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Molecular Cloning of a Chitinase Gene from the Ovotestis of Kuroda's Sea Hare *Aplysia kurodai*

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Abstract

In this study, we report that we successfully cloned and sequenced a chitinase gene from the ovotestis of Kuroda's sea hare *Aplysia kurodai*. By using reverse transcription-polymerase chain reaction (RT-PCR) and a system for the 5' and 3' rapid amplification of cDNA ends, we obtained a 1352 bp chitinase gene (*AkChi*) from the ovotestis of *A. kurodai*. *AkChi* contains a 1263 bp open reading frame that encodes 421 amino acids. The domain structure predicted from the deduced amino acid sequence was an N-terminal signal peptide and a catalytic domain of glycoside hydrolase (GH) family 18 chitinase. A comparative analysis of the deduced amino acid sequences of *AkChi* with those of the acidic mammalian chitinase of the California sea hare *Aplysia californica* revealed the highest homology at 83%. The purified chitinase from the ovotestis was digested by trypsin, and 119 residues of digested peptides were consistent with the deduced amino acid sequence of *AkChi*. We used RT-PCR to evaluate the expression of *AkChi* in various tissues of *A. kurodai*, and we observed that *AkChi* was expressed only in the ovotestis. A phylogenetic tree analysis, performed using the amino acid sequences of *AkChi* and known GH family 18 chitinases, showed that *AkChi* was separated from the molluscan chitinases with a chitin binding domain. To our knowledge, this is the first study demonstrating the cDNA cloning of an ovotestis chitinase from a sea hare.

Keywords

Chitinase, Molecular Cloning, Kuroda's Sea Hare *Aplysia kurodai*, Mollusc, Ovotestis, Phylogenetic Tree Analysis

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1. Introduction

Chitin, a major molecular constituent of the exoskeleton of insects and crustaceans, is a straight-chain homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine units [1]-[3]. Chitinases (EC 3.2.1.14) are enzymes that randomly hydrolyze the β-1,4 glycosidic bonds of chitin [4]. They have been found in various organisms, and they play important physiological roles in functions such as attack, defense, morphological changes, and digestion [5] [6].

The characterization and cDNA cloning of chitinases from several fishes have been reported [7]-[9]. The stomach chitinases of fish have been identified and are classified into two groups, acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2) based on the differences in their primary structure and the activity toward short substrates [8]. Chitinases from molluscs play important physiological roles in the digestion of food [10] [11], attacking crustaceans [12], and shell formation [13] [14]. However, reports on the distribution, characterization, and cDNA cloning of molluscan chitinases are limited [10]-[16]. In this study, we were using the Kuroda's sea hare, *Aplysia kurodai*. *A. kurodai* is a kind of herbivorous gastropoda seen in the vicinity of the coast from April to June. In addition, this creature was allowed to degenerate shells despite the shellfish. In a previous study, we detected chitinase activity in the ovotestis and egg of *A. kurodai* [16], whereas lysozyme activity (antibacterial enzyme activity) was not detected in all of the organs [16]. We also reported the purification and properties of a chitinase from the ovotestis of *A. kurodai* [16]. Together the results indicated that the physiological role of this chitinase was as a defense against nematodes and fungus which had chitin in the body wall as a structural component [16].

In the present study, we cloned the cDNA encoding chitinase from the ovotestis of *A. kurodai* and determined the primary structure of the chitinase.

2. Materials and Methods

2.1. Materials

Kuroda's sea hare *Aplysia kurodai* and laid egg were captured from the tide pools of Shimoda Bay (Shizuoka, Japan) in June.

