

Chapter 2

Purification of Antimicrobial Peptides from Human Skin

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Abstract

Human skin is a rich source of human antimicrobial peptides. Its cellular source is the keratinocyte, which terminally differentiates in the uppermost parts of the skin, eventually forming the stratum corneum, the horny layer. The easy availability of human stratum corneum makes it possible to identify and characterize human antimicrobial peptides with a biochemical approach. Moreover, the availability of lesional scales of patients with psoriasis, an inflammatory skin disease, allows the identification of human-inducible peptide antibiotics, which are absent in healthy skin. With this strategy, the beta-defensins hBD-2 and hBD-3, RNase-7 as well as psoriasin/S100A7 have been discovered as human antimicrobial peptides and proteins. A detailed description of the strategies and methods is presented, which allowed a successful identification and characterization of human antimicrobial peptides. We used various HPLC techniques, combined with antimicrobial testing as read-out system. In parallel, SDS-PAGE analyses as well as electrospray ionization mass spectrometry were used for further biochemical characterization as well as purity assessment.

Key words: Antimicrobial peptides, defensin, RNase-7, S100A7, skin, stratum corneum, purification, electrospray ionization mass spectrometry, high-performance liquid chromatography.

1. Introduction

Human skin is always in contact with the environment, but despite the presence of many favourable conditions for microbe growth, it is rarely infected. This unexpected observation led to the hypothesis that the uppermost skin layer contains a “chemical defence shield” consisting in antimicrobial peptides. Recent evidence indicates that antimicrobial peptides are synthesized by fully differentiated keratinocytes in the surface-exposed epidermis, a self-renewing stratified squamous epithelium composed of several layers of keratinocytes. Keratinocytes in the outmost

living cells containing layer (stratum granulosum) terminally differentiate and eventually form a physical barrier consisting of the horny layer (stratum corneum, SC). SC cells are sloughed off and replaced by newly differentiated cells originating from epidermal stem cells located in the basal layer. Thus, stratum corneum should be a rich source of human antimicrobial peptides (AMPs) and should allow its detection and characterization in an unbiased approach.

Purification of AMPs from skin requires special strategies. First of all, the detection system should be useful for screening to detect the antimicrobial peptide at low concentration. Several assay systems can be used for screening (1), e.g. radial diffusion assay (RDA) or microbroth dilution assay. The RDA assay is a highly sensitive assay which consumes only minimal amounts of peptides for testing (1). Unfortunately the microbroth dilution assay requires approx. 10 times more peptide than the RDA assay (1). Thus, due to the limited availability of human material, the RDA assay might be more useful for successful AMP purification from skin samples.

1.1. Extraction of AMPs from Stratum Corneum

SC is a rich source of various polar and non-polar lipids which make it less efficient to extract peptides and proteins. It might be thus useful to wash the material with a non-polar organic solvent such as ethyl acetate prior to peptide extraction. To achieve an optimal peptide extraction from SC, acidic buffers need to be used. The buffer system should be carefully chosen according to the strategy used for AMP purification: When reversed phase (RP)-HPLC is used as first and sole separation step (when amounts of only a few milligrams of SC are available), SC extraction with acetonitrile containing acidic buffers is recommended. When amounts > 500 mg SC are available, ethanolic citric acid buffers have been proven to be useful for extraction.

1.2. Chromatographic Separation

The SC amounts available will determine the purification strategy. Extracts from a few milligrams of SC will only allow the (partial) purification of the principle AMPs by RP-HPLC. This should be done with a micro-HPLC system. When amounts > 500 mg SC are available, a different, more complex purification protocol, which also includes cation exchange-HPLC, will allow the purification of the principal AMPs as well as most of the less abundant AMPs. Identification of less abundant AMPs requires an enrichment step by pooling the material from different HPLC runs.

For enrichment of cationic and amphipatic antimicrobial peptides (which represent by far the majority of all AMPs in SC), heparin-affinity chromatography is useful. The heparin-bound material is then separated by preparative RP-HPLC followed by micro-cation exchange-HPLC and finally micro-RP-HPLC.

