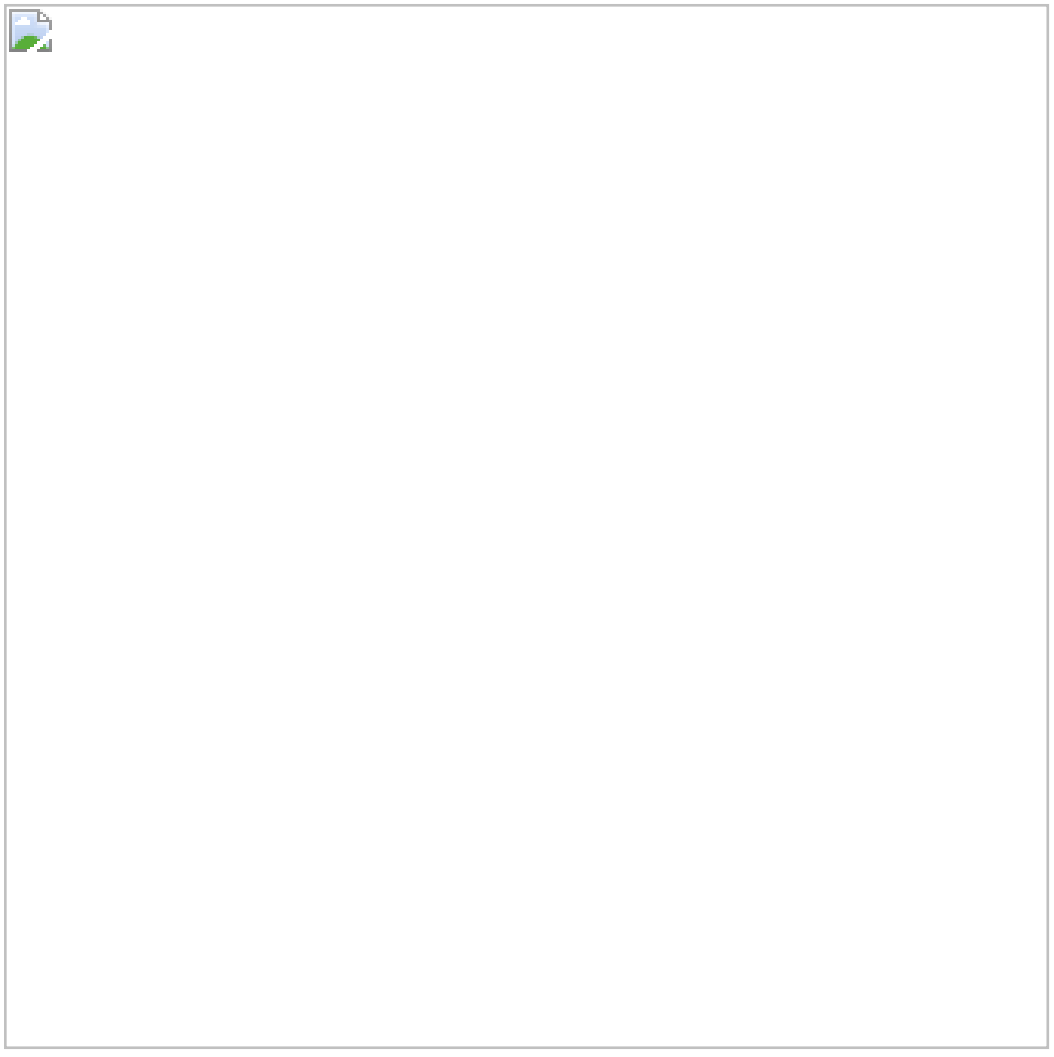

Humoral Defense of the Nematode Ascaris suum: Antibacterial, Bacteriolytic and Agglutinating Activities in the Body Fluid

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Humoral Defense of the Nematode *Ascaris suum*: Antibacterial, Bacteriolytic and Agglutinating Activities in the Body Fluid

