Antimicrobial properties of mucus from the brown garden snail *Helix aspersa*

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ABSTRACT

Research into naturally occurring antimicrobial substances has yielded effective treatments. One area of interest is peptides and proteins produced by invertebrates as part of their defence system, including the contents of mollusc mucus. Mucus produced by the African giant land snail, *Achatina fulica* has been reported to contain two proteins with broad-spectrum antibacterial activity. Mucus from the brown garden snail, *Helix aspersa*, appears to have skin regeneration properties. This study sought to investigate the antimicrobial properties of *H. aspersa* mucus. Mucus was collected from *H. aspersa* snails, diluted in phosphate-buffered saline (PBS), with the supernatant tested against a wide range of organisms in a disc-diffusion antimicrobial assay. This was followed with comparative experiments involving *A. fulica*, including bacteriophage assays. Mucus from both species of snail was passed through a series of protein size separation columns in order to determine the approximate size of the antimicrobial substance. Electrophoresis was also carried out on the *H. aspersa* mucus. Results indicated that *H. aspersa* mucus had a strong antibacterial effect against several strains of *Pseudomonas aeruginosa* and a weak effect against *Staphylococcus aureus*. Mucus from *A. fulica* also inhibited the growth of *S. aureus*, but the broad spectrum of activity reported by other workers was not observed. Antimicrobial activity was not caused by bacteriophage. Size separation experiments indicated that the antimicrobial substance(s) in *H. aspersa* were between 30 and 100 kDa. Electrophoresis revealed two proteins in this region (30–40 kDa and 50–60 kDa). These do not correspond with antimicrobial proteins previously reported in *A. fulica*. This study found one or more novel antimicrobial agents in *H. aspersa* mucus, with a strong effect against *P. aeruginosa*.

KEY WORDS: Anti-infective agents.
Helix aspersa.
Mucus.
Pseudomonas aeruginosa.

Introduction

The discovery of antibiotics in the middle of the 20th century significantly decreased the morbidity and mortality associated with infectious diseases. However, the ability of microorganisms to develop resistance to antimicrobial agents has been of concern since the first report of reduced susceptibility to penicillin of *Streptococcus pneumoniae* in 1965.[1] This has now become a major public health issue, noted by the Chief Medical Officer for England in her 2013 annual report,[2] and the subject of a World Health Assembly meeting which developed a global plan of action.[3]

Since many of the first antibiotics were naturally occurring substances, researchers have continued to investigate anecdotal evidence and folklore to find new antimicrobial agents. This has led to the discovery that, for example, a topical preparation of the essential oil 'tea tree' (*Melaleuca alternifolia*) from Australia has antiseptic and anti-inflammatory properties,[4] and that the Chinese herbal remedy qinghaosun (artemisinin) derived from *Artemisia annua* is an effective anti-malarial.[5] Copper, which was advocated by Hippocrates for the treatment of leg ulcers, is microbicidal and therefore has a useful role in infection control when incorporated into frequently touched surfaces in hospitals.[6]

One area of current interest is antimicrobial peptides (AMPs) in invertebrates. These are relatively small (5–15 kDa) molecules which are part of the animal's natural defence system.[7] Examples which have been characterised from molluscs include defensins, mytilins, myticins and mytimacins.[8,9] Most of the AMPs described to date are found in the haemolymph of the invertebrate and the literature suggests they have activity against an eclectic mix of microorganisms including bacteria, viruses and protozoa.[8]

A range of internal AMPs have been found in gastropods, but it is also likely that their external secretions would have components affording protection against potential pathogens, in a similar manner to the antimicrobial substances found in the mucus of fish and amphibians.[10,11]

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Terrestrial slugs and snails produce mucus which performs a variety of functions, including facilitating movement along the ground, communication and a non-specific, defensive response to physical or chemical irritation.[12]

The antimicrobial properties of the mucus collected from African giant land snails (*Achatina fulica*) were first described in the 1980s by researchers in Japan.[13,14] These authors reported finding that when mucus was mixed with water and centrifuged, the resulting supernatant exhibited antimicrobial activity in a standard disc-diffusion assay.[13] Biochemical investigations of the nature of the active ingredient led to the conclusion that it is a glycoprotein[13] of

