ASABF, a Novel Cysteine-rich Antibacterial Peptide Isolated from the Nematode Ascaris suum

PURIFICATION, PRIMARY STRUCTURE, AND MOLECULAR CLONING OF cDNA*

Yusuke Kato‡§ and Setsuko Komatsu¶

From the ‡Department of Insect Physiology and Behavior, National Institute of Sericultural and Entomological Science and the ¶Department of Molecular Biology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan

Previously, we reported antibacterial activity in the body fluid of the nematode Ascaris suum (Kato, Y. (1995) Zool. Sci. 12, 225–230). The antibacterial activity is due to a heat-stable and trypsin-sensitive molecule that was designated as ASABF (A. suum antibacterial factor). In the present study, the purification, determination of primary structure, and cDNA cloning of ASABF were carried out. The mature peptide of ASABF is a basic peptide consisting of 71 residues and containing four intramolecular disulfide bridges. The amino acid sequence of a precursor for ASABF, deduced from a cDNA clone, indicates that flanking peptides both at the N terminus and at the C terminus are eliminated by processing. ASABF exhibits potent antibacterial activity particularly against Gram-positive bacteria. ASABF has several features that resemble those of insect/arthropod defensins, whereas the statistical significance of the similarity is not observed on comparison of amino acid sequences. A search of data bases revealed ASABF homologues in Caenorhabditis elegans.

Antimicrobial peptides originating from multicellular organisms have been discovered, mainly in arthropods including insects, vertebrates, and plants (1). Interestingly, some antimicrobial peptides isolated from evolutionally distant origins are structurally similar. For example, defensins were originally found in mammalian neutrophil cells (2). Insect/arthropod defensins, isolated from the body fluid of insects and other arthropods, show a certain degree of sequence similarity with mammalian defensins (3). Both mammalian and insect defensins contain six cysteine residues contributing intramolecular disulfide bridges. Cecropins, linear and mostly helical antibacterial peptides without cysteine residues, were first detected in insects (4) and later isolated from porcine small intestines (5). Plant defensins are antifungal peptides with eight cysteine residues (6), and a homologue, drosomycin, was recently demonstrated in the fruit fly Drosophila melanogaster (7).

In addition, most immune proteins of insects, including antimicrobial peptides, are induced by bacterial challenge or wounding. The gene expression of these immune proteins is suggested to be regulated by transcription factors that resemble those controlling the genes for immunoglobulins and acute phase response proteins in vertebrates, e.g. NFκB. These results suggest that such regulatory systems are of evolutionally ancient origin, i.e. prior to the divergence of deuterostomes (e.g. vertebrates) from protostomes (e.g. insects) (8).

It is, therefore, possible to argue that some innate immune systems related to antimicrobial peptides may be evolutionally related. However, little has been experimentally studied on the early events in the evolution of the antimicrobial peptide-related defense systems. From this aspect, it is clearly important to explore how antimicrobial peptides and their gene regulation in lower invertebrates diverged during an ancient process of evolution. Although few fossil records are available, nematodes are thought to be of very ancient origin, at least comparable with the divergence time of the lines leading to vertebrates and to arthropods from an ancient group (9). The similarity of the antimicrobial peptide-related defense systems among evolutionally distant organisms, furthermore, encourages the application of model animals for studying the innate immunity. It has already been proposed that D. melanogaster may provide an excellent model for a molecular and genetic approach to innate immune reactions, including organisms other than insects (10). Similarly, the nematode, Caenorhabditis elegans, can also be another candidate for a model.

Parasitic nematodes in animal intestines can survive not only a hostile hydrolytic environment and host immune attacks but also a microbe-rich environment. Hence, the immune defenses against coliform microbes are essential for the parasites. We have already reported antibacterial, bacteriolytic, and agglutinating activities in the body fluid of the intestinal parasitic nematode, Ascaris suum (11). The antibacterial factor ASABF (A. suum antibacterial factor) is a heat-stable and trypsin-sensitive molecule, i.e. peptide/protein. In the present study, the purification, determination of primary structure, and cDNA cloning of ASABF were carried out. The results revealed that ASABF is a novel antibacterial peptide containing four intramolecular disulfide bridges and has several features similar to those of insect/arthropod defensins. ASABF homologues in C. elegans were, moreover, demonstrated by a computer-assisted search of data bases.

