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Identification and characterisation of anti - *Pseudomonas aeruginosa* proteins in mucus of the brown garden snail, *Cornu aspersum*

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ABSTRACT

Background: Novel antimicrobial treatments are urgently needed. Previous work has shown that the mucus of the brown garden snail (*Cornu aspersum*) has antimicrobial properties, in particular against type culture collection strains of *Pseudomonas aeruginosa*. We hypothesised that it would also be effective against clinical isolates of the bacterium and that investigation of fractions of the mucus would identify one or more proteins with anti-pseudomonal properties, which could be further characterised.

Materials and methods: Mucus was extracted from snails collected from the wild. Antimicrobial activity against laboratory and clinical isolates of *Ps. aeruginosa* was determined in disc diffusion assays. Mucus was purified using size exclusion chromatography and fractions containing anti-pseudomonal activity identified. Mass spectroscopy and high performance liquid chromatography analysis of these fractions yielded partial peptide sequences. These were used to interrogate an RNA transcriptome generated from whole snails.

Results: Mucus from *C. aspersum* inhibited growth of type collection strains and clinical isolates of *Ps. aeruginosa*. Four novel *C. aspersum* proteins were identified; at least three are likely to have antimicrobial properties. The most interesting is a 37.4 kDa protein whilst smaller proteins, one 17.5 kDa and one 18.6 kDa also appear to have activity against *Ps. aeruginosa*.

Conclusions: The study has identified novel proteins with antimicrobial properties which could be used to develop treatments for use in human medicine.

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Introduction

Antimicrobial resistance is a major public health problem [1] and scientists and clinicians need to devise protocols that encourage more efficient and sparing use of antimicrobial agents [2,3] in order to limit this. Multidrug resistant strains of many important pathogens, including *Pseudomonas aeruginosa* have emerged [4]. Thus, there is a requirement to develop new antimicrobial agents. One approach is to investigate mechanisms of resistance and thereby design drugs that can overcome them [5]. Another is to explore the reliability of accounts of the effectiveness of natural remedies. This has yielded some significant outcomes including the development of artemisinin to treat chloroquine–resistant strains of the malaria parasite *Plasmodium* spp [6]. and tea tree oil for skin infections [7].

Ps. aeruginosa is an opportunistic pathogen in humans; it is associated with a range of clinical presentations, including lower respiratory tract infections, septicaemia, wound infections and ear infections [8]. Nosocomial acquisition is a risk, especially in immunocompromised patients and those with severe burns [9]. Patients with cystic fibrosis are especially susceptible to respiratory infections with *Ps. aeruginosa*. Infection can be intractable, since the bacteria form biofilms within the lungs, which antibiotics cannot fully penetrate [10]. Multidrug resistant and extreme multidrug resistant strains of *Ps. aeruginosa* are of increasing concern [8,10]. The bacterium is ubiquitous in the environment and pseudomonal infections are found in vertebrates, invertebrates and plants [11,12]. Therefore, the strategies different organisms employ for defence against *Ps. aeruginosa* might be a source of naturally occurring antimicrobial agents, which could be used in human medicine.

The common brown garden snail, *Cornu aspersum* (*Helix aspersa*) has been valued for its healing properties since ancient times [13]. Snail-based remedies have been advocated for the treatment of gastrointestinal and respiratory conditions, infections characterised by rashes (including measles and smallpox), hernias and skin lesions [13]. Terrestrial molluscs secrete mucus, which is a viscous and biochemically complex substance. It facilitates locomotion, helps reduce water loss, acts as a defence against predators and its components enable communication between snails of the same species [14]. Among the constituents of snail mucus are glycoproteins, some of which are lectins with agglutination properties [15].

