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**Characterization of platelet-derived compounds
that induce antimicrobial peptides in human keratinocytes**

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presented by

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1 Introduction

Chronic, non-healing wounds are a clinically relevant problem resulting in high morbidity and mortality. Platelet formulations (PFs) such as platelet-rich-plasma have been used therapeutically for the treatment of various types of chronic wounds. In diabetic foot ulcer prospective clinical trials have shown promising results using PFs regarding wound closure and wound shrinkage [1], [2]. Larger retrospective studies have also shown that PFs are effective in the treatment of diabetic foot ulcer [3]. However, PFs did not influence healing rates in patients suffering from venous ulcers [4], [5], [6]. PF have been introduced as therapeutic option reducing deep and superficial wound infection in surgical wounds after sternotomy [7], [8] whereas attempts to applying PF onto surgical leg wounds have not reduced the occurrence of wound infections [9], [10]. The success in clinical application of PFs in the treatment of some types of chronic, non-healing wounds can be attributed, at least in part, to anti-infective properties of the PFs. These include direct antimicrobial effects that are mediated via platelet-bacterial interaction [11] or upon platelet-release of anti-microbial substances [12]. PFs are active against wound pathogens such as *methicillin-resistant Staphylococcus aureus (MRSA)*, *Escherichia coli (E.coli)* or *vancomycin-resistant Enterococcus (VRE)* [13], [14]. PFs may also display antimicrobial effects in a more indirect manner influencing tissues and cells in the wound environment. Recently a PF composed of released platelet factors (Platelet-released growth factors, PRGF) has been shown to induce several antimicrobial peptides such as human beta-defensin-2 (hBD-2), human beta-defensin-3 (hBD-3) and psoriasin in primary human keratinocytes representing the most abundant cell type of the epidermis [15], [16], [17]. HBD-2 exhibits antimicrobial activity against Gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa (P. aeruginosa)* [18]. Likewise HBD-3 has also been shown to be microbicidal against Gram-negative pathogens including *E. coli* and *P. aeruginosa* and is additionally active against Gram-positive bacteria such as *MRSA* and *VRE* [19]. PF contain a wide variety of factors such as growth factors and cytokines [20], [21] many of which have been shown to be released by the platelets themselves and are not derived from plasma [22]. The objective of this thesis is to identify factors derived from platelets that induce antimicrobial peptides (hBD-2 and hBD-3) in human keratinocytes *in vitro*. In order to rule out possible effects that might be mediated via platelet-keratinocyte interaction, platelet-released growth factors (PRGF) were used mimicking the situation in wounds after PF application and after platelet degranulation.

2 Material and methods

2.1 Material

<u>Chemicals</u>	<u>Supplier</u>
Calcium chloride (CaCl ₂)	PromoCell, Germany
cDNA-synthesis-kit	Takara Bio Europe/ Clontech, France
Crystal RNAmagic	Biolabproducts, Germany
Ethanol	Carl Roth, Germany
Isopropanol	Sigma-Aldrich, Germany
Keratinocyte Growth Medium 2 (KGM 2)	PromoCell, Germany
KGM 2 Supplement Mix	PromoCell, Germany
Sodium hydroxid (NaOH)	Merck, Germany
Sodium chloride (NaCl)	Merck, Germany
Phosphat-buffered saline (PBS)	Biowest, France
Penicillin/Streptavidin	Gibco, Germany
SYBR-Green Premix Ex Taq™ II	Takara Bio Europe/Clontech, France
Trifluoroacetic acid (TFA)	Sigma-Aldrich, Germany
Acetonitrile (ACN)	Sigma-Aldrich, Germany
Sodium citrate	Sigma-Aldrich, Germany
Ammonium acetat	Sigma-Aldrich, Germany
Ammonium sulfate	Sigma-Aldrich, Germany
<u>Enzymes</u>	<u>Supplier</u>
Reverse transcriptase	Thermo Fisher Scientific, Schwerte

Buffers	Supplier
Reverse transcriptase buffer	Thermo Fisher Scientific, Schwerte
Running buffer acetonitrile 0.05% TFA	Sigma-Aldrich, Germany
Running buffer H ₂ O 0.05% TFA	Sigma-Aldrich, Germany
Running buffer 80% isopropanol 0.05% TFA	Sigma-Aldrich, Germany
Storage buffer 65% acetonitrile in H ₂ O	Sigma-Aldrich, Germany
Running buffer 25 mM ammonium acetate	Sigma-Aldrich, Germany
Running buffer PBS 150mM NaCl	Sigma-Aldrich, Germany
MES buffer	
(2-(<i>N</i> -morpholino)ethanesulfonic acid) 20mM	Sigma-Aldrich, Germany
MES buffer	
(2-(<i>N</i> -morpholino)ethanesulfonic acid) 20mM,	
1 M NaCl	Sigma-Aldrich, Germany
NaH ₂ PO ₄ buffer 20 mM	Sigma-Aldrich, Germany
NaH ₂ PO ₄ buffer 20 mM, 0.4 M NaCl	Sigma-Aldrich, Germany

Cells	Supplier
NHEK f-c pooled,	PromoCell, Germany
Cat.NoC-12005, Lot.No 1040101	

Primer	Orientation	Sequence
hBD-2 forward	5'-3'	GCC TCT TCC AGG TGT TTT TG
hBD-2 reverse	3'-5'	GAG ACC ACA GGT GCC AAT TT

hBD-3 forward	5'-3'	TGTTTGCTTTGCTCTTCCTGT
hBD-3 reverse	3'-5'	CGCCTCTGACTCTGCAATAA
RP38 forward	5'-3'	TCAAGGACTTCCTGCTCACA
RP38 reverse	3'-5'	AAAGGTATCTGCTGCATCGAA

<u>Device</u>	<u>Supplier</u>
Incubator	Binder, Germany
Microscope cell culture	HundWetzlar, Germany
Nanodrop1000 spectrophotometer	Thermo Fisher Scientific, Germany
Step One Plus Realtime PCR-system	Applied Biosystems, USA
Sunrise microplatelet reader	Tecan, Germany
Thermocycler T3	Biometra, Germany
Centrifuge	Eppendorf centrifuge 5810R, rotor A-4-62
Centrifuge	Thermo Scientific Sorvall Legend XTR, Rotor 75003607
Centrifuge	Eppendorf centrifuge 5804R, rotor
Centrifuge RNA isolation	Eppendorf centrifuge 5417R, rotor F45-30-11
Shimazu chromatography system	GE-Healthcare
ÄKTA micro chromatography system	GE-Healthcare
ÄKTA purifier chromatography system	GE-Healthcare

<u>Column</u>	<u>Supplier</u>
HPLC RP4 column	GE Healthcare
HPLC RP8 column	GE Healthcare

HPLC PR18 column	GE Healthcare
Superdex 75 PC 3.2/30	GE Healthcare
Phenomenex Sec-C 2000	GE Healthcare
MONO S 5/50 GL	GE Healthcare
Amicon ultra-15, Centrifugal 10 kD-filter	Merck Millipore, Ireland
Vivaspin 500, Centrifugal 3 kD-filter	Sartorius, Germany

<u>Other</u>	<u>Supplier</u>
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Cell culture plates 24 well	Costar Corning Incorporated costar, USA
Steril filter 0,22µm	Sarstedt, Germany

2.2 Methods

2.2.1 Cell culture

Normal human epithelial keratinocytes (NHEK)

Keratinocytes are the most abundant cell type of the epidermis. Normal human epithelial keratinocytes (NHEK) are primary human keratinocytes that are isolated from human tissue such as juvenile foreskin or adult tissues like breast, abdomen or face and are used for *in vitro* experiments. NHEKs are commercially available as single-donor or pooled cell population. They are purchased as passage two or three and subsequently cultured. NHEKs are adherent cells that tend to form monolayers of cells showing typical epithelial morphology such as cobblestone patterns *in vitro*.

Cultivation of keratinocytes

NHEKs were store at -196°C, rapidly defrosted and carefully transferred to culture plates. After cultivation in Keratinocyte Growth Medium 2 (KGM 2) with supplements at 37°C the cells were used at 90-100% confluence. The morphology of the cells was monitored microscopically prior to stimulation and after 24 h of stimulation. Supernatants can be applied for protein level measurement via ELISA whereas the cells themselves were used for RNA isolation and subsequent quantitative RNA measurement through real-time polymerase chain reaction (rtPCR).

2.2.2 Preparation of samples

Preparation of platelet-released growth factors (PRGF):

PRGF solutions were obtained from fresh thrombocyte concentrates donated by the Institute of Transfusion Medicine in Kiel. Thrombocyte concentrates were centrifuged for 10 min at 2000 g, washed twice with sodium citrate buffer (0.011 M, pH 5.5, 37°C) and centrifuged for 10 min at 2000 g again. The thrombocyte pellets were resuspended in Keratinocyte Growth Medium 2, subsequently processed by ultrasound and stored at -80°C for 24 h. The solutions were defrosted, processed by ultrasound again and stored at -80°C for 24 h. Finally, the solutions were defrosted, centrifuged for 3 min at 18.000 g and the supernatants were stored at -20°C.

2.2.3 Separation, detection, processing and further analysis of samples

Chromatographical methods

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is used for separation of biosamples. It is a specific form of column chromatography. HPLC uses two different phases, the column acting as the solid phase and the solvent acting as the liquid phase. A widely used form of HPLC is the reversed-phase HPLC (RP-HPLC) where the column is made of a lipophilic material with aliphatic side chains and the liquid phase is a more hydrophilic agent such as water. The liquid phase is gradually mixed with a more lipophilic solvent such as acetonitrile eluting more lipophilic compounds gradually according to their increasing affinity to the liquid phase. In this study RP-HPLC was performed using RP-4, RP-8 and RP-18 columns with the number displaying the amount of C atoms the aliphatic side chains contain. The specific running conditions are noted in the underlines of the corresponding figures. Pure PRGF was acidified with trifluoroacetic acid (TFA) until the pH-value of the starting running buffer was reached. When samples derived from prior separation steps were further processed via HPLC, samples were first lyophilized and subsequently resuspended in running buffer. All samples prepared for HPLC were centrifuged prior to final application as only dissolved substances are suitable for this separation method.

Molecular weight filtration

Molecular weight filtration is a method that separates a sample into fractions according to the molecular weight by centrifuging the sample through a porous filter. The molecular weight cut-off (MWCO) is determined by the pore size of the filter leaving all components above the specific molecular weight in the concentrate and the ones below the molecular weight in the flow-through.

Here both 10 kD MWCO (Amicon ultra-15, Centrifugal 10 kD-filter) and 3 kD MWCO (Vivaspin 500, Centrifugal 3 kD-filter) filters were used according to the manufacturer's instruction. Briefly all molecular weight filtrations were done as following: 10 kD filtration, 90 min, 5000 g, 7°C; >10 kD washing 6 times 30 min, 5000 g, 7°C; <10 kD filtration, 60 min, 4000 g, 7°C; 10-3 kD washing 6 times 30 min, 4000 g, 7°C. Sequential molecular weight filtration with these 2 filters allowed separation into 3 fractions: Larger 10 kD, smaller 10 kD and between 10 kD and 3 kD.

Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) is a liquid chromatographical method that separates components by molecular weight. Columns used in SEC are typically composed of carbohydrates like dextran and agarose which cross-link and form porous matrices. Running buffers are commonly aqueous solutions. Buffer compositions are chosen such as to not affect the sample's stability and activity. Mild conditions regarding pH, ionic strength and temperature conditions are favorable in this regard. Ionic buffers (phosphate or ammonium acetate, e.g.) reduce possible nonspecific interactions of the wanted components in the sample and the column material such as ionic interactions. Molecules with high molecular weight do not fit into the pores of the column and will elute first. Intermediate size molecules fit into the pores and will elute accordingly, whilst the smallest molecules fit best into the pores and remain on the column the longest, eluting last. Here SEC was performed with a Superdex 75 PC 3.2/30 column. Prior to separation all samples were lyophilized and the precipitate was resuspended in water. Detailed running conditions are noted in the underlines of the corresponding figures.

Ion exchange chromatography

In ion exchange chromatography molecules can be separated according to their surface charge. Anionic exchange columns have positively charged functional groups such as quaternary ammonium ions that can capture anionic components whereas cationic exchange columns have negatively charged functional groups such as methylsulfonates that bind positively charged molecules. Here a Mono S GI 5/50 was used for cationic exchange chromatography. Prior to separations PRGF was acidified with 15% HCl in water until the pH-value was 6. After centrifugation the supernatant was injected. Detailed running conditions are noted in the underlines of the corresponding figure.

Ammonium sulfate precipitation

Precipitation of proteins using ammonium sulfate has been described as a method for purification and separation. If ammonium sulfate is added to water the dissolved ions ammonium and sulfate interact

with water molecules. The solubility of proteins in aqueous solutions is dependent on many chemical interactions such as hydrogen bonding between polar groups within the protein and water molecules. Gradual increase of ammonium sulfate concentration results in progressive competition of ammonium and sulfate ions against proteins for water molecules. Finally, this leads to protein precipitation. Some proteins require high ammonium sulfate concentrations in order to precipitate whereas other proteins may precipitate at lower ammonium sulfate concentrations. The tendency to precipitate is a property of the specific protein and is dependent on characteristics such as molecular weight. Therefore, in principle the method allows selective protein separation. The resulting precipitate can be resolubilized in buffers. Since the method utilizes reduced solubility not degradation biological activity of proteins can be preserved. Here 0.5 ml of PRGF were prepared with 145 μ l of 3.8 M aqueous ammonium sulfate solution (corresponding to 30% ammonium sulfate saturation), another 0.5 ml of PRGF were prepared with 335 μ l of 3.8 M aqueous ammonium sulfate solution (corresponding to 50% ammonium sulfate saturation) and another 0.5 ml of PRGF were prepared with 180 mg of ammonium sulfate and subsequently diluted with water until precipitation occurred upon centrifugation (corresponding to approximately 75% ammonium sulfate saturation). Precipitates were resuspended in KGM. Resuspended precipitates and supernatants were used for cell stimulation.