MATERIALS AND METHODS
Nematodes and Collection of Body Fluid

Adult female A. suum were obtained from Tokyo Shibaura Zohki, Tokyo, Japan. The nematodes were kept at 4 °C after isolation from pig small intestines, and body fluid was collected within 5 h as described previously (11). The collected body fluid was stored at −120 °C.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number D83529.

‡ To whom correspondence should be addressed: Laboratory of Metabolism, Dept. of Insect Physiology and Behavior, National Institute of Sericultural and Entomological Science, Oowashi 1-2, Tsukuba, Ibaraki 305, Japan. Tel.: 81-298-38-6106; Fax: 81-298-38-6028; E-mail: kato@nisesaffrc.go.jp.

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3 J. Moore, R. Parton, and M. W. Kennedy, personal communication.
Antimicrobial Assay

Inhibition zone assay was performed for the anti-\textit{S. aureus} assay, as described previously (11). Briefly, LB-agar plates (12) with small wells containing \(10^6\) colony-forming units/ml (final) logarithmic phase bacteria were prepared. Samples were poured into each well and incubated at 37°C for 18 h. Antibacterial activity was detected as clear zones around the wells after the incubation. The inhibition zone assay was also used for the antimicrobial assay. Fungi were inoculated on potato-dextrose agar plates (Difco). Wells cut at the edge of the developing fungal lawn received samples. The plates were incubated at 25°C for 2 days and were monitored at 225 and 280 nm. S-Pyridylethylated ASABF was eluted at 27% acetonitrile.

Protease Digestion

\textit{Lysyl Endopeptidase Digestion—}Twenty to forty \(\mu\)g of purified ASABF or S-pyridylethylated ASABF was dissolved in 100 \(\mu\)l of 200 mM Tris/HCl buffer, pH 8.5, containing 8 mM urea. The dissolved samples were diluted twice with ultrapure water, and 1 \(\mu\)g of lysylendopeptidase was added. The digestion was monitored at 225 and 280 nm. S-Pyridylethylated ASABF was dissolved in 200 \(\mu\)l of 50 mM ammonium bicarbonate buffer (pH 7.0) containing 1 \(\mu\)g of \textit{S. aureus} V8 protease and digested at 37°C for 18 h.

The fragments derived by protease digestion were separated by reversed-phase HPLC as described for S-pyridylethylated ASABF.

\textit{Determination of Amino Acid Sequence}

Purified ASABF, S-pyridylethylated ASABF, and the fragments derived by protease digestion were lyophilized and subjected to automated sequence analysis using an Applied Biosystems Procise™ or a Beckman LF3000.

Mass Spectrometry

The exact molecular mass of intact ASABF was determined by an ion spray ionization mass spectrometer (API 300 triple quadrupole mass spectrometer, Perkin-Elmer). The quadrupole was scanned over 500-2000 Da using a step size of 0.1 Da and a 1.0-ms dwell time/step. A matrix-assisted laser desorption ionization-time of flight mass spectrometry was used (Voyager™-RP, PerSeptive Biosystems) to determine the mass of the S-pyridylethylated fragment of ASABF, Arg<sub>99</sub>Gly<sub>100</sub>.

cDNA Cloning

A cDNA for ASABF was cloned using three-step PCR amplification.

