hBD-1: a novel β-defensin from human plasma

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Abstract **We report the isolation and characterization of a novel peptide with significant sequence homology to β-defensins from human blood filtrate. The human β-defensin-1 (hBD-1) is a short basic peptide of 36 amino acid residues. It contains six cysteines forming three intramolecular disulfide bonds. The molecular mass of hBD-1 is 3928.6 Da. Cloning of the specific cDNA confirmed the amino acid sequence of the native peptide. hBD-1 shares the nine conserved amino acids characteristic for β-defensins from respiratory epithelial cells and neutrophils of cattle and chicken leukocytes. hBD-1 is present in nanomolar concentration in human plasma.**

Key words: β-Defensin; Antimicrobial peptide; Hemofiltrate; Amino acid sequence; cDNA

1. Introduction

Small basic antibiotic peptides are part of the unspecific host defense system existing in all animals [1,2]. In vertebrates they are abundant in granules of phagocytic leukocytes as has been shown for protegrins [3], bactenecins [4], indolicidin [5], defensins [6], and β-defensins [7] of mammals and gallinacins from chicken [8]. In addition antimicrobial peptides are present in different epithelial cells establishing an antimicrobial barrier. For example, magainines have been identified in frog skin [9] and stomach [10], cecropin P1 in porcine small intestine [11], cryptdins in mouse small intestine [12], defensins in human small intestine [13], and tracheal antimicrobial peptide (TAP) in bovine trachea [14].

Recently we have improved the large-scale extraction of peptides from human hemofiltrate (HF) earlier described [15]. HF is obtained from patients with end stage renal disease (ESRD) undergoing hemofiltration for the elimination of uremic toxins [16]. HF quantitatively contains all small peptides present in plasma and can be considered to be a prepurified source of peptides from blood plasma [17]. We started a systematic investigation of peptides from HF [18]. In the course of this program a novel peptide hBD-1 homologous to β-defensins was isolated.

2. Experimental

2.1. Peptide extraction

HF from approximately 25 individuals was collected, cooled to 4°C, acidified to pH 3.0 with HCl, and diluted with deionized water to a final conductivity of 5.5 mS/cm. 800 litre equivalents of HF were applied to a cation-exchange column (25 × 7 cm, Fraktogel SP-650 (M), Merck, Darmstadt, Germany). Bound proteins and peptides were eluted in 5 litres with 1 M NaCl and precipitated with ammonium sulfate (680 g/l). Six precipitates representing 4800 litres of HF were dissolved in 2 litres of deionized water. Proteins were removed by ultrafiltration through a cellulose triacetate membrane with a cut-off of 20 kDa (Sartorius, Göttingen, Germany). The ultrafiltrate containing the peptides was further used in the isolation procedure.

2.2. Purification of hBD-1

Step 1. The ultrafiltrate was diluted with deionized water to a final conductivity of 5.5 mS/cm, pH 3.0. In two runs the ultrafiltrate was chromatographed on a cation-exchange column (6 × 20 cm; Fraktogel SP-650 (M)) equilibrated with buffer A (0.1 M acetic acid, 20% methanol, pH 3.0). After a 30 min wash the peptides were eluted in a linear gradient from 100% buffer A to 40% buffer B (0.5 M acetic acid, 20% methanol, 1 M ammoniumacetate, pH 5.5) in 60 min and 40% to 100% buffer B in 10 min. Absorbance at 280 nm and conductivity were monitored. The flow rate was 50 ml/min and starting at min 10, 2 min fractions were collected. Corresponding fractions of both runs were pooled and lyophilized.

Step 2. 100 mg from a total of 2.4 g of fraction 40 from step 1 were dissolved in 0.1% TFA and applied to a Parcosil C4 RP-HPLC column (10 × 125 mm, 5 μm, 300 Å; Biotek, Östringen, Germany). The mobile phases used for RP-HPLC were solvent A (0.1% TFA) and solvent B (0.1% TFA, 80% acetonitrile). Peptides were eluted with a linear gradient from 5% to 50% solvent B in 45 min. Absorbance was monitored at 214 nm and the flow rate was 2 ml/min. Fractions were collected manually.

Step 3. One rechromatography of fraction 35 from step 2 on a Vydac C18 RP-HPLC column (4.6 × 250 mm, 5 μm, 300 Å; MZ-Analysentechnik, Mainz, Germany) yielded highly purified hBD-1. The same solvents as in step 2 were used with a gradient from 25% to 50% solvent B in 25 min. Absorbance was monitored at 214 nm and the flow rate was 0.75 ml/min.