Detection

The device used for detection of separated compounds is a UV-spectrometer attached downstream of separation columns. Most proteins and peptides absorb UV light at the wavelength 215 nm resulting in a peak in the chromatogram. Peaks can be collected and used for further analysis.

Processing

Fractions are dried through lyophilization. Lyophilization is a particularly mild method to dry substances that might be degraded if stored in liquids over a longer period of time or might suffer from temperatures above 0°C. In this study proteins or peptides were suspected to be in the fractions. These can undergo degradation such as hydrolysis and thereby can be inactivated. If liquids are removed thoroughly these substances are much more stable and can be stored at -20°C. The principal in lyophilization is that liquids are removed from solved substances by first freezing the solution and then vaporizing the liquids. This process is called sublimation and is the transformation of solid state into vaporous state.

Quantitative and qualitative analysis

Quantitative analysis of components can be done by analyzing the size of UV-detected peaks, where the area under the curve (AUC) of a pure substance peak is directly proportional to the concentration. For exact calculation of the concentration a calibration with defined concentrations of the specific

substance is necessary. As in this study the substances corresponding to a defined peak are typically not known the calibration method is not possible. Here peaks from different experiments containing the same substances can be compared. Bioactivity of these peaks can be investigated in comparison to the approximate peak size/AUC presenting the approximate relative substance concentration.

Identification of components was performed using mass spectrometry (MS). MS allows the determination of the molar mass of biomolecules. For this, biosamples were first dried through lyophilization solved in methanol and inserted into the mass spectrometer in liquid phase. For MS, the molecules need to be ionized and the device allows to use either hard or soft ionization. To avoid fragmentation, the latter was chosen (here: ESI (*electrospray ionization*)). After ionization, the molecules are accelerated by an electric field and transmitted to Q-TOF (*quadrupole-time of flight*) analyzer. The quadrupole part of this analyzer consists of four parallel metal rods to which oscillating voltages are applied. This acts as a mass-to-charge filter: only molecules with a certain mass-to-charge ratio will be guided to the end of the analyzer. The other molecules travel on an unstable trajectory and collides with the surroundings before reaching the end. For the measurements in this thesis, the quadrupole filter was set to remove lighter molecules and transmit only the heavier proteins of interest. These molecules further proceed to the TOF unit, where they are accelerated for a short time and then drift along the flight tube with a constant velocity until they reach the detector. From the registered arrival time, the processor can calculate the mass-to-charge ratio of the detected molecules. Prior to the MS analysis, enzymatic digestion and cleavage of the proteins was performed which results in protein specific fragmentation. These fragment patterns can be analyzed by MS and compared to protein fingerprint databases. If the protein from the biosample is listed in these databases, the fragment pattern can be identified and assigned to a protein.

2.2.4 Biomolecular methods

RNA-isolation

For RNA isolation keratinocytes are lysed with 300 µl of CRYSTAL RNAmagic (Trizol) per well, transferred into tubes and 60 µl chloroform is added. Tubes are mixed through flipping a few times and centrifuged at 12000 g, 4°C for 15 min leaving 3 Phases: upper clear phase with RNA, middle white phase with protein and lower red phase with DNA. The upper phase is complemented with 150 µl of isopropanol for RNA precipitation and after centrifuging as described above for 10 min the pellet is washed with 300 µl of 75% ethanol. After 2 cycles of centrifugation for 5 min and washing as described above the supernatant is removed and the RNA dried at room temperature for 10 min. The pellet is resuspended in 12 µl of pure H₂O and subsequently used for total RNA measurement.

Total RNA quantification and RNA purity

In order to apply equimolar amounts of RNA for complementary DNA (cDNA) synthesis total RNA measurement is performed using a nano drop spectrometer. The RNA purity is evaluated by determining the ratio of absorbance at 260 nm and 280 nm as well as the ratio of absorbance at 230 nm and 260 nm. Nucleic acids like RNA, ssDNA and dsDNA absorb light at the wavelength of 260 nm. Phenols that are a key component of the CRYSTAL RNAmagic used for RNA-isolation absorb at wavelengths near 230 nm and at near 270 nm. Other possible contaminants such as proteins also absorb at wavelengths 230 nm or 280 nm. The RNA is considered pure if the 260/280 ratio is around 1.8 and the 260/230 ratio is around 2.0-2.2.

Reverse-transcription of RNA

For further analysis RNA must be reversely transcribed to complementary DNA (cDNA). Key components for this reaction are the enzyme reverse transcriptase (RT), deoxynucleotide triphosphates mix (dNTPs), RNase H, RNase inhibitor, oligo dT primers and reaction buffers. The primers bind to the Poly(A) tail sequence (which occurs in many RNAs) of the single stranded RNA template. RT catalyzes the DNA synthesis adding dNTPs. The RNase degrades the RNA template leaving single stranded cDNA.

Real-time-polymerase chain reaction (rtPCR)

Quantification of RNA is performed using real-time-polymerase-chain-reaction (rtPCR). Reagents needed for PCR are forward and reverse primers for the sequence (gene) of interest, an enzyme for DNA synthesis (for instance Taq DNA polymerase), deoxynucleotide triphosphate mix (dNTPs) as a substrate for the DNA polymerase and reaction buffers. Primers bind to the single stranded template DNA (ssDNA). DNA polymerase catalyzes the new DNA synthesis adding dNTPs. Newly synthesized double stranded DNA (dsDNA) is now denaturated at 94 °C leading to single stranded DNA being the starting point of the second cycle in the PCR process. In rtPCR reagents that intercalate dsDNA are added. These reagents (SYBR Green 2, e.g.) are fluorophores that emit light upon excitation. The intensity of this signal is detected while the amplification takes place in real time. In the linear range the intensity increases proportional to the amount of DNA. For relative quantification of DNA the threshold cycle (CT) is determined. CT is defined as the cycle when the fluorescence exhibits a defined value (threshold). In an untreated control the ratio of the CT value of a house keeping gene and the CT value for the gene of interest is set to 1. The same ratio is calculated for a treated sample. The quotient of these two ratios is the relative expression of the gene.

2.2.5 Statistical analysis

Data are presented as means \pm standard deviation of the mean. The number of independent experiments is indicated as “n”. Calculations were performed using GraphPad Prism 7 (Mac, GraphPad Software, La Jolla, USA). Analysis was performed with two-tailed unpaired Student’s t-test. P values less than 0.05 were considered statistically significant.

3 Results

3.1 Establishing separation methods for PRGF

3.1.1 Separation via reversed phase high-performance liquid chromatography (RP-HPLC)

Reversed phase-4 (RP-4)

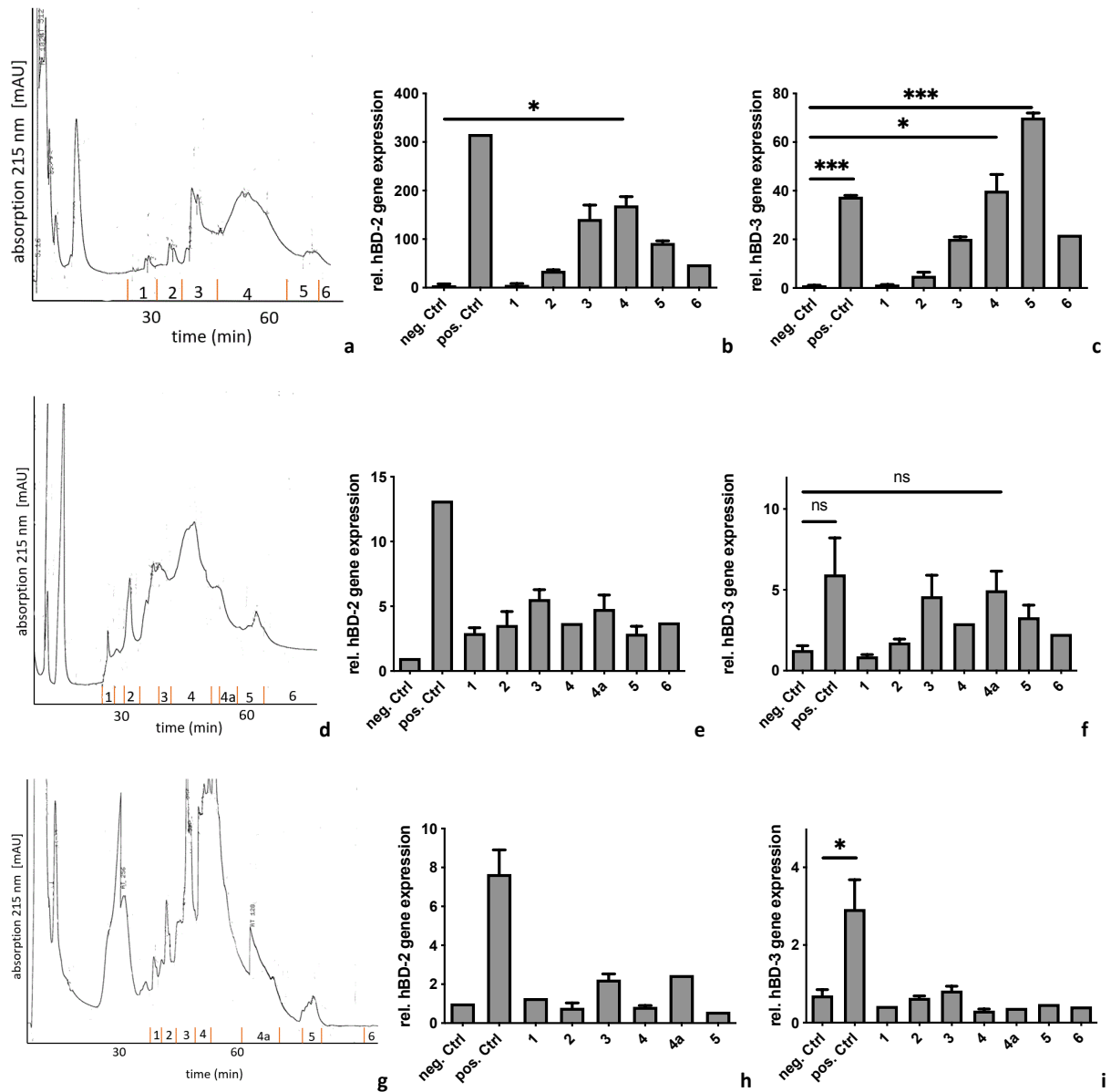


Figure 1 | Reversed phase-4 (RP-4) runs (a, d, g) and relative induction of hBD-2 (b, e, h) and hBD-3 (c, f, i) by fractions of the corresponding run. 1-3 earlier peaks, 4 main peak, 4a descending main peak, 5-6 later peaks, Ctrl=control, running conditions: buffer A: H₂O 0.05% TFA, B: acetonitrile (ACN) 0.05% TFA. Data are mean ± SD; *P<0.05; **P<0.01; *P<0.001 between selected relevant comparisons, two-tailed unpaired Student's t-tests.**

In order to separate components of PRGF at first a reversed phase-4 (RP-4) HPLC approach was chosen. This method separates components according to their hydrophobicity.

Fig. 1a, Fig. 1d and Fig. 1g show chromatograms of three individual RP-4 runs. Fig. 1a and Fig. 1d show similar chromatograms whereas Fig. 1g shows a chromatogram with longer running time (slower gradient). All three chromatograms share the following peaks: several immediate peaks after starting the separation (weak or no interaction with the column compared to interaction with aqueous running buffer), three earlier peaks (1-3), one main peak (4) and one later peak (5). Although this peak pattern can be considered similar there are discrepancies between the chromatograms regarding peak shape. Fig. 1g shows one large peak prior to peak 1. Individual peaks were activity tested regarding their ability to induce hBD-2 and hBD-3 in keratinocytes. In Fig. 1a the highest activity for hBD-2 gene expression is found in peak 3 and 4, for hBD-3 gene expression in peak 4 and 5. As Fig. 1d shows a shoulder in the descending part of peak 4, peak 4 was split into 4 (main) and 4a (descending). Here the highest activity for hBD-2 gene expression is found in peak 3 and 4a, for hBD-3 gene expression also in peak 3 and 4a. Because peak 4 was split into two peaks (peak 4 and peak 4a, Fig. 1d and Fig. 1g) overall activity of the main peak may have been reduced compared to the unsplit main peak 4 (Fig. 1a). In Fig. 1g biological activity was lost entirely.

In summary biological activity could be found in fractions of run Fig. 1a and run Fig. 1d for hBD-2 and hBD-3. However, these results could not be reproduced in run Fig. 1g.

