Molecular cloning of the defense factor in the albumen gland of the sea hare *Aplysia kurodai*

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Abstract **Aplysianin-A, an antibacterial glycoprotein in the albumen gland of the sea hare *Aplysia kurodai*, inhibited the growth of both Gram-positive and Gram-negative bacteria. Aplysianin-A cDNA clones were isolated from an albumen gland cDNA library. Sequence analysis reveals that aplysianin-A is produced as a precursor protein of 556 amino acid residues with a signal peptide of 19 amino acid residues and contains 6 potential *N*-glycosylation sites. Aplysianin-A mRNA was expressed tissue-specifically in the albumen gland. Homology search reveals that aplysianin-A has a 50% overall amino acid sequence homology to achacin, an antibacterial glycoprotein of the giant African snail *Achatina fulica*.**

Key words: Sea hare; Antibacterial glycoprotein; cDNA cloning; Defense factor

1. Introduction

Sea hares belong to the subclass Opisthobranchia of marine gastropods. They have only degenerated shells in their mantle cavity and expose their naked and soft bodies to the surroundings. However, there is no apparent predator which preferably preys on them. These observations have attracted investigators' interests. To date, various bioactive compounds such as toxins and antibiotics have been isolated from sea hares, mostly from their digestive gland. Most of these compounds are lipophilic and low molecular weight compounds derived from their algal diets and are discussed in connection with the defense system of the sea hares against potential predators and pathogenic microorganisms [1–3]. Recently, we have found that the sea hares possess potent antibacterial glycoproteins in various tissues [4–8]. Aplysianin-A is an active factor in the albumen gland of the sea hare *Aplysia kurodai* and consists of four identical subunits (85 kDa). It inhibited the growth of *Bacilus subtilis* and lysed murine MM46 tumor cells at low concentrations [9]. It is of interest to determine from a comparative biochemical point of view whether the antibacterial glycoproteins in the sea hares have sequences in common with other antibacterial factors present in invertebrates [10–13]. We isolated aplysianin-A cDNA from an albumen gland cDNA library. The deduced complete amino acid sequence of aplysianin-A showed a 50% overall amino acid sequence homology to achacin, an antibacterial glycoprotein in the mucus of the giant African snail which belongs to the subclass Pulmonata of terrestrial gastropods. Aplysianin-A inhibited the growth of bacteria whether they were Gram-positive or Gram-negative species. Here we describe the isolation of aplysianin A cDNA, the deduction of the complete amino acid sequence, and the antibacterial activity against various bacterial species.

2. Materials and methods

2.1. Animals

Sea hares were collected in Akita Prefecture, Japan. Each tissue used for the experiments was obtained from a live sea hare. Pooled albumen glands from more than 200 individuals were kept at −20°C until use.

2.2. Preparation of aplysianin-A

Aplysianin-A was purified from the pooled albumen gland tissue by a combination of gel filtration, ion exchange chromatography and chromatofocusing as described in the previous report [9].

2.3. Antibacterial activity of aplysianin-A

Antibacterial activity of the purified aplysianin-A was examined turbidimetrically according to the previous method [4]. Bacterial species tested were *Aeromonas salmonicida, A. hydrophila, B. subtilis, Escherichia coli, Edwardsiella tarda, Pseudomonas fluorescens, Streptococcus* sp. and *Vibrio anguillarum*.

2.4. N-terminal and internal amino acid sequence analysis

The N-terminal sequence of aplysianin-A was analyzed in Shimazu gas-phase sequencer PSQ-1. Aplysianin-A was cleaved with cyanogen bromide (CNBr) in 70% formic acid at room temperature for 15 h [14]. After dried under vacuum, the residue was applied to SDS-PAGE using 12% gel. The gel was electroblotted onto a polyvinylidene difluoride (PVDF) membrane [15], and a major stained band was analyzed for the amino acid sequence.

