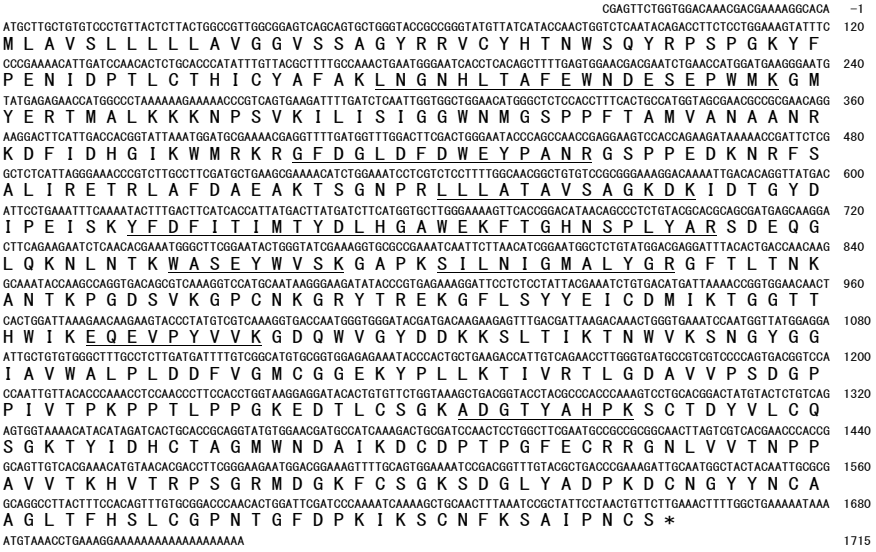


Figure 6. cDNA and deduced amino acid sequence of *SeChi*-**2**. DDBJ accession nos.LC319665. Underlined sequence show matching with the peptide fragments of the separated and trypsinized CBP-B (coverage: 21.01%, 116 residues). Calculated molecular weight: 61012.53. Isoelectric point: 8.79.

8.79, respectively. These values are approximate to those of chitinase isozymes purified from the liver of Japanese common squid, at 8.3 [28] and 9.2 [30], suggesting that chitinases from the liver of Decembrachiata are basic proteins.

SeChi-1 and SeChi-2 were found to consist of N-terminal signal peptides, a GH Family 18 catalytic domain, one chitin binding domain (CBD) for SeChi-1, and two CBDs for SeChi-2 (**Figure 7**). The domain structure of SeChi-2 was consistent with that of a squid chitinase previously reported to possess two CBDs [35]. SeChi-1, which has only one CBD, was revealed to be a new Decembrachiata chitinase.

Figure 8 compares the domain structure of mollusk and fish chitinases. Fish chitinases have previously been reported to contain one CBD [18] [19]. The domain structures of common mackerel stomach chitinases (*SjChi*-1 and *SjChi*-2) are shown in **Figure 8** as examples. Conversely, the domain structures of mollusk chitinases are diverse; a chitinase from the gonad of sea hare (*AkChi*) and a chitinase from Japanese oyster chitinase 3 (*CgChi*-3) possess no CBD; *S. esculenta* liver chitinase 1 (*SeChi*-1) and a snail *Biomphalaria glabrata* chitinase 3-like protein 1 (*BgChi*-3*lp*1) possess one CBD; and *S. esculenta* liver chitinase 2 (*SeChi*-2) and chitinase 3 from the mantle of *Hyriopsis cumingii* (*HcChi-3*) possess two CBDs. This suggests that mollusk chitinases have several domain structure, which correspond to their physiological roles.

3.4. Amino Acid Sequence of the Chitinases

Two CBPs (CBP-A and CBP-B) (**Figure 3** and **Figure 4**) obtained from the liver of *S. esculenta* were fragmented into peptides by trypsin treatment and compared with the amino acid sequences of SeChi-1 and SeChi-2 via proteome analysis.