1.3. Bioassays

Antimicrobial activity in HPLC fractions can be tested with various designer assays (1). HPLC solvents are usually not compatible with the AMP bioassays. In the case of volatile solvents (RP-HPLC steps), lyophilization of the samples will be sufficient to get rid of the problem. In the case of cation exchange-HPLC, salts are required for protein elution. Because several AMPs are salt sensitive, there will be a principal risk to get false-negative results.

In some cases it is possible to overcome this problem by using unconventional, pragmatic conditions for cation exchange-HPLC using volatile buffers such as ammonia salts at an acidic pH.

It is further important to choose the right bacterial target. Many of the AMPs have a restricted activity profile. It is therefore important to use the bacterial target of interest.

1.4. Biochemical Analyses

HPLC fractions should be analysed also by analytical biochemical techniques. Due to the highly cationic behaviour, conventional sodium dodecyl sulphate polyacryl amide electrophoresis (SDS-PAGE) analyses often did not work with AMPs. Instead, acidic acryl amide electrophoresis was initially used for checking purity of the AMPs (2). The Tricine-SDS-PAGE system (3) has been now successfully used for determination of the AMP's size (4–6). It is important to choose electrophoresis conditions which allow sufficient loading of SDS to the highly cationic AMPs and minimize formation of dimers and oligomers. Tricine buffers containing urea were found to be optimal.

Due to its high sensitivity, silver staining is preferred for detection of AMPs in the gel, because only nanogram amounts of AMPs are required.

RP-HPLC fractions can also be monitored by mass spectrometry. Electrospray ionization mass spectrometry (ESI-MS) is useful to directly determine the exact mass of AMPs in RP-HPLC fractions. Characteristic charge state patterns of AMPs upon ESI-MS analyses reveal further structural information about the AMPs isolated from SC. Because ESI-MS is highly sensitive towards the presence of salt, analyses of ion exchange-HPLC fractions requires elimination of anorganic salts, either by the use of volatile buffers or RP cartridges for each separate fraction.

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2. Materials

2.1. Extraction of AMPs from SC

1. Suspend stratum corneum or lesional psoriatic scales (e.g. 2.5 g in 100 mL of 0.1 M aqueous citric acid containing 50% (v/v) of 96% ethanol [denaturated with heptane]) (*see Note 1*).
2. Homogenizer (Ultraturrax[®]): use 2,000 rpm for 60 min and chilling in an ice–water mixture.
3. Ultrafiltration: YM3 filters, cutoff of 3 kDa (Millipore, Danvers, MA), are useful (*see Note 2*).

2.2. Chromatographic Separation of Antimicrobial Peptides

Use HPLC-quality solvents. All chromatographic steps are performed at room temperature (*see Note 3*).

1. Any HPLC or FPLC machine containing a pump, gradient mixer, UV detector, HPLC columns, and fraction collector can be used. UV detection should be done at either 215, 280 nm, or both. Use solvents that do not show absorbance at 215 nm (e.g. acetonitrile, water, and trifluoroacetic acid [TFA]) (*see Note 4*). For micro-HPLC we use a Smart[®]HPLC system (GE Healthcare Biosciences AB, Uppsala, Sweden) or an ETTAN LC[®]Purifier system (GE Healthcare).
2. For HPLC separation the following columns have been used:
 - a. Heparin Sepharose cartridge (Hi Trap, 10 × 5 mm, 1 mL volume, Amersham) (*see Note 5*).
 - b. Preparative wide-pore (300 Å) reverse phase (RP-8)-HPLC column (C8 Nucleosil with endcapping, 250 × 12.6 mm, 7 mm particle size, Macherey-Nagel, Düren, Germany) (*see Note 6*).
 - c. Micro-MonoS[®]-HPLC column for Smart[®]HPLC system (GE Healthcare) (*see Note 7*).
 - d. Micro-RP-18 (C2/C18) column for Smart[®]HPLC system (GE Healthcare) (*see Note 8*).