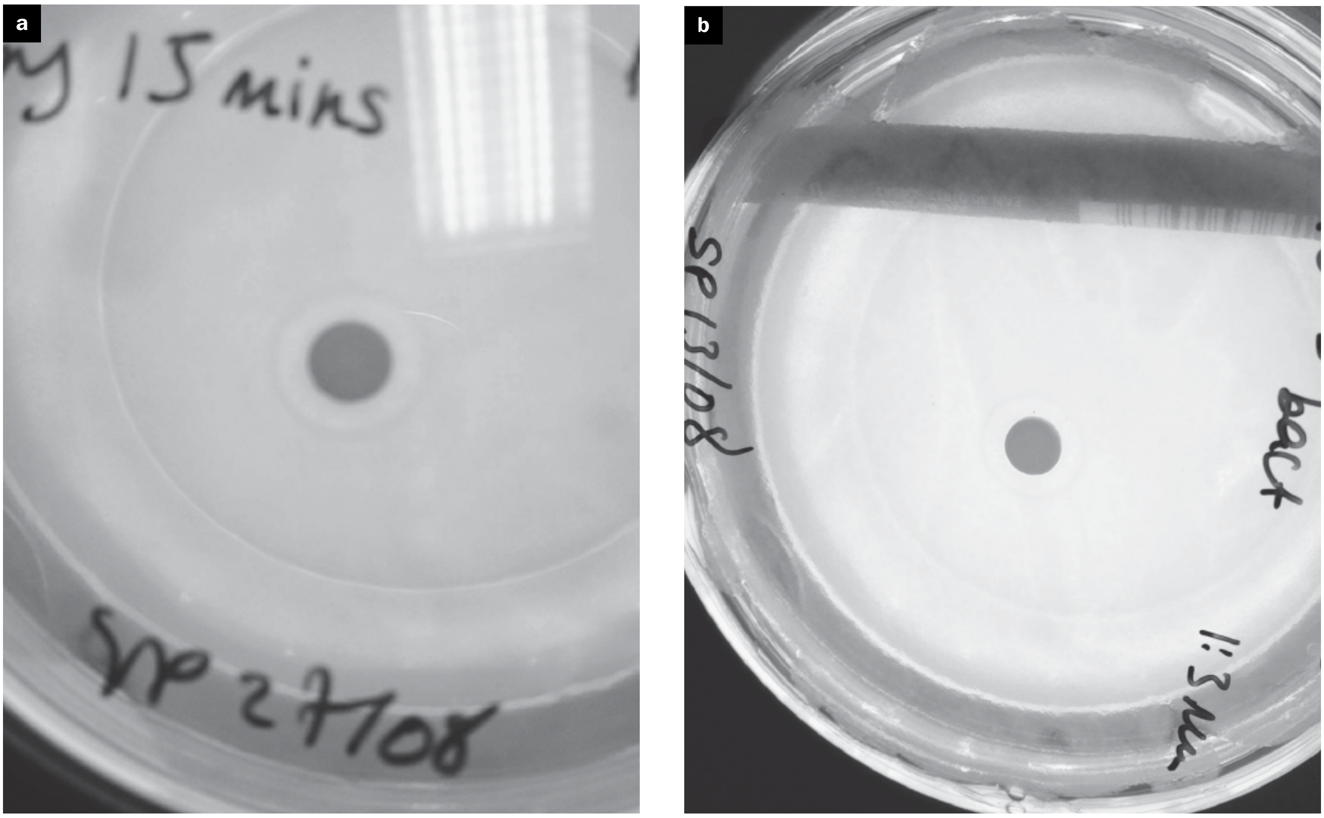

Fig. 1. Zones of inhibition observed after overnight incubation of 50 μL *Helix aspersa* mucus (diluted 1 in 3) on 5 mm disc on a lawn of **a)** *P. aeruginosa* NCIMB10548 and **b)** *P. aeruginosa* NCTC8626. *See these images in colour at www.bjbs-online.org*

around 140–160,000 Da,[14,15] which they subsequently named 'Achacin'.[15] They stated that Achacin inhibited the growth of a representative selection of Gram-positive and Gram-negative bacteria, namely *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*.[13–15] From their investigations of its mode of action, they concluded that Achacin is only effective against actively growing and dividing organisms.[15]

This work does not appear to have been followed up extensively, although Zhong *et al*.[16] found a smaller peptide (9700 Da) in *A. fulica* mucus, which they characterised as an AMP in the mytimacin family and therefore called 'Mytimacin-AF'. This was reported to have antimicrobial activity against *S. aureus*, several *Bacillus* spp., *Klebsiella pneumoniae* and *Candida albicans*,[16] but to be particularly effective in reducing growth of *S. aureus*. The authors did not discuss its possible mode of action or reasons for its selectivity. Similarly, Santana *et al*.[17] tested the *in vitro* activity of neat *A. fulica* mucus against *C. albicans*, *E. coli*, *S. aureus*, *S. epidermidis*, *Fusarium* spp. and *Salmonella* spp. They reported that it inhibited the growth of *Staphylococcus aureus* and *S. epidermidis*, but did not mention the results for the assays using the other organisms. In a separate set of experiments, Santana *et al*.[17] formulated mucus into a wound dressing preparation, which was applied to skin lesions on the backs of rats. They presented histological results suggesting that the *A. fulica* mucus promoted wound healing,[17] but specific antimicrobial activity *in vivo* was not reported.