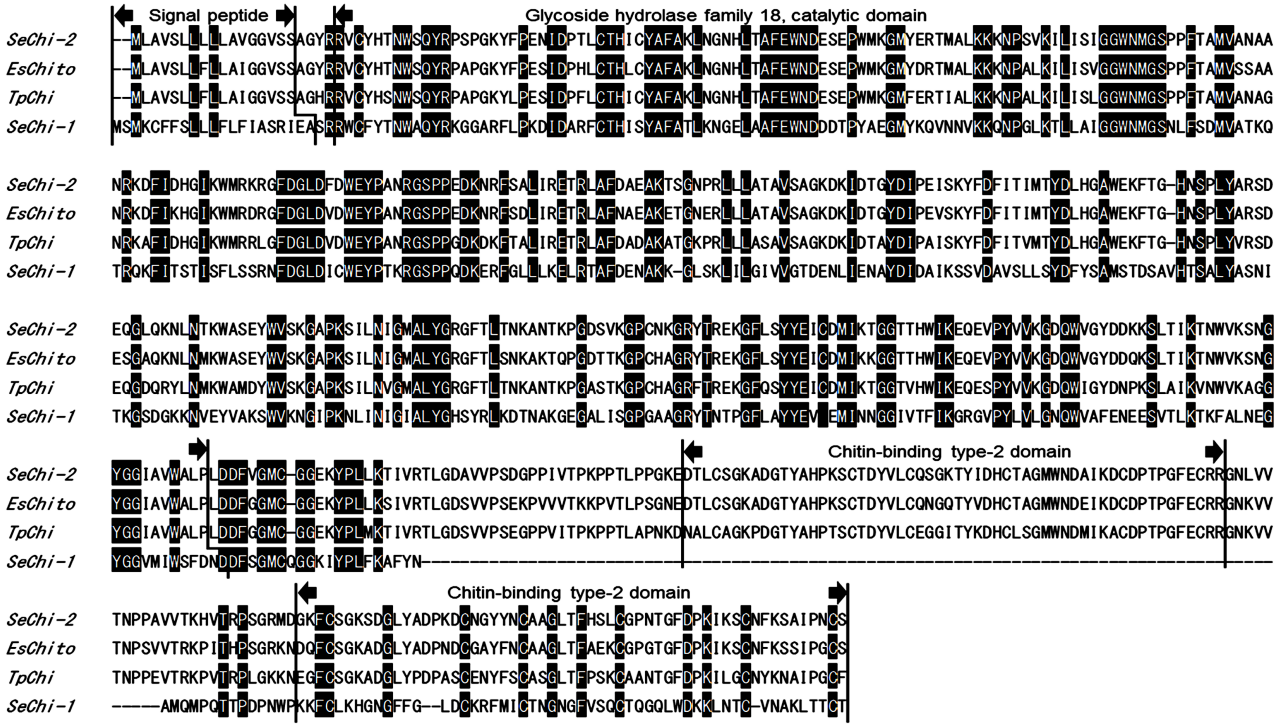


Figure 7. Multiple alignment of deduced amino acid sequences of *S. esculenta* Chitinases (*SeChi*-1 and *SeChi*-2) with *Euprymna scolopes* chitotriosidase (*EsChito*), and *Todarodes pacificus* Chitinase (*TpChi*). GenBank accession nos.: *EsChito*, AHM92582.1; *TpChi*, LC146770. Matched sequences are shown in black.

