Isolation and characterization of antimicrobial peptides from healthy male urine

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Abstract: A complex of low-molecular cationic peptides, extracted from human urine by a combination membrane ultrafiltration and weak cation exchange chromatography, was characterized in this study. It provides a simpler solution for the development of novel antimicrobial peptides from biological liquid waste.

Keywords: Antimicrobial peptides, human urine, protein purification, urinary cationic peptides.

INTRODUCTION

The female external urethral orifice is located relatively near to the anus. This situation facilitates the exposure of the urinary tract to potentially pathogenic microorganisms. However, despite this circumstance urine is normally sterile (Mulvey MA *et al*., 2000). A varied array of host defenses, such the flow of urine and multiple antimicrobial factors, help to maintain this sterility, strategically necessary for long-term survival. Epithelia lining of the urinary tract prevent adhesion of bacteria by the release of Tamm-Horsfall proteins, lactoferrin and lipocalin (Michael Z., 2007). Bactericidal proteins secreted by uroepithelia and the kidney or from the blood, are also relevant factors. The antimicrobial peptides found in the urine include α- and β-defensins and cathelicidin. Some multi-functional peptides can also exhibit antimicrobial activity. For example, hepcidin, a cysteine-rich antimicrobial peptide originated from the liver and found in urine, which regulates extra cellular iron concentration (Pigeon C *et al*., 2001).

Urine is approximately 95% water, with the remaining fraction being a wide range of metabolic wastes such as urea, dissolved salts, proteins and hormones, among other substances. The classic method to extract antimicrobial proteins from urine is using action exchange resin and further purification by RP-HPLC. Every peak is analyzed by acid urea polyacrylamide gel electrophoresis, and the fractions corresponding to the relevant molecular weight are tested for antimicrobial activity (Anderson RC *et al*., 2008; Christina HP *et al*., 2001; Erika VV., 1998). In this paper a simpler purification method was developed. This process combines membrane ultra filtration (Pellicon tangential-flow system) and weak cation exchange chromatography using CM Sepharose Fast Flow. The highest antimicrobial activity was presented by a complex of low-molecular cationic peptides purified from urine and are, therefore, suitable candidates for the development of novel reagents or therapeutic drugs.

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MATERIALS AND METHODS

Purification of urinary proteins from urine

The new purification process involves membrane ultrafiltration and weak cation exchange chromatography. This method reduces the required level of technical expertise for reproducibility of purification.

Procedure 1 Whole urinary proteins isolated from urine

Ten L of male urine were collected in a public toilet in one day. Microorganisms and other insoluble particulates were eliminated with a membrane (0.22μm, Millipore). Next, the filtrated urine was passed through a M_r 1000 cut-off membrane (Pellicon 0.5m^2, Millipore). The obtained retentate, which contains whole urinary proteins (WUP), was concentrated ~100-fold via constant volume diafiltration. Afterwards, to complete buffer exchange and pigment elimination, the concentrate was diafiltered against 10mM sodium phosphate buffer pH 7.5 (buffer A).

Procedure 2 Cut off by 50 kDa ultrafiltration

The M_r 1000 cut-off membrane retentate was then sent through a M_r 50,000 cut-off membrane (Pellicon 0.1m^2, Millipore). The obtained permeate (low molecular weight urinary proteins, LUP) is kept at constant volume by adding buffer A. The retentate contains high molecular weight urinary proteins (HUP). Every fraction (WUP, LUP and HUP) was then separated by 15% SDS-PAGE, and its antimicrobial activity verified against *E. coli* BL21 (DE3).

Procedure 3 Isolation of cationic peptides

The LUP fraction was directly loaded onto a CM Sepharose Fast Flow column at a flow rate of 2ml/min using a peristaltic pump set in a bed volume ~ 50ml (2.5 cm (ID) x 10cm (L)) and equilibrated with buffer A. Afterwards, it was washed with 500ml buffer A. To ensure an effective removal of unbound proteins, the A280 of the fraction was observed. The removal was considered completed when the absorbance almost

reached the baseline value. The absorbed cationic substances (urinary cationic peptides, UCP) was eluted with 5% acetic acid and then dialyzed against buffer A.