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ABSTRACT—Three humoral defense activities (antibacterial, bacteriolytic and agglutinating) were detected in the body fluid of the nematode *Ascaris suum*. Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) were more sensitive to the antibacterial activity than the Gram-negative bacteria (*Escherichia coli*). The antibacterial activity was heat stable and was lost by trypsin digestion. The molecular mass of the factor responsible for antibacterial activity was estimated as 6 kDa. The bacteriolytic activity against dried *Micrococcus luteus* was also detected. The bacteriolytic factor was 6–9 kDa in molecular mass, heat sensitive and trypsin sensitive. Both *E. coli* and glutaraldehyde-fixed trypsin-treated human A-type red blood cells were agglutinated in the body fluid. An analytical gel permeation HPLC revealed the agglutinating activity consists of at least two factors. Activities of both agglutinating factors were lost by heat treatment or trypsin digestion. The molecular masses estimated for the two agglutinating factors were 500 kDa and 25 kDa. Under experimental conditions, microbe-injection was not a prerequisite for the appearance of these defense activities.

INTRODUCTION

To date, little is known about the immunity of nematodes. However, as a lower invertebrate, nematodes can be a useful model to investigate common and primitive defense systems.

For example, insect immunity has been studied most prominently in invertebrate immunology. Some recent reports suggest common systems of immunity both in mammals and in insects, e.g. homologous antibacterial peptides [12, 14], *cis*-regulatory elements in their genes [20, 25] and common pathways of LPS detoxification [11] etc. These systems might have originated before the divergence of deuterostomes (e.g. vertebrates) from protostomes (e.g. insects). Nematodes are thought to be very ancient in origin, at least as the divergence of deuterostomes from protostomes, although the origin of nematodes in the evolutional tree is not completely determined because few fossil records are available. Recent reports on molecular evolution have been corroborating this view [4, 16, 27]. It is, thus, expected that a key to reveal the origin of such common immune systems will be provided by the investigation about the nematode immunity. In addition, higher animals have developed characteristic defense systems overlaying the common and primitive immunity, e.g. B- and T-cell based adaptive immunity in vertebrates [9] and prophenoloxidase cascades in arthropods and ascidians (it is also found in other invertebrates at a low level and the similar proteolytic cascades may be distributed widely in animals [24]), etc. These results also suggest the importance of investigation about the immunity of lower invertebrates as a model to study the common and primitive immunity.

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The utility of the experimental nematode *Caenorhabditis elegans* should be also mentioned. The fruit fly *Drosophila melanogaster*, another experimental animal, has been used as a model not only to explore the insect immunity but also to study the innate immune reactions in other animals including vertebrates [7, 26]. *C. elegans* might also have enough potential to provide a model system to study the common and primitive immunity from the aspect of molecular genetics [28].

In this study, I explored humoral defense activities in the body fluid of the parasitic nematode *Ascaris suum* as an initial study for the nematode immunity. To the best of my knowledge, this is believed to be the first report on the nematode immunity.

MATERIALS AND METHODS

Nematodes

Adult *A. suum* were obtained from a slaughterhouse, Gunma, Japan. Just after incision of small intestine of a pig, nematodes were isolated and washed 4 times with phosphate-buffered saline (PBS: 150 mM NaCl, 18 mM sodium-phosphate, pH 7.4) at room temperature. They were stored in PBS at 4°C and transported to the laboratory. To maintain nematodes viable in the laboratory, they were kept in Eagle's MEM (Nihonseiyaku, Tokyo, Japan) without any antibiotics at 37°C. The medium was changed every 6 hr. Female nematodes with >15 cm in body length were used within 24 hr of the incubation.

Bacteria

Escherichia coli JM109 was purchased from Takara (Kyoto, Japan). Other strains were gifts as following: *Staphylococcus aureus* (ATCC 6538P) from Dr. Masanori Yamamoto (Shikibo, Ltd., Osaka, Japan); *Micrococcus luteus* NISL 4106 (ATCC 398) and *Bacillus subtilis* NISL 4025 (IAM 1107=IFO 3026) from Dr. Sei-ichi Hara (Noda Institute for Scientific Research, Chiba, Japan). All strains were grown in LB medium [22] at 37°C with shaking.