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Cosmetic preparations containing extracts of *C. aspersum* mucus are currently widely marketed for the reduction of wrinkles and scarring [16,17]. However, mucus extracts also promote healing of burn wounds [18] and preliminary work indicates that they have anti-tumour properties and might have promise for treating melanogenesis [19]. To date, there have been few studies on the antimicrobial properties of the mucus of terrestrial molluscs [20] and most workers have focussed on the African Giant Land snail, *Achatina fulica*. Its mucus contains a 160 kDa glycoprotein 'achacin', which displays fairly broad-spectrum antimicrobial properties [20–23] and a 9 kDa antimicrobial peptide [24]. *C. aspersum* mucus also has antimicrobial activity, attributable to one or more proteins between 30 and 100 kDa in size [25,26]. There are two candidate proteins in this range – one approximately 50 kDa [25,26] and the other estimated as between 30 and 40 kDa [25] or 41 kDa [26].

In previous studies, preparations of *C. aspersum* mucus exhibited very clear activity against several laboratory-adapted culture collection strains of *Ps. aeruginosa* [26]. To investigate whether *C. aspersum* contains any antimicrobial agents which would be clinically useful, tests against isolates from patients are required. Testing of *Ps. aeruginosa* strains obtained from patients with cystic fibrosis would be the logical place to start this work. In addition, development of any such product requires identification and characterisation of the protein(s) responsible for the observed effects.

Therefore, the hypothesis for this present study was: *C. aspersum* mucus contains one or more proteins that have antimicrobial activity against *Ps. aeruginosa*.

Materials and methods

Twenty *Cornu aspersum* snails were collected from the wild in East Sussex, UK. They were placed in clear plastic tanks, part-filled with moist coconut husk substrate. These were kept in a room with ambient light and average temperature of 20°C. The snails were fed cucumber, butternut squash, green peppers, lettuce, and carrots and a provided with a source of calcium.

Mucus was collected by stimulating the snails with a cotton swab moistened in phosphate buffered saline (PBS) as described previously [25]. Mucus was harvested using a 5 mL plastic Pasteur pipette and, after settling at room temperature for 1–2 hours, placed into a 20 mL sterile plastic bijou bottle and diluted approximately 1:2 with PBS. The preparation method was modified to include an additional step of placing 2–4 8 mm glass beads into the bijou before shaking overnight (18–20 hours) at 4°C. The shaken mucus was centrifuged at 4000 rpm for 20 minutes to sediment large debris. The resulting supernatant was passed through a 0.45 micron filter after, which its protein content was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, East Grinstead, West Sussex, UK) and found to be between 4.0 and 8.0 mg/mL (n = 4).

As required, size separation was carried out in Vivaspin 500 columns (Sartorius Stedim Lab Ltd, Stonehouse, Gloucestershire, UK) at 1,000 kDa, 100 kDa, 30 kDa. The filtrate from the 1,000 kDa column was used to eliminate larger proteins. The 100 kDa and 30 kDa separation columns were used to concentrate the mucus fraction containing proteins between those two sizes, since previous work [25] had identified the active ingredient in *C. aspersum* to be in that size band.

Antimicrobial testing was as follows. Mucus was tested for anti-microbial activity against *Ps. aeruginosa* NCIMB 10548, *Ps. aeruginosa* NCTC 10662 and five well characterised clinical isolates of *Ps. aeruginosa*. These were isolates 2, 9, 10, 34 and 43 from the panel compiled by De Soyza et al. [27], which all originated from patients with cystic fibrosis. Disc diffusion assays were undertaken as previously described [26], using overnight Tryptone Soy Broth (Oxoid, Basingstoke, Hampshire, UK) cultures, diluted in PBS to between 10^6 and 10^7 colony forming units (cfu) per mL (verified by viable count). Aliquots of 100 µL were placed onto Isosensitest agar (ISA; Oxoid) plates and left to dry for 15 minutes. Then between three and six 5 mm sterile assay discs were placed on the plates and 50 µL of prepared mucus added to each disc. Every plate set up included a PBS control disc. Plates were left to dry for a further 40–60 minutes before incubation overnight (18–20 hours), aerobically at 37°C. Zones of inhibition were recorded in mm. A minimum of three replicates was tested against a particular strain of bacterium, but in most cases it was more than six.