The brown garden snail, *Helix aspersa*, has been used in human medicine since antiquity: Hippocrates recommended snail mucus for the treatment of protocoele, while Pliny stated that snail preparations could be employed in everything from childbirth to nosebleeds.[18] Recently, anecdotal reports of generic skin regeneration properties of the mucus from *H. aspersa* have been explored;[19] this has resulted in the commercial production of a topical preparation[20] claimed to have "wound healing" as well as anti-ageing properties. Tsoutsos *et al*.[19] tested the preparation on burns patients and while they noted that a range of opportunistically pathogenic bacteria were isolated from swabs collected from the wounds before treatment, this was not followed up with culture of post-treatment specimens. Thus, the specific antimicrobial properties of *H. aspersa* mucus do not appear to have been researched.

Therefore, the aim of this study is to investigate the antimicrobial properties of mucus from the common garden snail, *Helix aspersa*, and to characterise any active ingredient(s) discovered. As most of the previously published work in this area relates to *Achatina fulica*, analysis of mucus from this species of mollusc is also included for comparison.

Materials and methods

Snail care and mucus collection

Helix aspersa

Twenty snails were collected from the wild and kept indoors in a clear plastic tank (35 x 22 x 14 cm) at room temperature (~21 °C) and ambient light. They were fed on lettuce leaves, cucumber, carrots and apples and provided with water, along with a source of calcium. The tanks were cleaned weekly. Mucus production was encouraged by taking each individual snail and gently stimulating it with a cotton swab

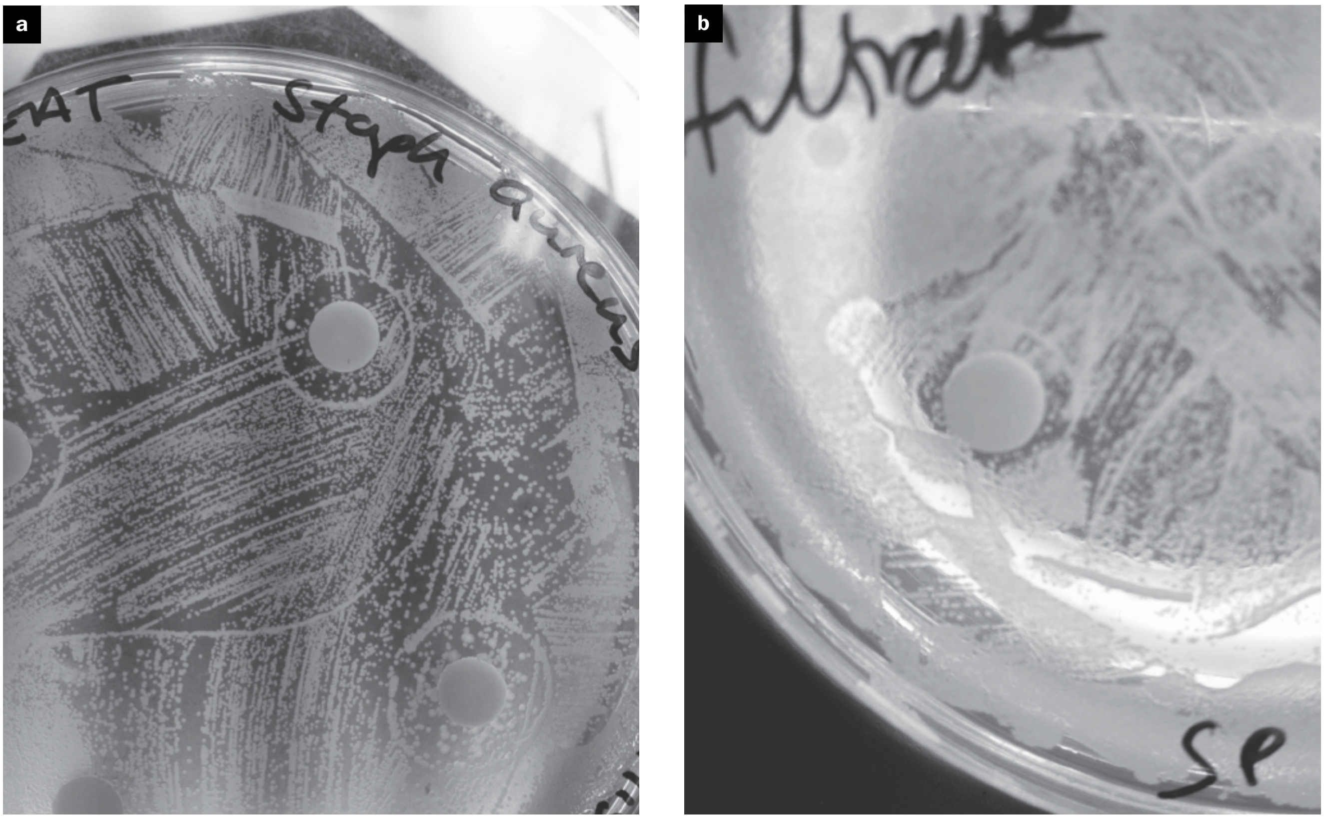

Fig. 3. Zones observed in the assay using filtrate from a 1000 kDa size separation column against *S. aureus* for **a)** *H. aspersa* mucus and **b)** *A. fulica* mucus, showing a defined ring with growth of organism within. *See these images in colour at www.bjbs-online.org*