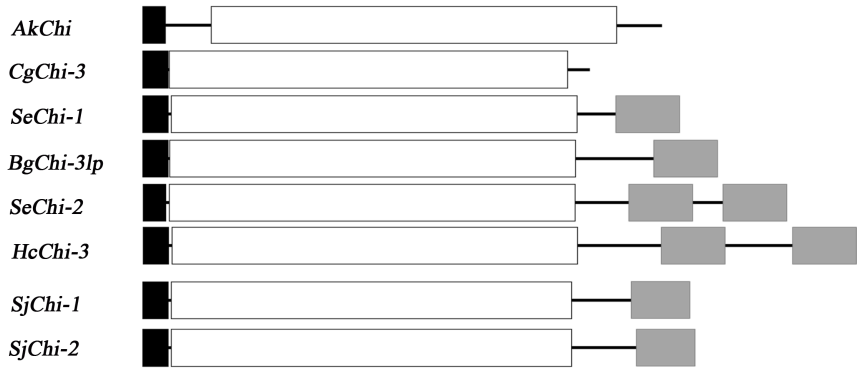


Figure 8. The schematic representation molluscan and fish chitinases. Black boxes show signal peptide. White boxes show GH family 18 catalytic domain. Gray boxes show the chitin-binding type-2 domain. GenBank accession nos.: *AkChi*, BAS44269; *CgChi*-3, AJ971239; *BgChi*-3*lp*, XP013090777; *HcChi*-3, AFO53261; *SjChi*-1, AB686657; *SjChi*-2, AB689022.

A sequence obtained from peptide fragments of CBP-A was consistent with the amino acid sequence of SeChi-1 (coverage: 27.23%, 125 residues) (**Figure 5**). A sequence obtained from the peptide fragments of CBP-B was consistent with the amino acid sequence of SeChi-2 (coverage: 21.01%, 116 residues) (**Figure 6**). These results indicate that SeChi-1 and SeChi-2 encode CBP-A and CBP-B, respectively. In other words, CBP-A was a protein band of chitinase isozyme SeChi-1 and CBP-B was a protein band of chitinase isozyme SeChi-2.

Trypsin is reported to cleave the C-terminal side of lysine (K) and arginine (R) [39]. It was confirmed that trypsin worked adequately at all cleavage sites because all of the obtained peptides ended with K or R.

3.5. Organ Expression of *SeChi*-1 and *SeChi*-2

The expression of *SeChi*-1 and *SeChi*-2 was investigated in eight *S. esculenta* organs, with both genes found to be expressed only in the liver (**Figure 9**). Expression of *SeChi*-2 was stronger than that of *SeChi*-1 (**Figure 9**). This result was consistent with findings from SDS-PAGE of CBPs isolated from the liver, such that the CBP-B band encoded by *SeChi*-2 was thicker than that of CBP-A encoded by *SeChi*-1 (**Figure 4**). Because *SeChi*-1 and *SeChi*-2 were found to be expressed in the liver, where chitinolytic activity was found, *SeChi*-1 and *SeChi*-2 are suggested to encode enzymes involved in chitin degradation in this organ. Furthermore, although high chitinolytic activity was detected in the posterior salivary gland, as shown in **Figure 2**, neither *SeChi*-1 nor *SeChi*-2 were expressed there. The presence of chitinases acting as poison in the posterior salivary gland of other cephalopods has been reported [33] [34]. Additionally, the presence of chitinases isozymes, which differ from SeChi-1 and SeChi-2, is suggested in the posterior salivary glandof *S. esculenta*.

3.6. Phylogenetic Analysis of *SeChi*-1 and *SeChi*-2

On the basis of amino acid sequence homology, phylogenetic analysis was conducted using SeChi-1, SeChi-2, GH family 18 chitinases of other organisms, and a GH family 18 chitinase of *Serratiamarcescens*, as an outgroup. SeChi-2 formed a group with other Decembrachiata chitinases, whereas SeChi-1 did not form a group with any of the mollusk chitinases (**Figure 10**). Considering that SeChi-1 is the first chitinase with one CBD to be identified in Decembrachiata (**Figure 8**), it was considered to be a new-type chitinase.