Protein assays

Protein determination and analysis

Each fraction was determined by BCA protein assay kit (Pierce Chemical) and quantified as BSA equivalents, based on a standard curve of 0, 0.5, 1, 2.5, 5, 10, 20, 40, 200 µg/ml of BSA. The purity and distribution of high molecular weight proteins were established by 15% SDS-PAGE and stained with Coomassie Blue. To verify the distribution of low molecular weight urinary proteins, the size-exclusion chromatography was used. In this study it was used an analytical chromatographic column Tosoh TSK G2000SWxl, pore size 125 Å, 7.8mm (ID) x 30cm (L). The mobile phase was isocratic and consisted of 25 mM sodium phosphate buffer 300mM NaCl pH 7.0, at a flow rate of 0.5 ml/min. The amino acid composition and the isoelectric point of the purified UPC were examined.

Biochemistrical characterization of urinary cationic peptides

Heat stability of UCP was tested during 30min at 100ºC, at acidic, neutral and basic pH. The protease susceptibility of UCP was tested. UCP were incubated with sufficient trypsin, proteinase K or pepsin under the optimal conditions. The antimicrobial test was prepared after proteinase heat inactivation. To emulate the characteristic of body fluids, NaCl in different concentrations was added. In some experiments, after the intramolecular disulfide bonds of UCP was reduced with DTT, substance mixed with assay mixture.

Antimicrobial assays

The colony forming units (CFU) assay was executed as described elsewhere (Porter EM *et al*., 1997). Briefly, static cultures were diluted with buffer A to give a microbe concentration of approximately 10^6 CFU/ml. 450µl bacterial suspension was distributed into each well of a sterile 24-well culture plate (Fisher). 50µl of the prepared sample was added to the bacterial solutions to obtain a significant terminal concentration in triplicates to evaluate their antibacterial activity. Controls in triplicates with 50µl of buffer A. After a 1 h incubation period at 37ºC, the reaction was ended by 1:100 dilution in ice-cold buffer A with 200mM NaCl. Then, it was sequentially diluted and plated on LB agar. The limit of detection was 10^3 CFU/ml. The counts of remaining live bacteria were calculated according to the number of colonies on the plates after overnight incubation at 37ºC.

RESULTS

Urinary proteins capture and antimicrobial activity

The use of a Millipore Pellicon device to prepare the sample appears to be a key part of the purification process. The first step of the procedure was a concentration by a M_r 1000 cut-off membrane ultra filtration. Then, the retentate was sent to a Millipore Pellicon equipped with a 50,000 M_r pore size membrane. The permeate fractions with low molecular weight were combined. Both, preliminary purification and concentration are done with the Millipore Pellicon. The molecular weight distribution of each fraction was shown in fig. 1A.

We tested the antimicrobial activity of the whole urinary proteins (WUP) enriched with a M_r 1000 cut-off membrane ultrafiltration (lane 2), the retentate HUP (lane 3) and the permeate LUP (lane 4) of a M_r 50,000 cut-off membrane against *E. coli* BL21(DE3) by the previously explained CFU assay. LUP fraction presented antibacterial activity, but HUP supported *E. coli* growth during 1 h of incubation at 37ºC (fig. 1B).