2.2. Cloning of the Chitinase cDNA from *A. Kurodai*

The sequences of all primers are presented in **Table 1**. Total RNA was extracted from the ovotestis of *A. kurodai* using ISOGEN II reagent (Nippon Gene, Tokyo) according to the manufacturer's instructions. First-strand cDNA was synthesized using 500 ng of total RNA and oligo dT primers with Prime Script Reverse Transcriptase (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Six degenerate primers were designed for the reverse transcriptase-polymerase chain reaction (RT-PCR) from conserved sequences of molluscan chitinase, including those from California sea hare (*Aplysia californica*; GenBank: XM_005112601), triangle sail mussel (*Hyriopsis cumingii*; GenBank: JN582038), Pacific oyster (*Crassostrea gigas*; GenBank: AJ971239), Hawaiian bobtail squid (*Euprymna scolopes*; GenBank: KF015222), and golden cuttlefish (*Sepia esculenta*; GenBank: AB986212).

The first PCR was performed using *A. kurodai* cDNA as a template and P1 and P2 as primers (**Figure 1**). The PCR parameters were as follows: 94˚C for 2 min, followed by 30 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s. Nested PCR was performed using the products of the first PCR as templates and P3, P4, P5, and P6 as primers, with the same PCR parameters as described above. The nucleotide sequence analysis of the RT-PCR amplified chitinase cDNA fragments from the ovotestis of *A. kurodai* detected one nucleotide sequence (*AkChi*).

For the 3' rapid amplification of cDNA ends (RACE), we designed primers specific to *AkChi* (*i.e.*, P7, P8, and P9, respectively; **Table 1**) based on the detected sequences. We amplified cDNA fragments encoding the 3' region of *AkChi* using *A. kurodai* cDNA as the template and the primer pairs P7 and 3R, P8 and 3R, and P9 and 3R (**Figure 1**). The PCR parameters were as follows: 94˚C for 2 min, followed by 30 cycles of 94˚C for 30 s, 56˚C for 30 s, and 72˚C for 30 s. For 5' RACE, specific primers (P10, P11, and P12 for *AkChi*; **Table 1**) were designed based on the nucleotide sequences obtained from RT-PCR. cDNA fragments encoding the 5' regions of *AkChi* were amplified using PCR. The first PCR was performed using the newly synthesized first-strand cDNA as a template and the primer pairs P10 and P11 for *AkChi*. Nested PCR was performed using the first PCR products as templates and the primer pairs P10 and P12 for *AkChi*. The PCR parameters were as follows:

Table 1. Primers used for PCR, RACE, and tissue-specific expression.

Primer	Sequence (5' → 3')	Purpose
P1*	TNGCNGCNTTYGARTGGAAYGA	Primary PCR
P2*	CATNCCNSWRAARTCRTCRTTRTC	Primary PCR
P3*	GGNGGNTGGAAYATGGG	Primary PCR
P4*	ACCCAYTGRTTNCCNARNACNA	Primary PCR
P5*	GNAAYTTYGAYGGNYTNGA	Primary PCR
P6*	TTDATCATYTCRCANACYTCRTARTA	Primary PCR
P7	GCCGGATACGAAGTGGAC	3' RACE
P8	GGAACTTAACGAGTACTT	3' RACE
P9	GACAGACGAGAGCGACTCTGGTCG	3' RACE
3R	CTGTGAATGCTGCGACTACGAT	3' RACE
P10	CACAATGACGTTGCAAG	5' RACE, Full-length PCR
P11	ATGGCCTGGGCTCATTTT	5' RACE
P12	TTATCCTCTGGAGGGCT	5' RACE
P13	CACGTTATGATTGCGAC	Full-length PCR
P14	TCTGCTGCTGTGAGTGCTGGCAAGG	tissue-specific expression
P15	GCATTTCGCACACCTCGTAGTAAGA	tissue-specific expression
β-actin-a*	GAYAAYGGNWSNGGNATGTG	tissue-specific expression
β-actin-b*	TCRAACATDATYTGNGTCAT	tissue-specific expression

Note: *Degenerate primers.