2.5. Isolation of aplysianin-A cDNA

Total RNA was extracted by the guanidium isothiocyanate method from tissues of the sea hare *A. kurodai* except for the albumen gland, and cytoplasmic RNA was prepared from the albumen gland as described [16]. Poly(A)$^+$ RNA was purified using Oligotex-dT30 (Takara Shuzo). Albumen gland cDNA was synthesized by oligo(dT) priming and ligated with *Eco*RI-digested λMOSSlox (Amersham) or pBluescript (Stratagene) after attachment of *Eco*RI adaptor. A partial aplysianin-A cDNA fragment was amplified by PCR from the albumen gland cDNA ligated with pBluescript using a 20-mer oligonucleotide (SK-20) (5′-CGCTCTAGAACTAGTGGATC-3′) which hybridizes with pBluescript DNA at a location similar to SK and a fully degenerate 20-mer antisense oligonucleotide corresponding to the amino acid sequence from 11 to 17 of aplysianin-A (see Fig. 1). PCR products were electrophoresed on 1% agarose gel, transferred to a nylon filter and probed with a fully degenerate 14-mer oligonucleotide corresponding to the amino acid sequence from 5 to 9 of aplysianin-A (see Fig. 1). An approximately 200 bp fragment which hybridized with this 14-mer oligonucleotide was gel-purified, subcloned, confirmed its identity to be the aplysianin-A cDNA by sequencing and used to screen the albumen gland cDNA library constructed with λMOSSlox DNA. Positive phage clones were converted to plasmid subclones by cre-mediated excision.

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Single-stranded DNA was prepared, and the nucleotide sequence was determined by the dideoxy method.

2.6. Northern blot analysis

1.0 μg of poly(A)$^+$ RNA from several tissues was fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel, transferrred to a nylon filter, fixed by Stratalinker (Stratagene) and probed with the aplysianin-A cDNA under the standard condition [16].

3. Results and discussion

3.1. Antibacterial activity of aplysianin-A

Aplysianin-A was purified and tested for its antibacterial activity using two Gram-positive species, *B. subtilis* and *Streptococcus* sp., and six Gram-negative species, *A. salmonicida, A. hydrophila, E. coli, E. tarda, P. fluorescens* and *V. anguillarum.*