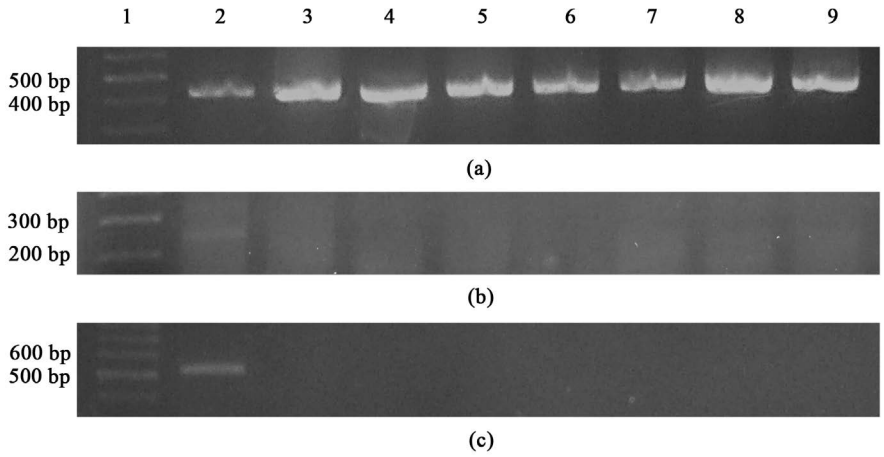
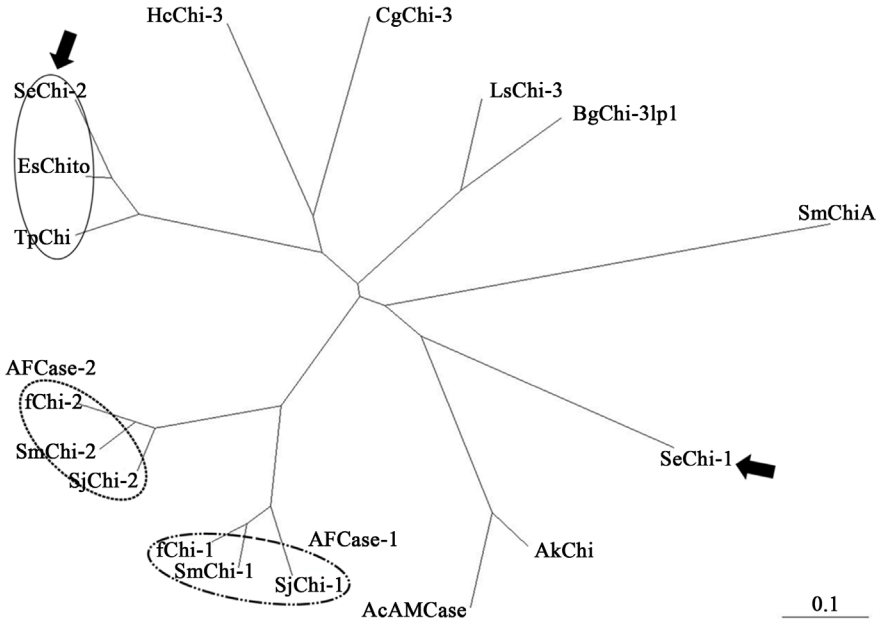


Figure 9. Chitinase and *β*-actin expressions in various organs. (a) *β*-actin; (b) *SeChi*-1; (c) *SeChi*-2. (M, markers; 1, liver; 2, heart; 3, branchial heart; 4, gill; 5, posterior salivary gland; 6, stomach; 7, caecum; 8, mantle).