For Size Exclusion Chromatography (SEC), prepared *C. aspersum* mucus was loaded on to a Superdex 75 16/60 column (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK), previously equilibrated with PBS. The column was run at 1 mL/min and fractions of 1.5 mL were collected. These fractions were submitted to SDS-PAGE and those showing the same protein pattern were pooled, concentrated using a 5 kDa cut-off Millipore spin filter and analysed by SDS-PAGE. Two proteins which appeared to be present in high concentrations were cut out from the gel and sent for analysis by Mass Spectroscopy.

This experiment was repeated using a Superdex 75 26/60 column (GE Healthcare Life Scientific), with the column run at 2 mL/min and fractions of 2 mL. The elution profile, monitored at 280 nm showed several peaks. Eluted fractions were bulked into pools

(labelled 1–6), based on protein pattern. Five of the resulting pools (2–6) were tested in duplicate against *Ps. aeruginosa* NCIMB 10548 in the disc diffusion assay described above and they were also examined by SDS-PAGE. One of the proteins observed was excised from the gel and analysed further by HPLC – chromatogram sequencing.

Proteins selected for further analysis by mass spectrometry were separated using SDS-PAGE taking account of precautions to minimise keratin contamination. The resulting gel was sent to the Centre of Excellence for Mass Spectrometry, King's College London. Two proteins from the SDS-PAGE were selected for MS analysis, one of approximately 50 kDa and the other estimated to be between 17 and 18 kDa in size. Selected proteins were excised from the gel and de-stained then reduced (with dithiothreitol {DTT}), treated with iodoacetamide (to alkylate any free cysteine residues) and digested overnight with trypsin. The resulting peptide fragments were washed and then lyophilised after which they were re-suspended in 50 mM ammonium bicarbonate. The suspensions were then subjected to reverse phase chromatography in an EASY NanoLC (Thermo Scientific) Ultra HPLC analyser and the eluted peptides ionised by electrospray and fragmented in an Orbitrap Velos Pro using collision-induced dissociation. The 20 ions that registered most strongly were analysed in the Proteome Discover V1.4 software and compared to peptide sequences in the Uniprot database. The data were also analysed with the PEAKS mass spectrometry software. Resulting peptides were compared with sequences in a *Helix pomatia* database (New England Biolabs, MA, USA).

For HPLC-chromatogram sequencing, prepared mucus was concentrated by passing it through 1 MDa and 100 kDa Vivaspin 500 columns (Sartorius) and running out in an SDS-PAGE gel. The fraction of interest (30–40 kDa) was cut and sent to The Proteome Factory AG, Berlin, Germany, where sequencing analysis was undertaken by Ms Julia Schendel. Using HPLC–chromatogram analysis a series of peptides was obtained after digestion with trypsin, chymotrypsin, elastase and LysC.

For RNA transcriptome sequencing, individual *C. aspersum* snails were stimulated to produce mucus as described above and then snap frozen in liquid nitrogen. After freezing, the shell was broken off and the body ground to a paste in a pestle and mortar; the pestle and mortar were kept cold throughout this process. Total RNA was extracted using the E.Z.N.A.(r) Mollusc RNA Kit (Omega BIO-TEK, Norcross, USA.), following the manufacturer's instructions for the long protocol. Total RNA was quantified for purity and integrity by UV-Vis spectroscopy (NanoDrop, Thermoscientific, Waltham, MA, USA). Samples were then stored at −80°C before transport to The University of Liverpool, Centre for Genomic Research. RNA was further purified using an RNA Clean & Concentrator kit (Zymo Research, CA, USA) and total RNA quantified using a 2100 Agilent Bioanalyzer (RNA Nanochip, Agilent Technologies, Waldbronn, Germany).

The sample was treated with a Teloprime full length cDNA amplification kit (Lexogen GmbH, Vienna, Austria) following the manufacturer's protocol. The product was checked for concentration and profile using a bioanalyser high sensitivity chip.

The ends of the sample were damage and end repaired using the Template preparation kit (Pacific Biosciences, CA, USA). After cleaning with Ampure XP beads (Beckman Coulter, IN, USA), the sample was ligated to PB adapter overnight at 25°C. The SMRTbell library was treated with exonuclease to remove non circular DNA. The SMRTbell library was cleaned and this was used to load sequel cells using diffusion loading at 6 pM on plate concentration and a 2 hour pre extension.