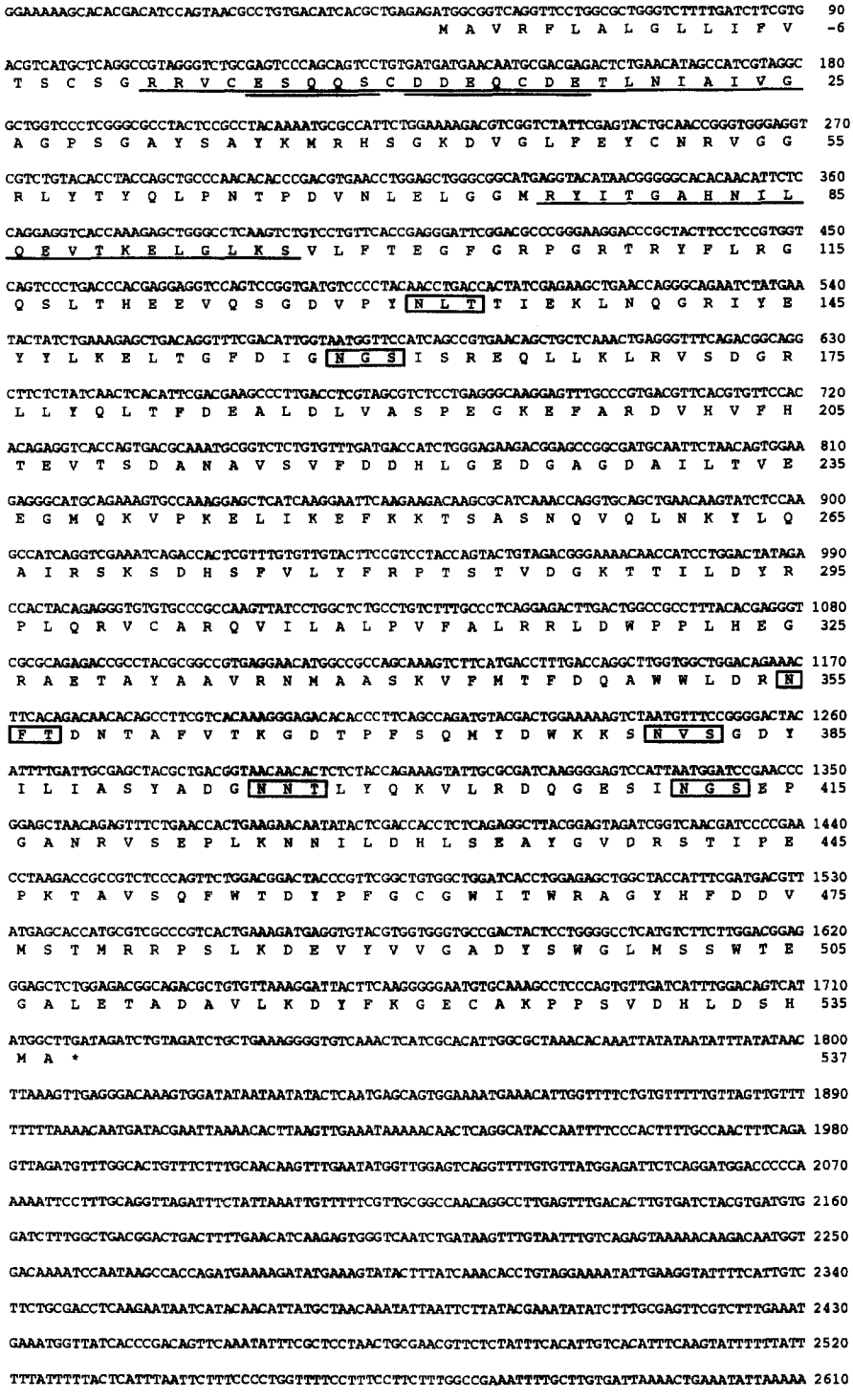

Fig. 1. The nucleotide and deduced amino acid sequence of sea hare aplysianin-A cDNA (pAPLY-A3T). The N-terminal amino acid residue is numbered as 1. The amino acid sequences determined by protein sequencing of the mature aplysianin-A and its CNBr-cleavaged fragment are underlined. The amino acid sequences used to design the oligonucleotides are doubly underlined. Potential *N*-glycosylation sites are boxed.

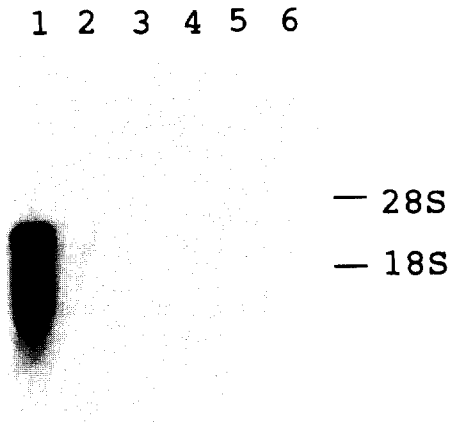

Fig. 2. Tissue-specific expression of aplysianin-A mRNA. 1.0 μg of poly(A)$^+$ RNA from albumen gland (lane 1), purple gland (lane 2), gonad (lane 3), midgut gland (lane 4), ctenidium (lane 5) and body wall (lane 6) was fractionated on a 1.0% agarose-formaldehyde gel and probed with the aplysianin-A cDNA. The positions of mouse 18S and 28S rRNA are indicated on the right.

Among them, *A. salmonicida* was most sensitive and the growth was inhibited by 50% at a concentration of 1.6 μg protein/ml or 5×10^{-9} M. *P. fluorescent* was most resistant (8 μg protein/ml for growth inhibition by 50%). The others were inhibited in the range of 2 to 7.4 μg protein/ml. The growth of the bacteria was depressed after addition of the aplysianin-A, and the period of growth depression depended on the amount of aplysianin-A added. After depression, the bacteria recovered their normal growth. In addition, there was no apparent decrease in turbidity (data not shown), suggesting that aplysianin-A has no bacteriolytic activity. These facts indicate that aplysianin-A was