Enzymes

Trypsin was obtained from DIFCO (Detroit, Mich.). Lysozyme from egg white was purchased from Wako (Osaka, Japan).

Collection of body fluid

A 26G hypodermic needle attached to a syringe (Terumo) was inserted into the pseudocoel in the anterior region of vulva. An aliquot of body fluid (about 700 μl/nematode) was collected by negative pressure. The collected body fluid was centrifuged at 5,000 rpm at 4°C for 10 min and the supernatant was stored at −30°C.

Antibacterial and lysis assay

Antibacterial activity was assayed by inhibition zone assay [8]. Twenty-five ml of LB medium (containing about 10^5 cfu/ml logarithmic-phase bacteria and 1.5% agar) was poured into each sterile Petri dish (9 cm in diameter). Samples (35 μl, each) were applied into each well (4 mm in diameter) in the LB agar plates and the plates were incubated at 37°C for 18 hr. Antibacterial activity was detected as clear zones after the incubation. To estimate streptomycin equivalent as control, the same procedure was applied to 3, 10, 30, 100, 300, 1,000, 3,000, 10,000 μg/ml streptomycin solution. The diameters of clear zones of streptomycin standards against *S. aureus* and *E. coli* were assessed and a calibration curve was prepared. Streptomycin equivalent was defined as streptomycin concentration which exhibited the same diameter as a tested sample in the clear zone assay.

Lysis activity was also estimated by clear zone against dried *M. luteus* in agar plates. Assay conditions were essentially those described by Mohrig and Messner [15].

Agglutination assay

The glutaraldehyde-fixed and trypsin-treated human A-type red blood cells (RBC) were used for agglutination assay described by Nowack *et al.* [17].

Trypsin digestion

Digestion was performed with 1 mg/ml (final) trypsin at 37°C for 3 hr in a modified saline (10 mM KCl, 110 mM NaCl, 22 mM sodium-phosphate, pH 6.7; this composition is the original *Ascaris saline* [5] with the exception that divalent cations were excluded). Before assay, 10 mM (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF, final) was added. A control experiment suggested that the proteolytic activity of trypsin was completely inhibited by *p*-APMSF at this concentration. The saline or 10 mM *p*-APMSF neither inhibited any antibacterial, bacteriolytic or agglutinating activities in the body fluid, nor exhibited these activities by themselves alone.

HPLC

HPLC was performed on a Pharmacia FPLC system. Samples were applied to a column (10 mm×300–310 mm) of Superdex 200 HR 10/30 or Superdex 75 HR 10/30 (Pharmacia LKB, Uppsala, Sweden) utilizing the modified saline as mobile phase at a flow rate 0.5 ml/min. To estimate molecular masses, a series of standards were used [thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa) and cyanocobalamin (vitamin B_{12}, 1.36 kDa)].

RESULTS

The body fluid of *A. suum* is thought to be nutritious [5] enough for microbes to grow. It is, therefore, expected that anti-microbe factor(s) may be present in the body fluid. In this study, I explored the presence of humoral defense activities in the body fluid. The results obtained by using natural body fluid of *A. suum* which were not inoculated with microbes or chemicals are presented here.

Antibacterial activity

Antibacterial activity was detected in the body fluid (Fig. 1). *S. aureus* (Gram-positive, coccus) was more sensitive than *B. subtilis* (Gram-positive, bacillus). The activity against *E. coli* (Gram-negative, bacillus) was relatively weak and detected only in some of tested individuals of nematodes (3 positive in 12). The activity in hemolymph was estimated as 10^3 μg/ml streptomycin equivalent against *S. aureus* and 10^2 μg/ml against *E. coli*. Both anti-*S. aureus* and anti-*E. coli* activity were stable against heat treatment of the body

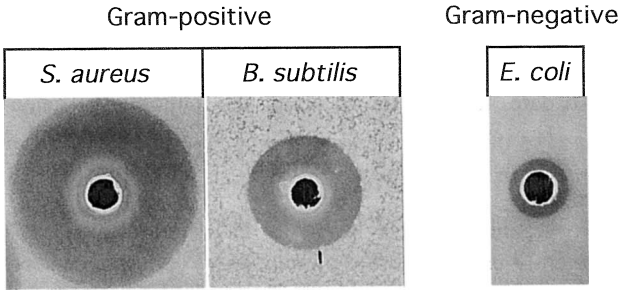

FIG. 1. Antibacterial activity of the body fluid. Antibacterial activity was assessed by inhibition zone assay. The body fluid was directly subjected to the test without any modifications.