2.3. SDS-PAGE Analysis

1. The method of Schagger and von Jagow (3) is used. As sample buffer 50 mM Tris–HCl, 4% (w/v) SDS, 12% (w/v) glycerol, pH 6.8, containing 8 M urea is used (*see Note 9*).
2. Gels with the dimension 130 × 100 × 1 mm are used and electrophoresis is done in the presence of 8 M urea for 2 h at 10 mA current, 30 V power (power limit 10 W) at room temperature (*see Note 10*).

3. Fixation of antimicrobial peptides is done for 30 min with aqueous 2-propanol (30% [v/v]) containing 10% (v/v) acetic acid and 0.3% (v/v) glutaraldehyde (*see Note 11*).
4. Proteins are stained with 0.03% (w/v) silver nitrate in deionized water followed by developing with a solution of 10% saturated aqueous Na₂CO₃ solution containing 0.1% (v/v) of saturated aqueous formaldehyde (40% [v/v]). Development is terminated by acetic acid (3% [w/v] in water) (*see Note 12*).

2.4. Electrospray Ionization Mass Spectrometry (ESI-MS)

1. We used a Q-ToFTM II mass spectrometer (Waters Micromass, Milford, MA).
2. As ESI gas nitrogen was used.
3. Nanoelectrospray ionization was used when sample amounts were limited or for MS/MS peptide sequencing experiments and were carried out after fitting the Q-ToFTM II mass spectrometer with a nano-Z spray source (*see Note 13*).
4. For nanoelectrospray MS analysis nanoflow-electrospray needles (Borosilicate metal-coated glass capillary, Waters Micromass, Milford, MA) were used. Salt containing samples are desalted using self-prepared μ -columns using constricted GelLoader tips (Eppendorf, Wesseling-Berzdorf, Germany) packed with a 2:1 mixture of Poros[®] 50R2 and Oligo R3 reversed phase medium (Applied Biosystems, Foster City, CA).
5. Acquisition and data analysis are performed using the MassLynx 4 software package supplied by Waters Micromass, Milford, MA.

2.5. Antimicrobial Activity Assay

The radial diffusion assay was used according to Steinberg and Lehrer (1) (*see Note 14*).

1. Trypticase soy broth (TSB, Difco, Detroit, MI): Full strength broth (30 g per litre deionized water), autoclaved, and stored at room temperature.
2. Sterile phosphate buffer, pH 7.4 or 6.5 (or as desired).
3. Agarose (Sigma): The use of a low electroendosmosis (EEO)-type agarose in place of standard agar is *critical* to limit electrostatic interactions between cationic AMPs and sulphated moieties of the agaropectin component of standard agar (1).
4. Underlay gels (1): Mix 50 mL of 100 mM sodium phosphate buffer with 5 mL full-strength trypticase soy broth, add 5 g agarose, and bring the volume to 500 mL with deionized distilled water. Adjust the final pH with NaOH or HCl, place the suspension on a hot plate, and stir it under heat until the

agarose dissolves. Dispense 50-mL aliquots, autoclave them at 121°C for 20 min, and store the sterilized media at room temperature. Before use, the solidified medium should be fluidized (we use a microwave oven) and placed it into a water bath maintained at 42°C.

5. Overlay agar (1): This agar contains 60 g (twice the customary amount) of trypticase soy broth (TSB, Difco) and 10 g agarose (Sigma) per litre of deionized water and is similarly treated as the underlay gels.
6. Microorganisms: *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* were used for routine AMP testing after determination of optical density (OD)/colony forming unit (CFU) ratio (*see Note 15*).
7. Peptides are introduced into 3 mm sample wells within the underlay gel.