The resulting PacBio READS where analysed at the University of Liverpool, Centre for Genomic Research using the ISO-seq pipeline (SMRT link software 5.0.1.9585) with the default parameter settings. The transcriptome was annotated using TransDecoder (version 3.0.1) for each strand and protein sequences were annotated using BLASTp (National Center for Biotechnology Information [NCBI], Bethesda, MD; http://www.ncbi.nlm.nih.gov/BLAST/). Protein sequences with a blast e-val below 0.01 were selected as best match.

The transcriptome was probed using the peptide sequences obtained from Mass Spectroscopy and HPLC using BLASTn. Matches to transcripts were further analysed using the EXPASY translate tool (https://web.expasy.org/translate/) to search for open reading frames. Proteins found to match to the relevant peptides were subjected to analysis using BLASTp.

Results

For antimicrobial spectrum of activity in *C. aspersum* mucus, zones of inhibition were observed in all cases when the mucus was tested against the laboratory strains and clinical isolates of *Ps. aeruginosa*. Figure 1 is an example of an anti-microbial disc diffusion assay plate where mucus was tested against *Ps. aeruginosa* NCIMB 10548. It shows a very narrow zone of clearing (<0.1 mm) around the PBS control and obvious zones of inhibition around the three control discs. The mean zones of inhibition recorded for the culture collection and clinical isolates of *Ps. aeruginosa* were all between 9 and 13 mm.

Size exclusion chromatography of *C. aspersum* mucus proteins was as follows. During the first Superdex column run, two peaks were eluted. Although one of these peaks appeared to represent three distinct protein fractions,

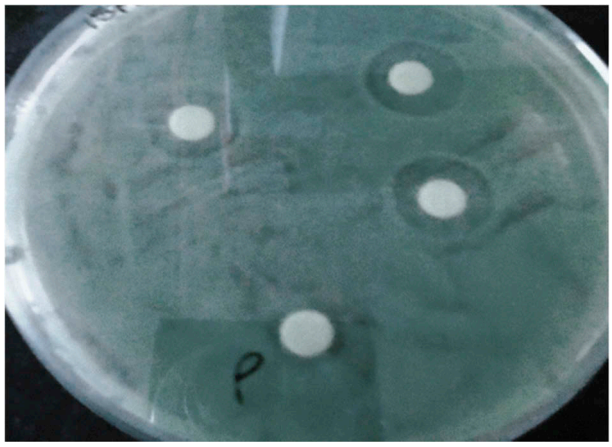
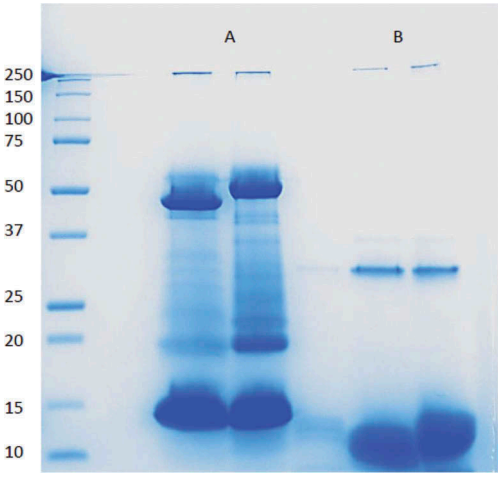


Figure 1. Antimicrobial disc diffusion plate assays using *Cornu aspersum* mucus demonstrating inhibition of *Pseudomonas aeruginosa* NCIMB 10548. Mucus preparations were tested in triplicate. P = phosphate buffered saline control.

