Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 791 (2003) 345–356
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Human hemoglobin-derived peptides exhibit antimicrobial activity: a class of host defense peptides

Cornelia Liepke*[,1], Susann Baxmann, Cornelia Heine, Nicole Breithaupt, Ludger Ständker, Wolf-Georg Forssmann

IPF PharmaCeuticals GmbH, Feodor-Lynen-Strasse 31, D-30625 Hannover, Germany

Received 23 December 2002; received in revised form 13 February 2003; accepted 13 March 2003

Abstract

Hemoglobin is a known source of biologically active peptides with various functions. In the present study, we report for the first time the existence of natural processed hemoglobin fragments exhibiting antimicrobial activity in humans. Two antimicrobial hemoglobin-derived peptides were purified from a human placental peptide library by consecutive chromatographic steps tracking the maximum growth inhibitory activity against *Escherichia coli* BL21. These peptides, consisting of 17 and 36 amino acid residues, were identified as being C-terminal fragments of γ-hemoglobin and β-hemoglobin, respectively. The antimicrobial β-hemoglobin fragment was also purified from lysed erythrocytes, demonstrating that proteolytic degradation of hemoglobin into small bioactive peptides already starts inside erythrocytes. The identified peptides inhibit the growth of Gram-positive and Gram-negative bacteria and yeasts in micromolar concentrations. Moreover, by LPS-binding, the β-hemoglobin fragment reduces biological activity of endotoxins. In contrast, even at high concentrations, the identified antimicrobial hemoglobin peptides do not exhibit toxic activity on human primary blood cells. We conclude that antimicrobial hemoglobin-derived peptides could be important effectors of the innate immune response killing microbial invaders.

Keywords: Peptides

1. Introduction

In addition to its primordial function as an oxygen carrier, hemoglobin is also a source of endogenous bioactive peptides [1]. The first hemoglobin-derived peptide with a discrete biological function was discovered in 1971 by Schally et al. [2]. They isolated a decapeptide with growth hormone-releasing activity from pig hypothalamus, which was identified as a 1–10 fragment of the β-chain of hemoglobin. In the following years, several hemoglobin-derived peptides with different biological functions were identified. The analgesic peptides kyotorphin [3] and neokyotorphin [4] corresponding to the 140–141 and 137–141 C-terminal sequences of α-globin were discovered in 1979 and 1982 from

*Corresponding author. Tel.: +49-511-5466-201; fax: +49-511-5466-132.
E-mail address: c.liepke@ipf-pharmaceuticals.de (C. Liepke).
[1]Cornelia Liepke and Susann Baxmann contributed equally to this work.

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00245-9

bovine brain. Brantl et al. [5] detected that proteolytic treatment of hemoglobin results in a series of peptides exhibiting opioid-like activity, called hemorphins. Since these findings, systematic studies of peptide composition of various tissue extracts have resulted in a large number of new peptides with a structure mainly originating from α- and β-globin chains [1]. Some of these peptides correlate with various disorders such as human lung adenocarcinoma [6], Alzheimer's disease [7], Hodgkin's disease [8] and brain ischemia [9] or even occur subsequently to physical exercise like long distance running [10]. In the latter study, it was noteworthy that the plasma level of the released hemorphin-7 exceeded that of β-endorphins by a factor of about 1000. Furthermore, it was shown that hemoglobin-derived opioid peptides have the capacity to release the classical opioid peptides from the pituitary gland into the circulatory system [11]. Therefore, there is increasing evidence that hemoglobin could serve in vivo as a powerful source of bioactive peptides playing a profound role in homeostasis [1].

In 1999, Fogaca et al. described the discovery of an antimicrobial peptide in the gut content of the cattle tick *Boophilus microplus* [12]. The peptide was identified as a fragment of bovine α-hemoglobin. They proposed that ticks utilize the host protein hemoglobin for their own defense against microorganisms. Recently, Froideveux et al. [13] described the isolation of another hemoglobin fragment with antibacterial activity derived from the N-terminus of bovine α-hemoglobin. The peptide was generated in vitro by peptic digestion of bovine hemoglobin [13]. In a more recent study, Parish et al. [14] show antimicrobial activity of human intact hemoglobin tetramers, of the separated alpha and beta subunits, and identified the carboxyl terminal 30 amino acids of the beta subunit as responsible for significant antimicrobial activity by mapping specific regions of the hemoglobin molecule using synthetic peptides. Up to now, it has not been known whether hemoglobin-derived peptides with antimicrobial function naturally occur in humans. Therefore, we screened for antimicrobially active compounds in human tissues. In the present work, we report the identification of antimicrobial hemoglobin-derived peptides from human placental tissue. Moreover, we present strong evidence that hemoglobin was already degraded inside erythrocytes into small peptides exhibiting antimicrobial activity.

2. Experimental

2.1. Preparation of a peptide library from human placenta

Human placental tissue (11 kg) obtained from healthy individuals in a maternity ward of a local hospital, was processed immediately after delivery and extracted in ice-cold 0.5 *M* acetic acid containing 10 m*M* ascorbate and 0.5 m*M* ethylenediaminetetraacetic acid (EDTA). The extract was homogenized with a blender for 2 min and the homogenate was stirred at 100 rpm overnight at 4 °C. After centrifugation (15 600×*g*, 20 min, 4 °C), the supernatant was filtered (7–12 μm, Schleicher and Schuell paper No. 1574, Dassel, Germany) and subsequently ultrafiltered using a Sartocon-Mini cross-flow system (0.1 m^2, polysulfone membrane, M_r cut-off: 50 kDa). The ultrafiltrate adjusted to pH 2.5 was subjected to cation-exchange chromatography enriching cationic peptides. It was loaded onto a strong cation-exchanger (Fractogel TSK SP 650 (M), 250×32 mm, Merck, Darmstadt, Germany) and peptides were subsequently eluted stepwise using consecutive buffers with increasing pH (pH 2.5–7.0) resulting in five fractions, designated as pH pools 0–4. Further subfractioning of each pH pool was done by reversed-phase (RP) chromatography. Each pH pool was applied to an RP column (Fineline RPC Source, 20×15.5 cm, 15 μm, Pharmacia, Freiburg, Germany) and subsequently eluted in a 60 min linear gradient from 0 to 50% B (solvent A: 0.01 *M* HCl, solvent B: 80% acetonitrile/0.01 *M* HCl). The collected fractions were lyophilized and stored at −20 °C, designated the peptide library.