3. Methods

3.1. Extraction of AMPs from Stratum Corneum

1. Wash 1 g heel stratum corneum (SC) with 50 mL ethylacetate and dry it under vacuum (*see Note 16*).
2. Suspend SC in acidic buffer, homogenize, and then add ethanol (*see Note 17*).
3. Centrifuge and use supernatant (*see Note 18*).
4. Concentrate the supernatant to ~ 5–10 mL, adjust to pH 8.0, then again to pH 4–4.5, centrifuge it again, and freeze it (at –20°C) until further use (*see Note 19*).
5. For further purification, thaw the sample, adjust the pH to pH 8.0, centrifuge it, and apply it to a heparin affinity column (*see Note 20*).
6. Wash the column with 3 volumes of equilibration buffer: 10 mM Tris–citrate, pH 8.0.
7. Strip the bound material from the column by the use of 3 mL 2 M NaCl in equilibration buffer (*see Note 21*).
8. Add trifluoroacetic acid (TFA) and adjust to pH 3.

3.2. Preparative RP-8-HPLC

1. Apply the heparin-bound material (from **Section 3.1**, Step 8.) to a preparative RP-8-HPLC column by the use of a 2–5 mL loop (*see Note 22*).
2. Separate proteins by elution with a gradient of increasing concentrations of acetonitrile. Turn the detector on 215 nm and – when possible also 280 nm – and choose the integrator

attenuation appropriate to protein amounts you expect (*see* **Note 23**).

3. Separate peaks – when possible – manually according to the appearance of UV-absorbing peaks and shoulders (*see* **Note 24**).
4. Place fractions immediately in the refrigerator.
5. Take off an aliquot of each fraction for the bioassay, SDS-PAGE analysis, or ESI-MS analyses using a microtitre plate (*see* **Note 25**).

3.3. Micro-MonoS[®] Cation Exchange HPLC

1. Combine fractions of RP-8-HPLC, which contain the same AMP and lyophilize them.
2. Dissolve the residue in 50–200 μL 20 mM ammonium formate buffer, pH 4.0, which contains 25% (v/v) acetonitrile.
3. Apply the sample to a micro-MonoS[®]-HPLC column, which has been equilibrated with the same acetonitrile-containing ammonium formate buffer, pH 4.0 (*see* **Note 26**).
4. Elute with a gradient (0–20 min) of increasing concentrations of NaCl (0–1 M) (*see* **Note 27**).
5. Take off an aliquot of each fraction for the bioassay, SDS-PAGE analysis, or ESI-MS analyses using a microtitre plate (*see* **Note 25**).

3.4. Micropore C2/C18-RP-HPLC

1. Combine fractions containing the same AMP and apply them directly onto a micropore C2/C18-RP-HPLC column (*see* **Note 28**).
2. Elute proteins with increasing concentrations of acetonitrile. Turn the UV detector on 215 nm (and 280 nm, if possible) and separate peaks manually.
3. Take off an aliquot of each fraction for the bioassay, SDS-PAGE analysis, or ESI-MS analyses using a microtitre plate (*see* **Note 25**).

3.5. Radial Diffusion Antimicrobial Activity Assay

1. Prepare organisms for the assay as well as the gel as outlined in **Section 2.5**.
2. Add 30 μL aliquots of preparative RP-8-HPLC fractions or 2 μL aliquots of micro-HPLC fractions to the wells of a round-bottom well microtitre plate. Lyophilize samples and add to each well 8 μL 0.01% (v/v) aqueous acetic acid and transfer 5 μL aliquots of the various samples into each well in the underlay gel.
3. Incubate the gel for 3 h in a 37°C incubator, then add the overlay, and incubate at 37°C overnight.

4. Calculate the results by measuring the diameter of the clearing zone (*see* **Note 29**).

3.6. SDS-PAGE Analyses

1. Mix fractions (30 μL of RP8-HPLC fractions, 2 μL of MonoS[®]-HPLC, and C2/C18-micro-HPLC fractions) with 10 μL sample buffer and boil for 10 min (*see* **Note 30**). Then load sample on the stacking gel and separate electrophoretically in the presence of 8 M urea (30 min at 100 V, 400 mA; then 90 min at 120 V, 400 mA) (*see* **Note 31**).
2. Fix peptides in the gel with fixation solution.
3. Stain proteins with silver nitrate solution (*see* **Note 32**).

