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ANTIBACTERIAL ACTIVITY OF SNAIL MUCUS MUCIN

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Abstract—1. An antibacterial activity was found in the mucin obtained from the body surface mucus of the African giant snail, *Achatina fulica Férussac*.

2. The water soluble fraction (WSF) and the mucin fraction (MF) of the mucus exhibited positive antibacterial activity both for the Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and for the Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, when assayed by the paper-disc method.

3. When MF was digested with a proteinase (Pronase), the activity was lost, while no changes in the activity was found on treatment with glycosidase. Thus, the antibacterial activity was ascribed to the protein moiety of the snail mucus mucin.

INTRODUCTION

The bodies of land pulmonata such as snails and slugs are characterized by rich mucus which covers their surface. Apparently, the mucus may serve in preventing the moisture evaporation, in helping smooth movements (Simkiss & Wilbur, 1977) and in protecting the body from mechanical injuries. In addition, some unknown biochemical function may be involved in the mucus, though nothing has been reported so far with this respect. Thus, in spite of the rather fragile structure and the wet condition of the cutaneous tissue, the animals are fairly resistant to infection by microorganisms. The existence of some antibacterial factor(s) is likely in the mucus.

In the present study, the antibacterial activity was surveyed in the aqueous extract and the mucin fraction of the mucus of African giant snails, *Achatina fulica Férussac*. On the four kinds of bacteria tested, including both the Gram-positives and the Gram-negatives, the snail mucus components demonstrated positive growth inhibition.

MATERIALS AND METHODS

Preparation of snail mucus mucin

African giant snails, *Achatina fulica Férussac*, were captured in Okinawa, transported via air to the laboratory, and fed. The shell size (height) of the snails ranged between 3.5 and 7.0 cm. Mucus secretion was stimulated by exposing the snails to an electric shock (5–10 V) at intervals of 30–60 sec and the mucus was collected into a pool for 10–20 heads. Each snail was used for 2–3 collections.

The water soluble fraction (WSF) of the mucus was obtained by the following procedures. Two volumes of water were added to the above mucus, stirred overnight, and centrifuged at 8000 *g* for 30 min. The supernatant was referred to as WSF.

The mucin fraction (MF) of WSF was obtained by use of the ethanol precipitation which is the most common isolation method for the mucins. Three volumes of ethanol were added to the WSF preparation and the mixture was centrifuged at 2500 *g* for 30 min. The precipitate obtained was redissolved in water and was referred to as MF.

Enzymic digestion of MF

Digestion of the protein moiety of MF was carried out as follows: Pronase E (Kaken Kagaku Co. Ltd, Tokyo), which is a non-specific proteinase, was added to the sample. The pH of the mixture was adjusted to 7.8–8.0 with 0.1 M NaOH and incubated at 40°C with a little toluol. During the incubation, pronase was supplemented twice and the pH was adjusted to 7.8–8.0 at intervals. After 74 hr incubation, when the pH ceased to fall, the reaction was stopped with trichloroacetic acid (TCA). The total amount of pronase added was twice the amount of mucin protein present (w/w). TCA in the mixture was removed by ether extraction. The aqueous fraction was used in the experiments after pH adjustment to 7.0.

The digestion of the carbohydrate moiety was carried out in an acetic acid–sodium acetate buffer solution (pH 4.0) at 37°C for 4 hr, using a glycosidase mixture (Glycosidase Mixed; Seikagaku Kogyo Co. Ltd, Tokyo). Enzyme, totalling 150% of the weight of mucin protein (w/w) present, was added in 4 portions at every hour. The digestion was stopped by addition of TCA and TCA was removed from the mixture in the same way as for protein digestion.

Determination of proteins and carbohydrates

Proteins and carbohydrates in each sample were determined by the micro-biuret method (Itzhaki & Gill, 1964) and the anthrone–sulphuric acid method (Trevelyan & Harrison, 1952). They were illustrated as equivalents of bovine serum albumin and glucose, respectively.

Examination of antibacterial activity

The antibacterial activity of each sample was examined by the paper-disc method as follows: two species of the Gram-positive bacteria, *Bacillus subtilis* (IFO 3515) and *Staphylococcus aureus* (IFO 12732), and 2 species of the Gram-negative bacteria, *Escherichia coli* (IFO 12734) and *Pseudomonas aeruginosa* (IFO 3080), were cultivated in liquid bouillon culture (containing meat extract 10 g, peptone 10 g, and NaCl 2.5 g/dm^3; pH 7.0) at 37°C for 20–24 hr, at a bacterial concentration of one platinum loop per 50 cm^3 of another bouillon medium (containing meat