moistened in phosphate-buffered saline (PBS). The resulting secretions were collected using 10 mL plastic Pasteur pipettes and pooled into one aliquot. The mucus was left to settle at room temperature for 1–2 hours before dilution (1 in 3) in PBS and centrifugation at 4500 rpm for 20 minutes. The supernatant was used in subsequent investigations.

Achatina fulica

Four captive bred juvenile snails were made available for this study courtesy of Dr Sally Willliamson, Liverpool John Moores University. They were maintained under the same conditions as the *H. aspersa* snails, except that the tank was slightly deeper (35 x 22 x 21 cm) and their diet was cucumber and butternut squash. Mucus production and collection was carried out in a similar manner to that described above for the garden snails. The mucus was diluted in PBS and processed to obtain supernatant as described above.

Initial antimicrobial assay

Supernatant from the diluted *H. aspersa* mucus was tested against a range of organisms: *Candida albicans* ATCC 10231, *E. coli* NCTC 10385, *K. pneumoniae* NCTC 11228, *Proteus mirabilis* NCTC 10823, *Pseudomonas aeruginosa* NCIMB 10548, *P. aeruginosa*, NCTC 8626, *S. aureus* NCTC 10788 and *Streptococcus pyogenes* NCIMB 13285, plus 'in house' isolates of *Acinetobacter* spp. (R4474), *Salmonella abony* and *Serratia marcescens*.

Organisms were grown overnight in broth cultures of tryptone soy broth (TSB; Oxoid, Basingstoke, Hampshire, UK) and diluted to between 10^6 and 10^7 colony forming units (cfu)/mL (verified by viable count). A 100 μL aliquot of the bacterial suspension was spread on an Isosensitest agar (ISA) plate (Oxoid) and left to dry for 15 minutes. Blood agar (prepared using Blood Agar Base No. 2 (Oxoid) plus 7% blood) was substituted for ISA in the *Streptococcus pyogenes* assay and Sabouraud Dextrose Agar (SDA; Oxoid) was used to test the activity against *C. albicans*. Three sterile paper assay discs (5 mm diameter) were applied to each plate and 50 μL mucus supernatant was added to two of them, while the third was treated with 50 μL PBS as a control. Plates were incubated at 37°C for 18–24 hours before being read. Zones of inhibition were recorded (in mm). For most of the organisms tested, the assay was performed twice and a mean zone of inhibition was calculated. For the two strains of P. *aeruginosa*, the assay was performed four times, generating eight readings from which the mean was taken.

After analysis of the results from the initial antimicrobial assay, the effect of *H. aspersa* mucus on *P. aeruginosa* was investigated in more detail and a third strain, namely *P. aeruginosa* NCTC 10662 was included. At this point, the *A. fulica* mucus was introduced into the study for comparison and both types of mucus were also tested against *E. coli* NCTC 10385, *Staphylococcus aureus* NCTC 10788 and *C. albicans* ATCC 10231.

Biochemical analysis

An aliquot of diluted *H. aspersa* mucus supernatant was analysed for protein content using the Biuret method. The result was obtained in g/L which was then calculated as mg/mL for the undiluted mucus.

Size separation assays

Approximately 500 μL aliquots of the mucus supernatant were processed in a series of Vivaspin 500 protein size separator columns (Sartorious, Epsom, Surrey, UK) at 1000 kDa, 100 KDa, 30 kDa and 10 KDa following the manufacturer's instructions (including pre-rinsing in distilled water to remove sodium azide). The resulting filtrate and concentrate were each tested in the antimicrobial assay against the three *P. aeruginosa* strains, *S. aureus*, *E. coli* and *C. albicans*. Each plate was set up with three test discs and one PBS control. All experiments were repeated at least once (i.e., at least six replicates); results were scored qualitatively.

Electrophoresis

H. aspersa mucus was processed through the 1000 kDa size separation column and the resulting filtrate was analysed by SDS-PAGE electrophoresis, by adding a 25 μL aliquot (in duplicate) to a 4–12% gradient NuPAGE Novex Bis-Tris Protein gel (Life Technologies, Paisley, UK). The gel was run in NuPAGE MOPS SDS Running Buffer at 200 V for 50 minutes. The Novex Sharp Pre-stained Protein Standard marker LC5800 (Life Technologies) was included in the run. The gel was then stained with 0.25% Coomassie Blue R250 dissolved in 50% methanol and 10% acetic acid for 2 h and destained overnight in several changes of solution containing 5% methanol and7.5% acetic acid.