\textit{Step 1. Reverse Transcriptase-PCR—}The poly(A)⁺ RNA isolated from the body walls of adult female \textit{A. suum}, as described by Kuramochi \textit{et al.} (15), was kindly given by Prof. Kyoshi Kita, Tokyo University. Single-stranded cDNAs were synthesized from 0.3 \(\mu\)g of the poly(A)⁺ RNA and oligo(dT)(25) adaptor primer, 5'-CTTTTCCGCTGTCGACATGCGG-3', using avian myeloblastosis virus reverse transcriptase. The cDNA coding Thr<sub>49</sub>-Arg<sub>50</sub> was amplified by PCR using a set of degenerate primers: the sense primer (29-mer) whose sequence is deduced from Cys<sub>47</sub>Gly<sub>48</sub> with a designed 5'-flanking sequence, 5'-GGCGCGCGCTG(T/C)(A/G)(A/G)AA(T/C)TG(T/C)GG-3', and the antisense primer (27-mer) whose sequence is derived from Pro<sub>44</sub>-Asp<sub>49</sub> with designed 5'-flanking sequence, 5'-AGCGAGCACG(A/G)TC(A/G)CA(A/G)AA(T/C)GCGG-3'. All reagents used in this step were obtained from an RNA LA PCR kit (AMV) (Takara). Denaturation was carried out at 95°C for 6 min (first cycle) or 1 min (second and following cycles), annealing at 35°C (initial 10 cycles) or 45°C (following 30 cycles) for 1 min, and polymerization at 72°C for 1 min. The total number of cycles was 40. Only the product of expected size was found. This product was subcloned into pGEM-T vector (Promega) and sequenced by a dye terminator system (PRISM™, Applied Biosystems) with an automated DNA sequencer (373A, Applied Biosystems).

\textit{Step 2. Amplification of 5'-End Using SL1 Primer—}Most of the mRNAs in \textit{Ascaris lumbricoides} are trans-spliced and acquire a common 22-nt SL1 sequence at the 5'-end (16). It is thus highly possible that cDNAs for ASABF contain the SL1 sequence. PCR was performed using a set of primers: the sense primer (22-mer) whose sequence is identical to the SL1 sequence, 5'-GTTTAAAATACCAAGATTTAGG-3', and the antisense primer (25-mer) whose sequence is identical to that for Thr<sub>49</sub>-Arg<sub>50</sub> revealed in “Step 1”, 5'-CGACTTCCAGGTCTTCAGCAGT-3'. All reagents used in this step were obtained from LA PCR kit Ver.2 (Takara). Denaturation was carried out at 95°C for 6 min (first cycle) or 1 min (second and following cycles), annealing at 55°C (following 30 cycles) for 1 min, and polymerization at 72°C for 1 min. The total number of cycles was 40. Only the product of expected size was found. This product was subcloned into pGEM-T vector (Promega) and sequenced by a dye terminator system (PRISM™, Applied Biosystems) with an automated DNA sequencer (373A, Applied Biosystems).

\textit{S-Pyridylethylated}

Twenty to forty \(\mu\)g of purified ASABF was dissolved into 200 \(\mu\)l of 0.25 M Tris/HCl buffer, pH 8.5, containing 8 mM guanidine hydrochloride, 1 M EDTA, and 0.1% 2-mercaptoethanol. The sample was flushed with nitrogen and incubated at 37°C for 2 h. Two \(\mu\)l of 4-vinylpyridine was added, and the sample was flushed with nitrogen again and incubated at room temperature for 2 h. S-Pyridylethylated ASABF was separated from the reagents by reversed-phase HPLC using a Sephadex C18 SC2.1/10 microbore column connected to a Pharmacia SMART system. Two linear gradient elutions were employed after an elution with ultrapure water for 3 min: 0–40% acetonitrile over 40 min and 40–100% acetonitrile over 5 min. Both the ultrapure water and acetonitrile used as mobile phases contained 0.05% trifluoroacetic acid. The flow rate was constant at 0.1 ml/min at ambient temperature. The elution pattern was monitored at 225 and 280 nm. S-Pyridylethylated ASABF was eluted at 27% acetonitrile.

The abbreviations used are: IC₅₀, 50% growth inhibitory concentration; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; SL, spliced leader; nt, nucleotide; kb, kilobase pair.

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quenced as described above. The nucleotide sequence deduced from the putative signal sequence and the N-terminal region of the mature ASABF, Step 3: Rapid Amplification of cDNA Ends—To determine the sequence of a full-length cDNA for ASABF, 3rd rapid amplification of cDNA ends was carried out using a set of primers: the sense primer (35-mer) whose sequence is identical to the 5′ untranslated region revealed in “Step 2,” 5′-GATATTCAGCAAAAAAGACAAAAACTCTGTCGACC-3′, and 5′-GTTTGGACTGTCGACC-3′. PCR conditions were identical to those described under “Step 2.” Major products were found to be 0.6 and 0.25 kbp. Their sequences revealed that the product of 0.6 kbp was the full-length cDNA for ASABF, except for the SL1 sequence.