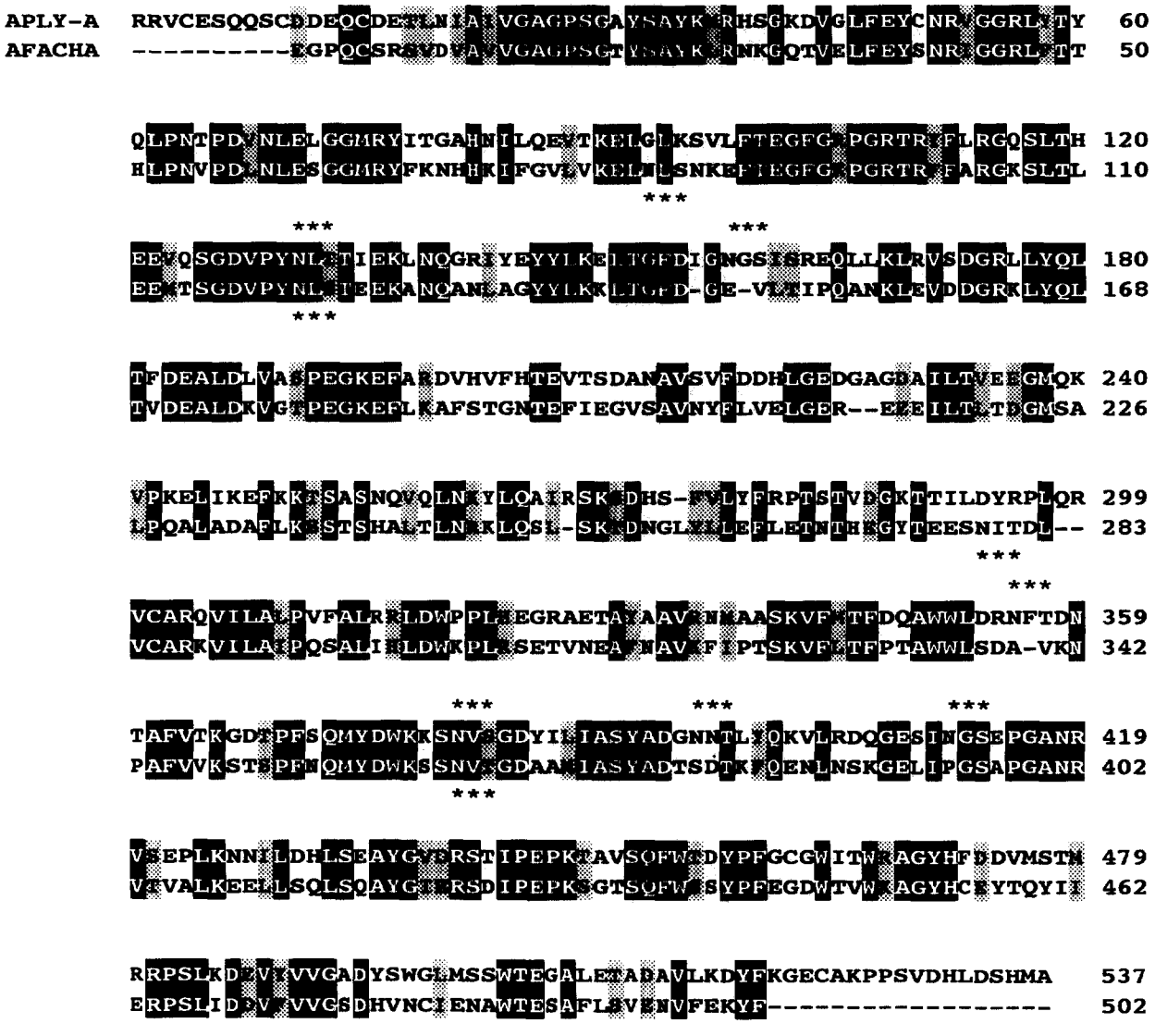

Fig. 3. Comparison of the amino acid sequences of aplysianin-A and achacin. Identical amino acids are shown in white against black, and conservative amino acids are shaded. Gaps introduced in the sequences to optimize the alignment are represented by dashes. Potential *N*-glycosylation sites are indicated by asterisks. Conservative amino acids are classified as follows: D and E; H, K and R; S and T; I, L, M and V; and F and Y.

not bacteriocidal, but bacteriostatic like aplysianin-E, an antibacterial glycoprotein in the egg mass of *A. kurodai* [1,17].