Bacteriophage screening assay

Aliquots of the diluted mucus from *H. aspersa* and *A. fulica* were screened for the presence of bacteriophage using a method modified from that described by Adams,[21] and tested against the following organisms: *C. albicans* ATCC 10231, *E. coli* NCTC 10385, *K. pneumoniae* NCTC 11228, *Proteus mirabilis* NCTC 10823, *Pseudomonas aeruginosa* NCIMB 10548, *P. aeruginosa* NCTC 8626, *P. aeruginosa* NCTC 10662, *S. aureus* NCTC 10788 and *Streptococcus pyogenes* NCIMB 13285, plus 'in house' isolates of *Acinetobacter* spp. (R4474), and *Serratia marcescens*.

For each organism, 100 μL of an overnight broth culture was spread on a tryptone soya agar (TSA) plate (Oxoid). A 5 μL drop of each type of mucus was spotted on the plate, which was allowed to dry before incubating for 24 h at 37°C. An enrichment culture method was also used, where 100 μL host bacteria and 5 μL mucus were inoculated into 10 ml TSB and incubated at 37°C for 18 h. After this, approximately 1 mL was passed through a 0.45 μm filter to remove the bacteria and 5 μL of the resulting suspension spotted on fresh bacterial lawns and the plates incubated for 24 h at 37°C. All plates were examined for any signs of clearing.

Table 1. Mean zones of inhibition in antimicrobial assay with 1 in 3 diluted *H. aspersa* mucus.

Organism	Mean zone of (SD) PBS control	Inhibition (mm) Mucus supernatant
Staphylococcus aureus NCTC 10788	0	0
Streptococcus pyogenes NCIMB 13285	5.5*	5.5
Candida albicans ATCC 10231	0	0
Escherichia coli NCTC 10385	0	0
Klebsiella pneumoniae NCTC 11228	0	0
Salmonella abony†	0	0
Proteus mirabilis†	0	0
Acinetobacter spp.†	0	0
Serratia marcescens†	0	0
Pseudomonas aeruginosa NCTC 8626	0	11.12 (2.57)
Pseudomonas aeruginosa NCTC 10548	0	11.63 (1.52)

*Non-specific zone of haemolysis noted in both control and test plates.
†'In-house' isolates with no type culture collection identification.

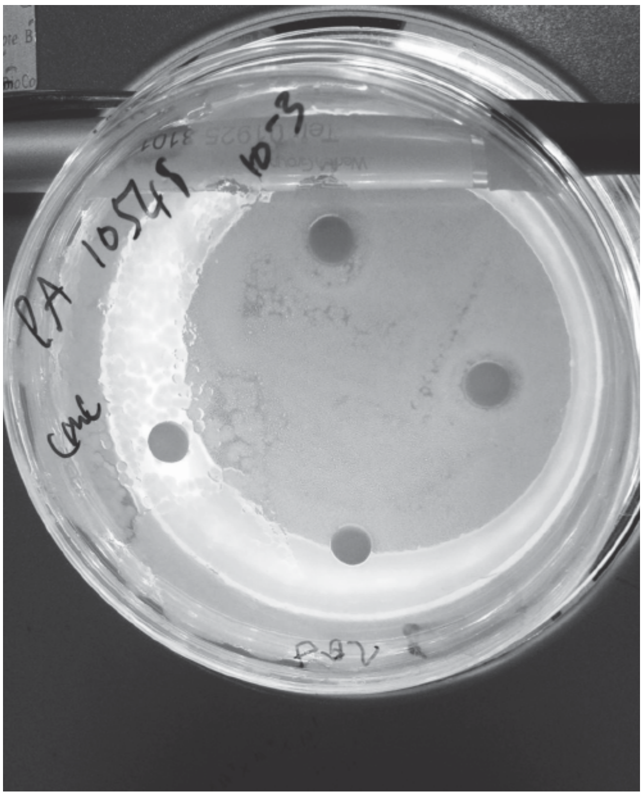

Fig. 2. Zones of inhibition with *P. aeruginosa* NCIMB 10548 after testing the filtrate obtained from passing the *H. aspersa* mucus though a 100 kDa column. *See this image in colour at www.bjbs-online.org*

Results

Small (between 500 μL and 1 mL) but sufficient quantities of mucus were successfully collected from the *H. aspersa* and the *A. fulica* snails each time experiments were conducted. The viscosity of the mucus varied between batches harvested on different days.

The results of testing the supernatant of the *H. aspersa* mucus after dilution (1 in 3) in PBS against a range of organisms are shown in Table 1. Clear, measurable zones of inhibition were obtained with the two *P. aeruginosa* strains (Table 1, Fig. 1), but no effect was observed for any of the other microorganisms.