Reversed phase-8 (RP-8)

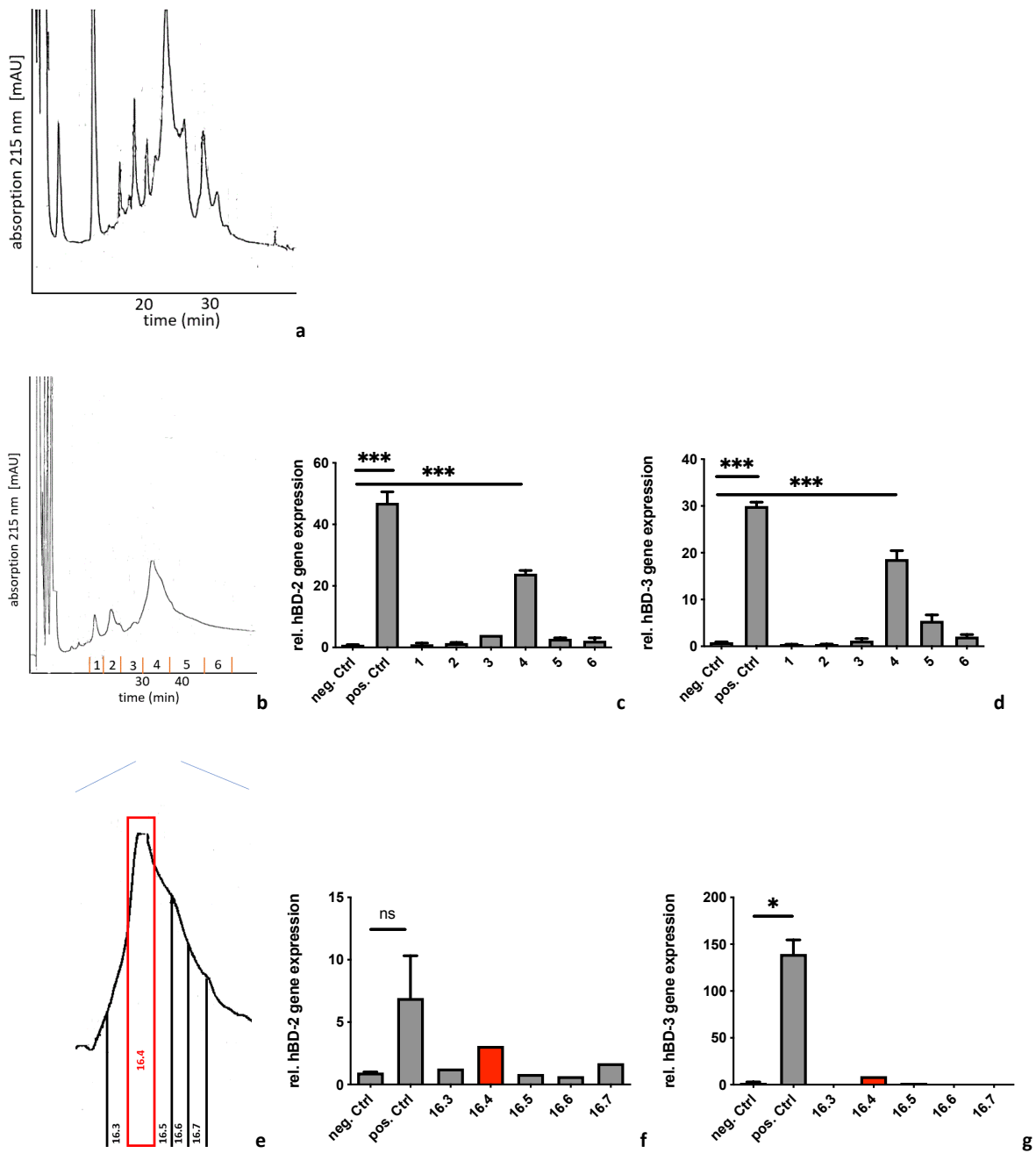


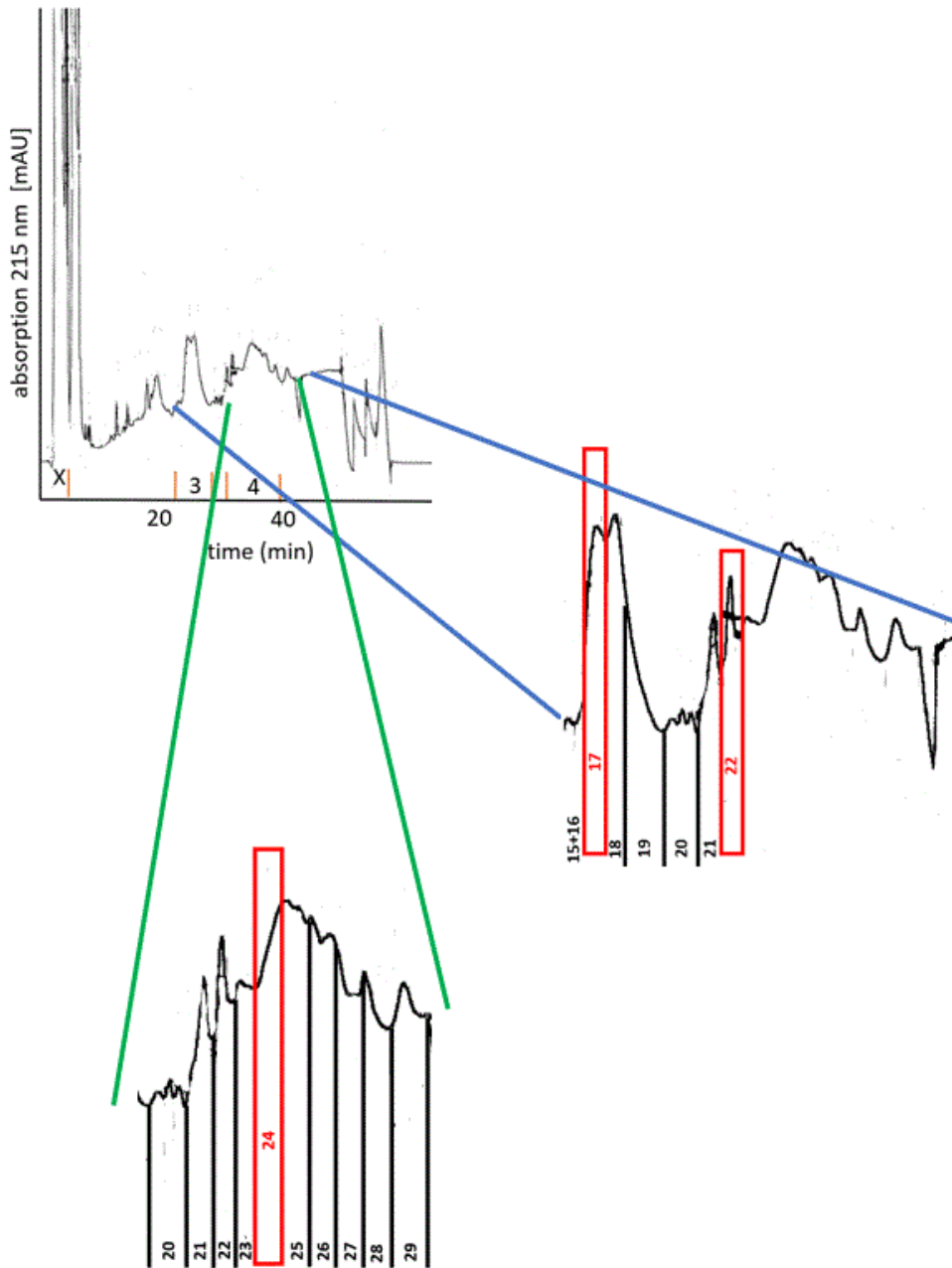
Figure 2 | RP-8 runs (a, b), enlarged peak 4 from run b (e) and relative induction of hBD-2 (c) and hBD-3 (d) by fractions of run b. Further analysis of fraction 4 from run b and relative induction of hBD-2 (f) and hBD-3 (g) by fractions of peak 4. Fraction 16.4 marked by red box with corresponding activity bars in red. 1-3 earlier peaks, 4 main peak, 5-6 later peaks, 16.3- 16.7 fractions of peak 4, Ctrl=control. a, running conditions: buffer A: H₂O 0.05% TFA, B: ACN 0.05% TFA, gradient: 0-5 min 10% B, 5-25 min 10-60% B, 25-35 min 60-100% B. b, running conditions: buffer A: H₂O 0.01% TFA, B: 80% isopropanol 0.01% TFA, gradient: 0-5 min 10% B, 5-25 min 10-60% B, 25-35 min 60-100% B. Data are mean ± SD; *P<0.05; **P<0.01; *P<0.001 between selected relevant comparisons, two-tailed unpaired Student's t-test.**

HPLC separation of PRGF with an RP-4 column resulted in relatively poor peak separation and inconsistent results regarding biological activity. RP-8 columns have longer hydrophobic side chains

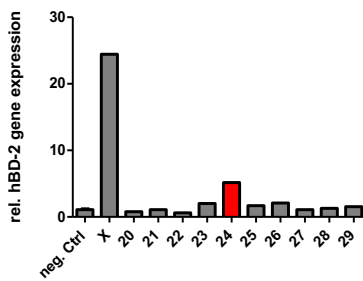
and can produce better peak separation. Therefore, an RP-8 column was applied under similar conditions regarding buffers (acetonitrile (ACN) 0.05% trifluoroacetic acid (TFA) and aqueous 0.05% TFA buffers) and gradient.

Fig. 2a displays improved peak sharpness and separation compared to the RP-4 chromatograms (Fig. 1a, Fig. 1d, Fig. 1g). However, biological activity could not be found in any peak (data not shown). Considering that organic solvents such as ACN and acidic running conditions could denature active PRGF components, milder conditions (regarding running buffers/solvents) were chosen. Accordingly, ACN was replaced by isopropanol (more hydrophilic) and the TFA concentration was reduced from 0.05% to 0.01%. Fig. 2b shows dramatically worse peak separation compared to Fig. 2a due to these modified running conditions. However, the approximate peak pattern from the RP-4 approach could again be found: weak binding immediate peaks, three earlier peaks (1-3), one main peak (4) and one later/descending peak (5). Fig. 2c and Fig. 2d show biological activity in the main peak (4) for both hBD-2 and hBD-3. Further analysis of peak 4 was performed by testing biological activity for the fractions of peak 4. Fig. 2e shows peak 4 from Fig. 2b (red box: fraction 16.4). One distinct fraction (fraction 16.4) shows relative induction by 3-fold for hBD-2 and 9-fold for hBD-3 (red bars, Fig. 2f and Fig. 2g).

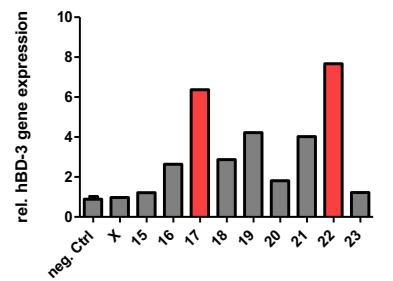
These results demonstrate that RP-8 separation of PRGF preserves bioactivity regarding the induction of hBD-2 and hBD-3 if mild conditions are applied. One distinct fraction in the ascending part of the main peak induces both hBD-2 and hBD-3. However, separation performance was reduced compared to RP separation with non-mild conditions.



a



b



c

Figure 3A | RP-8 run (a, upper left), enlarged peak 4 (a, lower left), enlarged peak 3 and 4 (a, right) and relative induction of hBD-2 (b) and hBD-3 (c) by fractions of the run. Fractions marked by red boxes correspond to red activity bars. X=first fraction of the run, 3 earlier peak, 4 main peak, 15-29 fractions of the run, Ctrl=control. a, b, running conditions: buffer A: H₂O 0.01% TFA, B: 80 % isopropanol 0.01% TFA.

RP-4 separation of PRGF components presented relatively poor peak separation and conflicting results regarding biological activity. RP-8 separation resulted in worsened peak separation when mild running conditions (more hydrophilic, less acidic) were chosen. However, one distinct fraction in the main peak was active both for induction of hBD-2 and hBD-3. To verify these findings in particular in order to decide whether the RP-8 separation was a viable separation method for PRGF another RP-8 separation was performed using a newer RP-8 separation column.

Fig. 3Aa upper left shows relatively poor peak separation. The main peak (4) and one earlier peak (3) are marked. Fig. 3Aa right and Fig. 3Aa lower left show peak 3 plus 4 and peak 4 respectively. One distinct fraction (red box in Fig. 3Aa lower left) of the main peak induces hBD-2 by 5-fold (red bar in Fig. 3Ab) and two distinct fractions (red boxes in Fig. 3Aa right) induce hBD-3 by approximately 7-fold (red bars in Fig. 3Ac). Notably one fraction of the earliest peaks (X) showed 25-fold induction of hBD-2.

Collectively this data demonstrates that PRGF-derived components can be separated successfully with a RP-8 separation approach whilst preserving bioactivity regarding the induction of hBD-2 and hBD-3. Bioactivity could be found in the ascending part of the main peak.