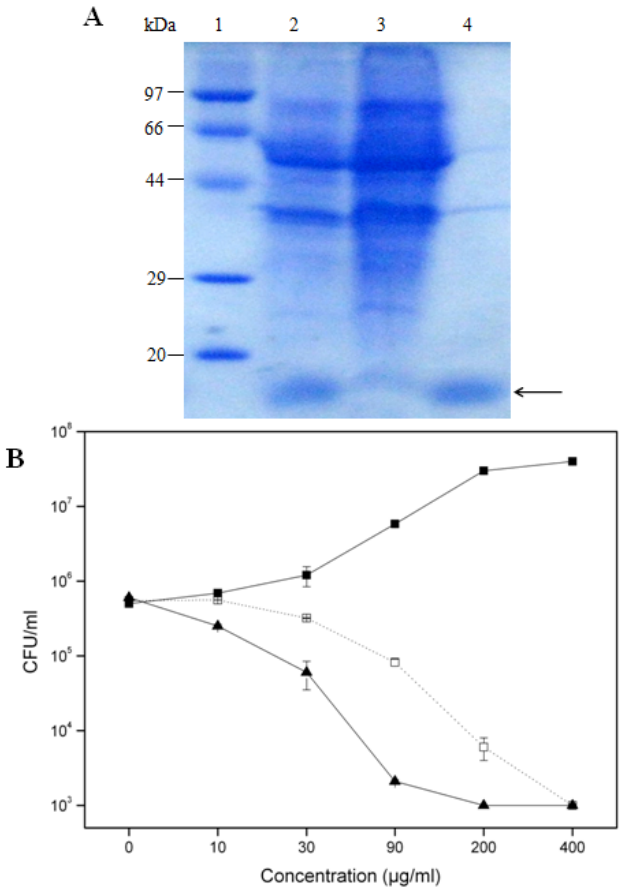

Fig. 1: Purification and characterization of human urinary proteins. (A) 15% SDS-PAGE analysis of purified urinary proteins with Coomassie Blue staining. Low molecular markers were run in lane 1 and their molecular mass values (in kDa) were indicated. Whole urinary proteins were enriched through ultrafiltration with a M_r 1000 cut-off membrane (lane 2). The retentate (lane3) and permeate (lane4) were obtained from a second ultra filtration with a M_r 50,000 cut-off membrane. Most of the permeate fractions had molecular weights lower than 20 kDa (arrow, lane4). (B) CFU assays of WUP, HUP and LUP

activity against *E. coli* BL21 (DE3). Each square contains plots of the mean ± standard error of triplicate CFU determinations on the vertical axis, at various concentrations of WUP, HUP and LUP shown on the horizontal axis. □,WUP; ■, HUP; ▲, LUP.

Isolation and analysis of urinary cationic peptides

Long-term activity can be maintained by isolation of cationic peptides using CM Sepharose Fast Flow, and then lyophilization from acetic acid solution. We verified the molecular size of the permeate (LUP) of 50,000 M_r cut-off membrane and the CM Sepharose Fast Flow elution fraction (UCP). Different molecular types were obtained from the 50,000 M_r cut-off membrane permeate in the gel filtration chromatography (fig. 2A). However, only one molecular species (fig. 2B) was obtained from the CM Sepharose Fast Flow elution. The elution time was comparable to the polystyrene standard (Mw 4000) (data not shown). The molecular weight distribution was in accord with typical antimicrobial peptides.

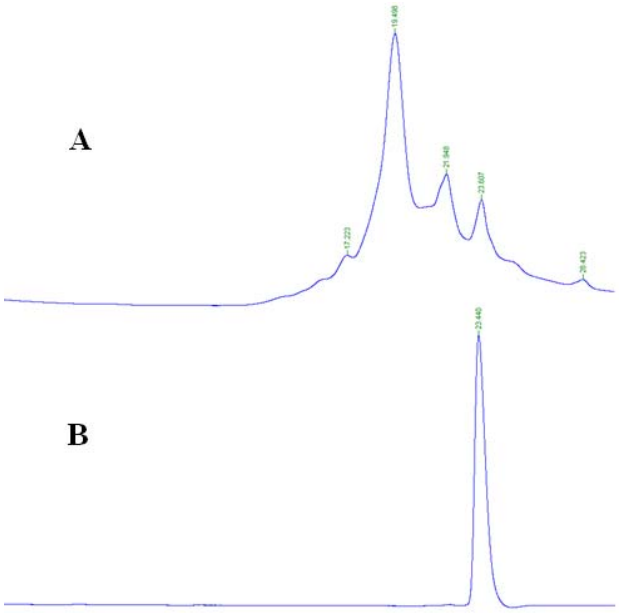

Fig. 2: Molecular size analysis by TSK G2000SWxl. The permeate of ultra filtration with the 50,000 M_r cut-off membrane gave several molecular species (A). CM Sepharose Fast Flow elution just gave a single peak (B).

In order to determine the relative amino acid composition of UCP, an amino acid analysis was used. The contents of aliphatic/aromatic, basic and acidic amino acids are 52%, 27% and 8%, respectively. Is notable the relatively high content (13%) of cysteine. This suggests that the cysteine linkage might be common in urinary antimicrobial peptides.

The pI (isoelectric point) of UCP was determined by isoelectric focusing with IEF pI markers (SERVA). The silver stain IEF gel image indicated that UCP were mainly composed of basic peptides, as the pI values were mainly shifted above pH 8.0 (data not shown).

Biochemical characterization of urinary cationic peptides

The antibacterial spectrum of UCP was screened. 10 and 30μg/ml UCP were tested for antimicrobial activity against gram negative bacteria (*E. coli* BL21(DE3), *Pseudomonas aeruginosa*), gram positive bacteria (*Staphylococcus aureus*), and yeast (*Candida albicans*). All three kinds exhibited sensitivity to UCP, and a dose-response effect was observed (fig. 3). *E. coli* BL21 (DE3) presented the highest sensitivity, and was therefore selected for further experimentation .