Tables 2a and 2b indicate the outcomes from protein size separation experiments. They show that the antimicrobial activity was found in the filtrate from the 1000 kDa column for both types of mucus and against the two organisms where zones of inhibition were observed. For the *H. aspersa* mucus, these results located the protein(s) of interest at between 30 kDa and 100 kDa in size (Table 2a), while the

Table 2a. The effect of passing *H. aspersa* mucus through protein size separator columns on the retention of antimicrobial activity, indicating zone of inhibition (+) or no zone of inhibition (–).

	10 kDa column		30 kDa column		100 kDa column		1000 kDa column	
	Filtrate	Concentrate	Filtrate	Concentrate	Filtrate	Concentrate	Filtrate	Concentrate
E. coli NCTC 10385	–	–	–	–	–	–	–	–
C. albicans ATCC 10231	–	–	–	–	–	–	–	–
S. aureus NCTC 10788	–	+	–	+	+	–	+	–
P. aeruginosa NCTC 8626	–	+	–	+	+	–	+	–
P. aeruginosa NCIMB 10548	–	+	–	+	+	–	+	–
P. aeruginosa NCTC 10662	–	+	–	+	+	–	+	–

In all cases, PBS control discs gave a zone size of 0 mm.

active ingredient in *A. fulica* mucus appeared to be around 100 kDa (Table 2b). When testing *H. aspersa* mucus against the strains of *P. aeruginosa*, clear zones of inhibition were obtained (Fig. 2). The activity against *S. aureus* found in both types of mucus became apparent after passing mucus through the 1000 kDa, but did not result in clear zones (Figs. 3and 3b).

The total protein content of the diluted *H. aspersa* mucus was 1.6 g/L. This was calculated to be 4.8 mg/mL protein in neat mucus.

The electrophoresis revealed eight protein bands in the *H. aspersa* mucus, including one between 50 kDa and 60 kDa and one at approximately 35 kDa (Fig. 4). Additional bands were noted at >260, 20, 15, 10, 12 and <10 kDa.

No bacteriophage activity was found in the *H. aspersa* or *A. fulica* mucus against the target organisms tested in either the direct or enrichment assay (Table 3). Although there was some faint clearing observed in the direct assay with *Serratia marcescens*, on further investigative sampling, no active bacteriophage were isolated.

Discussion

This study has shown that the mucus from the common brown garden snail, *H. aspersa*, has a demonstrable antimicrobial activity against several strains of *P. aeruginosa*. The bacteriophage assay results indicated that this is not an effect caused by bacteriophage in the mucus. Previous studies on the antimicrobial activity of snail mucus have not tested for this, although bacteriophages have recently been shown to adhere to the mucus from a wide of organisms, including humans, thus contributing to the antimicrobial response.[22] While the concentration of the active ingredient(s) was not ascertained, clear and repeatable zones of inhibition were observed when the mucus was diluted in PBS but not subject to any other preparation (Fig. 1).

The diameter of the assay disc was 5 mm, which meant that the specific antimicrobial effect accounted for over 6 mm in the recorded zones (Table 1). This effect against *P. aeruginosa* was initially found in the unseparated mucus supernatant and subsequent investigations indicated the active ingredient to be between 30 kDa and 100 kDa. As Table 2a shows, antimicrobial effect was found in the concentrate after the 30 kDa size separation and the filtrate after the 100 kDa step.

Electrophoresis results confirmed that two proteins were present in *H. aspersa* mucus in this size region (one between 50 kDa and 60 kDa and one approximately 35 kDa in size; Fig. 4).

Resistance by *P. aeruginosa* to currently available antimicrobials is an increasing problem in clinical practice.[23] Therefore, this is a significant result, which does not appear to have been reported previously.

In contrast, *A. fulica* mucus was not found to have activity against any of the strains of *P. aeruginosa* included in this study, either in the unseparated form or after any of the size separation processes. This was unexpected, as it contradicts previously reported studies,[13,14] which apparently showed a strong effect against this bacterium. It is not clear why this

Table 2b. The effect of passing *A. fulica* mucus through protein size separator columns on the retention of antimicrobial activity, indicating zone of inhibition (+) or no zone of inhibition (–).

	10 kDa column		100 kDa column		1000 kDa column	
	Filtrate	Concentrate	Filtrate	Concentrate	Filtrate	Concentrate
E. coli NCTC 10385	–	–	–	–	–	–
C. albicans ATCC 10231	NT	NT	NT	NT	–	–
S. aureus NCTC 10788	–	–	+	+	+	–
P. aeruginosa NCTC 8626	–	–	–	–	–	–
P. aeruginosa NCIMB 10548	–	–	–	–	–	–
P. aeruginosa NCTC 10662	–	–	–	–	–	–

In all cases, PBS control discs gave a zone size of 0 mm.
NT: not tested

should be the case, but the present findings appear to concur with those of Santana *et al*.,[17] who did not report any results from their tests using *P. aeruginosa* ATCC 1024.