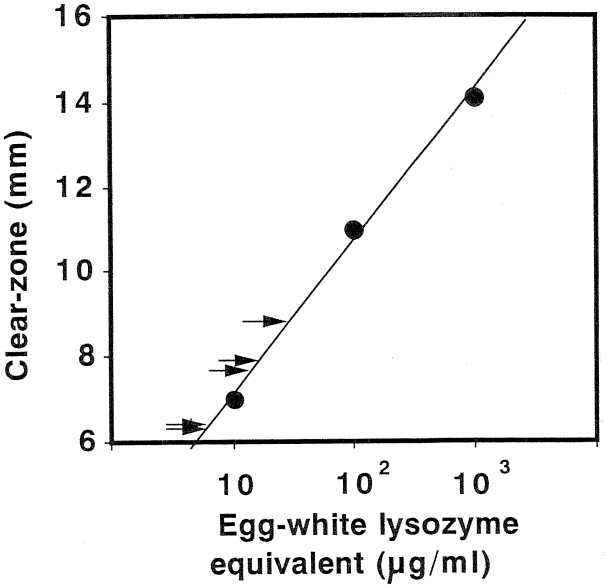

FIG. 2. Bacteriolytic activity in the body fluid. The activity was measured by clear-zone assay with agar gel containing dried *M. luteus*. Filled circles indicate the results of standard egg-white lysozyme. Each arrow indicates the activity in the body fluid obtained from an individual.

fluid at 100°C for 6 min. Molecular mass of anti-*S. aureus* factor was estimated to be 6 kDa as a single peak by gel permeation HPLC on a Superdex 200 HR 10/30 (Fig. 4) and a Superdex 75 HR 10/30 column (data not shown), at almost the same position as aprotinin (Fig. 4). This is designated as ASABF (*Ascaris suum* antibacterial factor). The ASABF was suggested to be a peptide (peptides) because ASABF was inactivated by trypsin digestion, and stable against heat treatment (Table 1).

Bacteriolytic activity

The bacteriolytic activity against dried *M. luteus* was also found. The activity was estimated as approximately 10 μg/ml egg white lysozyme equivalent (Fig. 2). The bacteriolytic factor, ASBLF (*Ascaris suum* bacteriolytic factor), was estimated 6–9 kDa by gel permeation HPLC on a Superdex 200 HR 10/30 column (Fig. 4) and on a Superdex 75 HR 10/30 column (data not shown). The bacteriolytic activity was lost not only by trypsin digestion but also by heat treatment (Table 1). Thus, ASBLF is suggested to be a different molecule from ASABF.