3.7. Electrospray Ionization Mass Spectrometry (ESI-MS) Analyses

1. For routine MS analysis, dilute aliquots (2–10 μL) of HPLC fractions containing the sample with 100 μL carrier solvent (50:50 acetonitrile:water containing 0.2% (v/v) formic acid) and infuse it into the electrospray source at a rate of 10–20 $\mu\text{L}/\text{min}$ (*see* **Note 33**). Sodium iodide can be used for mass calibration for a calibration range of m/z 100–2,000. The capillary potential is set to 3.5 or 4 kV and cone voltages between 25 and 75 V are chosen; cone temperature is set to 80°C; desolvation temperature is 150°C. The charge-to-mass ratio of ions is scanned within the range of 280–2,000.
2. For nanoelectrospray MS analysis a 1–3 μL aliquot of salt-free sample (*see* **Note 34**) is loaded into the nanoflow-electrospray needle. Salt containing samples are desalted using self-prepared μ -columns. Formic acid is added to the samples just before desalting to give a final concentration of 5% (v/v). After loading the column with the sample the column should be washed two times with 10 μL 5% formic acid. Peptides are eluted with 1–3 μL 60% methanol/5% formic acid directly into the nanoelectrospray needle and measured by nanoelectrospray ionization MS using a capillary voltage between 1 and 1.4 kV.
3. For mass mapping (mass fingerprint) when necessary, cysteine residues are reduced (by incubation of HPLC fractions containing 10–20 μg protein in 85 μL 100 mM NH_4HCO_3 , 10 mM dithiothreitol (DTT) for 20 min at 60°C) and subsequently alkylated (by incubation in 200 mM iodoacetamide for 20 min at RT) in the dark. Prior to digestion samples are desalted using 10 μL C₁₈ Poros[®] pipette tips or HPLC with C₂/C₁₈ columns.
HPLC sample fractions are either directly or after reduction and alkylation of cysteine residues subjected to trypsin digestion: lyophilized samples containing 2–4 μg protein are dissolved in 20 μL 100 mM NH_4HCO_3 , pH 8; tryptic digestion is started by adding 50 ng (with 1% acetic acid)

activated trypsin (modified trypsin, sequencing grade, Roche; 1:50 final molar enzyme:substrate ratio) and is allowed to proceed for at least 4 h at 37°C. Digests are either analysed directly or stored frozen until subsequent mass mapping: tryptic fragment masses are determined after desalting using self-prepared μ -columns (**Section 2.5**, Step 4) using ESI-MS.

4. Peptide sequencing by MS/MS is done by analyses of tryptic peptide fragments using a Q-TOF 2 mass spectrometer (Waters Micromass, Milford, MA) with nanoelectrospray ionization (*see* **Note 35**). Cone voltage and collision energy used to perform MS-MS are optimized for each peptide analysed. Collision energy varied between 17 and 40 eV with argon (14 psi) as the collision gas. Data for MS/MS are acquired over the mass range m/z 80–2,000.
5. Mass spectra are averaged typically over 2–10 scans (20–2 s/scan). The multiply charged raw data of intact proteins are background-subtracted and deconvoluted using MaxEnt1 to obtain singly charged ion mass spectra to determine average molecular masses of intact proteins.

The raw combined spectral data from small peptides or tryptic protein fragments or obtained after MS/MS fragmentation of selected precursor ions are background subtracted and subjected to Maximum Entropy 3 (“MaxEnt3”) deconvolution to determine monoisotopic molecular masses. Sample identity is determined by database search analysis of peptide fragment mass patterns and/or after de novo sequencing of tryptic peptide fragments. All mass fingerprint and MS/MS data are searched against the human protein database using the Mascot program (Matrix Science, Boston, MA). Peptide sequences are directly (“de novo”) determined from MS/MS data using the software program PepSeq from the MassLynx4 software package (Waters Micromass, Milford, MA). PepSeq-derived peptide sequences are analysed with the NCBI-BLAST protein database search program.