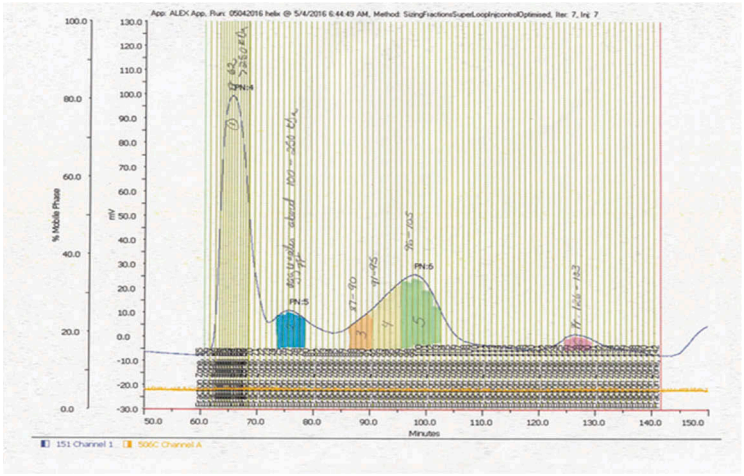
they all showed similar protein patterns on SDS-PAGE. One of these fractions (A) is shown in Figure 2(a), along with the proteins from the second peak (B). There were strongly staining bands at approximately 50 kDa and 17 kDa and these two bands were sent for Mass Spectroscopy.

Figure 2(b) shows the trace obtained from the second SEC procedure; the peaks are labelled 1–6, where 1 contains the protein aggregates. Figure 2(c) is the SDS-PAGE gel carried out on the fractions on the day of the SEC experiments, indicating the proteins detected in each fraction. Table 1 shows the estimated sizes of the proteins detected in each fraction and which ones were found to have antibacterial activity.

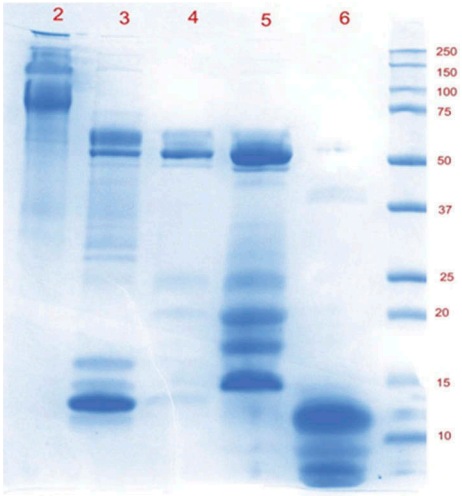
Peptides identified in C. aspersum mucus from mass spectrometry analysis and HPLC-chromatographic analysis were as follows. From the first SEC experiment the ~17 kDda and the ~50 kDa proteins (indicated in Figure 2(a)) were selected for Mass Spectrometry analysis. Table 2 shows the putative peptides obtained after deduction in comparison with a *H. pomatia* database. After the antimicrobial analysis of the fractions from the second SEC (Table 2), the protein of approximately 39 kDa was sent for the HPLC-chromatographic analysis; the peptides obtained from this are also shown in Table 2.



(a)



(b)



(c)

Figure 2. (a) SDS PAGE analysis of the protein profile of two pooled fractions (A and B) from the initial superdex 16/60 run, showing strongly staining bands at 50 kDa and 17 kDa in fraction A. Molecular weight ladder is on the left hand side. (b) Trace showing of fractions collected after running the mucus through a superdex 75 26/60 column. (c) SDS-PAGE analysis of proteins in peaks 2–6 from Figure 2(b). Molecular weight ladder is on the right hand side.

Using these peptides to probe the transcriptome database, four proteins were identified, as indicated in Table 2.

Transcriptome sequencing and assembly were as follows. Using the peptide sequences (Table 2) to probe the proteins identified in the RNA transcriptome revealed that peptide 1 aligned to a single match in the transcriptome. This sequence had 4 open reading frames, the longest of which is a protein sequence of 159 amino acids (17.5 kDa) (protein 1), (Figure 3). Protein 1 contains a conserved region of the complement Cq1 superfamily. Protein 1 has been submitted to the NCBI GenBank and allocated the accession number MK393387.

A combination of peptides 3, 5 and 6 matched to a 583 amino acid protein (64 kDa) that was identified as protein 2 (Figure 3).

Peptide 11 matched to a 169 amino acid protein (18.6 kDa) (Figure 3) that was identified as protein 3. Amino acids 1–155 map to a conserved region in the fibrinogen related domain (FReD) superfamily. Protein 3 has been submitted to the NCBI GenBank and allocated the accession number MK393388.

