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 pNP-(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 pNP-(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 Decabranchiata 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, (pNP-(GlcNAc)_n) (n = 2, 3) (Seikagaku, Tokyo, Japan) and pNP-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 pNP-(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 mM K_2[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     | CTGTGAATGGCGACTACGATTTTTTTTTTTTTTTTTTTTTTTTT | cDNA synthesis                       |
| Chi-a*       | GGNGGNTGGAAYATGGG                     | Primary PCR                          |
| Chi-b*       | TNGCNGCNTTYGARTGGAAAYGA               | Primary PCR                          |
| Chi-c*       | ANCANRANCNTTRGTYACCCA                 | Primary PCR                          |
| SeChi-1-1    | GCACCAAAGAAAGAGTGTGAT                | 3'RACE                               |
| SeChi-2-1    | GGACATAACAGCCCTCTG                   | 3'RACE                               |
| SeChi-1-2    | CTGTGAATGGCGACTACGAT                 | 3'RACE                               |
| SeChi-1-3    | GCACCAAAGAAAGAGTGTGAT                | 3'RACE                               |
| SeChi-1-4    | CCCATTCAAGTTGGACCAAAG               | 3'RACE                               |
| SeChi-2-2    | CGCTACCATGGCACTGCAAAGGG              | 3'RACE                               |
| SeChi-2-3    | CTCTATCCATGGTTGACTTTGACTTGACATTTC   | 5'RACE                               |
| AAP          | GGCCACGGCGTCGACTAGTACCGGGIIIGGGIIIGGIIG | 5'RACE                               |
| AUAP         | GGCCACGGCGTCGACTAGTAC                | 5'RACE                               |
| SeChi-1-5    | ACACATTACAGCAA                      | Full-length ORF                      |
| SeChi-1-6    | TAAATACAGCAAAATACAGTATTAT            | Full-length ORF                      |
| SeChi-2-4    | CAAGTCTCTGACGAGGTACAGGA             | Full-length ORF                      |
| SeChi-2-5    | GGCTGAAAATAAAAATATAGTT             | Full-length ORF                      |
| β-actin-a    | GGATGACGGAAAGCTGGGTATTT            | Organ expression                     |
| β-actin-b    | GTGGTGGACACCACACCTACCAGA            | Organ expression                     |
| SeChi-1-a    | GAAACTTTGTAGTGTGACCAT               | Organ expression                     |
| SeChi-1-b    | TGTTGTCATAAAGACGCAATTCC            | Organ expression                     |
| SeChi-2-a    | GAGAATACCACCACCTGCTGAAGA            | Organ expression                     |
| SeChi-2-b    | AGAAGATGTTAGGAATAGGGAT             | 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 up-
stream (5′) and downstream (3′) regions were amplified using the rapid amplifi-
cation 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 li-
gated into the pGEM-T Easy Vector (Promega). Full-length chitinase genes o-
btained from the liver of S. esculenta were amplified using platinum®Pfx DNA
polymerase (Invitrogen), which has proofreading activity. The reaction condi-
tions 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 Biosy-
systems, 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 am-
plified 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 reac-
tion 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 o-
btained 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 pNP-(GlcNAc)_2 and pNP-(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 sub-
strate, 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. (■) pNP-(GlcNAc)₂; (♦) pNP-(GlcNAc)₃; (□) pNP-(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 share homology with chitotriosidase of Hawaiian bobtail squid *E. scolopes* [35]. The upstream and downstream sequences of these 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 cdDNAs were amplified using Platinum®Pfx DNA polymerase.

Two full-length cdDNAs, *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. 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 8.87.

**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 7. The domain structure of SeChi-2 was obtained from the liver as examples. Conversely, the domain structures of mollusk chitinases have previously been reported to contain one CBD [18] [19]. The domain structures of decapod crustacean chitinases have previously been reported to possess two CBDs. This suggests that mollusk chitinases have several domain structures, which correspond to their physiological roles.

Figure 8. Amino Acid Sequence of the Chitinases

Two CBPs (CBP-A and CBP-B) (Figure 3 and Figure 4) were obtained from the liver of S. esculenta and two CBDs for SeChi-2 (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 is a squid chitinase, was revealed to be a new Decembrachiata chitinase.

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: 6102.53. 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 3-like protein 1 (BgChi-3lp1) 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 structures, 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-3lp, XP013097777; 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 chitinase isozymes, which differ from SeChi-1 and SeChi-2, is suggested in the posterior salivary gland of 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).
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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