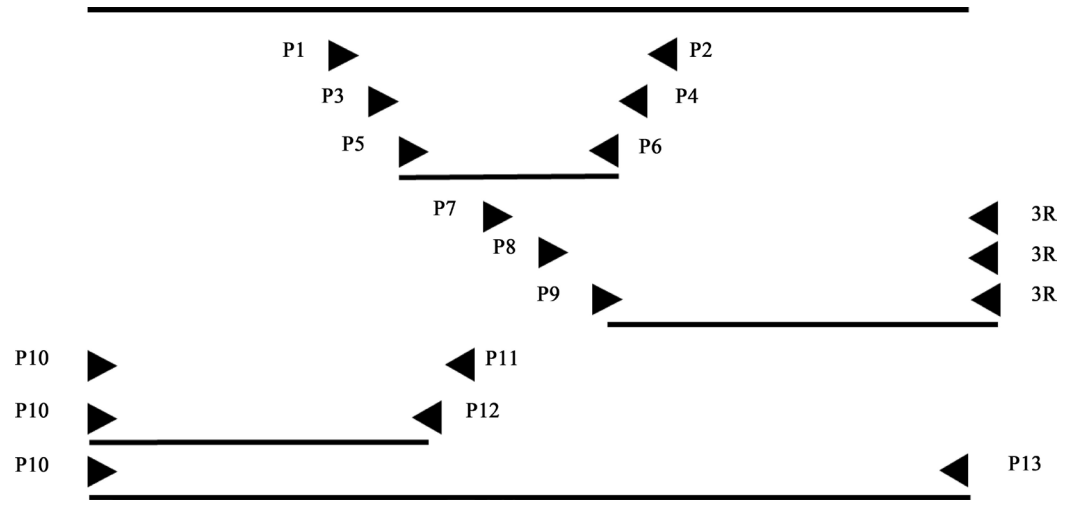


Figure 1. Schematic representation of the cDNA structure of *AkChi* and location of the primers. Arrowheads indicate the primers, and lines between the arrowheads indicate the amplified cDNA fragments.

94˚C for 1 min, followed by 30 cycles of 94˚C for 30 s, 49˚C for 30 s, and 72˚C for 30 s.

The nucleotide sequences of cDNA fragments containing a full-length open reading frame (ORF) were confirmed by PCR using specific primers (P10 and P13 for *AkChi*; **Table 1**) and Platinum *Pfx* DNA Polymerase (Invitrogen, Carlsbad, CA).

2.3. Nucleotide Sequence Analysis

The RT-PCR, 3' RACE, and 5' RACE amplification products, and the full-length amplification products were subcloned into pGEM-T Easy Vector (Promega, Madison, WI), according to the manufacturer's instructions.

Sequences were determined on an ABI PRISM 3130 genetic analyzer (Applied Biosystems, Foster City, CA) using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

2.4. Amino Acid Sequence of the Peptide of the Purified Chitinase from the Ovotestis of *A. kurodai*

A chitinase from the ovotestis of *A. kurodai* was purified as described [16]. The purified chitinase was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with AE-1360 EzStain Silver (ATTO, Tokyo). A gel slice was cut into small pieces and destained by destaining solution (15 mM $K_3[Fe(CN)_6]$, 50 mM $Na_2S_2O_3$). Destained gel pieces were trypsinized as described in the manual of In-Gel Tryptic Digestion Kit manual (Thermo Scientific, Waltham, MA). The peptide mixtures thus obtained were subjected to a nano-scale liquid chromatography-electrospray ionization-tandem mass spectrometry (nanoLC-ESI-MS/MS) analysis using a Q Exactive mass spectrometer (Thermo Scientific) equipped with a captive spray ionization source (Michrom Bioresources, Auburn, CA) and an Advance UHPLC System (Michrom Bioresources).

2.5. Tissue-Specific Expression of *AkChi*

Total RNA was prepared from the ovotestis, egg, skin, gill, crop, anterior gizzard, and posterior gizzard as described in the cloning methods section (2.2) above. First-strand cDNA was pre-cloned from the RNA isolated from each tissue and egg as described in the RT-PCR section (2.2) above. For tissue-specific expression, we designed primers specific to *AkChi* (P14 and P15, respectively; **Table 1**) based on the detected sequences. *AkChi* was amplified using the first-strand cDNA as template and the primer pairs P14 and P15 (**Table 1**). The PCR parameters were as follows: 94˚C for 1 min, followed by 35 cycles of 94˚C for 30 s, 62˚C for 30 s, and 72˚C for 30 s. To determine the amount of total RNA in each tissue, we amplified β-actin mRNA fragments using specific primer pairs (**Table 1**).