A combination of peptides 12, 13, 14, 15,16, 17 and 18 matched to a protein of 340 amino acids in length (37.4 kDa), labelled protein 4 (Figure 3). This sequence appeared in three separate places in the transcriptome. Two of these sequences have been submitted to the NBCI GenBank and allocated the accession numbers MK393385 and MK393386. Amino acids 150–340 map to a conserved region from the von Willebrand Factor A superfamily.

Table 1. Characteristics of each peak from Figure 2(b) in terms of activity against *Ps. aeruginosa* NCIMB 10548 and sizes of the proteins in each peak as determined by SDS-PAGE.

Peak	Mean zone of inhibition, mm	Protein sizes, kDa
2	0	250, 116, 72
3	14	56, 50,17,15, 12
4	0	60,50,23, 20,13
5	12	50, 24, 20, 17, 14
6	14.5	39, 12, 10, 5

Discussion

This study has confirmed the findings of Pitt et al. [25] and Bortolotti et al. [26] that *C. aspersum* mucus contains one or more proteins with anti-microbial activity against *Ps. aeruginosa*. The results for the antimicrobial plate assay were essentially qualitative, since the concentration of the active ingredient(s) within the mucus was not known. Nevertheless, clear and reproducible zones of inhibition were obtained not only with the type culture collection strains, but with all five clinical isolates which came from patients with cystic fibrosis.

This is an important finding as it indicates that further characterisation of the active ingredient(s) is justified. Previous work on *C. aspersum* mucus identified two possible proteins in the size range of between 30 kDa and 100 kDa on SDS-PAGE with anti-microbial activity against *Ps. aeruginosa* [25,26] and this was confirmed by repeated plate assays (S. Pitt, unpublished data) and Minimum Inhibitory Concentration experiments (J. Hawthorne, unpublished data). One of the proteins is between 50 and 60 kDa and the other between 30 and 40 kDa. Analysis by SDS-PAGE of the fractions of mucus obtained by the SEC procedure revealed proteins that corresponded with these sizes (Figures 2(a,c)). In addition, the SDS-PAGE of the unfractionated mucus showed clear bands in the 10–20 kDa size regions (Figures 2(a,b)). Some or all of these are likely to be lectins, which are common components of gastropod mucus. There are various classes of lectins and some of them function in the invertebrate immune response [28,29]. Lectins also play a role in mollusc mucus function, including binding/attaching to substrates and contributing to viscosity. They have also

Table 2. Peptides obtained from the 17 kDa, 50 kDa and 30–40 kDa proteins the matches in the transcriptome.

Peptide	Putative protein from Mass Spectrometry (MS)/HPLC-chromatogram (HPLC)	Sequence	Match to transcriptome database
1	17 kDa (MS)	LGNVAFTAAFTEDTSVAEGR	Protein 1
2	17 kDa (MS)	ANLLSGLLKTVVGLVGGLVKGDEVYVK	No match
3	50 kDa (MS)	GTYLALDYL	Protein 2
4	50 kDa(MS)	WWLVnLLmLVnESGSLGPPK	Protein 2
5	50 kDa(MS)	WWLVDLLFLVDESGSLGPPK	Protein 2
6	50 kDa(MS)	FGAVTFSDR	Protein 2
7	50 kDa(MS)	VFDWGVTTLNK	No match
8	50 kDa(MS)	LVLVTDGNSDSFLTNAAANLQK	No match
9	30–40 kDa (HPLC)	VVCGTWWDTLMYR	No match
10	30–40 kDa(HPLC)	QYLQVTWSSPR	No match
11	30–40 kDa(HPLC)	VGGEDAYYK	Protein 3
12	30–40 kDa(HPLC)	TNLGGLSSVHNEEGVVSCR	Protein 4
13	30–40 kDa(HPLC)	ATVGDLVKF	Protein 4
14	30–40 kDa(HPLC)	KLAKVF	Protein 4
15	30–40 kDa	ATAGDLVKF	Protein 4
16	30–40 kDa	TAGDLVKF	Protein 4
17	30–40 kDa	CGLEDEGLTGVSR	Protein 4
18	30-40 kDa	DVANTKNLRY	Protein 4