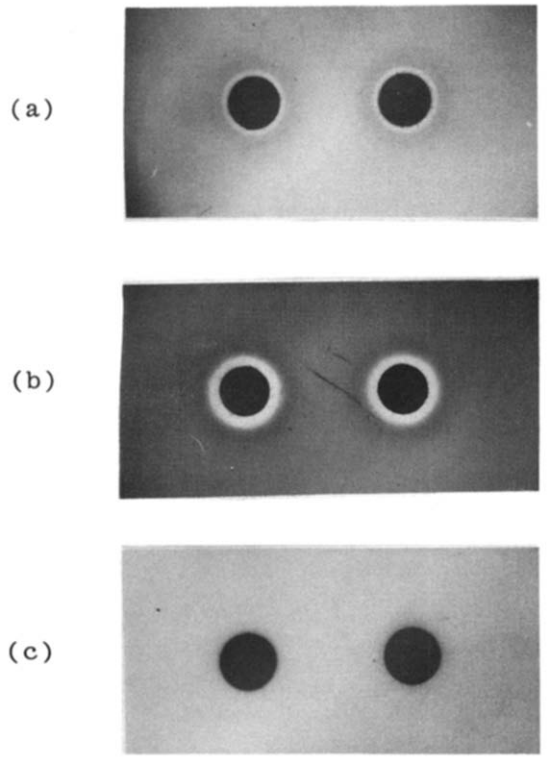

Fig. 1. Growth-inhibitory effects of sterile filtrate of snail mucus extract. The mucus was collected from the body surface of the African giant snail, *Achatina fulica Férussac*, and its water soluble fraction (WSF) was examined for antibacterial activity by a paper disc method after sterile filtration through an asbestos filter plate (No. 85SB, Toyo Roshi Co., Ltd, Tokyo). The bacteria used were, (a) *Bacillus subtilis* (IFO 3515) and (b) *Escherichia coli* (IFO 12734). The clear circular margins around the discs reveal the growth inhibition. Picture (c) shows the control experiment with water instead of WSF.

extract 5 g, peptone 10 g, NaCl 2.5 g, and agar 20 g/dm^3; pH 7.0). Each 4 cm^3 aliquot of the mixture was laid on the culture medium plate. Then a paper disc (filter paper with a diameter of 8 mm; paper disc for antibiotic examination, thick type, Toyo Roshi Co., Ltd, Tokyo) soaked in the sample was placed at the centre of each plate. After 24 hr incubation at 37°C, a growth-inhibitory circle was demonstrated around the discs for the positive samples and no clear margin was seen for the negative samples. The activity was described as the width of the growth-inhibitory clear margin around the disc.

RESULTS

Properties of the samples

The native mucus collected from the body surface of the snails was viscous but the viscosity differed from collection to collection. The mucus was slightly turbid and faintly coloured to yellow or brown depending on the different collection lots. The mucus contained about 99% moisture, 4 mg/cm^3 protein and 0.7–1.4 mg/cm^3 carbohydrate.

The water soluble fraction of the mucus (WSF) showed a pH value of 8.5–8.75 and gave positive tests for the biuret and the anthrone–sulphuric acid reactions. It exhibited a large precipitate on addition of ethanol, while no precipitate was formed by TCA. The WSF preparation showed a single peak (S = 2.7) in the ultracentrifugal pattern and containing about 1.4 mg/cm^3 protein, a value which suggests that nearly all of the mucus proteins were extracted with water. The carbohydrate content of the WSF varied from collection to collection, ranging from 0.24 to 0.47 mg/cm^3, and the ratio of carbohydrate to protein varied from 1:3 to 1:6 (w/w).

The ethanol precipitate of the WSF was jelly-like, transparently brown and readily soluble in water. The redissolved mucin precipitate (MF) was positive for both protein and carbohydrate tests, while the colourless ethanol supernatant was slightly positive to the biuret test.

Antibacterial activity of the WSF

The WSF preparation was sterile filtrated and submitted to the assay for the antibacterial activity. After a 24 hr incubation of bacteria at 37°C with the paper discs soaked in WSF, a marked growth-inhibitory circle was demonstrated around the discs for each of the tested bacteria as shown in Fig. 1.

Antibacterial activity of the MF

The MF preparation, the redissolved ethanol precipitate of the WSF, was submitted to the bioassay as for the WSF but with various sample concentrations (Table 1). It can be seen from the table that the MF preparation inhibited the growth of the four bacteria tested. The minimum effective concentration might be in the range of 175–210 μg/cm^3 for the Gram-positive bacteria and 105–126 μg/cm^3 for the Gram-negative bacteria in protein equivalents.

Antibacterial activity of MF after enzymic digestions

In the digestion by pronase as described in Materials and Methods, 40–45% of the protein positive to the biuret reaction was digested. In the carbohydrate digestion, 50% of the carbohydrate positive to the anthrone–sulphuric acid reaction was finally digested.

As shown in Table 2, the protein-digested sample did not inhibit the growth of any bacteria, even at a concentration high enough to show activity before digestion. The carbohydrate-digested sample did, however, cause growth inhibition.