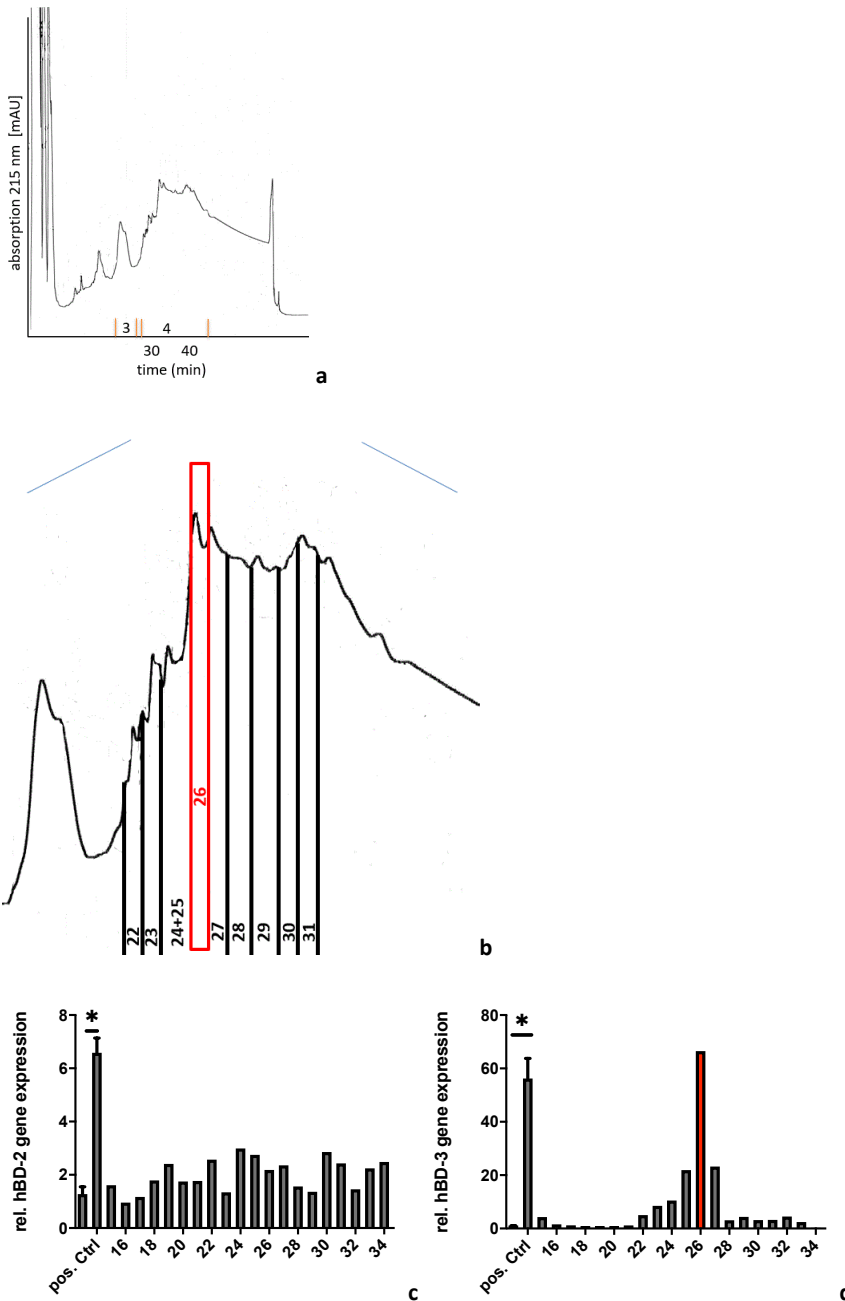


Figure 3B | RP-8 run (a), enlarged peaks 3 and 4 from run a (b) and relative induction of hBD-2 (c) and hBD-3 (d) by fractions of run. Fraction 26 marked by red box with corresponding activity bar in red. 3 earlier peak, 4 main peak, 16-34 fractions of the run, Ctrl=control. a, running conditions: buffer A: H₂O 0.01% TFA, B: 80% isopropanol 0.01% TFA. Data are mean \pm SD; *P<0.05; **P<0.01; *P<0.001 between selected relevant comparisons, two-tailed unpaired Student's t-test.**

In order to verify these results RP-8 separation was repeated under identical (mild) running conditions. Fig. 3Ba displays similar peak separation compared to the previous, identical RP-8 run. HBD-2 induction could not be found in any of the fractions (Fig. 3Bc). One specific fraction (fraction 26, red bar in Fig. 3Bd) induced hBD-3 by approximately 70-fold.

Three independent PRGF PR-8 separations were performed applying mild running conditions. In summary two out of three of these separations lead to fractions in the ascending part of the main peak that induced hBD-2. In one separation hBD-2 was not induced. HBD-3 was induced by distinct fractions of the ascending part of the main peak in all three experiments.

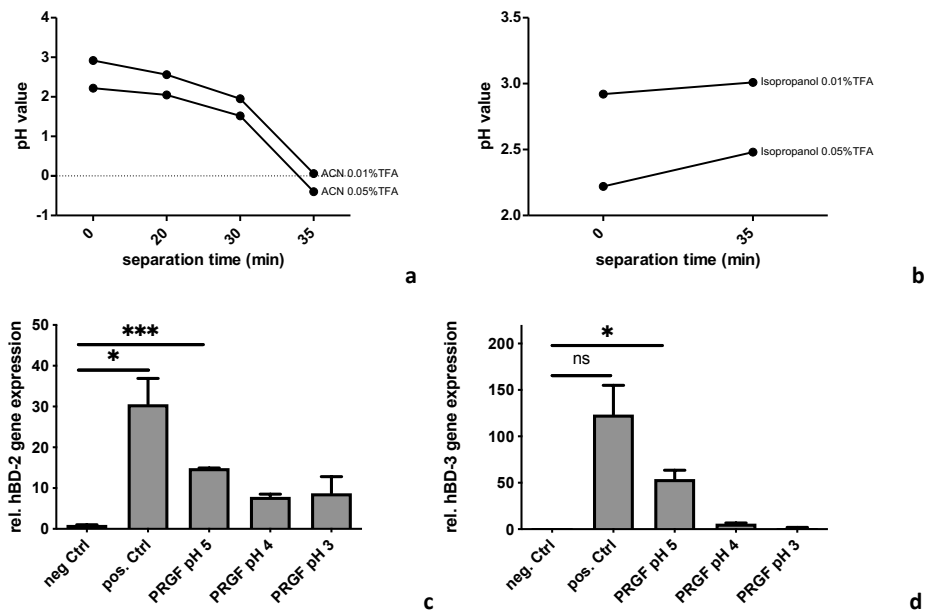


Figure 4 | Empirically determined pH-values during reversed phase separation with TFA in acetonitrile (ACN) running buffer (a) and TFA in isopropanol running buffer (b) and relative induction of hBD-2 (c) and hBD-3 (d) by PRGF pH 5, pH 4 and pH 3. a, b pH-values were determined empirically (single-rod measurement, glass electrode) for specified running timepoints. Data are mean \pm SD; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ between selected relevant comparisons, two-tailed unpaired Student's t-test.

In order to address the question whether acidic running conditions in RP-HPLC separation of PRGF decrease biological activity of PRGF components, pH-values under running conditions were first measured.

In both ACN and isopropanol pH-values were always below 3 at some point of the separation. PRGF was titrated to pH 5, pH 4 and pH 3 respectively and utilized for stimulation. Relative hBD-2 induction through pH 3 treated PRGF was 3 times lower than control. Relative hBD-3 induction under these conditions was lost entirely.

Biological activity of PRGF regarding the induction of hBD-2 and hBD-3 could not reproducibly be preserved when running buffers were composed of the organic buffer ACN and 0.05% TFA. When ACN was replaced by isopropanol and the TFA concentration was reduced to 0.01% biological activity both for hBD-2 and hBD-3 could be preserved in at least two independent experiments. Also, both experiments revealed one fraction of the main peak that induced either hBD-2 or hBD-3 or both.

3.1.2 Separation via molecular weight filtration

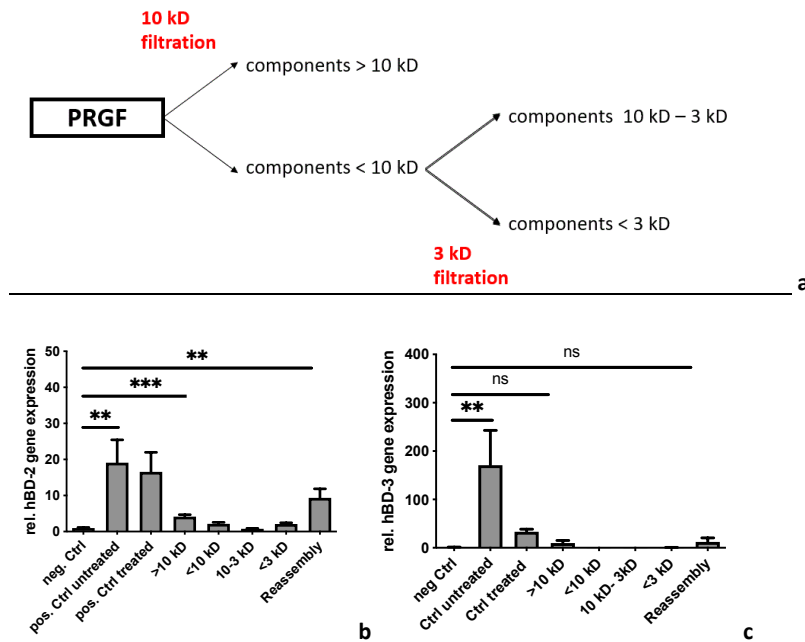


Figure 5 | Molecular weight filtration overview (a), relative induction of hBD-2 (b) and hBD-3 (c) after 10 kD and 3 kD molecular weight filtration. Ctrl=control, kD= kilo Dalton, Reassembly=fraction >10 kD + fraction between 10 and 3 kD + <3 kD, fraction pos. control treated was processed with the same conditions as fractions. Compound concentrations used for stimulation were kept constant in comparison to controls. **b**, n=3 independent experiments. **c**, n=1. Data are mean \pm SD; *P<0.05; **P<0.01; ***P<0.001 between selected relevant comparisons, two-tailed unpaired Student's t-test.

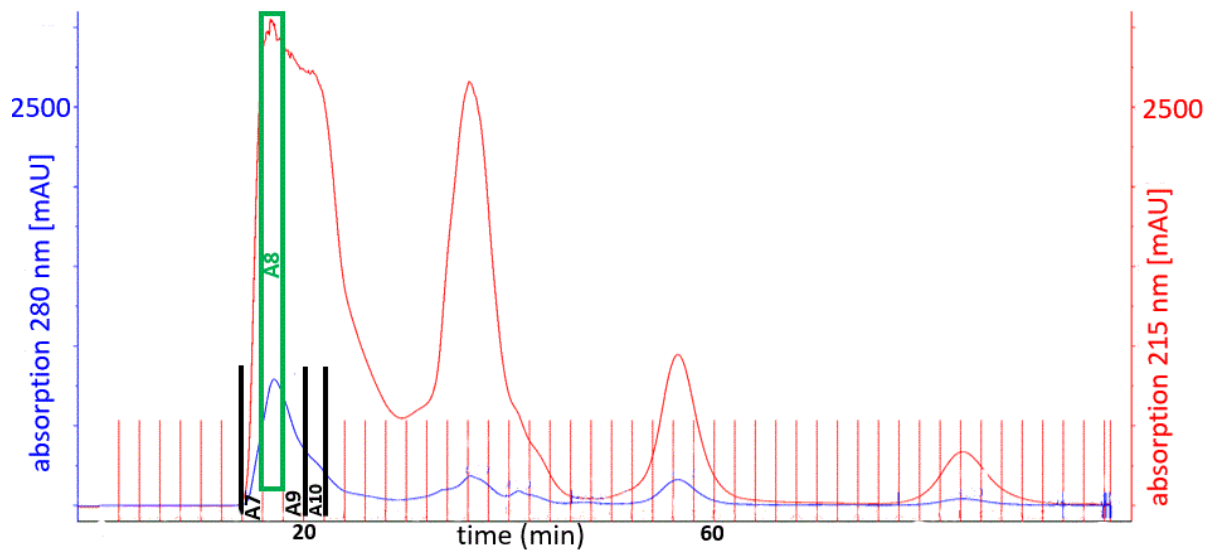
Platelet formulations are known to contain many diverse molecules, e.g. smaller and larger molecules. In order to address the question whether smaller or rather larger molecules derived from PRGF are inducers of both hBD-2 and hBD-3 in vitro molecular weight filtration was performed. Here molecules larger 10 kilodalton (kD) were separated, followed by 3 kD filtration resulting in 3 fractions. Larger 10 kD, smaller 3 kD and a fraction between 10 and 3 kD (Fig. 5a).

Mean relative hBD-2 induction through PRGF was approximately 15-fold. No significantly reduction of relative hBD-2 induction compared to the untreated control was observed for PRGF treated under filtration conditions. Fractions with molecules greater than 10 kD molecular weight induced hBD-2 by

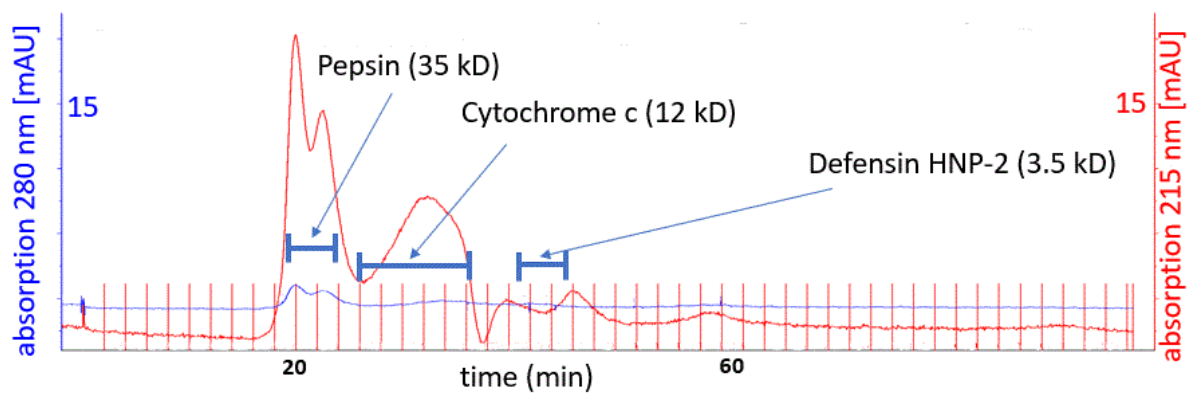
approximately 5-fold. Fractions with molecules below 10 kD molecular weight showed a weak mean increase by 2-fold. This effect could be attributed to the fraction lower 3 kD, since the fraction between 3kD and 10 kD was not active and the fractions lower 3 kD increased relative hBD-2 induction 2-fold by itself. Reassembly of all fractions (larger 10 kD, smaller 3 kD and fraction between 10 and 3 kD) showed relative mean induction by approximately 10-fold (Fig. 5b). Relative hBD-3 induction was more than 100-fold increased after PRGF stimulation. However, when PRGF was exposed to molecular weight separation conditions relative hBD-3 induction was reduced to 30-fold, fractions with molecules greater than 10 kD induced hBD-3 by 10-fold (Fig. 5c). Collectively these results demonstrate, that molecules larger 10 kD induce hBD-2. Notably reassembly of all three fractions (large, small and intermediate) results in higher hBD-2 induction than induction merely by molecules larger 10 kD. Thus, cooperative effects of molecules larger 10 kD and smaller 10 kD are possible. For hBD-3 the conditions of the filtration process itself reduced biological activity remarkably.