Computer-assisted Sequence Analysis

Standard sequence analyses were performed using Genetyx-Mac Ver. 7.3 (Software Development, Tokyo, Japan). The MPsech (Smith-Waterman algorithm, University of Edinburgh, U.K.) was used for searching the nucleic acid data bases at DDBJ, GenBank, and EBI Data Bank and the protein data bases at Swiss-Prot, Protein Information Resource, GenPept, and Protein Data Bank via the on-line E-mail server of the DNA Information and Stock Center, Tsukuba, Japan. Furthermore, the cDNA catalogue of C. elegans, including unpublished data, was searched using the BLAST algorithm (17) through the kindness of Prof. Yuji Kohara (National Institute of Genetics, Mishima, Japan). The statistical significance of sequence similarity was estimated by a jumbling test (18) using the program employed by Nagata et al. (19). The criteria described by Doolittle (20) were used to evaluate the score of the jumbling test.

RESULTS

Purification of ASABF—Because only a limited amount of A. suum body fluid was available, we selected a short step HPLC-based procedure to minimize loss. The body fluid was centrifuged to remove debris and directly subjected to gel permeation HPLC (Fig. 1A). The peak of antibacterial activity against S. aureus was detected at 6 kDa, estimated with a standard curve of molecular mass. Further purification was achieved by reversed-phase HPLC (Fig. 1B). The antibacterial activity against S. aureus was detected as a single peak. This peak was separated by tricine/SDS-PAGE, and a single band was detected at 8 kDa under non-reducing conditions or reducing conditions with β-mercaptoethanol (data not shown).

Primary Structure—The N-terminal sequence of intact, Ala15-Gly89, and S-pyridylethylated ASABF was determined using an automated gas-phase sequenator (Fig. 2). S-pyridylethylated ASABF was digested by lysyl endopeptidase, and the fragments Ala15-Lys85, Val86-Lys86, Phe87-Lys87, and Arg88-Lys89 were separated, whereas Gly84-Gly89 was not found. Furthermore, the fragments Ala15-Glu56, Lys57-Asp67, and Arg68-Arg89 were derived by the S. aureus V8 protease digestion of S-pyridylethylated ASABF. The sequence of these fragments was determined. The sequence from overlapping fragments was compared, and the entire amino acid sequence of mature ASABF was determined.

We next submitted the S-pyridylethylated fragment, Arg88-Gly89, to a matrix-assisted laser desorption ionization-time of flight mass spectrometry and obtained a molecular mass of 2419.9 Da. This is in good agreement with the mass calculated for this fragment, 2418.7 Da. Thus, the C-terminal amino acid residue is confirmed to be Gly89.

The mature peptide of ASABF contains eight cysteine residues. The molecular mass of intact ASABF was determined to be 7420.4 Da, i.e., the mass calculated for the intact ASABF is 7420.4 Da, i.e., in excess of 8 Da relative to the experimental mass. This difference of 8 Da is well explained if all of the eight cysteine residues contributed to the intramolecular disulfide bridges. This elucidation is also supported by the following experiment. Intact ASABF was digested with lysyl endopeptidase and subjected to reversed-phase HPLC. Only a single peak was detected in this case. However, after S-pyridylethylolation of this peak, the same profile with four major peaks was observed as for that of S-pyridylethylated ASABF digested with lysyl endopeptidase. These results suggest that the four regions, Ala15-Lys85, Val86-Lys86, Phe87-Lys87, and Arg88-Lys89, bind to each other with intramolecular disulfide bridges.