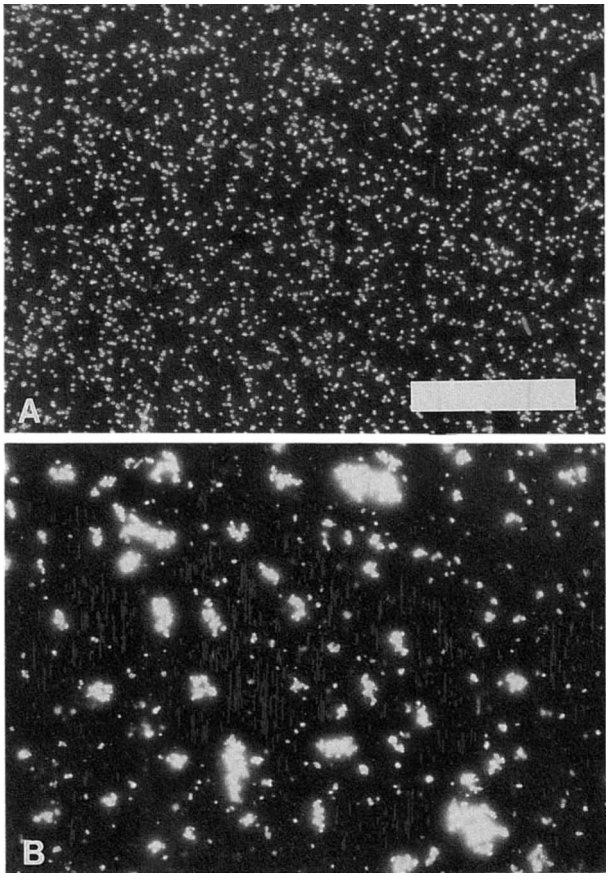

FIG. 3. Agglutinating activity of the body fluid against *E. coli*. Bacteria were treated without (A) and with (B) the body fluid at 37°C for 1 hr. Both photographs were taken with a light microscope (Optiphoto-2 with a plan 20X objective, a dark field condenser and a HFX-DX automatic photograph system, Nikon, Tokyo, Japan) at the same magnification. The white bar in A represents 10 μm.

Agglutinating activity

Agglutinating activity against bacteria was detected in the body fluid (Fig. 3A and B). The agglutinating factors against alien organisms in body fluid is important as the defense mechanism of invertebrates, e.g. non-self recognition [18] and opsonin activity [21], etc. Glutaraldehyde-fixed trypsin-treated human A-type RBC was also agglutinated in the body fluid (data not shown). The agglutinating activity against RBC was detected even in a 512–1024 fold diluted body fluid. Analytical gel permeation HPLC revealed two peaks of the agglutinating activity, at far different position from ASABF or ASBLF (Fig. 4). These agglutinating factors are designated as ASAGF-1 (*Ascaris suum* agglutinating factor-1; 500 kDa) and ASAGF-2 (25 kDa). The contribution of ASAGF-1 to total agglutinating activity was always much greater than that of ASAGF-2. The activities of both ASAGF-1 and 2 were lost by trypsin digestion or heat treatment (Table 1).

DISCUSSION

In this study, we detected three humoral defense activities, typical of invertebrates, in the body fluid of *A. suum*, i.e. antibacterial, bacteriolytic and agglutinating activity. Furthermore, the strength of these activities were estimated. *A. suum* is a parasitic nematode of pig intestine which lives in a microbe-rich environment. Hence, the biological defenses against the microbes are thought to be necessary. Our results indicate that the defense system against microbe infection involves a cell-free, humoral mechanism.

In some cases, the substantial nature of these activities overlaps each other. For example, lysozyme is a bacteriolytic molecule and also bactericidal against several kinds of Gram-positive bacteria [2], and some of the lectins are not only agglutinating factors but also cytotoxic or trigger to evoke subsequent defense responses [23]. In the case of *Ascaris suum*, however, ASABF, ASBLF, ASAGF-1 and ASAGF-2 are clearly suggested to be different molecules following the characterization with gel permeation HPLC, trypsin digestion and heat treatment.

It is difficult to compare directly the strength of antibacterial activities in body fluid among various animals, because antibacterial spectra are different among animals and assay conditions are not constant among reports. However, the anti-*S. aureus* activity in the body fluid of *A. suum* was estimated as 10^3 μg/ml streptomycin equivalent. In contrast, only faint antibacterial activity against Gram-negative bacteria was found. Similar incomplete antibacterial spectra were reported in other animals, e.g. no antibacterial activity against Gram-positive bacteria was detected in the cell-free hemolymph of horseshoe crab *Limulus polyphemus*, whereas it had strong effect against Gram-negative bacteria [10]. It is, nevertheless, an unusual way to assume that *A. suum* lacks the defense against Gram-negative bacteria. One of the possibilities is the contribution of cellular defense systems. In *Ascaris lumbricoides*, there are 4 large and highly branched cells (stellate cells) in the body cavity. It was reported that