2.2. Erythrocyte lysis in vitro

Peripheral venous blood was obtained from healthy volunteers. Erythrocytes were isolated from citrate-anticoagulated blood and separated from plasma by centrifugation at 1500×*g* for 10 min at 20 °C. The pellet obtained was washed twice with phos-

phate-buffered saline (PBS). For lysis of the isolated erythrocytes, the cell sediment was subjected to two cycles of freezing (−80 °C) and thawing (4 °C). Subsequently, at 4 °C cells were homogenized by sonication (20 min, 17 W) and centrifuged (10 000×*g*, 15 min). The supernatant was collected and stored at −20 °C. For HPLC purification, 2 ml of lysate were diluted with 200 ml deionized water and filtered (0.45 μm, Schleicher and Schuell, Dassel, Germany).

2.3. Peptide analysis

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) was carried out using a LaserTec RBT II (PerSeptive Biosystems, Framingham, MA, USA). The instrument is equipped with a 1.2 m flight tube and a 337 nm nitrogen laser. Positive ions are accelerated at 30 kV and up to 64 laser shots are automatically accumulated per sample position. α-Cyano-4-hydroxy-cinnamic acid (CHC, Sigma–Aldrich, Deisenhofen, Germany) was used as matrix. Accuracy of mass measurement was within 0.5%.

Purity of peptides was analyzed by capillary zone electrophoresis (CZE) with a model P/ACE 2100 CZE system (Beckman, Munich, Germany). Amino-acid sequencing was carried out on a gas phase sequencer (model 473 A, Applied Biosystems, Weiterstadt, Germany) by Edman degradation using the fast cycle protocol as recommended by the manufacturer.

2.4. Peptide synthesis

Peptides (hHEM-β (111–146), hHEM-γ (130–146) and MBI-28) were synthesized on an ABI 433A peptide synthesizer (Perkin-Elmer, Wellesley, MA, USA) using Fmoc solid-phase chemistry on a preloaded TentaGel F-PHB histidine TRT resin (Rapp Polymere, Tübingen, Germany). Crude peptides were purified by reversed-phase HPLC (conditions: column: Vydac C_{18}, 4.6×250 mm, 10 μm, 300 Å, MZ-Analysentechnik, Mainz, Germany; solvent A: 0.07% TFA/water, solvent B: 0.05% TFA in 80% acetonitrile; gradient: 10–70% B in 30 min, flow-rate: 0.8 ml/min, UV detection: 215/230 nm). Purity and identity of synthesized peptides were checked by analytical HPLC, mass spectrometry, and sequence analysis. Peptide content was quantified by amino acid analysis.

2.5. Antimicrobial assays

2.5.1. Microorganisms

Escherichia coli BL 21 was obtained from J. Alves (Hannover Medical School) and *Pseudomonas aeruginosa* BST from I. Steinmetz (Hannover Medical School). *Staphylococcus carnosus* TM 300 was kindly provided by F. Goetz, University of Munich. *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* (VRE) ATCC 51299, *Klebsiella pneumoniae* ATCC 10031 and *Saccharomyces cerevisiae* ATCC 9763 were purchased from the ''Deutsche Sammlung für Zellkulturen und Mikroorganismen'' (DSZM, Braunschweig, Germany).

2.5.2. Radial diffusion assay

For detection of antimicrobial activity in HPLC fractions, a sensitive radial diffusion technique was used as described earlier [15]. Lyophilized HPLC fractions were dissolved in sterile water and aliquots tested against *Escherichia coli* BL21 using a solid agarose medium. The agarose layer consisted of 30 mg/100 ml Tryptic Soy Broth (TSB, Sigma, Deisenhofen, Germany) in 10 m*M* sodium phosphate buffer (pH 7.2) with 0.02% Tween 20 (Sigma, Deisenhofen, Germany) and 0.8% GTG agarose (BioWhittaker Molecular Applications, Rockland, ME, USA). The plates were incubated at 37 °C for 16 h and diameters of inhibition zones were recorded.

2.5.3. Minimum inhibitory concentration (MIC)

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the antimicrobial peptides identified. Twofold serial dilutions of peptides were prepared in test medium containing 3 g/l TSB in 10 m*M* sodium phosphate buffer (pH 7.2). A standard inoculum of 50 μl bacteria suspension in test medium containing 2×10^5 to 5×10^5 CFU/ml was added to each well, containing 50 μl of the prepared peptide solution. After incubation for 16 to 20 h at 37 °C, bacterial growth was determined by visual analysis and ab-

sorbance measurement at 570 nm using a Dynatech microplate reader. The MIC was taken as the lowest peptide concentration at which bacterial growth was inhibited after 18±2 h.

2.5.4. Determination of LPS-binding by the chromogenic Limulus test

A quantitative chromogenic version of the Limulus amebocyte lysate (LAL) assay (QCL-1000) from Biowhittaker (Walkersville, MD, USA) was used. A standard solution of *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) with a concentration of 0.75 EU/ml was incubated with various amounts of the peptide hHEM-β (111–146) at 37 °C for 10 min in a sterile, endotoxin-free, 96-well microtiter plate. A total of 50 μl of LAL reagent was added to equal volumes of incubated test samples, LPS standards and hHEM-β (111–146) in endotoxin-free water, and the mixture was incubated for a further 10 min at 37 °C. Subsequently, 100 μl of the chromogenic substrate solution was added to each well. The reaction was terminated at 6 min by the addition of 25% acetic acid, and the absorbance at 405 nm was measured with a Dynatech microplate reader. Background absorbance at 405 nm, which for test samples included a component of absorbance due to hHEM-β (111–146), was subtracted from each reading. Free, bioactive LPS in test samples was quantified from standard curves, which were linear from 0.1 to 1.0 endotoxin units. The test was carried out in duplicate.

2.6. Cytotoxic assays

2.6.1. Determination of hemolytic activity

Human blood from a healthy donor was collected in vacuum tubes containing citrate (final concentration 0.106 *M*) as anti-coagulant. Erythrocytes were harvested by centrifugation for 10 min at 1500×*g* at 20 °C, and washed three times with PBS. PBS was added to the cell sediment to yield a 20% (v/v) erythrocyte/PBS suspension. This suspension was diluted 1:20 in TSB medium (3 g/l TSB supplemented with 287 m*M* glucose as an osmoprotectant) and 100 μl of the suspension obtained was subsequently added to 100 μl of peptide solution (TSB medium containing different peptide amounts) in a 96-well V-bottomed microtiter plate. Total hemolysis was achieved with 1% Tween 20 (Sigma, Deisenhofen, Germany). The plates were incubated for 1 h at 37 °C and centrifuged for 5 min at 1000×*g* at 20 °C. Finally, 150 μl of the supernatant fluid was transferred to a flat-bottomed microtiter plate, and absorbance was measured at 450 nm using a Dynatech microplate reader. The percentage of hemolysis was calculated by the equation: [(A_{450} of peptide-treated sample − A_{450} of buffer-treated sample)/(A_{450} of Tween 20-treated sample − A_{450} of buffer-treated sample)]×100.