In summary PRGF-derived components larger 10 kD induce hBD-2 and hBD-3. However, molecular weight filtration largely reduced the biological activity of PRGF-derived components.

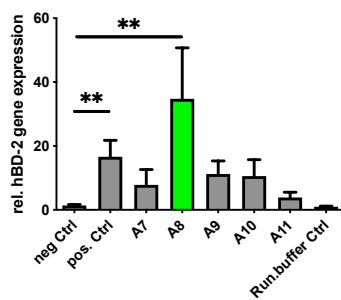
3.1.3 Separation via size exclusion chromatography (SEC)



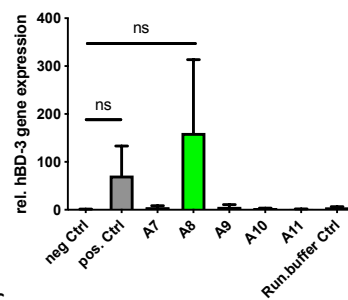
a



b



c



d

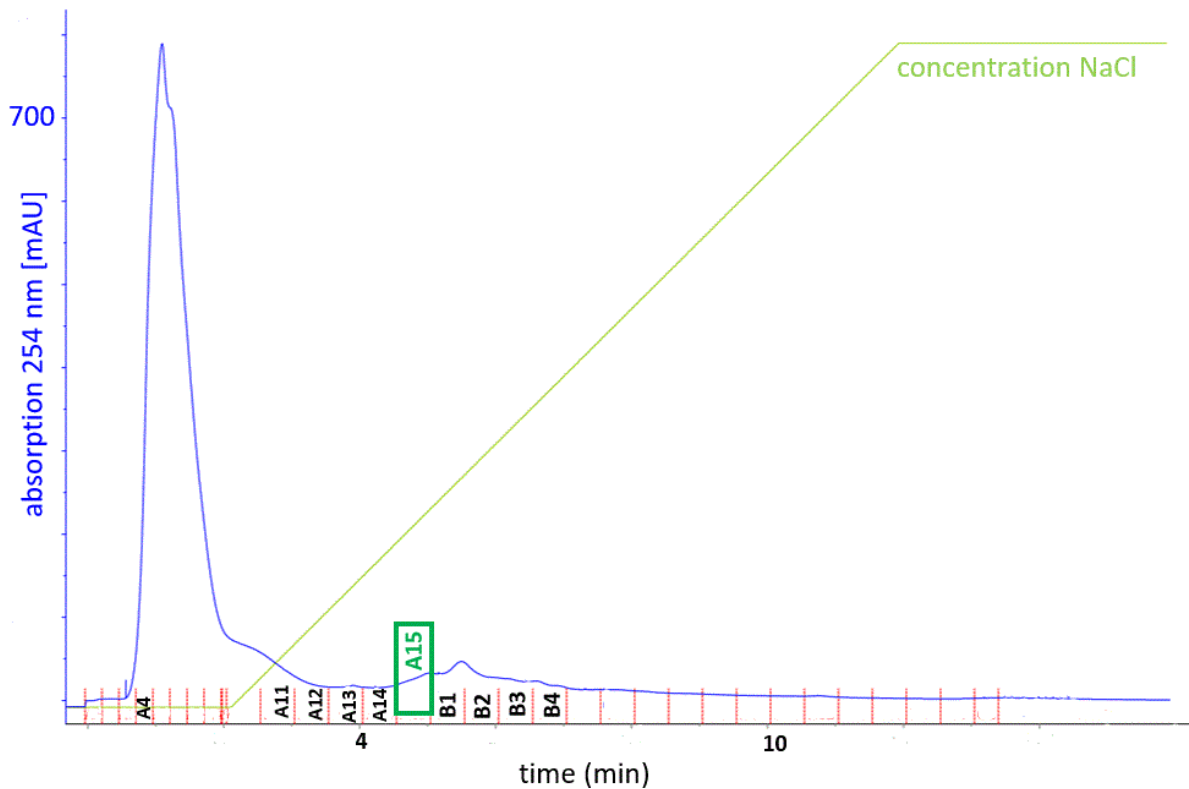
Figure 6 | Typical size exclusion chromatogram of PRGF (a), size exclusion chromatogram of 3 molecular weight markers (b) and relative induction of hBD-2 (c) and hBD-3 (d) by fractions of run a. Ctrl=control, Run. buffer Ctrl= running buffer control, kD= kilo Dalton. c, n=5 independent experiments. d, n=2 independent experiments. Running conditions: Running buffer: 25 mM ammonium acetate in water, flow: 50 μ l/min. Data are mean \pm SD; *P<0.05; **P<0.01; *P<0.001 between selected relevant comparisons, two-tailed unpaired Student's t-test.**

PRGF separation using molecular weight filtration demonstrated that PRGF-derived substances larger 10 kD are inductors of both hBD-2 and hBD-3. Such large molecules are likely protein or peptides. Protein purification from biosamples typically requires multistep protein separation strategies. As bioactivity of PRGF-components was reduced after molecular weight filtration and because molecular weight filtration has the disadvantage of separation into only a limited amount of fractions (here three) separation via size exclusion chromatography (SEC) was performed. This method allows separation according to molecular weight. This experiment aims towards establishing a second separation method besides RP-HPLC and getting further insight into what molecular weight PRGF-derived components have.

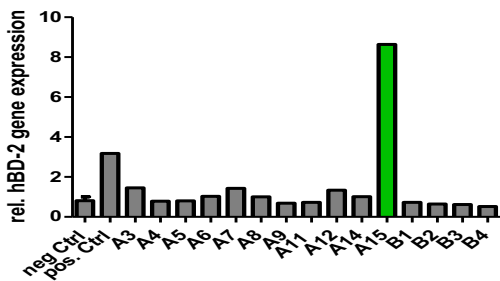
Fig. 6a shows a chromatogram of PRGF separated via SEC. Four larger peaks can be found at 215 nm absorption. The first peak displaying the largest molecules shows one or more shoulders. The latter three peaks display smaller molecules. The second peak also reveals two or more separate peaks at 280 nm wavelength whereas the last two peaks are relatively sharp. As PRGF is based on the cell culture medium KGM 2 pure KGM 2 was also separated via SEC in an independent run. The KGM 2 chromatogram also shows the latter 3 peaks whereas the first peak was not present (data not shown). Thus, the first peak contains only PRGF-derived components. One distinct fraction (A8, green box in Fig. 6a) induced hBD-2 by 35-fold and hBD-3 by 180-fold (Fig. 6c and Fig. 6d). In order to investigate how large the bioactive PRGF-derived components are, three different molecular weight markers were separated in an independent SEC run (Fig. 6b). Pepsin (35 kD), cytochrome c (12 kD) and defensin HNP-2 (3.5 kD) were chosen as molecular weight markers. As the fractions A13-B3 were colored violet this peak could be assigned to cytochrome c. The preceding peak was assigned to the larger molecule (pepsin, 35 kD) and the later peak to the smaller molecule (defensin HNP-2, 3.5 kD).

In summary these results demonstrate that several fractions of PRGF separated via SEC induce hBD-2 and one specific fraction of PRGF separated via SEC induces hBD-3. These fractions contain molecules with the molecular weight of roughly 35 kD or larger.

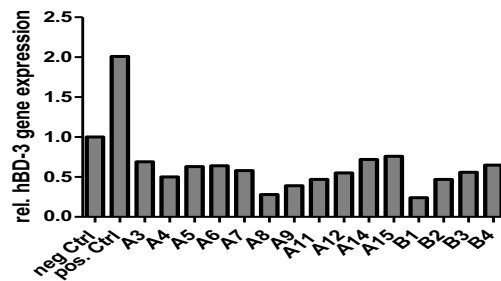
3.1.4 Ion exchange chromatography: Separation via cationic exchange chromatography



a



b



c

Figure 7 | Cationic exchange chromatogram of PRGF (a) and relative induction of hBD-2 (b) and hBD-3 (c) by fractions of the run. Ctrl=control. Running conditions: buffer A: 2-(*N*-morpholino)ethanesulfonic acid (MES) 20mM in water (pH 6.0), B: MES 20 mM plus 1 M sodium chloride (NaCl) in water (pH 6.0).

Separation of PRGF according to hydrophobicity and according to molecular weight were successful. Separation according to molecular charge is another useful separation technic that could potentially complement these two separation methods. Therefore separation via cationic exchange chromatography was performed.

Fig. 7a shows a chromatogram of PRGF separation according to positive charge. The first large peak (flowthrough) displays substances that do not bind to the column under the given separation condition (due to negative charge, e.g.). The two or three later peaks display substances that have bound to the column and are eluted during the run. Peak A15 (green box) shows approximately 8-fold induction of hBD-2 (Fig. 7b, green bar) whereas none of the fractions induce hBD-3 (Fig. 7c).

Consequently, PRGF separation via cation exchange chromatography results in one specific fraction that induces hBD-2.

3.2 Combining PRGF separation technics as a multi-step purification strategy

3.2.1 Ammonium sulfate precipitation followed by size exclusion chromatography and RP-18 separation

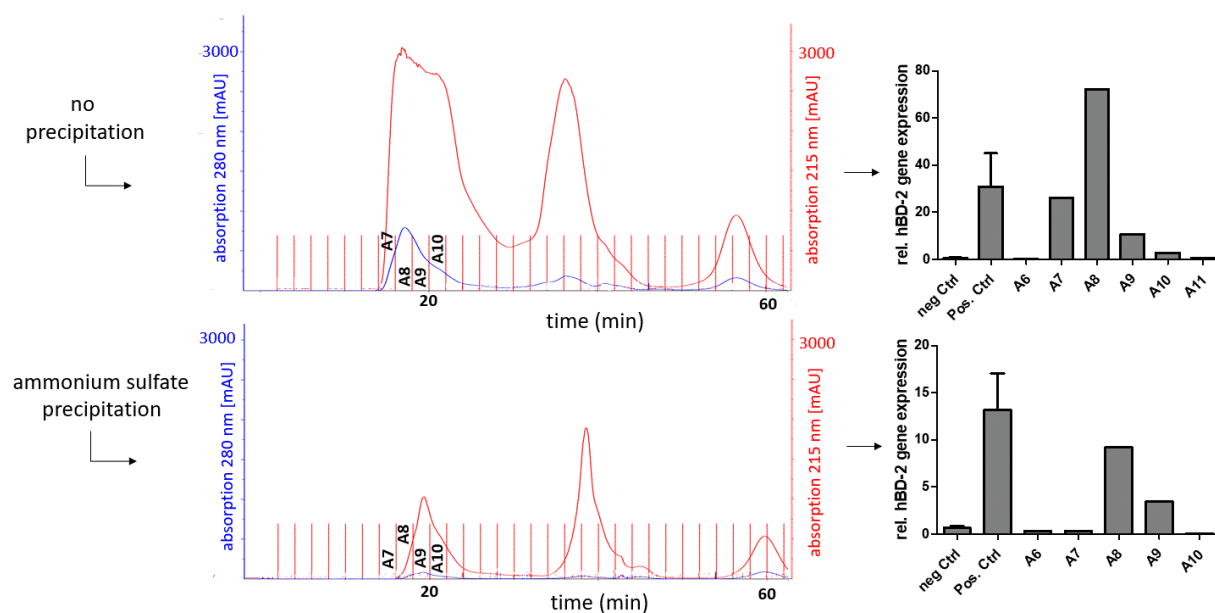


Figure 8 | No precipitation prior to size-exclusion chromatography (upper row) and ammonium sulfate precipitation (corresponding to 30% ammonium sulfate saturation) followed by size-exclusion chromatography (lower row) and relative induction of hBD-2 by fractions of runs. Running conditions: Running buffer: 25 mM ammonium acetate in water, flow: 50 μ l/min. Ctrl=control, data are mean \pm SD.

Precipitation of proteins with ammonium sulfate can be used for selective protein separation. Here PRGF was precipitated using ammonium sulfate followed by consecutive separation via size exclusion chromatography and measurement of bioactivity. Only the PRGF precipitate prepared with an amount

of ammonium sulfate corresponding to 30% ammonium sulfate saturation was bioactive regarding the induction of hBD-2, whereas the other precipitates with higher percentages of ammonium sulfate and the supernatants did not induce hBD-2 (data not shown). Therefore only the resuspended precipitate after the prior precipitation (30% ammonium sulfate saturation) was further analyzed with size-exclusion chromatography.

Fig. 8 shows that detection levels after precipitation were reduced indicating that some substances were successfully precipitated, resuspended and separated according to size whereas other substances had not been precipitated. Bioactivity (here only regarding induction of hBD-2) was reduced after precipitation but still measurable. Further analysis of fraction A9 with RP-18 HPLC (third separation step after precipitation and size exclusion chromatography) revealed low detection levels (not detectable versus background, data not shown).

In summary PRGF precipitation followed by size exclusion chromatography was a successful approach regarding selective precipitation and preservation of bioactivity. However further separation via RP-18 HPLC revealed low detection levels. This indicates that substance yield after these separation steps was too low for final qualitative analysis.