Abbreviation	Species	Accession number
SeChi-1	*Sepia esculenta*	AB986212
SeChi-2	*Sepia esculenta*	LC319665
TpChi	*Todarodes pacificus*	LC146770
EsChito	*Euprymna scolopes*	KF015222
HcChi-3	*Hyriopsis cumingii*	JN582038
CgChi-3	*Crassostrea gigas*	AJ971239
AcAMCase	*Aplysia californica*	XM005112601
AkChi	*Aplysia kurodai*	LC085435
BgChi-3lp1	*Biomphalaria glabrata*	XP013090777
LsChi-3	*Lymnaea stagnalis*	LC069028
SjChi-1	*Scomber japonicus*	BAL40979
SjChi-2	*Scomber japonicus*	BAL41779
SmChi-1	*Sebastiscus marmoratus*	AB686658
SmChi-2	*Sebastiscus marmoratus*	AB686659
fChi-1	*Paralichthys olivaceus*	AB121732
fChi-2	*Paralichthys olivaceus*	AB121733
SmChiA	*Serratia marcescens*	X03657

Figure 10. Phylogenetic analysis of chitinase amino acid sequences by the neighbor-joining method of the program ClustalW. A bacterial chitinase, *Serratia marcescens* chitinase, was used as the out group. The scale bar indicates the substitution rate per residue. The arrows show *SeChi*-1 and *SeChi*-2 obtained in the present study.

4. Conclusion

In this study, the distribution of chitinolytic enzyme activity in *S. esculenta* was measured and high chitinolytic activity was found in digestion-related organs such as the liver. These chitinases can potentially degrade ingested chitinous substances. In addition, high chitinolytic activity was observed in the posterior

salivary gland. Chitinases in the posterior salivary gland may act as a poison, as observed in other cephalopods. Chitinolytic activity in other organs suggests that chitinases are involved in defense against parasites and other activities. Two CBPs (CBP-A and CBP-B) with molecular weights of 52 and 62 kDa, respectively, were separated from the liver of *S. esculenta*. The molecular weight of CBP-B was consistent with that of SeChi, a chitinase previously purified from the liver of *S. esculenta*. CBP-A was suggested to be a chitinase isozyme obtained from the liver of *S. esculenta*. Full-length cDNAs (*SeChi*-1, *SeChi*-2) encoding two chitinases (SeChi-1, SeChi-2) were obtained from the liver of *S. esculenta*. The molecular weights of SeChi-1 and SeChi-2 calculated from their amino acid sequences were 51.2 and 61.0 kDa, respectively, and their isoelectric points were 8.87 and 8.79, respectively, indicating that they are basic proteins. SeChi-1 contained one CBD and SeChi-2 contained two CBDs. Peptide fragments of CBPs isolated from the liver of *S. esculenta* were analyzed and compared with the amino acid sequences of SeChi-1 and SeChi-2 by proteome analysis. A sequence obtained from peptide fragments of CBP-A was consistent with the amino acid sequence of SeChi-1 (27.23%) and a sequence obtained from peptide fragments of CBP-B was consistent with the amino acid sequence of SeChi-2 (21.01%). Among the *S. esculenta* organs studied, *SeChi*-1 and *SeChi*-2 were only expressed in the liver. This suggests that the two chitinases are involved in chitin degradation in the liver. The two chitinase genes were not expressed in the posterior salivary gland, where high chitinolytic activity was detected. This suggests that other chitinase isozymes are present in the posterior salivary gland. Phylogenetic analysis revealed that SeChi-2 formed a group with other Decembrachiata chitinases, whereas SeChi-1 did not group with mollusk chitinases. Considering that SeChi-1 represents the first chitinase to possess one CBD in Decembrachiata, SeChi-1 is considered to be a new-type chitinase.

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References

[1] Arbia, W., Arbia, L., Adour, L. and Amrane, A. (2013) Chitin Extraction from Crustacean Shells Using Biological Methods—A Review. *Food Technology and Biotechnology*, **51**, 12-25.

[2] Karthik, N., Akanksha, K., Binod, P. and Pandey, A. (2014) Production, Purification and Properties of Fungal Chitinases—A Review. *Indian Journal of Experimental Biology*, **52**, 1025-1035.