2.6. Phylogenetic Tree Analysis of *AkChi*

In order to classify the chitinase from the ovotestis of *A. kurodai* among the GH family 18 chitinases, we constructed a phylogenetic tree based on the enzyme precursor sequences by the neighbor-joining method, using the ClustalW program (http://www.genome.jp/tools/clustalw/). A bacterial chitinase (GenBank: X03657) was used as the out group.

3. Results and Discussion

3.1. Cloning of *A. kurodai* Chitinase cDNA

The structure of *AkChi* and the location of primer sequences are schematically represented in **Figure 1**. The internal sequence of the cDNA of *A. kurodai* ovotestis chitinase was amplified by RT-PCR using degenerate primers (from P1 to P6, respectively; **Table 1**); an amplified product of approx. 400 bp was obtained. The product was sequenced, and 86% homology with the acidic mammalian chitinase of *A. californica* was confirmed (accession no. XM_005112601). Because the sequence was part of ovotestis chitinase cDNA from *A. kurodai*, we used it to design gene-specific primers for 3’ and 5’ RACE (from P7 to P12; **Table 1**). An amplified product of approx. 430 bp was obtained by 3’ RACE, and its sequence contained a stop codon. An amplified product of approx. 520 bp was also obtained by 5’ RACE; its sequence contained a start codon. Based on these results, we designed full-length primers (P10 and P13; **Table 1**) to incorporate these start and stop codons. cDNA was amplified using the primers and the amplified product was sequenced.

The full-length cDNA of *A. kurodai* ovotestis chitinase (*AkChi*) was 1352 bp in length and contained an ORF of 1263 bp encoding 421 amino acids (**Figure 2**). The size of ORF of *AkChi* was smaller than it from *H. cumingii* [14], 1962 bp encoding 653 amino acids. A poly-A sequence in eukaryotes was detected at the 3’ end of *AkChi*. *AkChi*, which encodes *A. kurodai* ovotestis chitinase, has been registered in the database of the DNA Data Bank of Japan (DDBJ) (accession no. LC085435). We compared the deduced amino acid sequence of *AkChi* with that of other organisms using BLAST, and the highest homology, 83%, was confirmed with the acidic mammalian chitinase of *A. californica* (accession no. XM_005112601). **Figure 3** compares amino acid