2.6.2. Determination of toxic activity on PBMN cells

Cytotoxic activity of peptides on human peripheral blood mononuclear cells (PBMN) was measured by determining mitochondrial activity using the WST-1-assay. PBMN cells were freshly isolated from healthy human blood donors by Ficoll separation. After 2–3 h, the adherent cells (monocytes) were separated. Nonadherent cells were counted and viability was assessed by Trypan Blue exclusion. Cells were seeded in a density of 7×10^5 cells/ml and cultured for 4 days at 37 °C in a 5% CO_2 containing humidified atmosphere using RPMI 1640 medium supplemented with 1 m*M* L-glutamine, 25 m*M* Hepes, 10% fetal bovine serum, 10 μg/ml concanavalin A and 50 U/ml interleukin 2 (Sigma, Deisenhoff, Germany). Cytotoxic assays were carried out using PBMN cells at passage 2–4 in exponential growth phase with a viability >98%; 100 μl of a cell suspension containing 5×10^5 cells/ml were seeded per well in microtiter plates and incubated with test samples in different concentrations for 48 h. Then, 10 μl of WST-1 solution (Roche Diagnostics, Mannheim, Germany) was added to each well and following incubation for 4 h, absorbance was measured at 450/630 nm using a Dynatech microplate reader. The cytotoxic effect of 100 μg/ml cycloheximide (CHX) (Sigma, Germany) was used to decrease cell viability to zero. The viability of cells in percent was calculated by: [($A_{450/630\ nm}$ of peptide-treated sample − $A_{450/630\ nm}$ of CHX-treated sample)/($A_{450/630\ nm}$ of untreated cells − $A_{450/630\ nm}$ of CHX-treated sample)]×100.

3. Results

3.1. Purification and characterization of antimicrobial peptides from human placenta

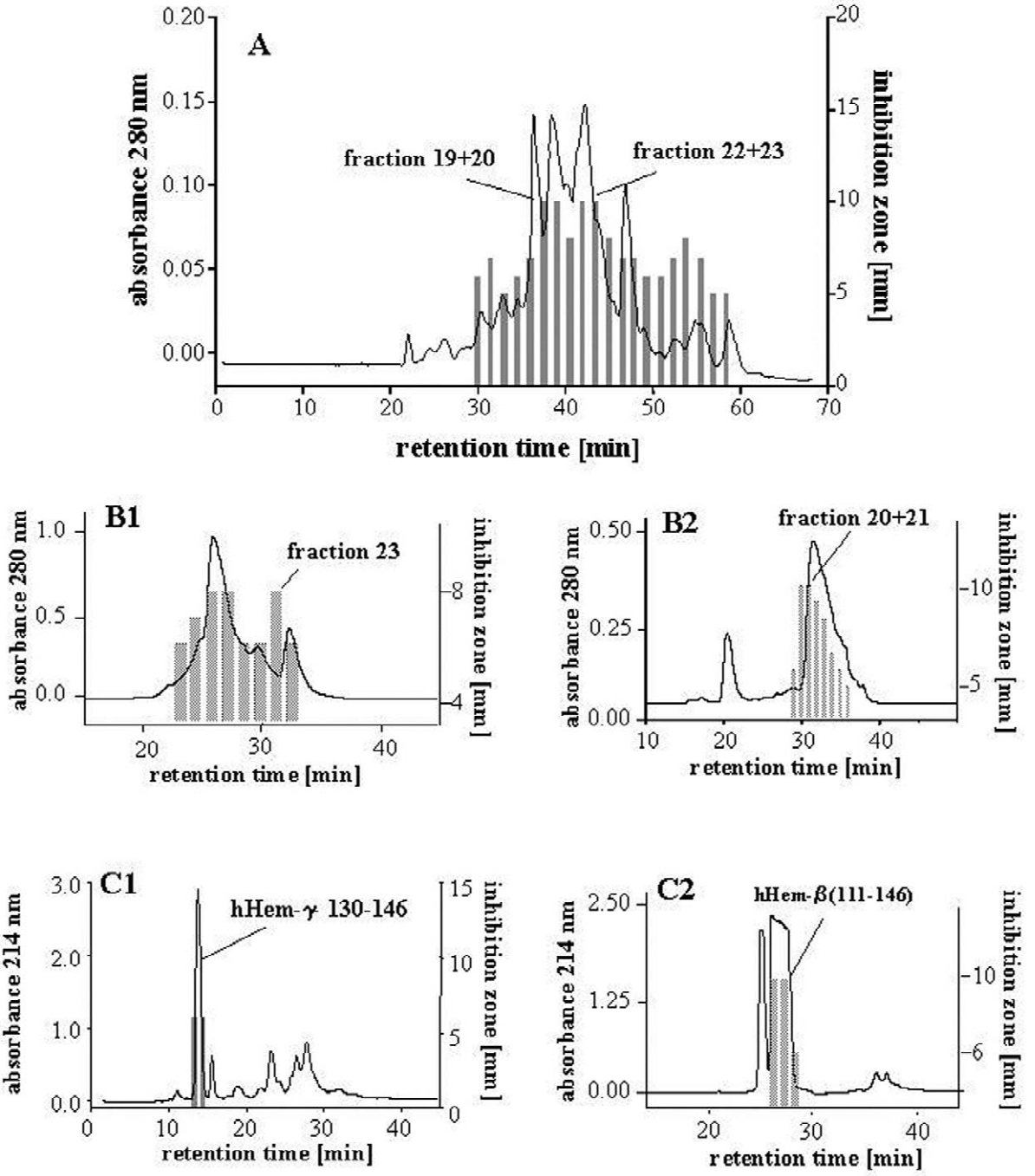
A peptide library from human placental tissue was screened for bacterial growth inhibitory activity against *Escherichia coli* BL21 using a sensitive radial diffusion assay [15]. Antibacterial activity was detected in several HPLC fractions of pH pool 4 (Fig. 1A). Pooled fractions 19 and 20 as well as pooled fractions 22 and 23, which exhibited the highest bacterial growth inhibitory activity were selected for further purification. To isolate the active compounds, two subsequent HPLC steps were carried out, tracking the maximum antibacterial activity within the resulting fractions.