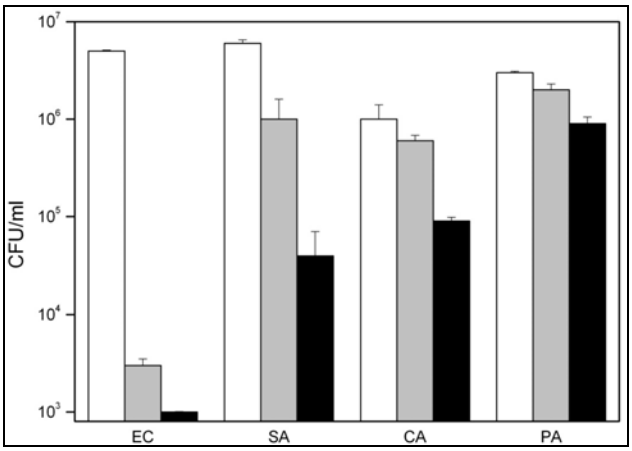

Fig. 3: Antibacterial spectrum of UCP. Various microbial strains were subjected to CFU assays using UCP at 10 and 30 μg/ml in buffer A after incubation for 1h. The organisms tested were *E. coli* BL21 (DE3) (EC), *Staphylococcus aureus* (SA), *Candida albicans* (CA), and *Pseudomonas aeruginosa* (PA). The count (CFU/ml) is indicated by the bars: *blank bar*, control (buffer A only); *gray bar* (UCP 10μg/ml); *black bar*, (UCP 30μg/ml).

The effect of pH on the heat stability of UCP was examined (fig. 4A). Basic pH markedly reduced the heat stability of UCP. The antibacterial capability of UCP was almost completely eliminated by heat inactivation at 100ºC for 30min at pH 10. In contrast, an acidic pH increased heat stability to the maximum. UCP reduced with DTT significantly decreased its effectiveness against *E. coli*, indicating that the disulfide bonds are necessary for its antibacterial capability.

UCP rich in basic amino acids were susceptible to trypsin, and the peptide bonds between basic amino acids were essential to keep antibacterial activity (fig. 4B). Notably, UCP were insensitive to proteinase K, especially to pepsin. Additional tests indicated that UCP could inhibit pepsin activity effectively *in vitro* (data not shown). To our knowledge, no pepsin inhibitor was found in the urine. With the identification of UCP, we will find the exact inhibitor in the complex cationic peptide mixtures.

The UCP activity was suppressed by raising the media conductance. At 24mS (fig. 4C) the activity level was almost negligible.