4. Notes

1. Heptane-denaturated ethanol is cheaper than non-denaturated ethanol. Highly volatile heptane does not interfere with any purification step. Instead of ethanol, acetonitrile can also be used. In the absence of organic solvents we were confronted with severe problems with the extracts, which gave turbid solutions after centrifugation

with a high content of fines (lipid drops mixed with solid particles). Ignoring this phenomenon has usually resulted in HPLC problems, such as high-pressure error and giving “memory compounds” on the HPLC column (7).

Protease inhibitor cocktails have not been added to the stratum corneum prior to extraction. We never observed proteolytic digests of the AMPs which we had isolated so far from stratum corneum. This includes hBD-2, hBD-3, RNase-7 (4–6), and psoriasin (8). In particular RNase-7 and psoriasin are extremely sensitive towards proteolysis. We therefore speculate that in skin endogenous protease inhibitors prevent proteolysis of AMPs.

2. The recovery of AMPs using Amicon (Danvers, MA) filters was found to be highest when acidic solutions containing a small percentage (20–30%) of water-soluble organic solvents were used. Use the right diameter of the filter to avoid losses of AMPs due to an un-appropriate ratio of filter diameter and (final) sample volume. Small extract volumes (100 μ L–1 mL) should be better either lyophilized or concentrated by vacuum evaporation or directly applied to the RP-HPLC column for separation. Losses of material seen in some cases usually come from sticking to the surfaces rather than degradation. Once cationic AMPs stuck to surfaces (glass, siliconized glass, plastic), we have been unable to reverse the process by washing with organic solvents. Therefore, our strategy to avoid sticking is to add organic solvents whenever possible at acidic pH to AMP-containing solutions.
3. It is our experience that chromatography at low temperature is not necessary for AMP purification. Skin-derived AMPs are remarkably stable at ambient temperature.
4. UV detection at 215 nm allows quantification of protein content and thus estimation of the AMP amounts in purified peaks. Although we used ubiquitin for calibration, the absolute amounts of known AMPs can be estimated from its calculated extinction coefficient.
5. For extracts from >10 g stratum corneum a 5 mL heparin-spharose cartridge is recommended.
6. A wide variety of reverse-phase columns obtained from different manufacturers can be used. Take care to use wide pore (300 Å) columns with endcapping. Most of the AMPs are very cationic. When there is no endcapping (which blocks the free silanol groups of the silica bead material), the highly cationic AMPs will show broadened peaks and often dramatic losses of recovery are observed. Choose RP-HPLC columns which have been optimized for the separation of basic compounds.

7. Do not use oversized cation exchange columns! For extracts obtained from >1 g stratum corneum we use the micro-MonoS[®]-HPLC column. For smaller amounts we are using a MiniS[®]-HPLC column.
8. C2/C18- μ HPLC columns give excellent resolution for several AMPs. When only little stratum corneum material is available and a single HPLC analysis is possible, a (micro)column with this material is recommended. In our hands classical wide pore C8-RP columns do not always allow complete separation of lysozyme from hBD-2. The use of a μ C2/C18 column leads to a retention time difference of about 10 min!
9. Addition of urea is essential for several AMPs to get bands at the expected size in the gel. For example, hBD-2 shows in "Phast[®]-System-high density gels", which lack urea, a 14 kDa band, just below that of lysozyme, whereas in Tricine gels with urea a band at 4 kDa can be observed.
10. With this method we obtained highest resolution of bands. We were able to separate the different 77, 72, and 69 residues containing forms of interleukin-8 (9).
11. Fixation can be a problem for low MW AMPs. The use of glutaraldehyde is compelling for detection of low amounts (< 10 ng per lane) of AMPs.
12. When highly sensitive detection is necessary, the gels can be destained with K₃[Fe (CN)₆] until background is cleared. Bands should never be completely destained! After rinsing the destaining solution, staining can be repeated as described. The band intensity does not reflect the relative amounts of the applied proteins.
13. Nanospray modus is recommended when very low peptide amounts are available. RP-HPLC fractions should be lyophilized and the (invisible) residue dissolved in 5 μ L ESI-MS solvent (*see* **Section 3.7**, Item 1). It is essential to completely evaporate the HPLC solvent, because otherwise remaining TFA will affect sensitivity. Take care to avoid the presence of detergents, which make analyses impossible.
14. This assay is highly sensitive and needs only minimal amounts of the preparation being tested. With appropriate solvent controls it is possible to test micro-HPLC fractions without any lyophilization. In some cases it could be necessary to protect HPLC fractions from losses by irreversible adherence to the plate. When this is a problem, add 5 μ L 0.1% (w/v) bovine serum albumin in 0.01% aqueous acetic acid to each well of the microtitre plate prior to adding HPLC fractions.