The antimicrobially active fractions 19 and 20 (Fig. 1A) were loaded onto an RP column. Elution of bound material was carried out by linearly increasing the amount of solvent B. Bacterial growth inhibitory activity eluted over a broad range (Fig. 1B1), and fraction 23 was selected for further purification. Since it is known that most antimicrobial peptides are of cationic nature, a strong cation-exchange column was used for final purification. The resulting HPLC fractions were desalted and subsequently tested for the presence of antimicrobial activity, which could be detected in two fractions corresponding to the main peak (Fig. 1C1). Analysis of active fractions by CZE revealed a compound of high purity (data not shown). The molecular mass of the purified peptide was determined to be 1935 Da measured by MALDI mass spectrometry (data not shown). Edman degradation yielded the following peptide sequence of 17 amino acid residues: WQKMVTAVASALSSRYH. Comparison of the amino acid sequence obtained with the SwissProt Database showed 100% identity to the human γ-hemoglobin fragment 130–146 (hHEM-γ (130–146)). Based on the isolated peptide amount, it was calculated that 1 kg of the analyzed placental tissue contained 10 mg of hHEM-γ (130–146).

Additional chromatography of the pooled fractions 22 and 23 (Fig. 1A) using RP chromatography resulted in one major peak (Fig. 1B2). The corresponding fractions were shown to inhibit the growth of *Escherichia coli* BL21. In the final purification step, fractions 20 and 21 exhibiting the highest antibacterial activity were pooled and separated using an RP C_4 column. Antimicrobial activity was detected in two fractions corresponding to the main peak (Fig. 1C2). The purity of the active fractions was proved by CZE (data not shown). MALDI mass spectrometric analysis revealed a molecular mass of 3902 Da for the purified antimicrobial peptide. Edman degradation yielding the peptide sequence VCVLAHHFGKEFTPPVQAAYQKVVAG-VANALAHKYH and subsequent database analysis led to the identification of the human β-hemoglobin fragment 111–146 (hHEM-β (111–146)). In 1 kg of human placental tissue, 360 mg hHEM-β (111–146) was detected, corresponding to 250 mg per placenta.

3.2. Purification of a hemoglobin-derived peptide exhibiting antimicrobial activity from erythrocyte lysate

It is known that extensive proteolytic degradation of hemoglobin takes place inside erythrocytes [1]. Therefore, we analyzed whether antimicrobially active hemoglobin fragments occur inside erythrocytes. Using the radial diffusion assay, we were able to show that lysed erythrocytes inhibit the growth of *Escherichia coli* BL21. For purification of antimicrobial peptides from erythrocyte lysate, RP chromatography was used as a first separation step. Antimicrobial activity was detected in two fractions (Fig. 2A). The fraction exhibiting the highest bacterial growth inhibitory activity was applied to an analytical cation-exchange column. Elution was carried out increasing linearly the amount of solvent B resulting in a highly effective separation of inactive material from the antimicrobial compound (Fig. 2B). Final purification was carried out using an analytical RP column resulting in the purification of an antimicrobial peptide to homogeneity (Fig. 2C). By Edman degradation and molecular mass analysis (Fig. 2D), the peptide was identified as the human β-hemoglobin fragment 111–146, which we had already purified before from human placenta. Based on the isolated peptide amount, it was calculated that 1 l human blood contained 4 mg hHEM-β (111–146).


A
fraction 19+20
fraction 22+23
absorbance 280 nm
inhibition zone [mm]
retention time [min]
B1
fraction 23
absorbance 280 nm
inhibition zone [mm]
retention time [min]
B2
fraction 20+21
absorbance 280 nm
inhibition zone [mm]
retention time [min]
C1
hHem-γ 130-146
absorbance 214 nm
inhibition zone [mm]
retention time [min]
C2
hHem-β(111-146)
absorbance 214 nm
inhibition zone [mm]
retention time [min]

3.3. Functional characterization of synthetic antimicrobial peptides

To confirm the identity and biological properties of the isolated peptides hHEM-γ (130–146) and hHEM-β (111–146) both were chemically synthesized. To specify the spectrum of activity of the peptides, their antimicrobial activity was determined against several bacterial strains and yeast. MIC values of various antimicrobial peptides obtained from the literature cannot be easily compared, as different strains of microorganisms and experimental conditions are used. Therefore, the antimicrobial activity of both hemoglobin fragments were analyzed, and compared to that of MBI-28 (CEMA), a synthetic peptide, whose amino acid sequence was derived from parts of silk moth cecropin and bee melittin. MBI-28 is known for its potent broad spectrum antimicrobial, strong hemolytic, and antiendotoxic activity [16]. The identified hemoglobin peptides exhibit a broad spectrum of activity, inhibiting the growth of Gram-positive and Gram-negative bacteria and yeasts in micromolar concentrations (Table 1). Even human pathogens such as *Staphylococcus aureus* (e.g. wound infections), *Enterococcus faecalis* (VRE) (e.g. endocarditis), *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (e.g. lung infections) were susceptible to the isolated peptides. Antimicrobial potency of hHEM-β (111–146) is higher than that of hHEM-γ (130–146). In comparison to MBI-28, the peptide hHEM-β (111–146) show strain-dependently a 2- to 25-fold weaker growth inhibitory activity.

Addressing the question whether hHEM-β (111–146) acts as antiendotoxic agent by binding to Gram-negative endotoxin (LPS), inhibition of the biological activity of LPS was analysed using the chromogenic LAL assay. hHEM-β (111–146) inhibits LPS-induced activation of a proenzyme in LAL in a dose-dependent manner, which is visualized by inhibition of the chromogenic LAL response to LPS (data not shown). At 6.5 μ*M*, the IC_{50} is within the range of concentrations necessary for bacterial growth inhibition.

In order to determine whether hHEM-β (111–146) and hHEM-γ (130–146) are able to lyse the membrane of erythrocytes, their hemolytic activity was assessed. In contrast to the highly hemolytic peptide MBI-28, both hemoglobin peptides in bactericidal concentrations show only moderate hemolytic activity (Fig. 3A). Toxic activity against human primary blood cells was analyzed applying both hemoglobin peptides to human peripheral blood mononuclear cells (PBMN). In contrast to MBI-28, no toxic activity was observed applying the identified hemoglobin peptides in concentrations as high as 130 μ*M* (hHEM-β (111–146) and 260 μ*M* (hHEM-γ (130–146), respectively (Fig. 3B).