3.2.2 RP-8 separation followed by PR-18 separation

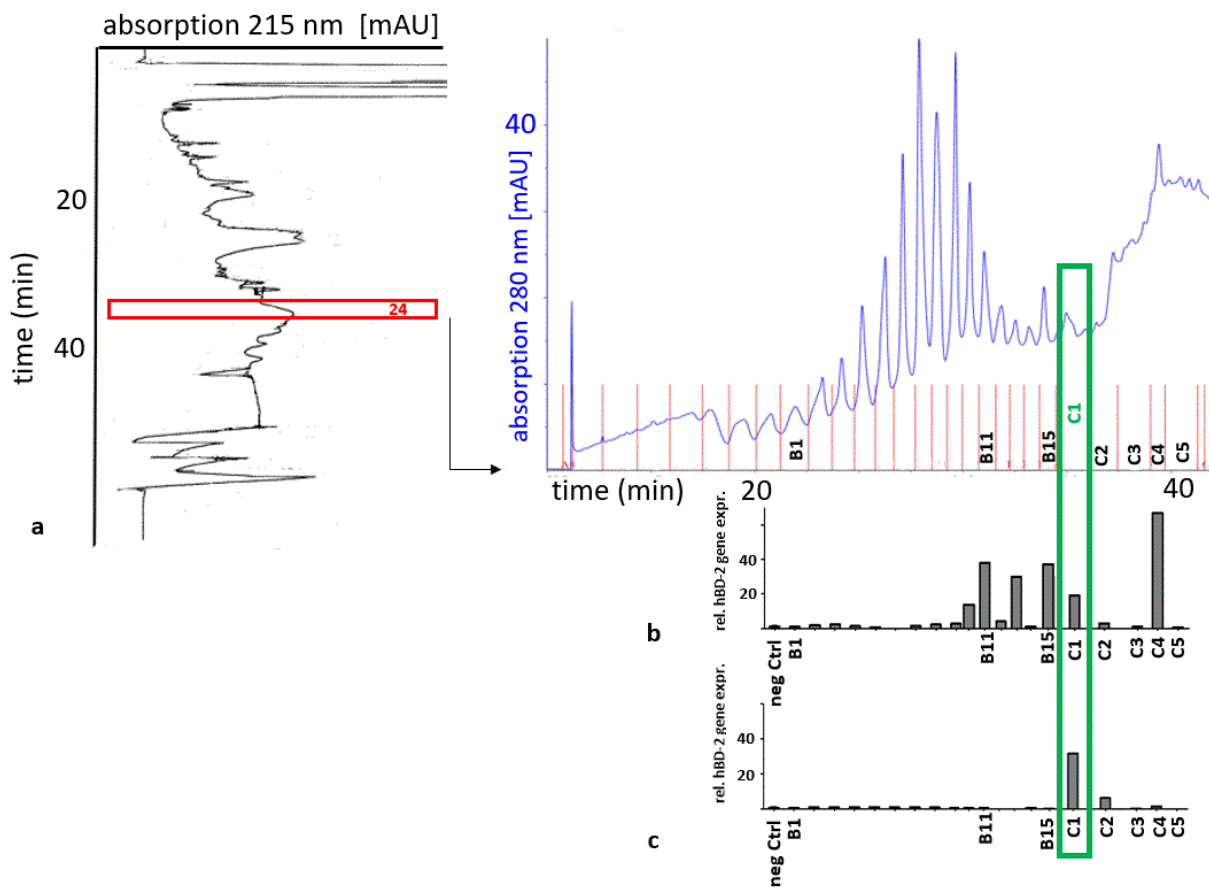


Figure 9 | RP-18 run (a, right) of fraction 24 from Fig. 3A (a, left), relative induction of hBD-2 by fractions of the run (b) and relative induction of hBD-2 by fractions of the run stimulating with half the concentration compared to b (c).

Ctrl=control. a. running conditions: buffer A: H₂O 0.01% TFA, B: 80% isopropanol 0.01% TFA.

PRGF separation using RP-8 columns with mild running conditions (isopropanol, 0.01% TFA) showed relatively poor peak separation. However, bioactivity regarding the induction of both hBD-2 and hBD-3 could be found in distinct fractions of the separation. In two independent experiments both hBD-2 and hBD-3 were induced by specific fractions of the ascending part of the main peak (Fig. 2 and Fig. 3A). In order to further specify these active fractions, fraction 24 from Fig. 3Aa was further analyzed by an RP-18 separation approach (using mild running conditions) and consecutive activity testing.

Fig. 9a (left) shows separation via RP-4 (active fraction 24, red box), Fig. 9a (right) shows several uniformly shaped and pulsed peaks followed by one more irregularly shaped peak (fraction C1) and one late broad peak (fraction C2 onwards). Activity testing (here induction of hBD-2) revealed several active fractions (Fig. 9b). Activity testing was repeated using half the concentration of PRGF-derived

components (Fig. 9c). Here one fraction (C1) induced hBD-2 by approximately 30-fold. The following fraction (C2) induced hBD-2 by approximately 6-fold. Notably activity testing in Fig. 9b revealed induction by fraction C1 by approximately 19-fold for hBD-2 and by approximately 15-fold for hBD-3 although the initial fraction 24 did not induce hBD-3 (data not shown). Fraction C1 was tested by mass spectrometry. Several masses could be found demonstrating that two RP separation steps were not sufficient in regard to substance purity (data not shown).

In summary these findings demonstrate that RP-8 separation followed by RP-18 separation is a PRGF separation strategy that preserves bioactivity regarding the induction of hBD-2. One specific fraction (C1) showing absorption at 280 nm could be collected and induced hBD-2 reproducibly. C1 also induced hBD-3 in one activity testing experiment.

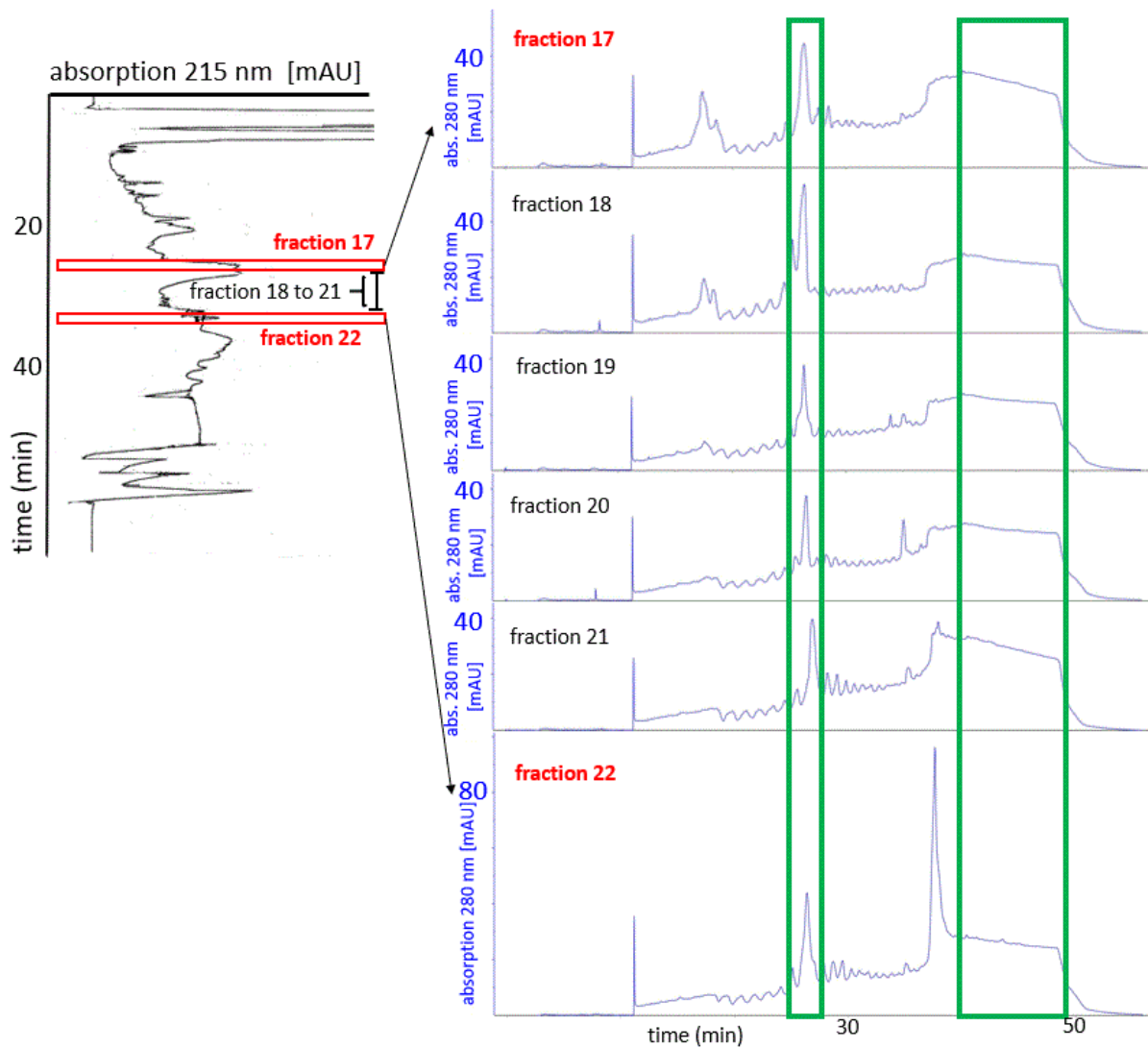


Figure 10 | RP-18 runs (right) of fraction 17 to 22 from Fig. 3A (left), fractions 17 to 22 were active fractions inducing hBD-3, red boxes fraction 17 and 22 (most active fractions). Green boxes: two peaks shared by all RP-18 runs from fraction 17 to 22, running conditions: buffer A: H₂O 0.01% TFA, B: 80% isopropanol 0,01% TFA.

RP-8 separation of PRGF resulted in several hBD-3 inducing fractions (Fig. 3Ac). In order to further analyze these fractions RP-18 separation was performed with each of these active fractions.

Fig. 10 (right) shows six chromatograms of active fractions. Fraction 17 and 22 were the most active fractions. The upmost chromatogram shows fraction 17, the lowest chromatogram shows fraction 22 and the intermediate chromatograms show the according intermediate fractions. All chromatograms share one relatively sharp peak (narrow green box) and one broad peak (wider green box). Active compounds may be located in these peaks. However, fraction 20 induced hBD-3 by only 2-fold and also shows these peaks. Individual fractions of the upper run (fractionated fraction 17) were not active regarding the induction of hBD-3 (data not shown).

Collectively RP-8 separation of PRGF followed by RP-18 separation did not preserve bioactivity regarding the induction of hBD-3.

3.2.3 Size exclusion chromatography followed by PR-18 separation

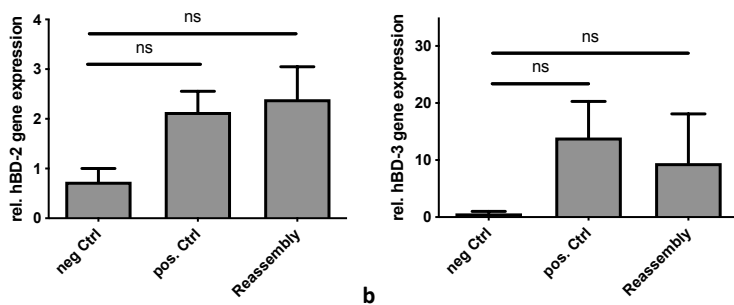
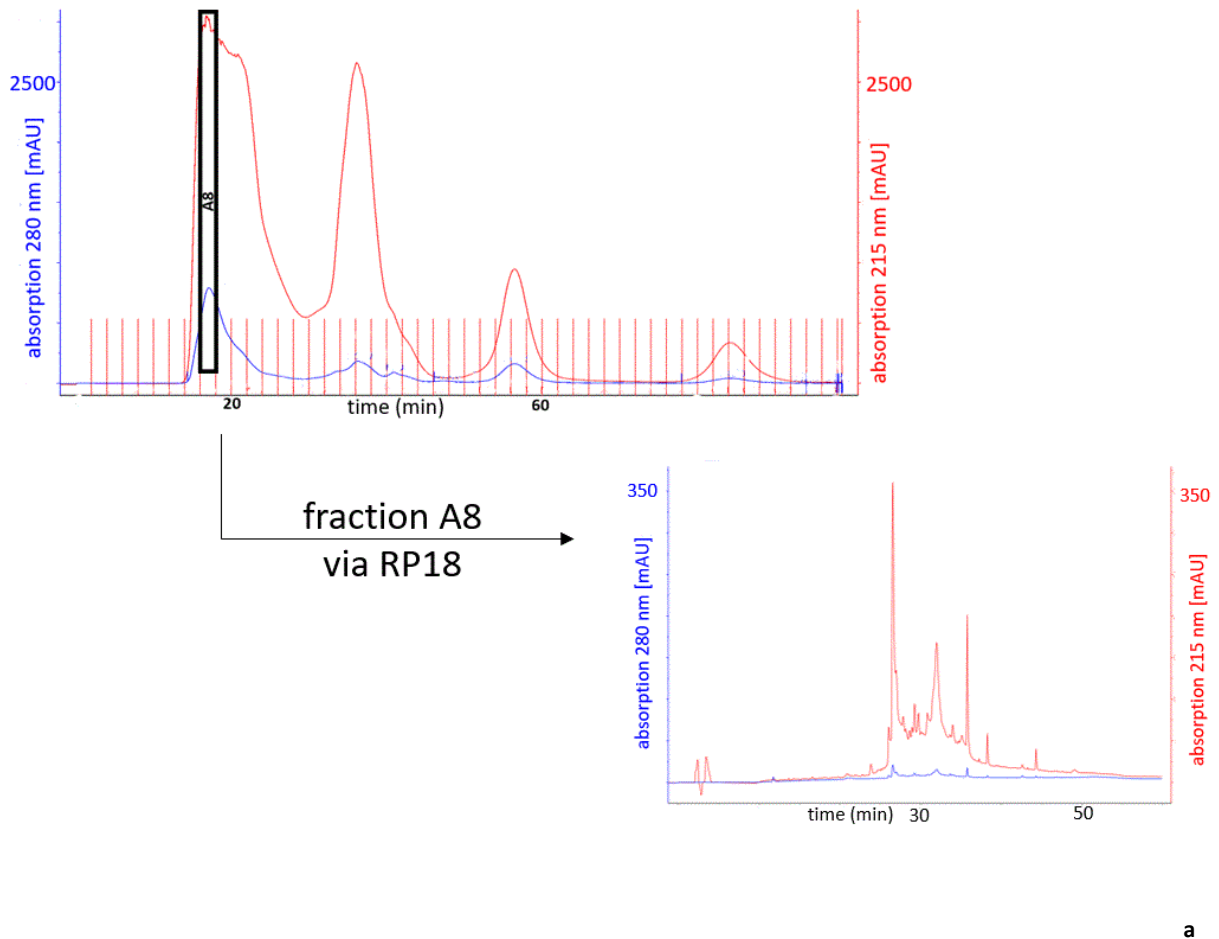


Figure 11 | Size exclusion chromatography (upper) followed by RP-18 separation (lower) of fraction A8 (a), relative induction of hBD-2 (b) and hBD-3 (c) by reassembled fractions of the lower run. Ctrl=control. a. Size exclusion chromatography running conditions: 25 mM ammoniumacetate in water; RP-18 running conditions: buffer A: H₂O 0.05% TFA, B: ACN 0,05% TFA. Data are mean \pm SD; *P<0.05; **P<0.01; ***P<0.001 between selected relevant comparisons, two-tailed unpaired Student's t-test.