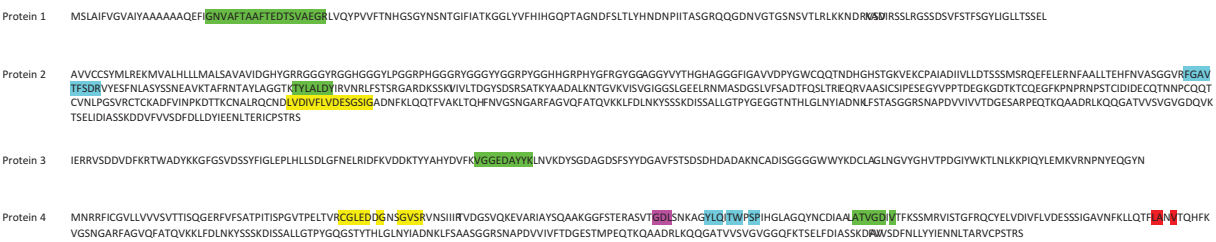


Figure 3. Protein sequences 1–4 peptide sequences are highlighted.

been found to have unexpected properties with respect to binding mammalian cells. For example, *C. aspersum* mucus extracts promote wound healing by stimulating the proliferation and migration of mammalian fibroblasts [30]. Similarly, the ability of the *H. pomatia* agglutinin (HPA) to attach to mammalian cells expressing the N-acetylgalactosamine surface protein, which is a marker of malignancy. A 12 kDa lectin with similar properties is present within *C. aspersum* mucus. It binds to very specific mammalian cell surface markers and has been extensively characterised with a view to using it in diagnostic assays for cancers and other conditions [15].

Previous size separation experiments with *C. aspersum* mucus found that component proteins less than 30 kDa in size did not appear to express antimicrobial activity [25]. Therefore, the identification of a 17.5 kDa protein ('protein 1') with possible anti-microbial activity (Table 1, Table 2, Figure 3) which appears to be a sialic acid binding lectin was an unexpected finding (Figure 5(a)). Some lectins have pathogen recognition receptor (PRR) properties [30] and this could contribute to the anti-microbial activity. Gastropod lectins have been known about for some time but to date none have been exploited for antimicrobial treatments. Another unexpected finding was the 18.6 kDa protein ('protein 3') within the 30–40 kDa mucus fraction (Table 2, Figure 3, Figure 4(c)). The sequence suggests that it has a region consistent with the fibrinogen related domain (FreD). While fibrinogens are well known and characterised in mammals for their role in coagulation, they are also part of the invertebrate defence mechanism. FReDs work as part of the internal immune system and they have agglutination and lectin-like binding properties [31]. However, some FReDs with PRR and bacteriolytic properties – at least *in vitro* – are present in animals including shrimp [32] and amphioxus [33]. Although the presence of the 18.6 kDa protein in the anti-pseudomonal fraction of the mucus noted here could be co-incidental, this might equally play an important function in the observed anti-bacterial effect.

The initial SEC experiments suggested that the protein of around 50 kDa was present in large concentrations. It was therefore selected for sequencing by mass spectroscopy. The second SEC and testing of the fractions against *Ps. aeruginosa* indicated that this protein probably lacks antimicrobial activity. As Table 1 shows, the 50 kDa protein was present in fractions 3, 4 and 5, although only the first and last of these showed anti-bacterial activity. Probing of the RNA transcriptome with the resulting peptides revealed a slightly larger protein of 64 kDa, 'protein 2' (Table 2, Figure 3). It remains unclear whether this is the protein identified as 50 kDa in the first SEC experiment. Protein 2 seems likely to be an extracellular matrix protein.