DISCUSSION

The WSF preparation was positive both to protein and carbohydrate tests and was precipitated with ethanol but not with TCA. These results suggested the presence of carbohydrates, proteins and/or polypeptides, but most likely mucin, i.e. the conjugate form of carbohydrates and proteins. The variation seen in the ratio of carbohydrate to protein in the WSF might reflect the differences in the length of the carbohydrate chains.

The sterile-filtrated WSF clearly inhibited growth in all the bacteria tested. This suggested that there exists some antibacterial factor(s) in the water-soluble fraction of the snail mucus.

The antibacterial factor was sought in the mucin fraction of the WSF by use of ethanol precipitation.

Table 1. Antibacterial activity of mucin fraction (MF) of snail mucus

MF concentration		Growth inhibition			
Protein ($\mu g/cm^3$)	Carbohydrate ($\mu g/cm^3$)	*B. subtilis*	*S. aureus*	*E. coli*	*P. aeruginosa*
1770	589	+ + +	+ + +	+ + +	+ + +
1089	211	+ + +	+ + +	+ + +	+ + +
900	375	+ +	+ +	+ +	+ +
421	81	+ +	*	*	+ +
350	116	+ +	+ +	+ +	+ +
263	51	+ +	*	*	+ +
210	86	+	+	+ +	+ +
175	58	−	−	+	+
151	62	±	−	+	+
126	52	−	−	±	±
105	43	−	−	−	−
71	29	−	−	−	−
35	11	−	−	−	−
17	6	−	−	−	−

* Data lacking.

Each judgement is based on data from 4 plates.

The mucin fraction was precipitated with ethanol from the crude water extract (WSF) of the mucus collected from the body surface of the African giant snail, *Achatina fulica Férussac*. The antibacterial activity of the MF preparation was assayed by a paper disc method. Serially varying amounts of the MF preparation were applied. The inhibitory activity is expressed in 5 grades judged by the width of the clear margin around the paper disc. + + +, over 3 mm; + +, 2–3 mm; +, 1–2 mm; ±, 0–1 mm; −, none.

As the MF preparation also inhibited the growth of the bacteria, the active factor may have mucin structure. The activity was similar against both the Gram-positive and the Gram-negative bacteria, which have quite different cell wall structures, and so the effect is probably not at the cell surface. The MF preparation does not show any lysozymic activity (unpublished data).

In order to know whether the antibacterial effect of the mucin is due to its protein or carbohydrate moiety or both, the effects were tested after digesting one or the other of the moieties. The results shown in Table 2 suggest that the protein or polypeptide moiety is essential, or at least related, to the antibacterial activity of the snail mucus mucin, whereas the carbohydrate moiety does not seem to be involved in the activity.

Few studies have been reported so far on the components of snail mucus (Levene, 1925; Suzuki, 1941; Masamune & Yoshizawa, 1956), but the existence of such an antibacterial factor in the mucus mucin appears a novel finding. The antibacterial factor might be functioning to protect the wet-skinned animal from external infection. As most of the antibiotics so far found are the products of microorganisms, i.e., fungi and bacteria, it is interesting to find such antibiotic activity in the normal secretion of a mollusc. The present result is closely related to a recent report on the presence of an antibiotic factor in the marine

Table 2. Antibacterial activity of mucin fraction (MF) of snail mucus before and after enzymic digestion

Sample	Sample concentration		Growth inhibition			
	Protein ($\mu g/cm^3$)	Carbohydrate ($\mu g/cm^3$)	*B. subtilis*	*S. aureus*	*E. coli*	*P. aeruginosa*
Before digestion (MF)	481	92	+ + +	+ + +	+ + +	+ + +
After pronase digestion	500	147	−	−	−	−
After glycosidase digestion	574	50	+ + +	+ + +	+ + +	+ + +

Each judgement is based on data from 4 plates.

The preparation methods of MF and the judgement of the antibacterial activity are the same as described in the legend of Table 1. The digestions were carried out as described in Materials and Methods. The concentration of the protein-digested preparation was adjusted to equal the protein level of MF, and the figure for its protein concentration should include peptides released from the mucin by digestion.

molluscs (Mori *et al.*, 1980). Another report describes how a bactericidal factor is induced in the haemolymph of fly larvae when they are injured (Natori, 1977). Such factors may play an important role in the primitive defence mechanisms of lower animals.

On the other hand, it is well known that the albumin gland secretion, the embryos, and the haemolymph of snails contain agglutinins which can distinguish the human ABO blood system (Bhatia *et al.*, 1967; Kothbauer & Schenkel-Brunner, 1971; Uhlenbruck *et al.*, 1972). Though both these factors and the present antibacterial factor(s) are related to mucins, or glycoproteins, their relationships are still obscure. Further study will be carried out in detail on the mechanism of the growth inhibition by the antibacterial factor(s) in the snail mucus mucin.

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