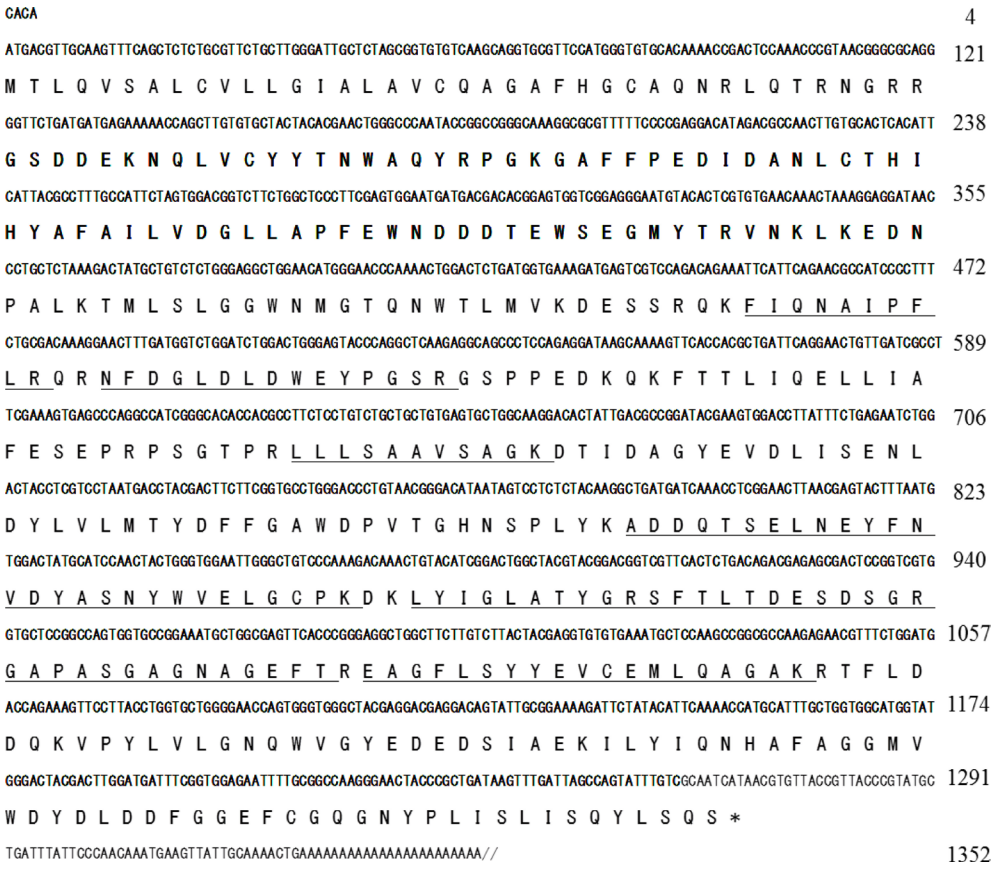


Figure 2. cDNA and deduced amino acid sequences of *AkChi*. Underlined sequences show matching with the peptide fragments of the purified and tripsinized enzyme (coverage: 35.39%, 119 residues).

sequences from *AkChi* and some other known molluscan chitinases (*A. californica*, *H. cumingii*, *C. gigas*, *E. scolopes*, *and S. esculenta*). The deduced amino acid sequence of *AkChi* was shown to have a structure of the GH family 18 chitinase, with an N-terminal signal peptide and a GH 18 catalytic domain. The catalytic domain also contained an active site that is a conserved sequence of GH family 18 chitinases (**Figure 3**). Though the chitinase of *H. cumingii* [14] and *E. scolopes* [15] had two chitin binding domains (CBDs) and the chitinase of *S. esculenta* had one CBD, *AkChi* lacked a CBD. It was reported that fish chitinases have one CBD [8]. This result suggests that the structure of molluscan chitinase is diverse compared to the fish chitinases.

3.2. Amino Acid Sequence of the Chitinase

We analyzed the sequences of the peptide fragments obtained by the tryptic treatment of the purified chitinase from the ovotestis of *A. kurodai* [16] were analyzed and compared them to the deduced amino acid sequence of *AkChi*. The obtained sequences from peptide fragments were consistent with the deduced amino acid sequence of *AkChi* (coverage: 35.39%, 119 residues) (**Figure 2**). This result suggests that *AkChi* is a gene coding the purified enzyme. In addition, trypsin is cut the C-terminal side of lysine and arginine. In this result, it was confirmed that the trypsin is working properly in the all of cleavage site.

3.3. Tissue-Specific Expression of *AkChi*

We investigated the tissue-specific expression of *AkChi* in *A. kurodai* by RT-PCR using the housekeeping *β*-actin gene as a control (**Figure 4**). It is reported that fish express chitinase to the digestive organs for digestion of chitin from food [17]. The expression profile results indicated that *AkChi* was present only in the ovotestis. We previously detected chitinase activity in the ovotestis and egg from *A. kurodai* [16], whereas lysozyme activity (antibacterial enzyme activity) was not detected in any of the organs [16]. *A. kurodai* has to prey on seaweed.

