BRITISH JOURNAL OF BIOMEDICAL SCIENCE, 2016
VOL. 73, NO. 1, 49–50
http://dx.doi.org/10.1080/09674845.2016.1155377

Letter to the Editor: Antimicrobial properties of mucus from the brown garden snail *Helix aspersa*

Daria Bortolotti[a], Claudio Trapella[b], Tatiana Bernardi[b] and Roberta Rizzo[a]

[a]Department of Medical Sciences, Section of Microbiology and Medical Genetics, University of Ferrara, Ferrara, Italy; [b]Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy

ARTICLE HISTORY Received 11 February 2016 Accepted 15 February 2016

Dear Andrew Blann

We have read with interest the paper by Pitt et al. [1] reporting the ability of snail mucus of *Helix aspersa* to exhibit antimicrobial activity due to the presence of antimicrobic substances. We have developed an efficient technique to purify *H. aspersa* mucus to improve purity without affecting its antimicrobial effects. The use of NaCl to induce mucus production deeply affects protein content and consequently mucus quality. For this, we have standardised an extraction method with low concentrations of NaCl (3%) and the use of an extractor machine (Beatrix®; Colognesi industries; Ferrara, Italy) that collects about 600 mL of crude extract using 500 snails (about 10 Kg) after 45 min. Filtration and lyophilisation (Lio 5P liophilyzer (Pascal s.r.l.; Milan, Italy)) procedures eliminate mucus impurities, preserve mucus quality and obtain a solid product that could be easily shipped and conserved. The obtained lyophilised mucus presented no microbial contaminations, maintaining antimicrobial effects.

To evaluate the antimicrobial effect of lyophilised *H. aspersa* mucus, we selected different micro-organisms: Gram negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC BAA-47); Gram positive bacterium *Staphylococcus aureus* (ATCC 25923); fungi *Candida albicans (*ATCC 10231). The bacteriostatic activity of mucus suspension was determined by adding suspended mucus to liquid bacterial cultures (Lauria broth (LB) medium). Bacterial growth was evaluated by reading at 600 nm every 30 min for one hour. We observed, in agreement with Pitt et al. [1], a higher bacteriostatic effect of the mucus towards Gram negative bacteria. Moreover, we evaluated the minimal inhibitory concentration (MIC) and IC50 (half maximal inhibitory concentration) in liquid LB medium. MIC was performed using a broth microdilution method. In brief, serial dilution of mucus (50–3.4 ug/uL) was added to test organisms, prepared at a final concentration of 10^4 Colony-forming unit (CFU)/mL. The MIC was defined as the lowest concentration of suspended mucus that inhibits micro-organism growth (absence of turbidity in the culture) after overnight incubation at 37 °C. IC50 was evaluated treating test micro-organisms (10^4 CFU)/mL) with serial dilution of mucus (range: 50–3.4 ug/uL). After overnight incubation, IC50 was determined by interpolation after reading at 600 nm.

We observed, for Gram negative bacteria, a MIC value of 25 ug\uL and an IC50 value of 28 and 41 ug\ul for *E. coli* and *P. aeruginosa*, respectively. MIC value for Gram positive bacterium (*Staphylococcus aureus*) and fungi (*C. albicans)* was >50 ug\uL and IC50 value reached concentrations over 100 ug/uL.

Since *H. aspersa* mucus is widely used as a component of dermatological substances and over-the-counter drugs, as cosmetic creams and cough syrups, we evaluated the cytotoxic activity of the mucus on human T-cell line (Jurkat, ATCC TIB-152), by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, MO, USA). Briefly, Jurkat cells were plated in a 96-well plate at the concentration of $1x10^6$ cells\mL in a final volume of 100 ul. Cells were then treated with different concentrations of suspended mucus for 1hr at 37 °C. After incubation, 10ul of MTT was added to each well and incubated again at 37 °C for 4 h. Cells were then homogenised with 100ul of solvent and the plates read with a microplate reader (Multiskan Plus MK II, Labsystems; Milano, Italy) at 570 nm. Our results showed the absence of any critical effect of the lyophilised mucus on human cells.