[3] Komatsu, M., Son, J., Matsushita, N. and Hogetsu, T. (2007) Fluorescein-Labeled Wheat Germ Agglutinin Stains the Pine Wood Nematode, *Bursaphelenchusxyiophilus*. *Journal of Forest Research*, **13**, 132-136. https://doi.org/10.1007/s10310-008-0065-9

[4] Khoushab, F. and Yamabhai, M. (2010) Chitin Research Revisited. *Marine Drugs*, **8**, 1988-2012. https://doi.org/10.3390/md8071988

[5] Patil, R.S., Ghormade, V.V. and Deshpande, M.V. (2000) Chitinolytic Enzymes: An Exploration. *Enzyme and Microbia Technology*, **26**, 473-483. https://doi.org/10.1016/S0141-0229(00)00134-4

[6] Slámová, K., Bojarová, P., Petrásková, L. and Kren, V. (2010) *β-N*-acetylhexosaminidase: What's in a Name…? *Biotechnology Advances,* **28**, 682-693. https://doi.org/10.1016/j.biotechadv.2010.04.004

[7] Gooday, G.W. (1999) Aggressive and Defensive Roles for Chitinases. *Cellular and Molecular Life Sciences*, **87**, 157-169.

[8] Henrıssat, B. and Baıroch, A. (1993) New Families in the Classification of Glycosyl Hydrolases Based on Amino Acid Sequence Similarities. *Biochemical Journal,* **293**, 781-788. https://doi.org/10.1042/bj2930781

[9] Fukamizo, T., Koga, D. and Goto, S. (1995) Comparative Biochemistry of Chitinases-Anomeric Form of the Reaction Products. *Bioscience Biotechnology Biochemistry*, **59**, 311-313. https://doi.org/10.1271/bbb.59.311

[10] Koga, D., Yoshioka, K. and Arakane, Y. (1998) HPLC analysis of Anomeric Formation and Cleavage Pattern by Chitinolytic Enzyme. *Bioscience Biotechnology Biochemistry*, **62**, 1643-1646. https://doi.org/10.1271/bbb.62.1643

[11] Shinya, S., Nagata, T., Ohnuma, T., Taira, T., Nishimura, S. and Fukamizo, T. (2012) Backbone Chemical Shifts Assignments, Secondary Structure, and Ligand Binding of a Family GH-19 Chitinase from Moss, Bryumcoronatum. *Biomolecular NMR Assignments*, **2**, 157-161. https://doi.org/10.1007/s12104-011-9346-x

[12] Ikeda, M., Miyauchi, K., Mochizuki, A. and Matsumiya, M. (2009) Purification and Characterization of Chitinase from the Stomach of Silver Croaker *Pennahiaargentatus*. *Protein Expression and Purification*, **65**, 214-222.

[13] Ikeda, M., Miyauchi, K. and Matsumiya, M. (2012) Purification and Characterization of a 56 kDa Chitinase Isozyme (PaChiB) from the Stomach of Silver Croaker-*pennahiaargentatus*. *Bioscience Biotechnology Biochemistry*, **76**, 971-979. https://doi.org/10.1271/bbb.110989

[14] Kurokawa, T., Uji, S. and Suzuki, T. (2004) Molecular Cloning of Multiple Chitinase Genes in Japanese Flounder, *Paralichthysolivaceus*. *Comparative Biochemistry and Physiology B*, **138**, 255-264.

[15] Ikeda, M., Kondo, Y. and Matsumiya, M. (2013) Purification, Characterization, and Molecular Cloning of Chitinases from the Stomach of the Threeline Grunt *Parapristipomatrilineatum*. *Process Biochemistry*, **48**, 1324-1334.