3.2. Sequence determination and cDNA cloning of aplysianin-A

Partial amino acid sequences of aplysianin-A were determined using the mature protein and its CNBr fragment, and the sequences are shown in Fig. 1. To obtain a partial aplysianin-A cDNA fragment, PCR was carried out using albumen gland cDNA ligated with pBluescript as a template with SK-20, a 20-mer oligonucleotide which hybridizes with pBluescript DNA near the *Eco*RI site (see section 2), and a fully degenerate 20-mer antisense oligonucleotide corresponding to the amino acid sequence from 11 to 17 of aplysianin-A (see Fig. 1). PCR resulted in production of an approximately 200 bp fragment which hybridized with a fully degenerate 14-mer oligonucleotide corresponding to the amino acid sequence from 5 to 9 of aplysianin-A (see Fig. 1). This fragment was isolated, subcloned and its sequence was determined. The fragment contained the nucleotide sequence coding for the N-terminal amino acid sequence shown in Fig. 1. Using this fragment as a probe, an albumen gland cDNA library was screened, and several positive hybridizing plaques were obtained and converted to plasmid subclones. The clone with the longest cDNA insert (pAPLY-A3T) was selected and sequenced on both strands.

The nucleotide and deduced amino acid sequence are shown in Fig. 1. The cDNA is 2605 bp in length, not including poly(A) tail, and contains an open reading frame coding for 556 amino acid residues. The N-terminal arginine residue determined by sequencing the mature protein is numbered as 1 in Fig. 1. The amino acid sequence from 1 to 25 and from 76 to 96 agreed with the protein sequence data. The nucleotide sequence from 49 to 106 codes for 19 amino acid residues rich in hydrophobic amino acid residues, which is considered to constitute a signal peptide. Aplysianin-A contains 9.8% neutral sugar [9], and there are six potential *N*-glycosylation sites, which could account for the sugar content.

3.3. Tissue-specific expression of aplysianin-A mRNA

To examine the tissue-specific expression of aplysianin-A mRNA, poly(A)$^+$ RNA was prepared from albumen gland, purple gland, gonad, midgut gland, ctenidium and body wall, and Northern hybridization was carried out (Fig. 2). Aplysianin-A mRNA was detected only in the albumen gland, and its size was calculated to be approximately 2.5 kb. Thus, aplysianin-A is considered to be produced as a precursor specifically in the albumen gland.

3.4. Homology search

The deduced amino acid sequence of aplysianin-A has a significant overall homology to achacin, an antibacterial glycoprotein isolated from the body surface mucus of the giant African snail *Achatina fulica* [13] (48% identity and 59% similarity) (Fig. 3). Aplysianin-A and achacin share several features: they exist as a multimer composed of four or two identical subunits, respectively; they are considered to be produced as a precursor protein and secreted; they are glycoproteins, and the two potential *N*-glycosylation sites are located at similar amino acid positions when aligned. However, aplysianin-A mRNA is expressed in the albumen gland, while achacin mRNA is expressed in the collar tissue, not in the albumen gland. In addition, although achacin is bacteriocidal [18], aplysianin-A is bacteriostatic, not bacteriocidal. These differences are likely to imply their divergent physiological roles and may reflect the distinct modes of life as the sea hare is aquatic and the snail terrestrial.

In our screening of the antibacterial glycoproteins among marine gastropods, only the members of Aplysiidae in the Opisthobranchia were recognized to have similar glycoproteins so far [7,8,19]. The present study has revealed that the two species of gastropods which are phylogenically close to each other but have different modes of life possess similar antibacterial glycoproteins. The information regarding the distribution of aplysianins, achacin, and related antibacterial glycoproteins would be important for understanding the evolution of the defense molecules in gastropods.

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