15. Vigorous mixing of bacterial suspensions (especially for *Pseudomonas aeruginosa* and after centrifugation) is important to disperse any clumps and provide an even inoculum suspension (1).
16. It is our experience that ethylacetate treatment of stratum corneum enhances recovery of AMPs, possibly by dissolving non-polar lipids and therefore increasing access of aqueous solutions to stratum corneum particles, which contain high amounts of lipids. Treatment with acetone is not recommended because most of the AMPs are soluble in acetone and thus would be lost. Alternatively diethylether could be used, but this requires extreme care!
17. Instead of ethanol, acetonitrile can also be used. In the absence of organic solvents, we often have had problems with the extracts giving turbid solutions after centrifugation with a high content of fines (lipid drops mixed with solid particles). Ignoring this phenomenon has usually resulted in HPLC problems, such as high-pressure error and giving “memory compounds” on the HPLC column.
18. The recovery of AMPs using Amicon (Danvers, MA) filters was found to be highest when acidic ($\text{pH} < 3$) solutions containing a small percentage (20–30%) of water-soluble organic solvents were used. Use the right diameter of the filter to avoid losses due to inappropriate ratio of filter diameter and (final) sample volume.
19. pH adjustment to pH 8 has always been done after thawing of frozen acidic samples. It is our experience that AMP recovery from samples which have been stored at pH 8 were always lower than those stored at acidic pH. After adjustment of extracts to an acidic pH often very fine precipitates occur. It is recommended to centrifuge these concentrated extracts again to avoid problems with HPLC analyses.
20. The size of the heparin affinity cartridge should be appropriate to the amount of extracted material. For extracts obtained from stratum corneum amounts < 1 g we always used a 1 mL cartridge. A 5 mL cartridge was used for higher amounts of extracts. It could be important to perform a second heparin affinity chromatography to see whether the whole heparin-binding material was indeed bound in the first chromatography step. Initially we had diafiltered the extracts to omit low MW compounds. Today we directly apply the extracts onto the heparin column. If extracts are directly (without diafiltration) applied, be aware that also low MW cationic compounds (which could represent antibiotic substances) will bind to the heparin

column! If your antimicrobially active HPLC fractions do not show strong silver stained bands and no masses > 2 kDa upon ESI-MS, look for the raw data (single charged species) of the ESI-MS analyses to identify possibly a low MW antibiotic compound!

21. It may occur that not all AMPs are efficiently stripped from the column with 2 M NaCl. We have observed that most of the hBD-3 and parts of HNP-1 stuck to the heparin column. hBD-3 could be eluted with acidic buffers like 0.1 M glycine/HCl buffer, pH 2 and HNP-1 with 0.1 M NaOH. Be aware that a repeated use of these eluents may destroy AMP-binding capacities of the heparin columns when often re-used!
22. Instead of concentrating the samples to a very low volume we used injection loops with the capacity for several millilitre sample volume. Broadening of peaks was never found to be a problem, except when the samples contained organic solvents >10%. When very polar AMPs are expected, it is recommended to lyophilize or "SpeedVac" the (acidic) sample and dissolve the residue in a small volume of water.
23. Detection at 215 nm is recommended, because some of the AMPs show no or a very low absorbance at 280 nm. When possible, absorbance at three wavelengths (215, 254, 280 nm) should be measured because the absorbance ratio is very characteristic for each peptide. This UV absorbance profile together with the retention time will often give sufficient initial information about the AMP that eluted in given HPLC fractions.
24. We use manual separation of HPLC fractions. Often AMPs do not represent the major UV-absorbing peaks, but instead shoulders within the peak. These can be easily separated manually and then analysed for its specific AMP activity. If this is higher in the shoulder than in the major UV-absorbing peak, it indicates that most of the contaminating protein has already been separated. It is important for a successful purification of AMPs to separate as early as possible contaminating non-active impurities.
25. We take, just after the RP-HPLC step, aliquots of each HPLC fraction and place it into round-bottomed microtitre plates (for each assay system one plate to avoid repeated freezing and thawing), which then will be stored frozen (-20°C). Immediately before biological testing will be performed, samples are lyophilized and then dissolved in appropriate solvents.