Fig. 1. Purification of antimicrobial peptides from a human placenta peptide library by consecutive chromatographic steps. Each purification step was guided by the radial diffusion assay for detection of antimicrobial activity. (A) Human placenta peptides (pH pool 4) were separated by RP chromatography. The bars show the diameters of inhibition zones indicating the antimicrobial activity of the resulting fractions against *E. coli* BL21. Applying a test amount equivalent to 220 mg placental tissue, fractions 19+20 and 22+23 were identified exhibiting maximum bacterial growth inhibitory activities, and were selected for further purification. (B1) Fractions 19 and 20 were pooled and fractionated using RP chromatography (conditions: column: YMC Gel C_8–C_{18}, 47×300 mm, 300 Å, 15–30 μ*M*, Waters/Millipore, MA, USA; solvent A: 0.01 *M* HCl, solvent B: 80% acetonitrile/0.01 *M* HCl; gradient: 20–70% B in 37.5 min; flow-rate: 40 ml/min). An amount equivalent to 550 mg placental tissue of each HPLC fraction was tested for antimicrobial activity. (B2) Fractions 22 and 23 were pooled and applied to an RP column (conditions: column: PrepPak, 47×300 mm, 300 Å, 15–30 μm, Baker, NJ, USA; solvent A: 30% methanol/0.01 *M* HCl, solvent B: 0.01 *M* HCl in methanol; gradient: 20–70% B in 47.5 min; flow-rate: 40 ml/min). An amount equivalent to 550 mg placental tissue of each HPLC fraction was tested for antimicrobial activity. (C1) Final purification of the antimicrobial substance was carried out, separating fraction 23 of (B1) using a strong cation-exchange column (conditions: column: Parcosil Pepkat, 4×50 mm, 300 Å, 5 μm, Biotek, Östringen, Germany; solvent A: 10 m*M* phosphate buffer (pH 4.5), solvent B: the same buffer with 1 *M* NaCl, gradient: 0–60% B in 60 min, flow-rate: 0.75 ml/min). Resulting fractions were desalted and tested for antimicrobial activity applying an amount equivalent to 2.2 g placental tissue. (C2) Rechromatography of fractions 20 and 21 of (B2) using an analytical RP C_4 column resulted in the purification of an antimicrobially active peptide to homogeneity (conditions: column: Biotek Silica C_4, 20×250 mm, 100 Å, 5 μm, Östringen, Germany; solvent A: 0.1% TFA, solvent B: 80% acetonitrile/0.1% TFA; gradient: 20–50% B in 50 min, flow-rate: 10 ml/min). An amount equivalent to 550 mg placental tissue of each HPLC fraction was tested for antimicrobial activity. (D1/D2) Sequence analysis of the pure antimicrobial peptides by Edman degradation led to the identification of the 130–146 fragment of γ-hemoglobin (D1) and the 111–146 fragment of β-hemoglobin (D2). Amino acid sequences are shown by the one letter code.

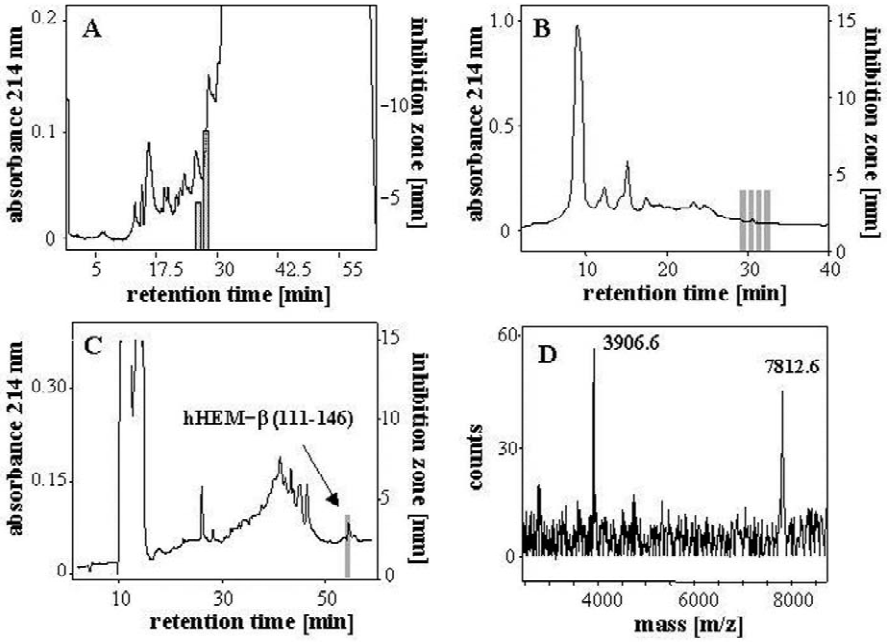


Fig. 2. Purification of an antimicrobially active hemoglobin-derived peptide from erythrocyte lysate by stepwise chromatographic separation. Each purification step was guided by the radial diffusion assay for detection of antimicrobial activity. (A) 2-ml erythrocyte lysate were separated by RP chromatography (conditions: column: YMC Gel C_8–C_{18}, 47×300 mm, 300 Å, 15–30 μ*M*, Waters/Millipore, MA, USA; solvent A: 0.01 *M* HCl, solvent B: 80% acetonitrile/0.01 *M* HCl; gradient: 0–100% B in 46.5 min; flow-rate: 30 ml/min). The bars show the diameters of inhibition zones indicating the antimicrobial activity of the resulting fractions against *E. coli* BL21. Antimicrobial activity was detected in two adjacent fractions. (B) The fraction exhibiting highest antimicrobial activity in (A) was applied to an analytical cation-exchange column (conditions: column: Parcosil Pepkat, 4×50 mm, 300 Å, 5 μm, Biotek, Östringen, Germany; solvent A: 10 m*M* phosphate buffer (pH 4.5), solvent B: the same buffer with 1 *M* NaCl, gradient: 0–60% B in 60 min, flow-rate: 0.75 ml/min). Elution resulted in a highly effective enrichment of the antimicrobial peptide. (C) Final purification was carried out by means of micro scale RP chromatography using an analytical column (conditions: column: Reprosil-Pur, 1×250 mm, 100 Å, 3 μm, Dr Maisch, Ammerbuch-Entringen, Germany; solvent A: 0.06% TFA, solvent B: 0.05% TFA in acetonitrile; gradient: 10–55% B in 50 min; flow-rate: 20 μl/min). Edman degradation of the pure peptide led to the identification of hHEM-β (111–146), also purified from human placenta. (D) Analysis of the pure peptide by MALDI–MS revealed a molecular mass of 3906 Da. A peptide dimer was detected with the corresponding mass of 7812 Da.