During the study, it was discovered that the use of the 1000 kDa column was a useful concentration step; the active ingredient(s) were retained and it enhanced the effect of both types of mucus against *Staphylococcus aureus*. (Tables 2a and 2b; Figs 3a and 3b). Using this processing prior to the antimicrobial assays, it was found that *H. aspersa* mucus also had an inhibitory effect against *S. aureus*, although it was less marked (Fig. 3a). A similar effect was noted with the *A. fulica* mucus (Table 2b, Fig. 3b). The fact that *A. fulica* mucus was found to be active against *S. aureus* does concur with previous work,[13–17] although none of the other authors report the relatively weak effect found in the present study. However, apart from Santana *et al*.,[17] all describe isolating the active ingredient (i.e., Achacin[13–15] or Mytimacin-AF[16]) before testing it against the bacteria.

Santana *et al*.[17] seem to have followed a very similar antimicrobial assay method to that employed in the present study, with the exception of their use of Mueller-Hinton agar instead of ISA and wells in the agar instead of discs. They reported that an aliquot of 5 µL of *A. fulica* mucus did not inhibit growth of *S. aureus*, but that an effect was noted at 10 µL and 20 µL.[17] They did not attempt to separate the protein content or concentrate the mucus in any way, so this supports the idea that the effect is enhanced when more of the active ingredient is available to interact with the bacterium.

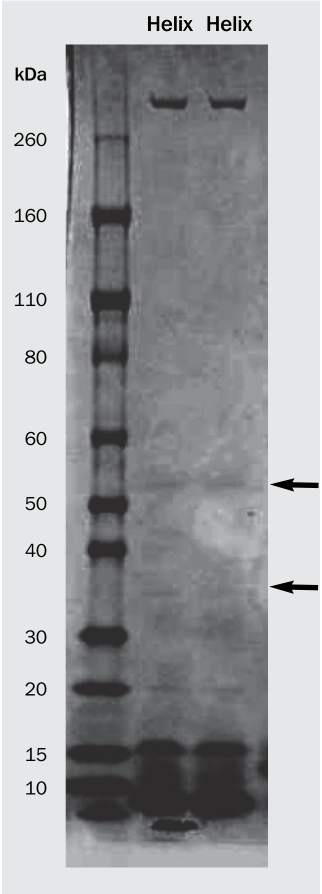

Fig. 4. Results obtained from SDS-PAGE electrophoresis using the filtrate obtained from passing *H. aspersa* mucus through a 1000 kDa size separation column.

The *H. aspersa* mucus was not found to be effective against a range of other bacteria or *C. albicans*. Initially this finding was rather unexpected, as components of *A. fulica* mucus have previously been reported to inhibit the growth of *E. coli*,[15,16] a number of *Bacillus* spp.[13,16] and *K. pneumoniae*[16] as well as *C. albicans*.[16] Extrapolating from the literature, it seemed possible that *H. aspersa* mucus might have a similar broad spectrum of activity, which is why various organisms were tested in the initial antimicrobial assay. However, this study was not able to reproduce the antimicrobial effect of *A. fulica* mucus against *E. coli*, *C. albicans* or *P. aeruginosa*. It is possible that this discrepancy is due to variations in methodology or the strains of organisms used. However, Santana *et al*.[17] also tested *A. fulica* mucus using a simple method (involving whole mucus rather than particular fractions) against a range of organisms and similarly reported an effect for *Staphylococcus* species only. They used different type culture collection organisms for *E. coli*, *P. aeruginosa* and indeed *S. aureus* to the present study,[17] which shows that strain variation is unlikely to be the explanation. By coincidence, they used the same *C. albicans* (ATCC 10231) and found the same (negative) result.

In this study, size separation was used to isolate and concentrate the active ingredient (see below), but no effect was observed when *A. fulica* mucus was tested against *E. coli*, *P. aeruginosa* or *C. albicans* (Table 2b). This suggests that the finding of a limited activity for *H. aspersa* mucus is likely to be genuine.

The *H. aspersa* mucus contained 4.8 mg/mL protein, which is comparable to the 4 mg/mL reported by Iguchi *et al*.[13] in *A. fulica* mucus. The size separation experiments (Table 2a) indicated that there were no proteins of less than 30 kDa active against the microorganisms under test in this study. This size and specificity means that it is unlikely that the antimicrobial activity is associated with lysozymes, which are known to be present in snails and their mucus.[24] Invertebrate lysozymes are usually small proteins with molecular weights of around 15 kDa. It also indicates that an antimicrobial compound analogous to Mytimacin-AF[16] or any other AMP[8] is unlikely to be present.

In this study, the substance which was active against *S. aureus* in *A. fulica* was present in both the filtrate and the concentrate after separation in the 100 kDa column. This was a surprise, as Achacin is reported to be a protein of 160 kDa, formed of two subunits of 70–80 kDa each.[14] It would be expected that only the whole molecule would be active in the antimicrobial assays and that the effect would therefore have been seen solely in the 100 kDa concentrate, but it was not (Table 2b).