Strong anti-pseudomonal activity was observed with fraction 6 (Table 1) which contained the protein between 30 and 40 kDa. The work following this up has identified a 37.4 kDa protein, 'protein 4' (Table 2, Figure 3). It was found in three separate places in the transcriptome, which suggests it is important for the snail. Reports of proteins in this mass range within invertebrates are limited, but they function as part of the mammalian innate immune response and some have anti-microbial properties. For example, cathelicidin LL-37 has activity against *Escherichia coli, Candida albicans* and *Staphylococcus aureus* [34]. Another protein, CAP-37 [35] appears to target Gram negative bacteria and has a particularly strong effect on *Ps. aeruginosa*. The 37.4 kDa protein in *C. aspersum* mucus shares homology with a predicted collagen 6 (IV) protein in another species of snail, *Biomphalaria glabrata*, and a match to conserved domains of the von Willebrand factor A superfamily. Although it is an extracellular matrix protein, collagen 6(IV) from mammals has antibacterial properties and the significance of the vWFA domains in this effect was recently demonstrated [36]. Peptides containing the relevant domains are reported to have good activity against selected bacteria, including *Ps. aeruginosa*. This may provide a partial explanation for the antimicrobial properties of this novel protein, although BLAST results showed mapping to only 55% of the *B. glabrata* protein.

The reason for the presence of a substance with targeted anti-pseudomonal activity in *C. aspersum* mucus is not immediately clear. Although *Ps. aeruginosa* is a common soil organism, to which terrestrial snails are continually exposed, there are many other potential pathogens in the soil environment. *Ps. aeruginosa* is a known pathogen of insects, within which it employs similar virulence factors to those in mammalian hosts [37]. *Pseudomonas* spp. thrive in the environmental conditions provided by human mucus, especially in the lungs of patients with cystic fibrosis

[10,38]. It is therefore possible that snail mucus has similar constituents that can enhance the growth of these bacteria, which necessitates specific protection. Also, a specific immunological interaction between this particular mollusc and bacterium has been previously noted. Repeated injection with suspensions containing high titres of *Ps. aeruginosa* have been shown to stimulate a measurable immune response in *C. aspersum*, while preparations containing other microorganisms did not [39]. Thus, it appears that the anti-microbial proteins identified here could have a particular function in contributing to protection of *C. aspersum* against *Ps. aeruginosa* infections. If this could be ascertained, it may be possible to exploit it for development of treatment of human infections with this bacterium. As the results presented here indicate, there appears to be promise for these proteins as treatment against *Ps. aeruginosa* strains implicated in serious respiratory infections, but they could also be used to in preparations to target infections in deep wounds. Therefore, further characterisation of the form and function of all these proteins would be beneficial. Production of purified versions of the proteins would make them available in greater concentration than has been possible from crude mucus preparations. This would allow quantitative antimicrobial assays to be conducted as opposed to the qualitative results presented here. Re-investigation of the antimicrobial activity to confirm the specificity against *Ps. aeruginosa* could be conducted. Detailed observations of the site within the bacterial cell and mechanism of action of each of these proteins could be carried out.

Although four novel proteins have been identified here, the 37.4 kDa protein, to be named Aspernin, appears to be the most significant finding and it could be exploited to develop an antimicrobial substance for use in human medicine. Because *C. aspersum* is farmed commercially in several countries, it is already possible to source large numbers of snails at low cost. Therefore, unlike many naturally-derived pharmaceutical compounds, sourcing the raw material would not be a problem and the antimicrobial components might be separated out when the snails are processed for other purposes. This work represents an advance in biomedical science because it has identified a potential novel antimicrobial treatment against the clinical important pathogen *Ps. aeruginosa*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Summary table

What is known about this subject:

- *Cornu aspersum* mucus has anti-microbial properties
- It is particularly effective against *Pseudomonas aeruginosa*
- The antimicrobial effect is attributable to one or more proteins between 30 and 100 kDa in size

What this paper adds:

- *Cornu aspersum* mucus contains several proteins with anti-microbial properties
- Three such proteins have been identified with masses of 17.5 kDa, 18.6 kDa and 37 kDa respectively, the most important of these is the 37 kDa protein, Aspernin
- The mucus is effective against both type culture collection and clinical isolates of *Ps. aeruginosa*

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