[16] Kakizaki, H., Ikeda, M., Fukushima, H. and Matsumiya, M. (2015) 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 (*Pennahiaargentata*). *Open Journal of Marine Science*, **5**, 398-411. https://doi.org/10.4236/ojms.2015.54032

[17] Kawashima, S., Ikehata, H., Tada, C., Ogino, T., Kakizaki, H., Ikeda, M., Fukushima, H. and Matsumiya, M. (2016) Stomach Chitinase from Japanese Sardine *Sardinopsmelanostictus*: Purification, Characterization, and Molecular Cloning of Chitinase Isozymes with a Long Linker. *Marine Drugs*, **14**, 1-13. https://doi.org/10.3390/md14010022

[18] Matsumiya, M. Kakizaki, H. and Ikeda, M. (2017) Prosperity Strategy and Chitinase in Fish. *Chitin and Chitosan Research*, **23**, 4-16.

[19] Ikeda, M., Kakizaki, H. and Matsumiya, M. (2017) Biochemistry of Fish Stomach Chitinase. *International Journal of Biological Macromolecules*, **104**, 1672-1681.

[20] Wang, G.L., Xu, B., Bai, Z.Y. and Li, J.L. (2012) Two Chitin Metabolic Enzyme Genes from *Hyriopsiscumingii*: Cloning, Characterization, and Potential Functions. *Genetics and Molecular Research*, **11**, 4539-4551. https://doi.org/10.4238/2012.October.15.4

[21] Yonezawa, M., Sakuda, S., Yoshimura, E. and Suzuki, M. (2016) Molecular Cloning and Functional Analysis of Chitinases in the Fresh Water Snail, *Lymnaeastagnalis*. *Journal of Structural Biology*, **196**, 107-118.

[22] Li, H., Wang, D., Denq, Z., Huang, G., Fan, S., Zhou, D., Liu, B., Zhang, B. and Yu, D. (2017) Molecular Characterization and Expression Analysis of Chitinase from the Pearl Oyster *Pinctadafucata*. *Comparative Biochemistry and Physiology Part B*: *Biochemistry & Molecular Biology*, **203**, 141-148.

[23] Badariotti, F., Lelong, C., Dubos, M.P. and Favrel, P. (2007) Characterization of Chitinase-Like Proteins (Cg-Clp1 and Cg-Clp2) Involved in Immune Defence of the Mollusk *Crassostreagigas*. *The FEBS Journal*, **274**, 3646-3654. https://doi.org/10.1111/j.1742-4658.2007.05898.x

[24] Badariotti, F., Thuau, R., Lelong, C., Dubos, M.P. and Favrel, P. (2007) Characterization of an Atypical Family 18 Chitinase from the Oyster *Crassostreagigas*: Evidence for a Role in Early Development and Immunity. *Developmental & Comparative Immunology*, **31**, 559-570.

[25] Gao, L., Xu, G. J., Su, H., Gao, X.G., Li, Y.F., Bao, X.B., Liu, W.D. and He, C.B. (2014) Identification and Expression Analysis of cDNA Encoding Chitinase-Like Protein (CLP) Gene in Japanese Scallop *Mizuhopectenyessoensis*. *Genetics and Molecular Research*, **13**, 10727-10740. https://doi.org/10.4238/2014.December.18.14

[26] Matsunaga, G., Karasuda, S., Nishino, R., Fukushima, H. and Matsumiya, M. (2016) Molecular Cloning of a Chitinase Gene from the Ovotestis of Kuroda's Sea Hare *Aplysiakurodai*. *Advances in Bioscience and Biotechnology*, **7**, 38-46. https://doi.org/10.4236/abb.2016.71005

[27] Yang, B., Zhang, M., Li, L., Pu, F., You, W. and Ke, C. (2015) Molecular Analysis of Atypical Family 18 Chitinase from Fujian Oyster *Crassostreaangulata* and Its Physiological Role in the Digestive System. *PLoS ONE*, **10**, e0129261. https://doi.org/10.1371/journal.pone.0129261

[28] Matsumiya, M. and Mochizuki, A. (1997) Purification and Characterization of Chitinase from the Liver of Japanese Common Squid *Todarodespacificus*. *Fisheries Science*, **63**, 409-413. https://doi.org/10.2331/fishsci.63.409