Size separation experiments indicated that the antimicrobial effect in the *H. aspersa* mucus was due to one or more proteins of between 30 kDa and 100kDa in size (Table 2a) and electrophoresis revealed no clear band present in the 110–160 kDa region (Fig. 4). Bands were visible at between 30 kDa and 40 kDa and between 50 kDa and 60 kDa (Fig. 4), but they were comparatively faint, suggesting that these proteins were present in relatively low concentrations. It therefore could be that *H. aspersa* mucus does indeed contain an antimicrobial protein which corresponds to Achacin and is of a similar size, but that it was not detectable by the biochemical methods used in this study. This idea is supported by the fact that mucus from both species of snail produced a similar, albeit relatively weak, antimicrobial effect against *S. aureus* in the assay used here (Figs. 3a and 3b) and Achacin is reported to be effective in killing this Gram-positive coccus.[15] However, the ingredient active against *S. aureus* in the *H. aspersa* mucus was clearly within the 30–100 kDa range and it is possible that one of the two proteins identified in this region is responsible for the antistaphylococcal effect.

The activity of the *H. aspersa* mucus against *P. aeruginosa* appears to be stronger and there are two possible explanations for this. One is that there is an Achacin-like protein and that it interacts differently with *P. aeruginosa*. This is plausible as Otsuka-Fuchin *et al*.[15] suggested that Achacin could be targeting cell wall synthesis, and *P. aeruginosa* is a Gram-negative bacillus, with a different cell wall composition to *S. aureus*. Alternatively, as antimicrobial agents exploit peculiarities in prokaryotic metabolism, this protein could be

Table 3. Results from direct and enriched phage assays against a range of target organisms.

	H. aspersa mucus		*A. fulica* mucus	
Organism	Direct phage assay	Enriched phage assay	Direct phage assay	Enriched phage assay
Pseudomonas aeruginosa NCIMB 10548	No zone	No zone	No zone	No zone
Pseudomonas aeruginosa NCTC 8626	No zone	No zone	No zone	No zone
Pseudomonas aeruginosa NCTC 10662	No zone	No zone	No zone	No zone
Staphylococcus aureus NCTC 10788	No zone	No zone	No zone	No zone
Streptococcus pyogenes NCIMB 13285	No zone	No zone	No zone	No zone
Proteus mirabilis NCTC 10823	No zone	No zone	No zone	No zone
Klebsiella pneumoniae NCTC 11228	No zone	No zone	No zone	No zone
Escherichia coli NCTC 10385	No zone	No zone	No zone	No zone
Serratia marcescens	No zone*	No zone	No zone	No zone
Acinetobacter R4474	No zone	No zone	No zone	No zone
Candida albicans ATCC 10231	No zone	No zone	No zone	No zone

*Some signs of clearing but no phages were obtained when the zone was isolated.

interfering with a species-specific pathway. The other possibility is that one or both of the two smaller proteins (30–40 kDa and 50–60 kDa in size) identified as potential antimicrobials in *H. aspersa* mucus are affecting the *P. aeruginosa*. The strength of the effect on bacteria of this species (Figs. 1a, 1b and 2) could be because the two substances interact to achieve the antimicrobial effect. Further work to fully characterise the active ingredient(s) and exploration of the specific antimicrobial effects should clarify this

Although the results presented here were repeatable, it was not possible to confirm many of the previously published findings with *A. fulica*. This could be due to the low concentration of the active ingredient(s) in the mucus as it was collected and processed in this study. It is therefore possible that *H. aspersa* has a broader spectrum of activity than found here, analogous to that reported by other authors for *A. fulica*. Once the active ingredient has been fully characterised, tests with the wide range of microorganisms used in initial antimicrobial assay in this study could be repeated.

Further work would include obtaining a profile of the protein content using, for example, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) and then isolating fractions of the mucus protein without denaturing them using, for example, high-performance liquid chromatography (HPLC) or native gel electrophoresis; the individual fractions could then be put into the antimicrobial assay in order to determine the exact size of the active ingredient(s). Once known, the protein could be sequenced and fully characterised, which would allow elucidation of its site and mode of action against relevant microorganisms.

In this study, *H. aspersa* mucus was found to be equally effective against three laboratory strains of *P. aeruginosa*. The next phase, therefore, would be to test it (*in vitro*) against clinical isolates. If this proves successful, then the effectiveness of the topical preparation, such as the one already available commercially,[19] could be explored.

The antimicrobial activity in *H. aspersa* mucus appears to be caused by one or more novel substances with molecular weight between 30 kDa and 100 kDa. There is a particularly strong inhibitory effect against *P. aeruginosa* and further investigation is warranted. □

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