No modification, e.g., glycosylation or phosphorylation, was indicated based on the data of the HPLC profiles of the sequenator and the mass spectrometry. The pI calculated from the entire sequence of mature ASABF is 8.7, i.e., ASABF is a cationic molecule.

cDNA Cloning and Deduced Precursor—A cDNA for ASABF was cloned using a three-step PCR-based approach with poly(A)+ RNA from the body walls of A. suum as a template (Fig. 2; and see “Materials and Methods”). A precursor peptide for ASABF is deduced from the nucleotide sequence of the cDNA. From the amino acid sequence of the precursor, mature
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ASABF was indicated to be flanked by a hydrophobic putative signal peptide, Met^1-Ala^{18}, at the N terminus. In addition, a four-residue peptide, Arg^{90}-Ser^{93}, was found at the C terminus as a flanking peptide that should be eliminated by processing. Some pre-mRNAs in nematodes are processed by trans-splicing and acquire a common 22-nt SL sequence from a small SL RNA (16). In the cloning procedure, the PCR amplification of the 5'-end was achieved using the SL1 sequence as a sense primer. The 22-nt trans-spliced leader sequence SL1 is shown as (\(\text{SL}\)). The termination codon is marked with an asterisk. The polyadenylation consensus signal is double underlined.

**Antimicrobial Activity**—Purified ASABF was tested for antibacterial activity (Table I). Gram-positive bacteria, *S. aureus*, *M. luteus*, *E. coli*, *P. vulgaris* were very sensitive to ASABF. Their IC\(_{50}\) were estimated to be 0.6–5 \(\mu\)g/ml, i.e., 0.08–0.7 \(\mu\)M. Gram-negative bacteria *E. coli* and *P. vulgaris* were less sensitive than Gram-positive bacteria. S-pyridylethylated ASABF exhibited no antibacterial activity against *S. aureus*. No antifungal activity was detected against the tested fungi, *A. brassicicola*, *S. tritici*, and *T. virens*.

**DISCUSSION**

This paper describes the purification, primary structure, and cDNA cloning of the novel antibacterial peptide ASABF discovered in the body fluid of the nematode *A. suum*. ASABF has been confirmed as a peptide, and it is thus strongly suggested that antibacterial peptides contribute to the immune defense of the ancient animal nematodes. Mature ASABF is a basic 71-residue peptide containing eight cysteines engaged in intramolecular disulfide bridges. To the best of our knowledge, this is the first report on the structure of an antibacterial protein in a nematode.

In some trials, a protein exhibiting weak antibacterial activ-

**TABLE I**

| Bacteria                  | IC\(_{50}\) μg/ml |
|---------------------------|------------------|
| **Gram-negative bacteria**|                  |
| *Escherichia coli* (JM109) | 50               |
| *Proteus vulgaris* (ATCC 13315) | 10               |
| **Gram-positive bacteria**|                  |
| *Staphylococcus aureus* (ATCC6538P) | 0.6             |
| *Bacillus subtilis* (ATCC6633) | 1.2             |
| *Micrococcus luteus* (ATCC398) | 0.8             |

Fig. 2. Nucleotide sequence of a cDNA clone for ASABF. The deduced amino acid sequence of a precursor is represented below the nucleotide sequence. The region corresponding to mature ASABF is underlined. The primary structure of mature ASABF was determined using an automated gas-phase sequenator and was completely identical to this underlined sequence. The 22-nt trans-spliced leader sequence SL1 is shown as (\(\text{SL}\)). The termination codon is marked with an asterisk. The polyadenylation consensus signal is double underlined.

* Y. Kato, unpublished data.
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FIG. 3. Sequence comparison of ASABF with tenecin 1. Identical residues are dark-stippled, and similar residues are light-stippled. The following sets of amino acids are considered similar: G, A, C, S, T, E, D, Q, N; R, K, H; V, M, L, I; and F, Y, W. The two cysteine residues of ASABF do not correspond to the conserved cysteine array of tenecin 1 (asterisks). Numbers below the alignment represent the position of the amino acid residues of ASABF.