4. Discussion

4.1. Purification and characterization of antimicrobial hemoglobin fragments

The in vitro antimicrobial activity of whole hemoglobin has been known since the studies of Hobson and Hirsch published in 1957 [17]. According to these authors, the antibacterial activity of hemoglobin is restricted to a precise set of conditions in vitro and the antimicrobial function in vivo is considered to be unlikely. However, the existence of proteolytically generated hemoglobin fragments with antimicrobial activity was not discovered until 1999 [12]. Fogaca et al. purified a peptide, corresponding to the 33–61 fragment of the α-chain of bovine hemoglobin, from the gut of the tick *Boophilus microplus*. They supposed that proteolytic degradation of hemoglobin resulting in the production of antibacterial peptides takes place inside the tick gut for its own defense against microorganisms. Interestingly, Parish et al. in a recent study demonstrated that human intact hemoglobin tetramers and the separated alpha and beta subunits exhibit considerable antimicrobial activity against Gram-positive, Gram-negative bacteria and fungi [14]. Mapping specific regions of the human hemoglobin molecule for antimicrobial activity using synthetic peptides led

Table 1
Antimicrobial activity of synthetic hemoglobin fragments

Microorganism	MIC (μ*M*)		
	hHEM-β (111–146)	hHEM-γ (130–146)	MBI-28
Gram-positive bacteria			
M. luteus	8	100	4
S. carnosus	10.25	52	2
S. aureus	38.5	77.5	2
E. faecalis (VRE)	25.6	>130	8.2
B. subtilis	16	52	1
Gram-negative bacteria			
E. coli BL21	16	52	1
K. pneumoniae	51.3	>130	2
P. aeruginosa BST	32	>130	8.2
Yeast			
S. cerevisiae	12.8	52	1

Minimum inhibitory concentrations (MICs) of synthetic antimicrobial peptides were determined by liquid growth inhibition. The MIC was taken as the lowest peptide concentration at which bacterial growth was inhibited following an incubation time of 18±2 h. The highest concentration tested was 250 μg/ml, corresponding to 64 μ*M* hHEM-β (111–146), 130 μ*M* hHEM-γ (130–146), and 81 μ*M* MBI-28.

to the identification of the carboxyl terminal 30 amino acids of the beta subunit as responsible for significant antimicrobial activity. However, until now, the natural occurrence of antibacterial hemoglobin-derived peptides in humans is unknown.

With the aim of identifying naturally occurring antimicrobially active peptides in humans, we established a peptide library from human placental tissue as a known source for low molecular mass antimicrobial components [18]. Purification procedures led to the isolation of two hemoglobin-derived peptides with antibacterial properties. The peptides correspond to the C-terminus of γ-hemoglobin (fragment 130–146), and β-hemoglobin (fragment 111–146), respectively. The identification of these C-terminally derived peptides exhibiting antimicrobial properties is in accordance with the findings of Parish et al. [14] showing antimicrobial activity of the synthetic peptide hHEM-β (116–146). Furthermore, from human cerebellum a peptide was isolated with a sequence starting like hHEM-β (111–146) at amino acid residue 111 of β-hemoglobin. However, the C-terminal amino acid residues are unknown, because the amino acid sequence could not be completely determined [7]. Therefore, it is possible that the human cerebellum peptide and the antimicrobial hHEM-β (111–146) are identical. Interestingly, the human cerebellum peptide is increased fivefold in the cerebellum of patients suffering from Alzheimers's disease (AD), and no antimicrobial activity was described, until now.

The γ-hemoglobin combined with α-hemoglobin make up the fetal hemoglobin F, which consists of two γ- and two α-chains. In contrast, adult hemoglobin is formed by two β- and two α-chains. The fetal hemoglobin is expressed until 4 or 5 weeks after birth, and its most important property is its high oxidizability [19]. During gestation, the erythroid cells reduce the production of γ-chains and switch to form β-chains [20]. Amino acid sequences of γ-hemoglobin and β-hemoglobin show 73.1% identity, revealing a conserved primary structure of the molecules. According to our results, the C-terminus of both hemoglobin subunits is a precursor for antimicrobial peptides. Therefore, independent of age, proteolytic degradation of hemoglobin can lead to the generation of smaller peptides with antibacterial activity.

In a recent review, Ivanov et al. [1] discussed the role of hemoglobin as a source of endogenous bioactive peptides and developed the concept of a tissue-specific peptide pool. They proposed that enzymatic hemoglobin degradation starts inside erythrocytes leading to relatively long peptides con-

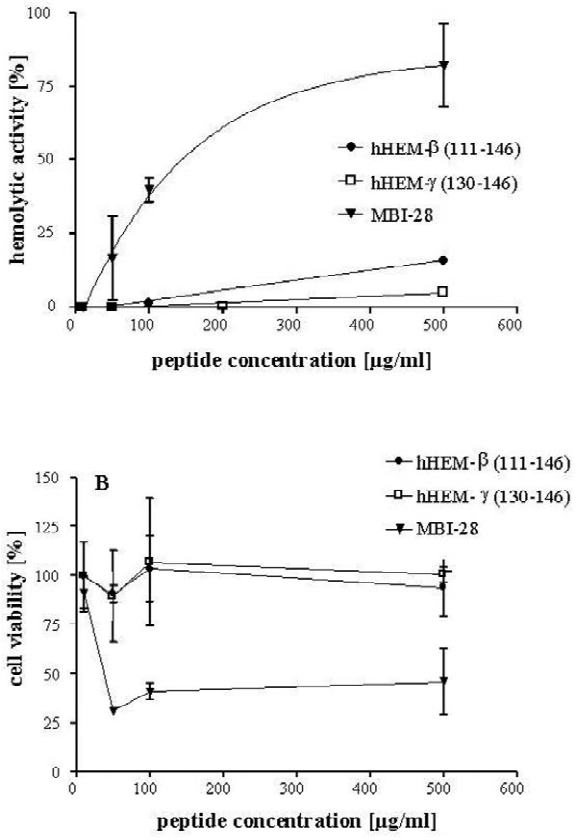


Fig. 3. Cytotoxic activities of hHEM-β (111–146) and hHEM-γ (130–146). (A) Synthetic hHEM-β (111–146) and hHEM-γ (130–146) in high concentrations weakly permeabilize membranes of erythrocytes leading to release of hemoglobin from the cells. The hemolytic activities of hHEM-β (111–146) and hHEM-γ (130–146) are shown as percentage of total hemolysis, which was achieved by treatment with 1% (v/v) Tween 20. Values are mean±SEM of two independent experiments. (B) Synthetic hHEM-β (111–146) and hHEM-γ (130–146) do not exhibit toxic effects on PBMN cells. Viability of PBMN cells following a 48-h incubation with peptides was determined using the WST-1 assay, measuring mitochondrial activity of cells. Values are mean±SEM of two independent experiments.