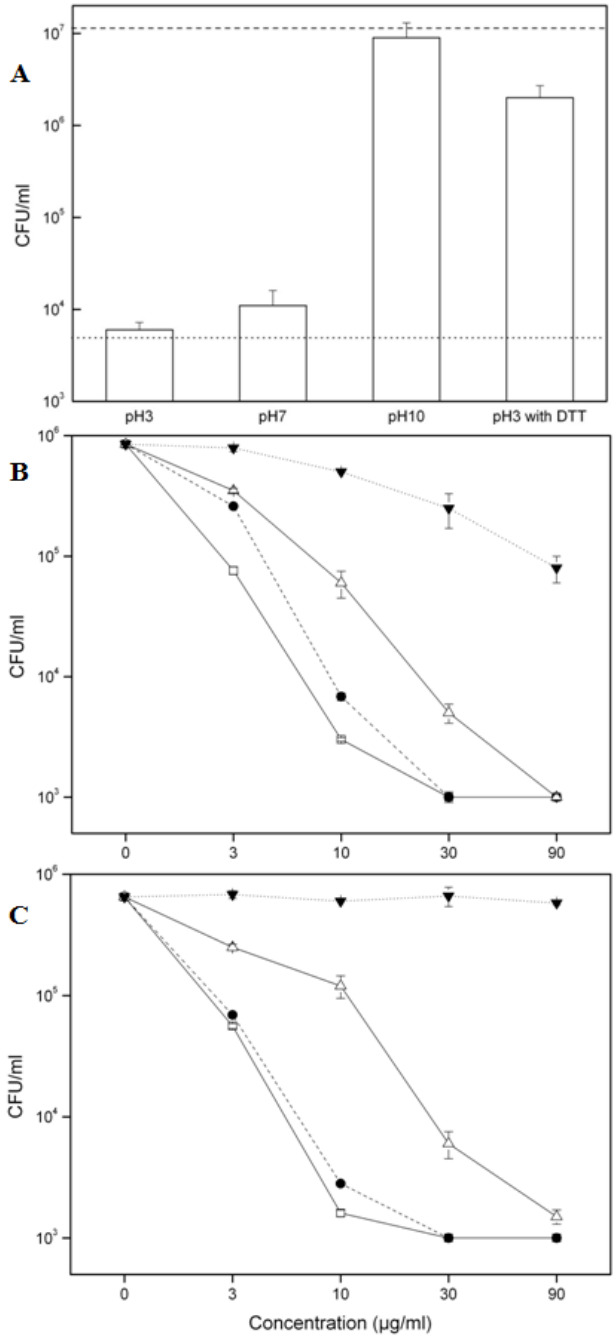

Fig. 4: Biochemical characterization of UCP. (A) The effect of pH, heat and reducing agent on the activity of UCP. UCP were treated at 100ºC for 30min under various conditions. *E. coli* was incubated with 10µg/ml of UCP at 37ºC for 1h and then the CFU assays were carried out as described above. The positive control (UCP without any treatment) is indicated by the dotted line and the negative control (microbial input) by the dashed line. (B) Protease susceptibility of UCP. UCP were incubated with trypsin, proteinase K and pepsin under the optimal conditions and then the CFU assays at various concentrations of UCP were performed. ϒ, control (without any treatment); ●, pepsin; △, proteinase K; ▼, trypsin. (C) The effect of solution conductance on antimicrobial activity. Assay conditions: □, control (Buffer A); ●, Buffer A and conductance 6 mS; △, Buffer A and 12 mS; ▼, Buffer A and 24 mS.

DISCUSSION

One proposed solution involved the use of macro porous adsorbing resin to capture trace urinary proteins from a volume of urine. For instance, Amberlite XAD-7 (Rohm & Haas), followed by diafiltration to eliminate pigments after elution from resin, proved to effectively capture even LUP. Absorbing resin is suitable for large scale production. In addition, we screened several molecular weight cut-off ultra filtration cassettes, 10k, 30k, 50k and 100kDa. The 50 k cut-off membrane provided the highest purity and the maximum yield of LUP.

The body of most living creatures has an innate host defense, evolved to combat microbial infections and other exogenous and endogenous elements that could threaten the host health, and thus its survival chances. The widely distributed and abundant cationic antimicrobial peptides are the main parts of the host innate defense.

Cationic peptides, abundant elements in plants and animals, possess a significant degree of antimicrobial capabilities. Proteinase inhibitors, such as trypsin and chymotrypsin can be found in some plants, and their presence has been linked to higher plant resistance to infections (Kim JY *et al*., 2009). In mammals an analogous connection could exist. For instance, a trypsin inhibitor such as the glycoprotein ulinastatin can be obtained from human urine. Several forms of pancreatitis and toxic shock, among other medical conditions, can be treated by this inhibitor. The experiments we have conducted indicate that small amounts of ulinastatin can hinder the growth of bacteria although *in vitro,* even elevated quantities of ulinastatin have shown not to be an effective bactericide (data not shown).

Some authors have previously considered the use of proteinase inhibitors as bacteriostatic agents in plants via an antifeedant effect (Vernekar JV *et al*., 2001). According to our findings, some urinary cationic peptides fractions could inhibit pepsin *in vitro*. This could indicate that the antimicrobial activity of urinary peptides could be caused by another different mechanism, besides its well known ability to break the integrity of bacterial cell membrane. Further research is needed before a definite conclusion can be drawn.

Some areas of eastern China are among the most densely populated in the world. In those regions there are an elevated number of public toilets, allowing for large-scale urine collection. Many prescription drugs (e.g. urokinase, ulinastatin, kallikrein) that are derived from urine have been produced on a commercial scale in China for a long time (Deng LJ *et al*., 2006). After whole urinary proteins are captured, low molecular weight urinary proteins are usually removed as impurities during the first stages of the treatment process. This paper seeks to explore a new approach to a more efficient utilization of the urine source, since the naturally occurring human peptides are excellent candidates for the development of novel antibiotic agents or immunomodulators.

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