2.3. Capillary zone electrophoresis

Purity of fractions was controlled by capillary zone electrophoresis (CZE) with a P/ACE 2000 system and System Gold software (Beckman, München, Germany). Samples were injected by pressurization for 1–10 s. The diameter of the uncoated capillary was 75 μm and the effective and total length were 50 cm and 57 cm, respectively. Peptides were separated at a constant current of 120 μA and detected at 200 nm. The buffer consisted of 0.1 M sodium phosphate, pH 2.5, containing 0.2 g/l hydroxypropylmethylcellulose.

2.4. Reduction and S-alkylation

100 μg of purified hBD-1 were dissolved in 95 μl alkylating buffer (250 mM Tris-acetate, 6 M guanidinium-HCl, 2 mM EDTA, pH 8.0). After addition of 5 μl reducing solution (400 mM DTT in alkylating buffer) and incubation for 30 min at 40°C, 20 μl of 500 mM iodoacetamide in alkylating buffer were added. Incubation was continued at room temperature for 30 min in the dark. The reaction was stopped with 0.1% TFA and the carboxamidomethylated peptide (CAM-peptide) was immediately purified by RP-HPLC.

2.5. Peptide sequence analysis

The amino acid sequence of the native and *S*-alkylated peptide was determined by the edman degradation method on a 473 A sequenator (ABI, Weiterstadt, Germany) equipped with MicroCartridge (ABI) following the instructions of the manufacturer. Programming, data collection and evaluation were perfomed using Model 610A Data Analysis software (ABI).

2.6. Amino acid analysis

Gas-phase hydrolysis was carried out with a Waters Pico Tag Work-

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station (Millipore, Eschborn, Germany) with 6 M HCl for 1 h at 160°C. Amino acids were analyzed on a 1090L AminoQuant Analyzer (Hewlett Packard, Waldbronn, Germany) using precolumn derivatisation with OPA and Fmoc [17]. Norvaline and sarcosine were used as internal standards at final concentrations of 50 μM. Data were collected and evaluated on a HP 300 ChemStation (Hewlett Packard).

2.7. Mass determination

Molecular masses of the native, reduced, and *S*-alkylated peptide were measured on a Sciex API III triple-stage quadrupole mass spectrometer with electrospray interface (Perkin-Elmer, Überlingen, Germany) in positive-ion mode. Samples were applied with a Harvard infusion pump (FMI GmbH, Egelsbach, Germany) at a flow rate of 5 μl/min. Data were collected and evaluated with MacSpec Software (Perkin-Elmer).

2.8. Cloning and characterization of cDNA

RNA-purification, cDNA first strand synthesis, polymerase chain reaction (PCR), and DNA sequencing was performed as described [19]. A degenerate PCR sense primer (BDNT; 5′-CCCGGGAATTCTAGAGAYCAYTAYAAYTGYGT-3′) was derived from the N-terminus of the peptide sequence. The antisense primer was Unip-2 (5′-CCTGAATTCTAGAGCTCAT$_{17}$-3′), the same oligo(dT) primer used for cDNA first strand synthesis. Both primers contained an add-on sequence with the recognition sequence for *Eco*RI. PCR-fragments were cleaved with *Eco*RI and purified from non-incorporated primers and small restriction fragments with Magic-PCR-Preps (Promega, Heidelberg, Germany). Purified PCR-fragments were ligated in the *Eco*RI restriction site of pBluescript II SK+ (Stratagene, Heidelberg, Germany). Then *E. coli* XL1-Blue cells were transformed with the products and recombinant clones were selected by the blue/white color srceening on indicator plates containing IPTG and X-gal.

3. Results and discussion

We report the isolation and structural characterization of a novel human peptide present in plasma. It shares some characteristic properties with bovine neutrophil β-defensins, TAP from bovine trachea, and gallinacins from chicken. Therefore this peptide is named human β-defensin-1 (hBD-1) and represents the first human member of the family of β-defensins.