[29] Matsumiya, M., Miyauchi, K. and Mochizuki, A. (2002) Characterization of 38kDa and 42kDa Chitinase Isozymes from the Liver of Japanese Common Squid *Todarodespacificus*. *Fisheries Science*, **68**, 603-609. https://doi.org/10.1046/j.1444-2906.2002.00467.x

[30] Matsumiya, M., Miyauchi, K. and Mochizuki, A. (2003) Purification and Some Properties of a Chitinase Isozyme from the Liver of Japanese Common Squid *Todarodespacificus*. *Fisheries Science*, **69**, 427-429. https://doi.org/10.1046/j.1444-2906.2003.00640.x

[31] Nishino, R., Suyama, A., Ikeda, M., Kakizaki, H. and Matsumiya, M. (2014) Purification and Characterization of a Liver Chitinase from Golden Cuttlefish, *Sepia esculenta*. *Journal of Chitin and Chitosan Science*, **2**, 238-243. https://doi.org/10.1166/jcc.2014.1065

[32] Kondo, H., Morita, T., Ikeda, M., Kurosaka, C., Shitara, A., Honda, Y., Nozaki, R., Aoki, T. and Hirono, I. (2010) Identification of Enzyme Genes in the Liver of the Bleeker's Squid *Loligobleekeri* by Expressed Sequence Tag Analysis. *Fisheries*

Science, **76**, 161-165. https://doi.org/10.1007/s12562-009-0182-1

[33] Fry, B.G., Roelants, K. and Norman, J.A. (2009) Tentacles of Venom: Toxic Protein Convergence in the Kingdom Animalia. *Journal of Molecular Evolution*, **68**, 311-321. https://doi.org/10.1007/s00239-009-9223-8

[34] Ruder, T., Sunagar, K., Undheim, E.A., Ali, S.A., Wai, T.C., Low, D.H., Jackson, T.N., King, G.F., Antunes, A. and Fry, B.G. (2013) Molecular Phylogeny and Evolution of the Proteins Encoded by Coleoid (Cuttlefish, Octopus, Squid) Posterior Venom Glands. *Journal of Molecular Evolution*, **76**, 192-204. https://doi.org/10.1007/s00239-013-9552-5

[35] Kremer, N., Philipp, E.E., Carpentier, M.C., Brennan, C.A., Kraemer, L., Altura, M.A., Augustin, R., Häsler, R., Heath-Heckman, E.A., Peyer, S.M., Schwartzman, J., Rader, B.A., Ruby, E.G., Rosenstiel, P. and McFall-Ngai, M.J. (2013) Initial Symbiont Contact Orchestrates Host-Organ-Wide Transcriptional Changes that Prime Tissue Colonization. *Cell Host & Microbe*, **14**, 183-194.

[36] Alves, D.M., Cristo, M., Sendao, J. and Borges, T.C. (2006) Diet of the Cuttlefish *Sepia officinalis* (Cephalopoda: Seplidae) off the South Coast of Portugal (Eastern Algarve). *Journal of the Marine Biological Association of the United Kingdom*, **86**, 429-436. https://doi.org/10.1017/S0025315406013312

[37] Matsumiya, M., Miyauchi, K. and Mochizuki, A. (1998) Distribution of Chitinase and *β-N*-Acetylhexosaminidase in the Organs of a Few Squid and a Cuttlefish. *Fisheries Science*, **64**, 166-167. https://doi.org/10.2331/fishsci.64.166

[38] Ohtakara, A. (1988) Chitinase and *β-N*-acetylhexosaminidase from *Pycnoporus-cinnabarinus*. *Method in Enzymology*, **161**, 462-470.

[39] Olsen, J.V., Ong, S.E. and Mann, M. (2004) Trypsin Cleaves Exclusively C-Terminal to Arginine and Lysine Residues. *Molecular & Cellular Proteomics*, **3**, 608-614. https://doi.org/10.1074/mcp.T400003-MCP200