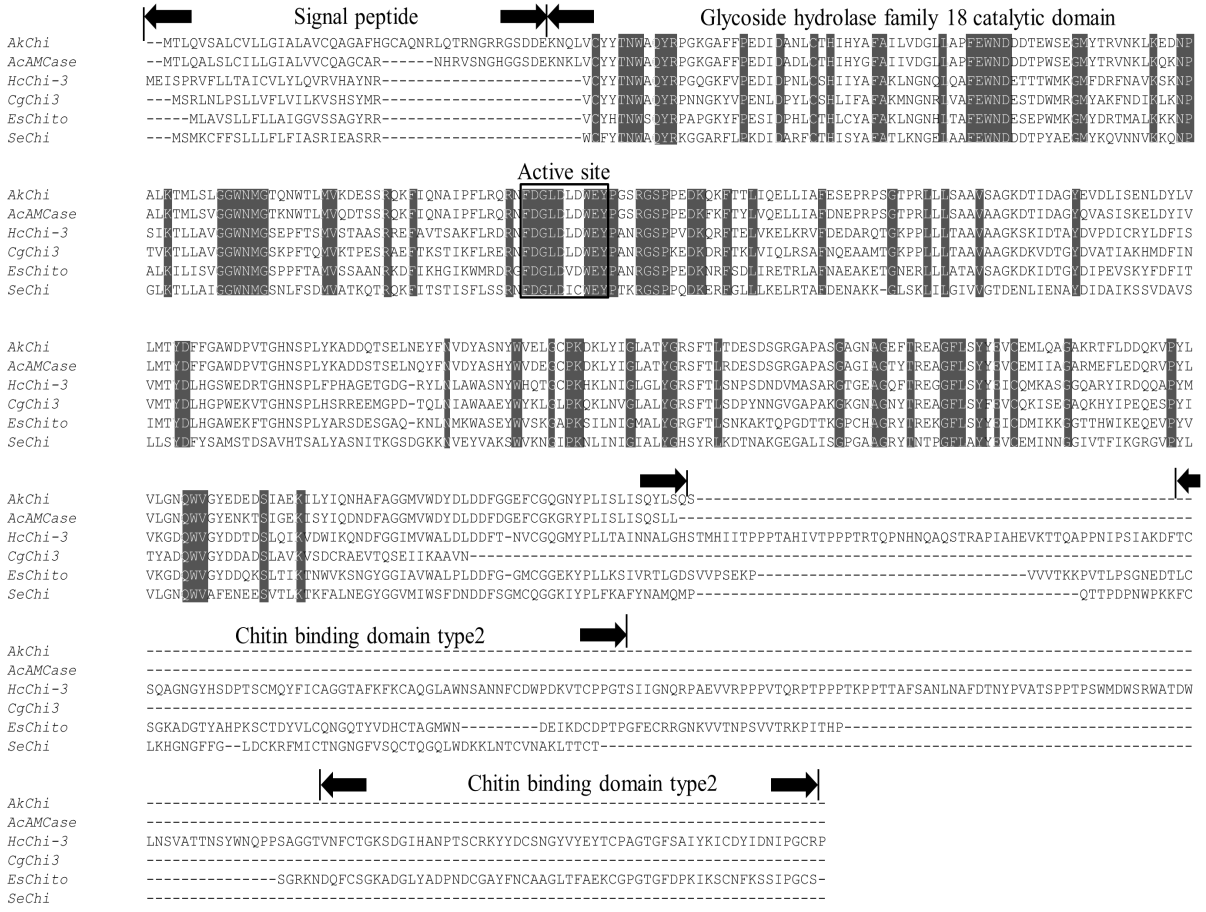


Figure 3. Multiple alignment of duduced amino acid sequences of *A. kurodai* chitinase (*AkChi*) with *Aplysia californica* acidic mammalian chitinase (*AcAMCase*), *Hyriopsis cumingii* chitinase-3 (*HcChi*-3), *Crassostrea gigas* Chit3 protein A (*CgChi*3), *Euprymna scolopes* chitotriosidase (*EsChito*), and *Sepia esculenta* chitinase (*SeChi*). GenBank accession nos.: *AcAMCase*, XM_005112601; *HcChi*-3, JN582038; *CgChi*3, AJ971239; *EsChito*, KF015222; *SeChi*, AB986212. Matched sequences are shown in black.