The peptide was isolated from 4800 litres of human hemofiltrate by a combination of batch extraction, cation-exchange chromatography, and RP-HPLC. Peptides were recovered with 1 M NaCl after absorbtion to a cation-exchange column and precipitated with ammonium sulfate. Ultrafiltration through a membrane with a cut-off of 20 kDa was carried out to separate

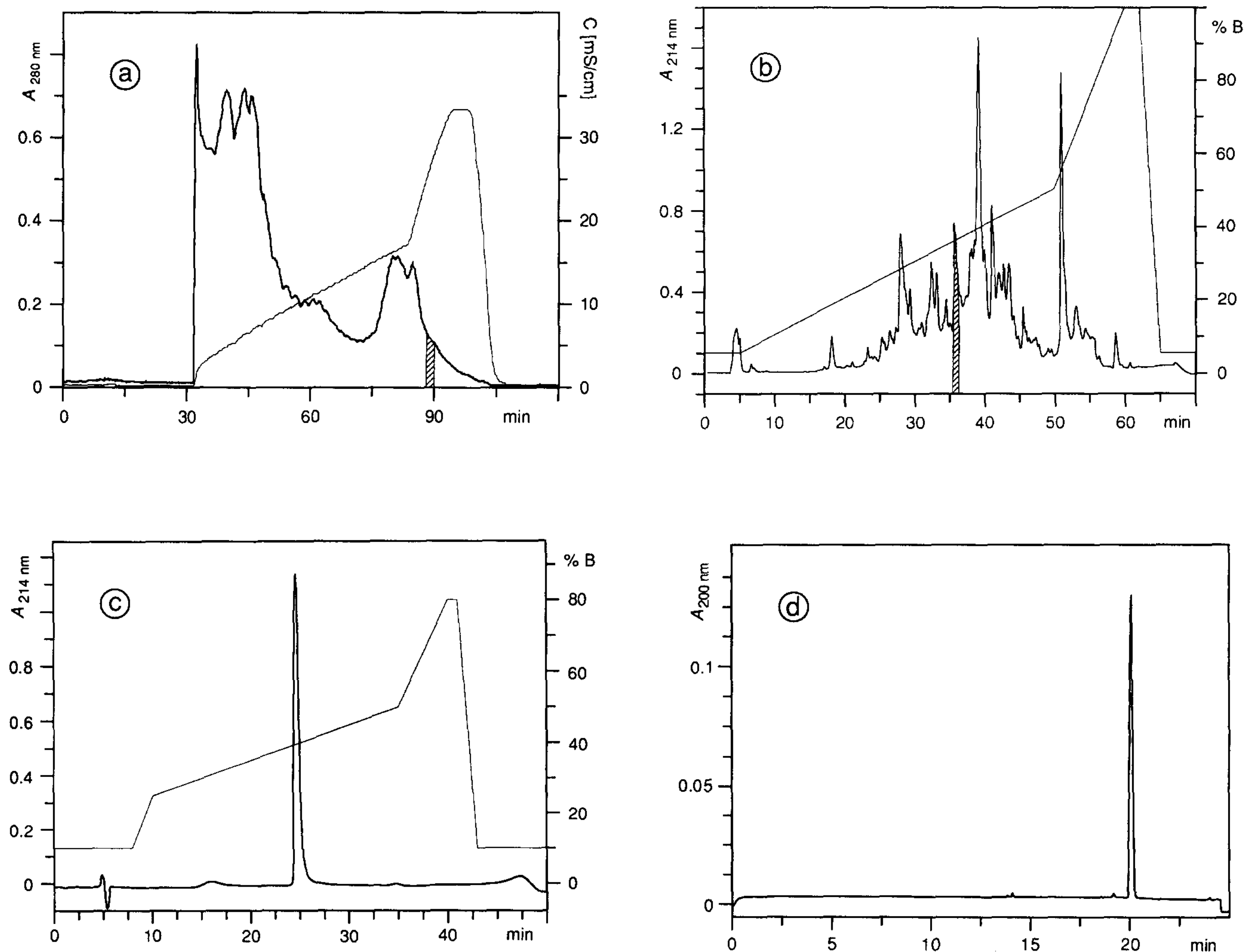

Fig. 1. Purification of hBD-1. (a) Chromatogram of the preparative cation exchange chromatography (— absorbance at 280 nm, —— conductivity). The marked fraction 40 is further processed. (b) Preparative RP-C4 chromatography of fraction 40 (— absorbance at 214 nm, —— gradient in % B). The marked fraction 35 contains hBD-1. (c) Analytical RP-C18 chromatography of fraction 35 (— absorbance at 214 nm, —— gradient in % B) and (d) electropherogram of hBD-1.