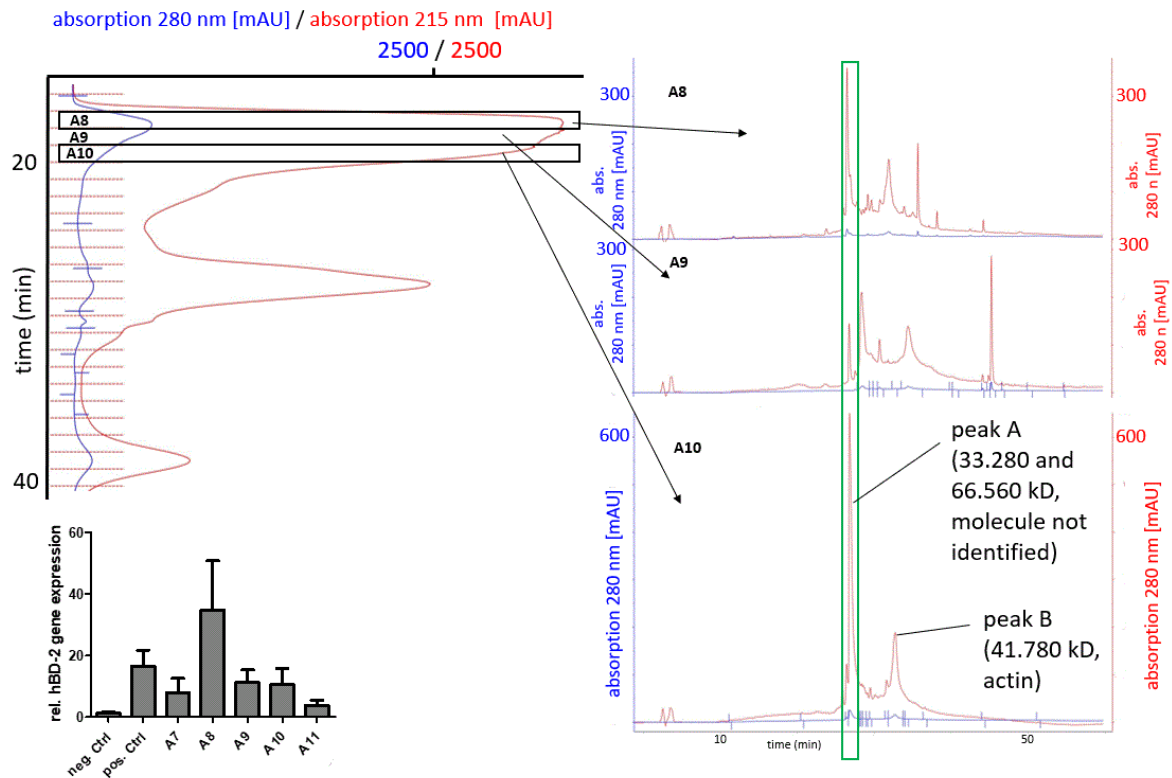
PRGF separation via size exclusion chromatography and via RP-HPLC were successful as isolated separation technics. Therefore, the combination of the two methods was performed. Here size exclusion chromatography was followed by RP-18 separation under non-mild conditions (acetonitrile and 0.05% TFA). Non-mild conditions allow better peak separation. Albeit bioactivity could not reproducibly be preserved when non-mild conditions were applied, RP-18 separation under non-mild conditions was chosen for better peak sharpness. This allows higher resolution determination of how many substances can be found in active fractions. Fraction A8 from size exclusion chromatography was active in five independent experiments and was consequently separated according to hydrophobicity. Fractions from the RP-18 run were pooled (reassembled) and bioactivity regarding the induction of hBD-2 and hBD-3 was tested.

RP-18 separation of active fraction A8 shows a chromatogram with several sharp peaks (Fig. 11a lower) indicating that the fraction contains several substances. However total UV absorption at 280 nm was relatively low compared to the detection level in the size exclusion run. This could be due to substance lost in the separation process (e.g. sedimentation upon acidification with TFA prior to separation). Reassembly of all fractions obtained from the RP-18 run shows low activity regarding induction of hBD-2 and some activity regarding hBD-3 (Fig. 11b and c). However, hBD-3 induction within the experiment was relatively variable (one value high, one value low).

In summary active fraction A8 contains several substances. Overall yields after size exclusion followed by RP-18 separation was relatively low. Bioactivity after these two separation steps could not be found conclusively.

3.3 PRGF separation technics combined with qualitative analysis (identification)

3.3.1 Size exclusion chromatography followed by PR-18 separation and mass spectrometry



a

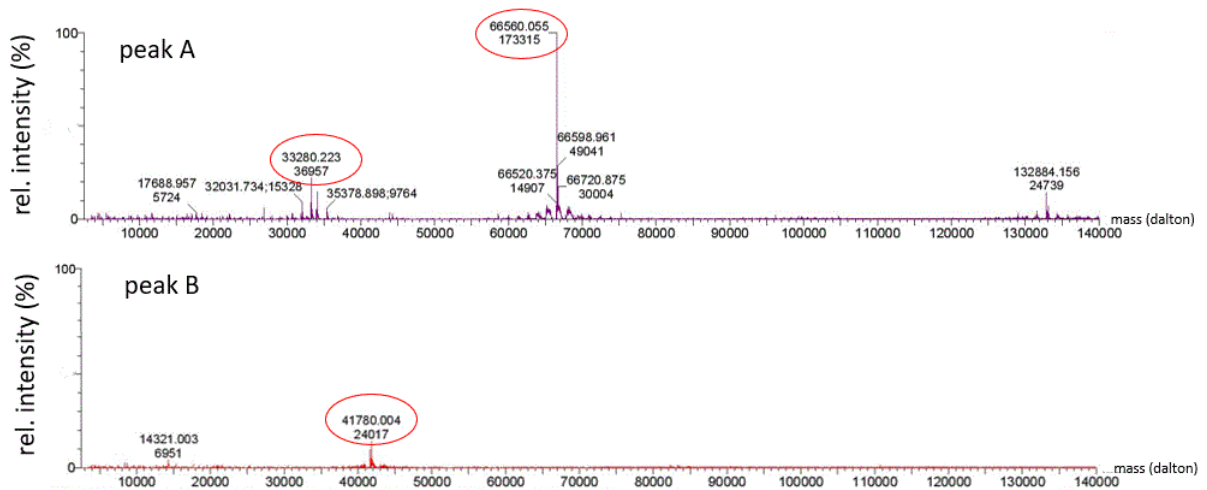


Figure 12 | Size exclusion chromatogram (a, upper left) followed by RP-18 separation (a, three chromatograms, right) of fraction A8, A9 and A10, relative induction of hBD-2 by fractions of the size exclusion chromatogram (a, lower left), mass spectrometry of peak A and peak B (b). SEC running conditions: Running buffer: 25 mM ammonium acetate in water, flow: 50 μ l/min. RP-18 running conditions: running buffers: A: H₂O 0.05% TFA, B: ACN 0,05% TFA; green box: peaks shared by all analyzed fractions. Data are mean \pm SD.

PRGF separation via size exclusion chromatography resulted in several hBD-2 inducing fractions with one fraction displaying 40-fold relative activity (A8, Fig. 6c). The following two fractions show relative induction by around 10-fold (A9 and A10). In order to further specify these fractions RP-18 separation using non-mild running conditions was performed. Qualitative analysis using mass spectrometry with and without trypsin proteolysis was performed for final substance identification.

RP-18 chromatograms of the fractions A8, A9 and A10 (Fig. 12a right) share one peak (green box). The others peaks are not definitively found in all three chromatograms simultaneously. As all three fractions show some bioactivity regarding the induction of hBD-2 the shared peak is a candidate for a hBD-2-inducing substance. Mass spectrometry of the two peaks from fraction A10 (peak A and peak B) revealed two dominant masses (33.280 kD and 66.560 kD peak A and 41.780 kD peak B). Trypsin proteolysis of these peaks resulted in identification of actin (peak B). Analysis of peak A did not result in a specific molecule.

These results show that identification of molecules derived from PRGF using size exclusion chromatography and RP-18 separation followed by qualitative analysis using trypsin proteolysis is possible as the molecule actin could be identified. However, another peak (peak A), candidate for an hBD-2 inductor, could not be identified using this approach.

3.3.2 Size exclusion chromatography followed by mass spectrometry

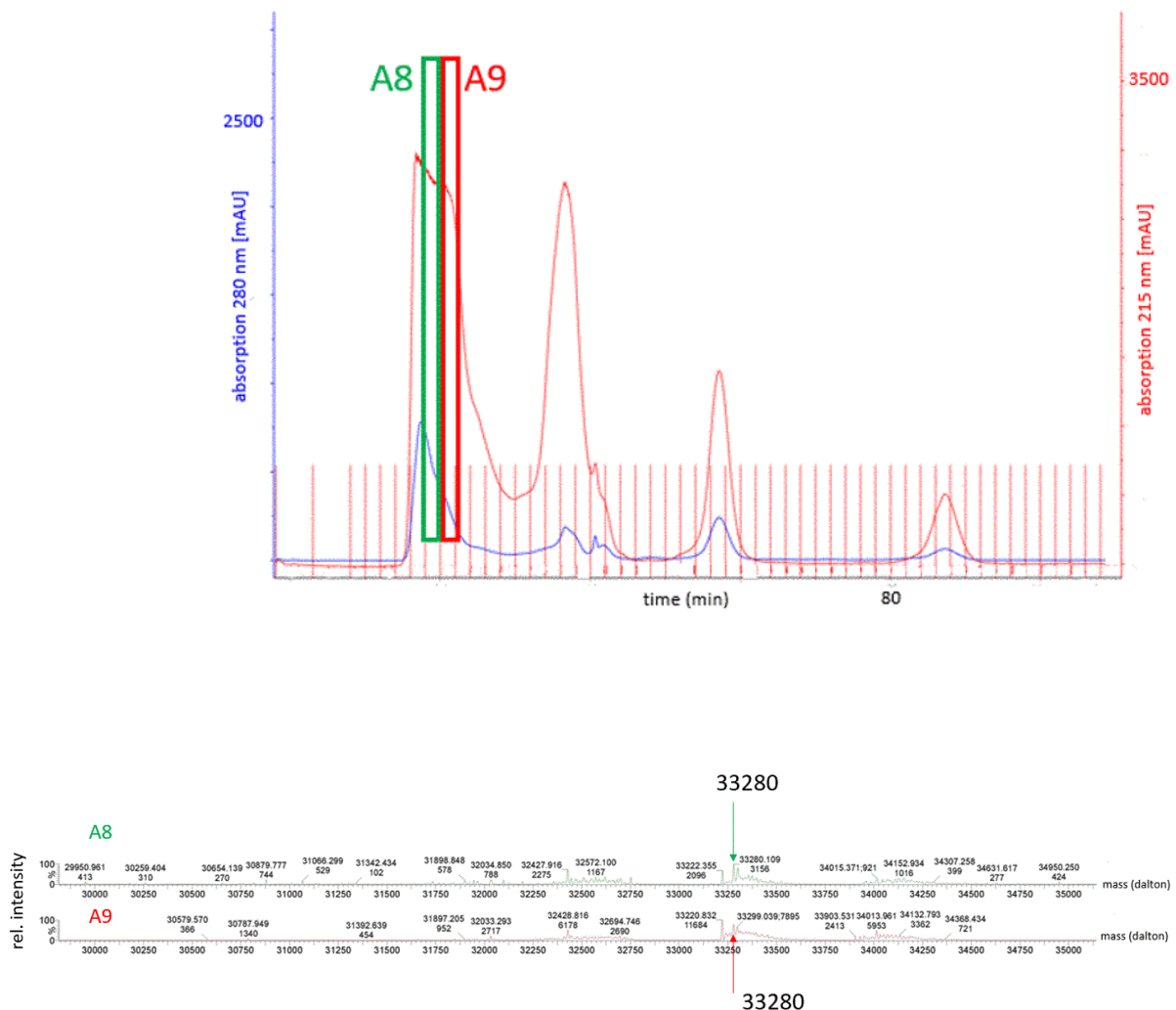


Figure 13 | Size exclusion chromatography followed mass spectrometry of fractions A8 and A9. Running conditions: Running buffer: 25 mM ammonium acetate in water, flow: 50 μ l/min. Green box: Fraction A8, red box fraction A9.

To verify whether fractions preceding fraction A10 also contain the candidate substance with the molecular weight 33.280 kD, PRGF-fractions obtained from size exclusion chromatography were analyzed with mass spectrometry.

Fig. 13 shows PRGF separation via size exclusion chromatography. Fractions A8 and A9 also contain molecules with the mass 33.280 kD (arrows). Note that fraction A8 does not correspond perfectly with fraction A8 from Fig. 12. Fractions are shifted slightly. Therefore, fraction A8 from Fig. 13 corresponds more accurately to fraction A9 from Fig. 12.