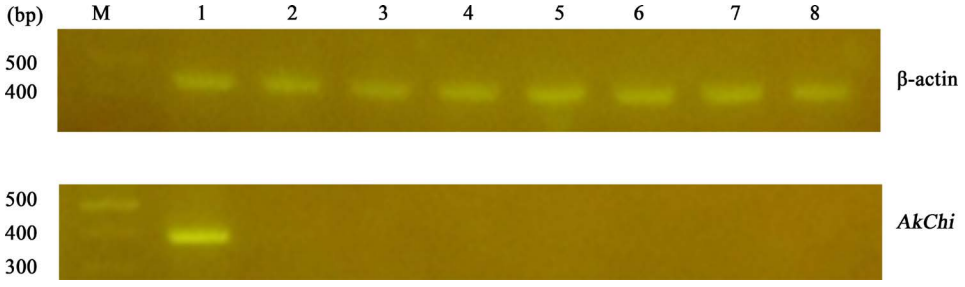
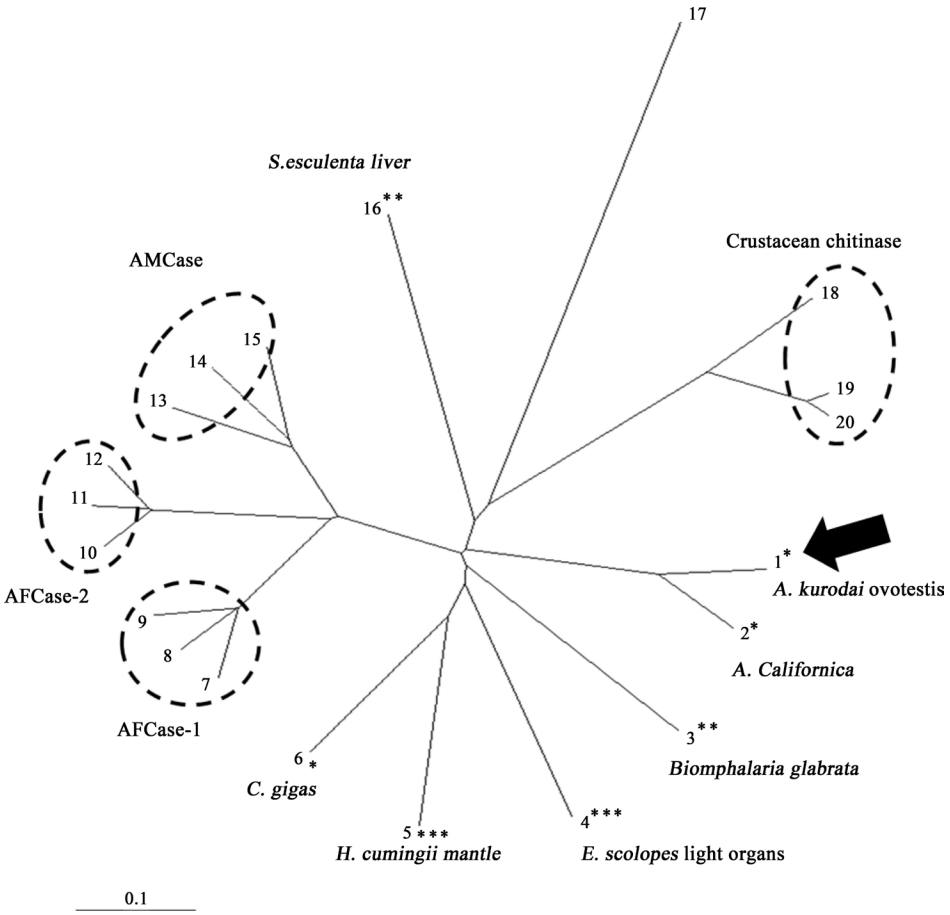


Figure 4. Expression profiles of *AkChi* and *β*-actin mRNA in tissue using RT-PCR. M, markers; 1, ovotestis; 2, egg; 3, skin; 4, gill; 5, buccal mass; 6, crop; 7, anterior gizzard; 8, posterior gizzard.