taining about 30 amino acid residues. This primary proteolysis is followed by the next degradation step coupled with release of newly formed shorter peptides from red blood cells. The released peptides are hydrolysed by tissue-specific proteases resulting in the tissue-specific peptide pool. The antimicrobial hemoglobin fragments identified in the present study were isolated from human placental tissue, which contains high amounts of both fetal and maternal blood providing the fetus with oxygen and nutrients. In order to avoid proteolysis during placental tissue extraction, acidic conditions and a temperature of at least 4 °C were selected, far from conditions optimal for protease activity. Therefore, we are positive about the occurrence of antimicrobial hemoglobin fragments not being an artefact of peptide library generation. Nevertheless, to make sure and to identify the point of origin of these peptides, we wanted to clarify if proteolysis of hemoglobin leading to antimicrobial fragments already takes place inside erythrocytes, which consist of up to 34% hemoglobin. Indeed, we detected bacterial growth inhibitory activity in the cell lysate of human erythrocytes. This finding conflicts with the experiments of Fogaca et al. [12], who were not able to detect antibacterial activity in lysed erythrocytes. However, deviating from our experiments, they analyzed bovine red blood cells for antimicrobial activity, and moreover, they used a different test system and a different bacterial test strain. In the present work, further purification of erythrocyte lysate by means of HPLC monitoring antimicrobial activity within the resulting fractions led to the isolation of hHEM-β (111–146), which we have already identified in human placental tissue. Therefore, we conclude that proteolytic degradation of hemoglobin inside erythrocytes forms antimicrobially active peptides. Our data provide strong evidence that the peptide hHEM-β (111–146) is already generated inside erythrocytes. The occurrence of peptides similar to hHEM-β (111–146) in erythrocytes is also proved by Karelin et al., who isolated a peptide from erythrocyte lysate matching the sequence of hHEM-β (111–146) except for the absence of the first four N-terminal amino acids [21]. However, for this peptide no antimicrobial activity has been hitherto described.

Comparison of the peptide amounts detected in placental tissue (250 mg per placenta, corresponding to 500 mg/l placental blood) and in lysed erythrocytes (4 mg/l peripheral venous blood) indicates that referring to the blood content, placental tissue contains approximately 100 times more hHEM-β (111–146) than erythrocytes obtained from circulating blood. Therefore, we suppose that hemoglobin degradation started inside erythrocytes, but the main degradation leading to antimicrobially active pep-

tides takes place by further proteolytic cleavage in placental tissue. The enzyme(s) responsible for the release of hHEM-β (111–146) from hemoglobin are as yet unknown. Cleavage of the peptide bond Leu_{110}–Val_{111} within the hemoglobin β-chain, resulting in the formation of hHEM-β (111–146), resembles the enzymatic processing of hemoglobin leading to the release of the opioid peptide VV-hemorphin (cleavage of peptide bond Leu_{32}–Val_{33}). Hemorphins and related fragments are isolated from several tissues, such as pig and bovine hypothalamus, bovine brain, human liquor, human pituitary gland and human plasma (reviewed in Ref. [1]). In our laboratory, hemorphins were also isolated from the same placenta peptide library we used for the isolation of hemoglobin-derived antimicrobial peptides [22]. The purification of these similarly processed hemoglobin peptides from one tissue extract indicates that they may be generated by the same proteinase. Recent studies demonstrated a possible role of the lysosomal enzyme cathepsin D in the specific release of VV-hemorphin-7 from hemoglobin [23]. However, it can be noted that proteolytic cleavage leading to the release of hHEM-β (111–146) and hemorphins must be catalyzed by a protease able to cleave between two hydrophobic amino acid residues. Further investigation is required to identify the enzyme(s) involved in the generation of hHEM-β (111–146).

4.2. Biological activity of hHEM-β (111–146) and hHEM-γ (130–146)

The isolated peptides in micromolar concentrations exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria, and yeasts. The antimicrobial potency of the identified hemoglobin peptides is lower than that of MBI-28, and is at the lower extremity of the activity range described for antimicrobial peptides (MIC 8–100 μ*M* for the isolated hemoglobin peptides, compared to MICs<10 μ*M* for the most active peptide antibiotics). However, the amount of hHEM-β (111–146) detected in human placental tissue was about 250 mg per placenta, about 500 mg/l placental blood (260 μ*M*), meaning that the detected peptide concentration is higher than concentrations necessary for antimicrobial activity (Table 1). Even at this high concentration of 500 mg/l, no toxic effects of both hemoglobin peptides on human blood cells, such as PBMN cells were observed. But the peptide hHEM-β (111–146) shows weak membrane-permeabilizing activity on erythrocytes in high concentrations. It is possible that, once available in the bloodstream, hHEM-β (111–146) can cause the release of more antimicrobial peptides from erythrocytes by membrane permeabilization. The occurrence of non-toxic antimicrobial hemoglobin peptides in remarkably high concentrations gives strong evidence for the in vivo activity and possible importance of these peptides for host defense.