These results demonstrate that a candidate substance with the molecular weight of 33.280 kD can be found in an active fraction of two independent experiments (different PRGF from different donors).

4 Discussion

Purifying specific substances from crude biomaterials can be challenging. For instance the purification of platelet-derived growth factor (PDGF) from platelet rich plasma required a five step purification strategy combining several different separation methods [23]. Each separation step needs to fulfill the following criteria: sufficient substance yield (either for further separation or for final identification, here: detection level after separation) and preservation of bioactivity. Also, the separation itself has to be feasible in terms of successful removal of unwanted substances from the biosample. Here separation technics were evaluated with regard to the mentioned criteria. PRGF induction of both hBD-2 and hBD-3 is at least partially mediated via the epidermal growth factor receptor (EGFR) [15], [16]. As many EGFR ligands are proteins [24] applied separation methods should be suitable for protein separation.

Reversed phase HPLC (RP-HPLC) is an established separations technique that offers extremely high resolution. In principal the method is suitable for protein separation with excellent peak separation and high substance recovery [25]. Here RP-4 separation of PRGF was performed using standard separation conditions (running buffer acetonitrile (ACN) and water, 0.05% trifluoroacetic acid, Fig. 1 a, d, g). Detection levels were high, however peak separation was relatively poor and bioactivity could not be found reliably after separation (Fig. 1b-c, e-f, h-i). Isopropanol may have less effect on bioactive PRGF components compared to acetonitrile and less acidic running conditions could likewise preserve bioactivity. PH-values during separation were determined empirically (Fig. 4a, b). Isopropanol/water running buffer reduced acidity by 20-30-fold compared to ACN/water running buffer. Reducing TFA concentration from 0.05% to 0.01% further reduced acidity by 5-fold (Fig. 4c, d). Therefore, running buffer isopropanol/water combined with 0.01% trifluoroacetic acid was evaluated. Two independent experiments applying these milder conditions resulted in reproducible preservation of bioactivity of PRGF-derived component regarding the induction of hBD-2 and hBD-3. When bioactivity was lost in a third experiment this might have been due to weak responsiveness of the cells that were used (weak induction by positive control, Fig. 3Bc, e.g.). Active fractions always displayed detectable levels of substances while peak separation was acceptable (Fig. 3Ba, e.g.). In order to further purify PRGF components initial RP-8 separation was followed by an RP-18 separation step (Fig. 9). Bioactivity regarding both the induction of hBD-2 and hBD-3 could be found. Active fractions displayed low but measurable detection levels. One specific peak was further analyzed by mass spectrometry. Final substance identification was not possible using this separation approach as several masses were detected. This demonstrates that substance purity was not high enough.

In summary RP-HPLC could be established as a separation method for PRGF components that induce hBD-2 and hBD-3 and is suitable for a PRGF separation strategy.

Platelets contain a wide variety of substances. Some of which are small molecules such as serotonin (176 Dalton) [26] others large molecules such as Ral GTP-binding protein (27 kD) [27]. Molecular weight filtration (Fig. 5) demonstrated that hBD-2 induction was mainly promoted by substances larger 10 kD. However, hBD-2 induction levels by PRGF-derived substances larger 10 kD was approximately 4-fold lower compared to unseparated PRGF. This is either due to substance loss during molecular weight filtration or due to cooperative effects of molecules larger 10 kD and molecules smaller 10 kD. Interestingly reassembly of PRGF components larger 10 kD and smaller 10 kD after molecular weight filtration partially restored the hBD-2 induction capacity. Substance loss possibly in combination with cooperative effects of molecules larger 10 kD and molecules smaller 10 kD is responsible for this observation. When pure PRGF was treated in the same manner (temperature, centrifugation) as the PRGF separated by molecular weight filtration hBD-3 induction was reduced at least by 3-fold (Fig. 5c). Induction of hBD-3 by PRGF molecules larger 10 kD was even further reduced compared to the treated positive control. However, hBD-3 induction was still measurable. Cooperative effects of small molecules and large molecules as observed for hBD-2 induction were not observed for hBD-3 induction. PRGF directly or indirectly induces both defensins at least partially via the EGRF [15], [16]. These results indicate that both hBD-2 and hBD-3 are induced by PRGF derived substances larger 10 kD that are likely proteins. These proteins might be EGRF ligands.

Although induction levels overall were reduced both for the induction of hBD-2 and hBD-3 likely due to the separation process itself molecular weight filtration can be considered in a multi-step PRGF separation strategy as bioactivity could be preserved in general.

Size exclusion chromatography (SEC, Fig. 6a, c, d) confirmed that molecules larger 10 kD are inductors of both hBD-2 und hBD-3. Molecular weight markers (Fig. 6b) indicate that these inductors are even larger (>30 kD). Detection levels in active fractions were high (high yield) and bioactivity for both defensins was overall higher than measured in the positive control. As expected for SEC as a method in general peak separation was not as accurate compared to RP-HPLC.

SEC can be used as a separations step for PRGF purification. Particularly it could be considered as a last polishing step e.g. to separate proteins from small molecules such as running buffer components as KGM 2 components were successfully separated from active PRGF components.

Cationic exchange chromatography has been used successfully as a separation method for protein isolation from platelets [23]. Here cationic exchange chromatography was applied resulting in one specific detectable peak that induced hBD-2 but not hBD-3 (Fig. 7). One large peak, the flowthrough of

unbound substances, was not active. Hence the method offers potential in regard to the wanted effect of isolating active components from a large quantity of inactive molecules. To investigate the method's suitability for PRGF separation in more depth, further separations using cationic exchange chromatography will be necessary.

Precipitation of proteins has been shown to be a useful method in a multi-step separation strategy. Interleukin (IL)-1 β has successfully been isolated from *E. coli* supernatant through several separation steps including ammonium sulfate precipitation followed by size exclusion chromatography (SEC) [28]. PRGF precipitation followed by SEC resulted in reduced total substance yield compared to non-precipitated PRGF separated merely by SEC. HBD-2 activity was found in a distinct fraction (Fig. 8), whereas hBD-3 induction was not observed. One active fraction was further analyzed by RP-18-HPLC separation. After this third separation step no substances could be detected.

Ammonium sulfate precipitation of PRGF was not further investigated. Relevant substance loss was observed after precipitation. Although bioactivity regarding the induction of hBD-2 was preserved, final substance recovery was not successful after two additional purification steps.

In summary RP-HPLC, SEC and cationic exchange chromatography demonstrated suitability for PRGF separation, whereas molecular weight filtration as well as ammonium sulfate precipitation could not be established for PRGF separation due to low substance yield or largely reduced bioactivity after separation. In order to further purify hBD-2- and hBD-3-inducing substances derived from PRGF combinations of the suitable separation techniques were investigated.

In two out of three RP-8 runs hBD-2 induction was found in the ascending part of the main peak (Fig. 3Aa, c, e.g.). Further separation via RP-18 of these active fractions revealed one specific peak that reproducibly induced hBD-2 (Fig. 9b, c). HBD-3 induction was not found after these two RP-HPLC separation steps (Fig. 10). These findings must be confirmed in additional identical experiments in order to evaluate if two-step RP-HPLC is reproducibly suitable for PRGF separation.

SEC separation of PRGF resulted in several active fractions in regard to induction of hBD-2 (Fig. 6c). One fraction (A8) was most active whereas adjacent fractions were less active. However, fractions A7, A9 and A10 were active regardless. For hBD-3 the most active fraction was A8 with no activity found in preceding and following fractions (Fig. 6d). The most active fraction A8 was further analyzed using RP-18 separation (Fig. 11a). The RP-18-chromatogram reveals several sharp peaks demonstrating that fraction A8 contains several distinct substances. These findings are expected as SEC is a separation method with low sharpness in regard to peak separation. Also, substance yield was low compared to substance yield observed in fraction A8. This indicates that substance loss arises by the RP-18 separation itself e.g. substances remain bound to the column or are precipitated upon acidification

prior to the separation. All fractions of the RP-18 separation were collected and reassembled. Activity for both hBD-2 and hBD-3 was not found conclusively (Fig. 11b, c). Notably RP-18 separation was performed using non-mild running conditions (standard acid concentrations and acetonitrile running buffer). Further experiments combining SEC and RP-HPLC need to show if the combined separations steps are suitable for PRGF fractionation in regard to preservation of bioactivity. Mild RP-HPLC-running conditions are favorable in this setting as substance yield was low and bioactivity was not preserved when not-mild running conditions were chosen. Further these results demonstrate that SEC is more suited as a polishing step after initial separation steps as SEC followed by RP-HPLC revealed that the most active fraction both in regard to induction of hBD-2 and hBD-3 consists of several distinct substances (low purity after merely SEC separation). Four fractions from SEC were active in regard to induction of hBD-2. Three of these fractions were analyzed further (Fig. 12 a). Fraction A8 contained at least eight distinct substances as at least eight peaks can be distinguished (Fig. 12 right side upper chromatogram). Likely fraction A8 contains even more than eight substances, as some peaks have shoulders and could contain more than one substance and as some substances are precipitated prior to separation by acidifying the sample. Fraction A9 contains at least five substances (Fig. 12 right, middle chromatogram), some of which do not appear in fraction A8. Fraction A10 shows a minimum of two substances (Fig. 12 right, lower chromatogram). As all three fractions (A8, A9 and A10) induced hBD-2 common peaks are candidate peaks for hBD-2 inducers. Only one peak could clearly be found in all three fractions (peak A, green box). If this peak includes a specific hBD-2 inducer the peak size should be largest in the peak found in fraction A8. However, the peak size of peak A in fraction A10 is at least as large as the corresponding peak in fraction A8. The two peaks found in fraction A10 were further analyzed by mass spectrometry. In peak A two leading masses were found: 33.280 kD and 66.560 kD. As the latter mass is double of the lower mass these masses could indicate that the identified molecule is present as a monomer and as a dimer. Peak B revealed one leading mass: 41.780 kD. Further analysis of both peaks using enzymatic digestion followed by comparison with protein fingerprint database revealed actin in peak B. The protein from peak A could not be identified either due to the fact that the specific protein is not implemented in the data base or due to disulfide bounds within the protein. Larger, possibly incompletely digested fractions, found in peak A (data not shown) could be a sign of disulfide bounds as tertiary structure element of the proteins found in the peak. For hBD-3 activity was only found in fraction A8. Candidate peaks are therefore the non-common peaks from the three fractions A8, A9 and A10 (not peak A). One explanation for this could be the existence of distinct PRGF-derived inducers of hBD-2 and hBD-3. As distinct fractions after RP-HPLC separation (Fig. 3A and Fig. 3B) and after cationic exchange chromatography separation (Fig. 7) induced either hBD-2 or hBD-3 (not both) the existence of distinct hBD-2 and hBD-3 inducers in PRGF is further supported by these findings. Crucially none of the peaks derived from fraction A8, A9 or A10 have been

shown to be bioactive in regard to induction of either of the two defensins. Bioactivity was not tested for these peaks as reassembly of all peaks from the most active fraction A8 did not induce either of the defensins conclusively (Fig. 11). Fraction A8 and fraction A9 were directly analyzed by mass spectrometry. Both fractions revealed many masses as expected from previous experiments showing that many molecules are present in both fractions. Among these masses the previously found mass 33.280 kD was found in both fractions (Fig. 13). Further experiments are necessary to show that combined RP-HPLC and SEC preserves bioactivity before attempting to identify substances using mass spectrometry.

Based upon these findings a multi-step separations strategy sequence for hBD-2 could be:

Cationic exchange chromatography → RP-8-HPLC → SEC → MS (identification).

For hBD-3 the following sequence could be considered:

RP-8-HPLC → RP-18-chromatography → SEC → MS (identification).

5 Abstract

The use of formulations based on thrombocyte concentrate lysates (platelet-released growth factors, PRGF) revealed promising effects in the treatment of chronic wounds. The underlying mechanisms are not well understood. We could show that PRGF induced the expression of antimicrobial peptides (AMPs) such as the human beta-defensin-2 and -3 (hBD-2, hBD-3) in keratinocytes. This may contribute to the observed beneficial effects of PRGF-based formulations in wound treatment. Given that it is unclear which components of platelets induce AMPs in keratinocytes we aimed to biochemically identify PRGF-derived factors that induce hBD-2 and hBD-3 in human keratinocytes. As PRGF contain a wide variety of different molecules we first investigated if larger (>10 kD), medium (10 to 3kD) or smaller (<3 kD) molecules induced hBD-2 and hBD-3. Using molecular weight filtration, we found that the fraction >10 kD was most active although induction levels were lower compared to native PRGF. Notably, reassembly of all three fractions (>10 kD, 10 to 3 kD and <3 kD) restored the effect of the native PRGF indicating a synergistic action of different PRGF-components to induce hBD-2 in keratinocytes. For hBD-3 reassembly did not restore the effect. Gel filtration experiments combined by mass spectrometric analyses revealed the presence of several proteins between 30-40 kD in the hBD-2 and the hBD-3 inducing fraction. Further purification of the hBD-2 inducing protein(s) by conventional reversed-phase (RP)-HPLC failed due to acid labile properties of the hBD-2-inducing molecules. Optimized RP-HPLC separation strategies using low acidic conditions revealed a distinct hydrophobic fraction containing hBD-2- but no hBD-3-inducing activity. Interestingly, a less hydrophobic fraction contained hBD-3-, but no hBD-2-inducing activity suggesting that PRGF contains distinct proteins that selectively induce hBD-2 and hBD-3 in keratinocytes. Using sequential two-step RP-HPLC separation one distinct fraction reproducibly induced hBD-2 and is a candidate fraction for further analysis such as final compound identification. These experiments are the basis for the final identification of PRGF-derived mediators that support healing of chronic wounds by their capacity to induce the expression of AMPs in keratinocytes. These mediators could be promising lead substances for the development of drugs that promote wound healing and display anti-infective properties via a novel mechanism that induces defensins in human skin.

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