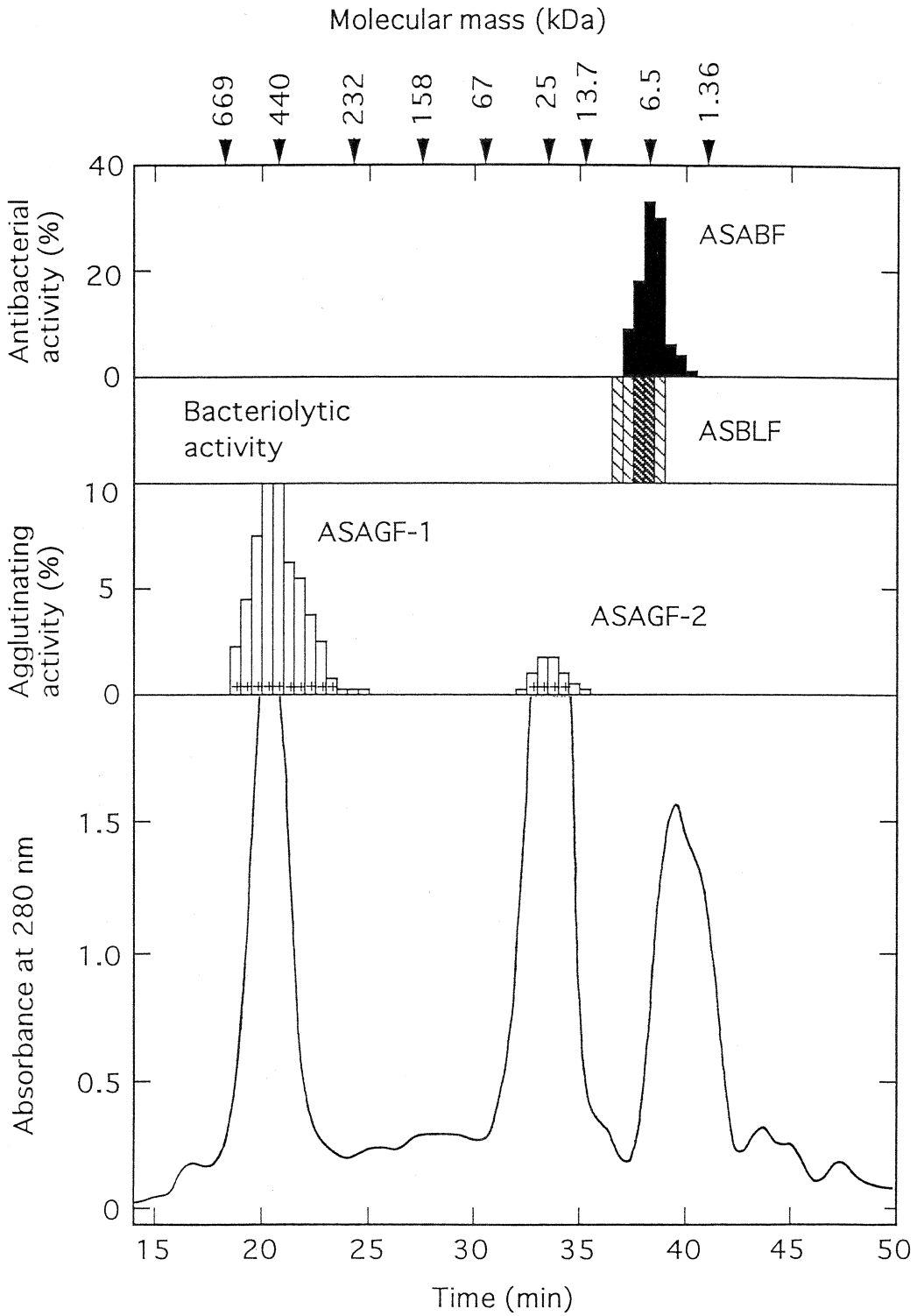

FIG. 4. Profile of the body fluid proteins using gel permeation HPLC on a Superdex 200 HR 10/30 column. Approximately 500 μl of the body fluid was loaded. A modified *Ascaris saline* (see the Materials and Methods) was used as mobile phase at a flow rate 0.5 ml/min. The fractions were subjected to antibacterial, bacteriolytic and agglutinating assay without any modification. The antibacterial and agglutinating activities are represented as relative value (the activity of parent body fluid=100). Plus marks (+) in the columns of agglutinating activities are the fractions agglutinating *E. coli*. The bacteriolytic activities are represented as dark-hatched (strong activity) or light-hatched (weak activity) boxes. Molecular masses indicated in the upper site are the elution time of standards. N.D., no data.

TABLE 1. The properties of humoral defense factors in the body fluid of *A. suum*.

	Antibacterial factor	Bacteriolytic factor	Agglutinating factors	
	ASABF	ASBLF	ASAGF-1	ASAGF-2
Molecular mass (kDa)	6	6–9	500	25
Trypsin digestion	inactivated	inactivated	inactivated	inactivated
Heat stability*	stable	inactivated	inactivated	inactivated

* Heat treatment was performed at 100°C for 6 min.

these cells were covered with injected samples, including india ink and *E. coli*, 6 hr after the injection. Although distinct evidence of actual phagocytosis was not observed, the stellate cells were suggested to play a role in defense [3].

The origin of ASABF should be investigated in future study. Many antibacterial peptides which have almost the same molecular weight and heat stability as ASABF were found in pig intestine [1]. The chance of contamination of body fluid with gut contents during collection was, if there is any, reduced to a minimum level by following the method described in this work. However, the possibility that the nematode could absorb the antibacterial peptides from host pigs cannot be excluded. This question should be answered after a complete purification and characterization of ASABF.