26. We use unusual conditions for cation exchange-HPLC for AMP separation! These conditions were found to be optimal for separation of various skin proteins (4–8) and allowed us good recoveries of highly cationic AMPs. It is our experience that textbook conditions (neutral pH, anionic buffers) gave lower AMP resolution (broad peaks!) and lower recoveries. Highly cationic AMPs, which are eluting only with high NaCl content, often cannot be detected in antibacterial assay systems due to AMP's salt sensitivity. Therefore, it has been sometimes our strategy to separate all, at high NaCl eluting proteins by subsequent (C2/C18)-RP-HPLC and to test RP-HPLC fractions for AMP activity.
27. Instead of acetonitrile also 30% (v/v) ethanol (denaturated with heptane) can be used. Addition of organic solvents to the elution buffer is essential for high AMP recovery. It is believed that amphipathic AMPs otherwise stick to the cation-exchanger matrix.
28. Fractions need not to be concentrated to decrease the volume, when an injection loop of appropriate size is available. It is recommended to add 1 μ L TFA/1 mL fraction.
29. Depending on the peptide, sometimes a completely clear zone, a clear zone with bacterial growth within the clearing zone or a complete clear zone, which is surrounded by a concentric zone of partial clearing owing to a reduction in microcolony density, was observed. We found the different patterns as AMP-specific parameters, which also depended on the concentration of the AMP as well as the assay condition, including the test organism.
30. We always performed SDS-PAGE analyses in the absence of reducing agents, because we never saw better band resolution in its presence. In contrast, some AMPs showed broader bands in the presence of dithiothreitol or mercaptoethanol.
31. Urea is necessary to reduce or prevent formation of dimers and oligomeric peptides. HBD-2 (MW \sim 4 kDa) migrates in the absence of urea like a 16 kDa protein. HBD-3 (MW \sim 5 kDa) still migrates in the presence of urea like a 9 kDa peptide.
32. Note a different band shape (even, convex, or concave) and band staining colours of different AMPs, e.g. defensins always showed a grey colour, whereas psoriasin and lysozyme were stained red-brown.
33. Take care that samples do not contain too much TFA, which would cause low signal intensity. It may be helpful to lyophilize (or "SpeedVac") the sample prior to

ESI-MS analyses and dissolve it in ESI-MS solvent. Samples originating from MonoS[®]-HPLC cannot be measured directly upon ESI-MS (due to the salt content). Intensive signals of anorganic salt clusters do not allow detection of far less intensive protein signals. NaCl-containing cation exchange-HPLC fractions therefore require a desalting process, otherwise no protein signals are visible. Alternatively, cation exchange-HPLC can be performed with volatile buffers (e.g. 1 M ammonium formate) as eluents. When adducts of ammonium formate adducts are still observed, we recommend to repeatedly add water to the samples and repeatedly lyophilize them until no crystalline residue (from salt) is visible.

34. Nano-ESI-MS is recommended when only low AMP amounts are expected. Because for nanospray-ESI-MS analyses only a few microlitres are required, due to the high TFA content it is essential to lyophilize the HPLC fractions prior to analyses! Dissolve the residue in sample solvent.
35. Peptide digests should be passed through a self-prepared μ -column and then eluted with acetonitrile. This procedure gives far less background and therefore much better results than the direct application of digests due to enrichment as well as elimination of contaminants.

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