Thus, *A. kurodai* is not necessary chitinase in digestion and attack of food as squid [10] [11] and octopus [12], respectively. In addition, there is not necessary to shell formation because it does not even have shells. These results suggest that the role of this chitinase is as a defense against nematodes and fungus which have chitin in the body wall as a structural component.

3.4. Phylogenetic Tree Analysis of *AkChi*

We performed a phylogenetic tree analysis of GH family 18 chitinases and *AkChi* (**Figure 5**). Acidic mamma-



No.	Species	Genbank accession number
1	*Aplysia kurodai* (chitinase)	LC085435
2	*Aplysia californica* (acidic mammalian chitinase)	XM_005112601
3	*Biomphalaria glabrata* (chitinase-3-like protein 1)	XP_013090777
4	*Euprymna scolopes* (chitotriosidase)	KF015222
5	*Hyriopsis cumingii* (chitinase-3)	JN582038
6	*Crassostrea gigas* (Chit3 protein)	AJ971239
7	*Epinephelus coioides* (chitinase1)	AB686658
8	*Sebastiscus marmoratus* (chitinase1)	FJ169895
9	*Parapristipoma trilineatum* (chitinase1)	AB642677
10	*Epinephelus coioides* (chitinase2)	FJ169894
11	*Parapristipoma trilineatum* (chitinase2)	AB642678
12	*Sebastiscus marmoratus* (chitinase2)	AB686659
13	*Bos Taurus* (chitin binding protein b04)	AB051629
14	*Mus musculus* (acidic chitinase)	EF094027
15	*Homo sapiens* (acidic mammalian chitinase)	AF290004
16	*Sepia esculenta* (chitinase)	AB986212
17	*Serratia marcescens* (chiA protein precursor)	X03657
18	*Portunus trituberculatus* (chitinase1)	AB874469
19	*Portunus trituberculatus* (chitinase2)	AB890123
20	*Scylla serrata* (chitinase)	EU402970

Figure 5. Phylogenetic tree analysis of chitinase amino acid sequence by the neighbor-joining method of the program Clustal W. A bacterial chitinase, *Serratia marcescens* chitinase, was used as the out group. The scale bar indicates the substitution rate per residue. The arrow shows *AkChi* obtained in the present study. * Molluscan chitinase without a CBD; ** Molluscan chitinase with one CBD; *** Molluscan chitinase with two CBDs.

lian chitinases (AMCases) have been found in the stomach of mammals. Two chitinase groups with different structures and activity toward short substrates, AFCase-1 and AFCase-2, have been found in the stomach of fish [8]. Crustacean showed a chitinase group [18]. In contrast, molluscan chitinases did not show clear chitinase groups. The reason for this might be the differences in the chitinase domain structure that are due to the presence or absence of a CBD and the number of CBDs. We previously detected chitinase activity in the ovotestis and oviduct from the Walking sea hare *Aplysia juliana* [16]. If the success in cloning the chitinase from *A. juliana*, it will be conceivable to form a group of sea hare chitinase.

4. Conclusion

The cDNA of the ovotestis chitinase obtained from *A. kurodai* contained a 1263 bp open reading frame with a coding potential for 421 amino acid peptides. *AkChi* had the structural motifs of GH family 18 chitinase, but it did not have chitin binding domain. This study is the first report of the cloning of chitinase from the ovotestis of a sea hare.

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Abbreviations

RT-PCR: reverse transcription-polymerase chain reaction;
RACE: rapid amplification of cDNA ends;
GH: glycoside hydrolase;
AFCase-1: acidic fish chitinase-1;
AFCase-2: acidic fish chitinase-2;
CBD: chitin binding domain;
$K_3[Fe(CN)_6]$: potassium ferricyanide;
$Na_2S_2O_3$: sodium thiosulfate.