The bacteriolytic activity was detected in the body fluid of *A. suum* as around 10 μg/ml hen egg white lysozyme equivalent. The estimation of bacteriolytic activities in body fluid as hen egg white lysozyme equivalent were reported for the annelide *Eisenia felide andrei* [13] and for various species of insects [15]. These reports suggest that the bacteriolytic activity of *A. suum* is almost within the limit of that of reported animals under normal conditions (i.e. without any inoculation or infections). However, bacterial inoculation induces a higher bacteriolytic activities in these reported animals (e.g. 20-fold for *E. felide andrei* and the insect *Galleria mellonela*).

The agglutinating activities in the body fluid of invertebrates have been reported (a list was described in the review by Ratcliffe *et al.* [19]). The agglutinating activity of *A. suum* was estimated as 512–1024 for a hemagglutination titer. It could be suggesting that the agglutinating activity of *A. suum* plays a role in humoral defense because its hemagglutination titer is within the range of that of the other studied animals against the tested erythrocytes which have the greatest affinity, and *E. coli* is also agglutinated in the body fluid of *A. suum*. A β-galactoside-binding lectin was isolated from *C. elegans* [6]. Since we cannot, so far, obtain distinct results of monosaccharide inhibition assays or analyses on divalent cation dependency for partially purified ASAGF-1 or -2 with gel permeation HPLC, the relationship between the agglutinating activities in the body fluid of *A. suum* and the lectin from *C. elegans* is unclear. This is, however, the first evidence of existence of agglutinating activities in the cell-free body fluid of nematodes, because the lectin reported by Hirabayashi *et al.* [6] was extracted from whole bodies of *C. elegans*.

The body fluid of *A. suum* had strong defense activities which were observed without a bacterial inoculation. Not only inducible but also constitutive defense factors have been reported in invertebrates [19], so it is possible to argue that nematodes might have at least constitutive mechanisms. However, because of the possibility of natural immunization, it should not be concluded that the nematode has or has no constitutive defense factors. This question should be answered with experiments under microbe-free condition. *A. suum*, however, is seemed to be unsuitable for such experiments because it is difficult to achieve the microbe-free condition without antibiotics which might be absorbed into the body cavity and might affect the condition of defense activities.

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