The evaluation of the protein content of the lyophilised mucus by SDS–PAGE (BioRad TGX Precast Gel 10%, Biorad, CA, USA) and Coomassie Blue R250 staining confirmed the presence of two sets of proteins, one above 50 kDa and one between the range of 40–30 kDa (Figure 1(a)). We extracted the protein bands and analysed them by ESI-Q-TOF-HPLC-MS instrument (Agilent 6520-nano-HPLC Chip cube; CA, USA) (Figure 1(b) and (c)). The protein bands were extracted using a 0.2 M NaOH solution, the extract was filtered off using a 0.22-micron regenerate cellulose filter and directly injected into HPLC auto sampler. Five microlitres of

CONTACT Daria Bortolotti brtdra@unife.it

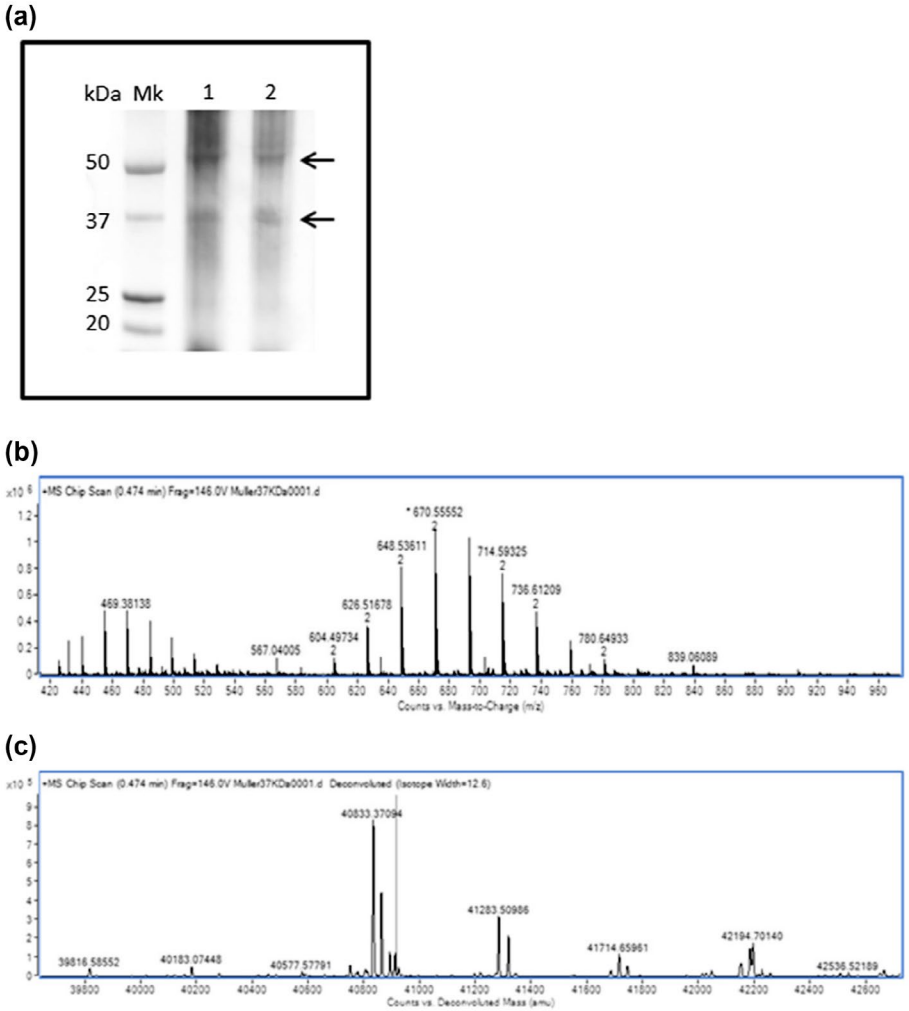

Figure 1. Analysis of protein content in lyophilised *H. aspersa* mucus. (a) SDS–PAGE and Coomassie Blue R250 staining of two samples (1, 2) of lyophilised mucus. Two sets of proteins, one above 50 kDa and one between the range of 40–30 kDa are shown. Mk: standard protein marker (Precision Plus Protein Prestained Standards, Biorad). (b) Mass spectra of multicharge protein between 40 and 30 kDa. (c) Deconvolution analysis of the multicharge spectra.

the above solution was injected using a linear gradient from 0% solution B (97% water, 3% acetonitrile 0.1% formic acid) to 90% solution B, 10% solution A (97%aceonitrile, 3% water, 0.1% formic acid) in 15 min using a Zorbax 300SB-C18 column (43 mm × 75 µm, Enrichment 4 mm × 40 nL) at 400 nL per minute. The 50-kDa protein band was composed by several proteins, difficult to be discriminated. On the contrary, the protein band in the range of 40–30 kDa was composed by a single protein of 41 kDa (Figure 1(b)), as calculated by the deconvolution software from the multi-charge pattern showed between 604.49734 and 780.64933 Da in the mass spectra (Figure 1(c)).

In conclusion, Pitt and co-authors [1] and our new data are perfectly in agreement on the antimicrobial properties of *H. aspersa* mucus.[2–6] We believe that our results point out also the importance of standardised purification procedures to maintain mucus quality and properties and to avoid human cell cytotoxicity. Furthermore, new studies will be of importance to identify the potential antimicrobial properties of the identified mucus proteins.

Disclosure statement

No potential conflict of interest was reported by the authors.

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