Both hemoglobin fragments are cationic peptides with a calculated p*I* of 9.5 (hHEM-β (111–146)) and 10.9 (hHEM-γ (130–146)), respectively. The predicted secondary structure (PredictProtein, EMBL, Heidelberg, Germany) of the peptides displays an alpha-helical character typical of basic antimicrobial pore-forming peptides [24]. This is in accordance with the known three-dimensional structure of human hemoglobin, which shows an alpha-helical region within the C-terminal part of the hemoglobin β-chain (Protein Data Bank: file 2HHB). The structure of the peptides gives rise to the hypothesis that their mechanism of action resembles that of known antimicrobial peptides such as magainins and defensins permeabilizing bacterial membranes [25]. Another function of several antimicrobial cationic peptides is the ability to neutralise LPS and prevent endotoxinaemia [26,27]. Therefore, we were interested in analyzing the LPS-binding capability of hHEM-β (111–146). Indeed, in bacterial growth inhibitory concentrations hHEM-β (111–146) inhibits the LAL proenzyme activating effect of LPS. In contrast, using the same chromogenic LAL assay, Kaca et al. showed that binding of the entire hemoglobin tetramer to LPS leads to enhancement of LPS biological activity [28]. In a more recent study, Jürgens et al. described that LPS-induced tumor necrosis factor (TNF-α) production of mononuclear cells is enhanced by hemoglobin, whereas in the LAL assay, a LPS concentration-dependent increase or decrease in endotoxin activity by hemoglobin was observed [29]. However, the hemoglobin concentration tested was much lower than in the study of Kaca et al. To evaluate a potential role of hHEM-β (111–146) in the host defense system for prevention

of endotoxic shock reactions, a more detailed investigation of its antiendotoxic effect, e.g. reduction of LPS-induced TNF-α release, is necessary.

In conclusion, we have shown for the first time that hemoglobin is a source of antimicrobial peptides naturally occurring in humans. We were able to provide strong evidence that hemoglobin proteolysis in human erythrocytes and in human tissues such as placenta leads to the formation of peptides effectively inhibiting the growth of microorganisms. In accordance with the data of Parish et al. [14] we identified the region comprising antimicrobial fragments located at the C-terminus of the amino acid sequences of β-hemoglobin or γ-hemoglobin. Due to their broad antimicrobial action against Gram-positive and Gram-negative bacteria and yeast even against human pathogens, and to the endotoxin-binding capability, these antimicrobial peptides could be important effectors of the innate immune response, which effectively kills microbial invaders. Hemoglobin fragments are available in all human tissues, even in infective lesions, via the bloodstream. Therefore, we suspect that hemoglobin-derived peptides may also play an important role in the wound healing process.

References

[1] V.T. Ivanov, A.A. Karelin, M.M. Philippova, I.V. Nazimov, V.Z. Pletnev, Biopolymers 43 (1997) 171.
[2] A.V. Schally, Y. Baba, R.M. Nair, C.D. Bennett, J. Biol. Chem. 246 (1971) 6647.
[3] H. Takagi, H. Shiomi, H. Ueda, H. Amano, Nature 5737 (1979) 410.
[4] K. Fukui, H. Shiomi, H. Takagi, K. Hayashi, Y. Kiso, K. Kitagawa, Neuropharmacology 22 (1983) 191.
[5] V. Brantl, Ch. Gramsch, F. Lottspeich, R. Mertz, K.H. Jaeger, A. Herz, Eur. J. Pharmacol. 125 (1986) 309.
[6] Y.X. Zhu, K.L. Hsi, Z.G. Chen, H.L. Zhang, S.X. Wu, Z.Y. Zhang, F.P. Fang, S.Y. Guo, Y.S. Kao, K. Tsou, FEBS Lett. 208 (1986) 253.
[7] J.R. Slemmon, C.M. Hughes, G.A. Cambell, D.G. Flood, J. Neurosci. 14 (1994) 2225.
[8] A.V. Pivnik, N.A. Rasstrigin, M.M. Philippova, A.A. Karelin, V.T. Ivanov, Leuk. Lymphoma 22 (1996) 345.
[9] J.R. Slemmon, T.M. Wengenack, D.G. Flood, Biopolymers 43 (1997) 157.
[10] E.L. Glamsta, L. Morkrid, I. Lantz, F. Nyberg, Regul. Pept. 49 (1993) 9.
[11] F. Nyberg, K. Sanderson, E.L. Glamsta, Biopolymers 43 (1997) 147.
[12] A.C. Fogaca, P.I. da Silva Jr., M.T.M. Miranda, A.G. Bianchi, A. Miranda, P.E.M. Ribolla, S. Daffre, J. Biol. Chem. 274 (1999) 25330.
[13] R. Froidevaux, F. Krier, N. Nedrar-Arroume, D. Vercaigne-Marko, E. Kosciarz, C. Ruckebusch, P. Dhulster, D. Guillochon, FEBS Lett. 24617 (2001) 1.
[14] C.A. Parish, H. Jiang, Y. Tokiwa, N. Berova, K. Nakanishi, D. McCabe, W. Zuckerman, M.M. Xia, J.E. Gabay, Bioorg. Med. Chem. 9 (2001) 377.
[15] R.I. Lehrer, M. Rosenmann, S.S.L. Harwig, R. Jackson, P.J. Eisenhauer, J. Immunol. Methods 137 (1991) 167.
[16] K.L. Piers, M.H. Brown, R.E.W. Hancock, Antimicrob. Agents Chemother. 38 (1994) 2311.
[17] D. Hobson, J.G. Hirsch, J. Exp. Med. 107 (1958) 167.
[18] T.M.N. Scane, J.F. Guest, D.F. Hawkins, Br. J. Obstet. Gynaecol. 95 (1988) 633.
[19] N. Bogair, Med. Hypotheses 34 (1991) 105.
[20] J. De Simone, A.L. Mueller, Blood 53 (1979) 19.
[21] A.A. Karelin, M.M. Philippova, V.T. Ivanov, Peptides 16 (1995) 693.
[22] H.P. Lammerich, A. Busmann, C. Kutzleb, M. Wendland, P. Seiler, C. Berger, P. Eickelmann, M. Meyer, W.G. Forssmann, E. Maronde, Br. J. Pharmacol. 138 (2003) 1431.
[23] I. Fruitier, I. Garreau, J.M. Piot, Biochem. Biophys. Res. Commun. 246 (1998) 719.
[24] G. Saberwal, R. Nagaraj, Biochim. Biophys. Acta 1197 (1994) 109.
[25] M. Vaara, Microbiol. Rev. 56 (1992) 395.
[26] M. Gough, R.E.W. Hancock, N.M. Kelly, Infect. Immun. 64 (1996) 4922.
[27] B.P. Giroir, P.A. Quint, P. Barton, E.A. Kirsch, L. Kitchen, B. Goldstein, B.J. Nelson, N.J. Wedel, S.F. Carroll, P.J. Scannon, Lancet 350 (1997) 1439.
[28] W. Kaca, R.I. Roth, J. Levin, J. Biol. Chem. 269 (1994) 25078.
[29] G. Jürgens, M. Müller, M.H.J. Koch, K. Brandenburg, Eur. J. Biochem. 268 (2001) 4233.