FIG. 4. Alignment of ASABF and the homologues in C. elegans. The conserved arrays consisting of eight cysteine residues are marked with asterisks. Identical residues are dark-stippled, and similar residues are light-stippled.

ity was evaluated by a jumbling test. The normalized alignment score was estimated to be 3.82 of the standard deviation, and its evaluation is “marginal” (20). All of the normalized alignment scores between ASABF and other insect/arthropod defensins are 3.0 of the standard deviation, i.e. “improbably significant,” except for sapecin B (3.01 of the standard deviation).4 In conclusion, the significant sequence similarity is not verified, whereas some similar features are observed between ASABF and insect/arthropod defensins. Moreover, ASABF contains eight cysteine residues, whereas the number of cysteine residues is six, without exception, in insect/arthropod defensins (24). We thus propose to classify ASABF into a novel group of antibacterial proteins in the present situation. However, we are not rejecting the proposal that ASABF and insect/arthropod defensins are possibly related by common ancestry. Are they evolutionally related? This question is curious from the aspect of a search for the origin of cysteine-rich antibacterial peptides. Insect/arthropod defensins show a certain degree of sequence similarity with mammalian defensins as mentioned in the Introduction. It is unclear whether these antibacterial peptides diverged from a common ancestor molecule. The discovery of ASABF, however, suggests that the cysteine-rich antibacterial peptides could be very ancient in origin. Further studies on antibacterial proteins in lower invertebrates should elucidate the evolutional relationship among ASABF, insect/arthropod defensins, and mammalian defensins. In addition, antifungal peptides containing eight cysteine residues have been also reported, i.e. plant defensins and drosomycin (see Introduction). ASABF exhibits no potent antifungal activity and no significant sequence similarity to these antifungal peptides. However, allowing for several gaps, the array consisting of eight cysteine residues seems to be arranged in a similar pattern between ASABF and these antifungal peptides. Interestingly, it has been suggested that structural and functional properties of plant defensins resemble those of insect and mammalian defensins (6). Revealing the relationship between ASABF and these antifungal peptides might be another key to studying the evolutional relationship among cysteine-rich antimicrobial peptides.

One of our goals is to introduce the nematode C. elegans as a model animal for investigation on innate immunity, as mentioned in the Introduction. From this aspect, it is very curious regarding whether ASABF homologues exist in C. elegans. A cDNA catalogue by Prof. Yuji Kohara was searched. BLAST data base searches revealed significant sequence identity with a deduced protein from the cDNA sequence, yk150c7 (Fig. 4). In the optimum region corresponding to Leu-Gly of ASABF, yk150c7 exhibits 42% identity and 57% similarity with a normalized alignment score of 5.61 of the standard deviation, i.e. “probably significant.” Furthermore, nucleic acid data bases and protein data bases were searched. The protein deduced from the putative gene, T22H6.5, was found to be a protein most similar to both ASABF and yk150c7 by the MPsrch™ data base search (Fig. 4). In the optimum region corresponding to Leu-Cys of ASABF, T22H6.5 exhibits 39.4% identity and 54.5% similarity with a normalized alignment score of 4.61 of the standard deviation. T22H6.5 is also similar to yk150c7 with 48.6% identity and 67.6% similarity in the optimum region corresponding to Phe-Cys of ASABF. T22H6.5 contains nine cysteine residues, and one of the cysteines is found in the highly hydrophobic putative signal sequence at the N-terminal region. The array consisting of eight other cysteine residues is similar to that of ASABF. It is noteworthy that the highly similar region among ASABF, yk150c7, and T22H6.5 is almost identical to the region overlapping insect/arthropod defensins (Figs. 3 and 4). The function of the deduced proteins from yk150c7 and T22H6.5 has been unknown and is not predicted. Further experimental analyses are necessary to confirm the function of these ASABF homologues in C. elegans.

To date, a number of antimicrobial proteins were isolated from multicellular animals. Most of them are, however, derived from higher animals, e.g. vertebrates and arthropods. The higher animals seem to develop characteristic defense systems, e.g. B- and T-cell-based adaptive immunity in vertebrates and prophenoloxidase cascades in arthropods, overlaying primitive immunity as described previously (11). Studying the immune defense of lower invertebrates, such as nematodes, could be a way to isolate the primitive systems from these additional systems. The present work was carried out as the initial step of this project.

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*The jumbling test was carried out for the insect/arthropod defensins listed in Ref. 24.
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