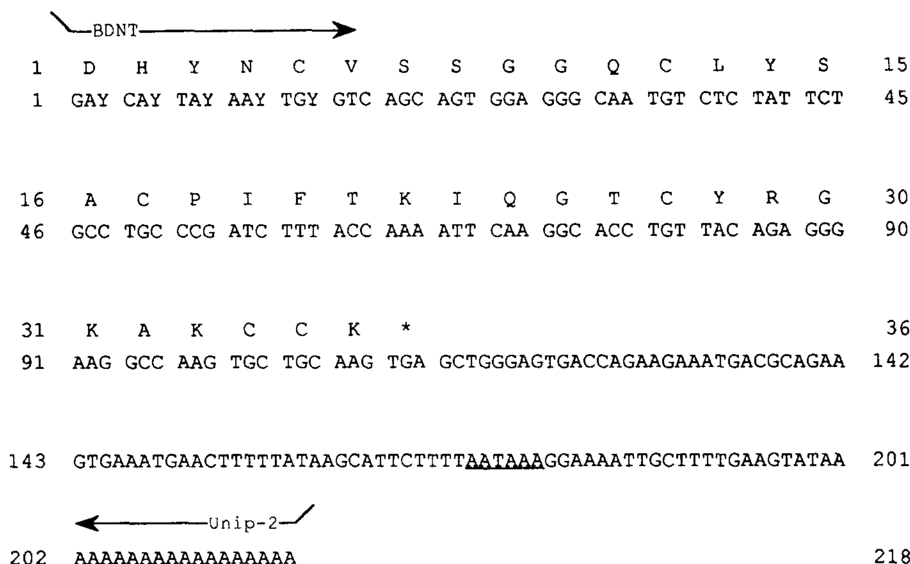

Fig. 2. Amino acid sequence and partial cDNA of hBD-1. The upper line represents the amino acid sequence in single letter code. The primers BDNT and Unip-2 used for cloning are shown as arrows. The add-on sequences are indicated as small flags. The stop codon is marked with an asterisk and the putative polyadenylation signal is underlined. The first 17 nucleotides origin from the degenerate primer BDNT so all possible nucleotides for the third codon position are listed in the sequence (Y = C or T).

remaining plasma proteins from peptides. In two runs the ultrafiltrate containing the peptides was chromatographed on a preparative cation-exchange column and eluted in a linear gradient (Fig. 1a). hBD-1 elutes in fraction 40 at 90 min at high salt concentration. Further purification by semi-preparative RP-HPLC of fraction 40 on a C4 column resolved hBD-1 in a peak at 38.8 min in fraction 35 in the complex chromatogram (Fig. 1b). Rechromatography on an analytical RP-C18 column resulted in a pure and homogenous peptide (Fig. 1c) as judged by capillary zone electrophoresis (Fig. 1d).

Sequence analysis of native hBD-1 resulted in a peptide sequence of 36 amino acids. Positions 5, 12, 17, 27, 34 and 35 showed no PTH-amino acid and were proposed to contain cysteines, which are not detected by standard edman degradation. Positive identification of cysteines was performed sequencing reduced and *S*-alkylated hBD-1. All amino acids including the six CAM-cysteines at the supposed positions 5, 12, 17, 27, 34 and 35 were readily detected (Fig. 2).

hBD-1 was subjected to amino acid analysis to establish the amino acid composition. Table 1 is a comparison of the values obtained by amino acid analysis and sequence analysis. The results of both experiments are consistent. Slightly lower values for serine, histidine, threonine and tyrosine in amino acid analysis are due to partial destruction during acid hydrolysis.

Molecular masses of 3928.0 ± 0.5 Da for the native peptide and 4276.5 ± 0.6 Da for the reduced and alkylated peptide were measured by electrospray mass spectrometry (Fig. 3). The mass difference of 348 Da results from addition of six carboxamidomethylene groups ($-CH_2CONH_2$, 58 Da) to the six cysteines in hBD-1. Alkylation without prior reduction was not successful and the same mass as for the native peptide was determined. Reduction without alkylation resulted in a molecular mass of 3934.2 ± 0.8 Da, a difference of 6 Da relative to native hBD-1 (data not shown). The calculated average masses of hBD-1 with and without three disulfide bridges are 3928.6 Da and 3934.6 Da, respectively. This is in good agreement with the measured masses of the native and reduced peptide. These data support the presence of six cysteines as demonstrated by amino acid sequence analysis. All cysteines are involved in three intramolecular disulfide bonds.

Based on the peptide sequence a degenerate sense primer BDNT was designed with an additional 5′ add-on sequence used for subcloning. BDNT covers the first six N-terminal

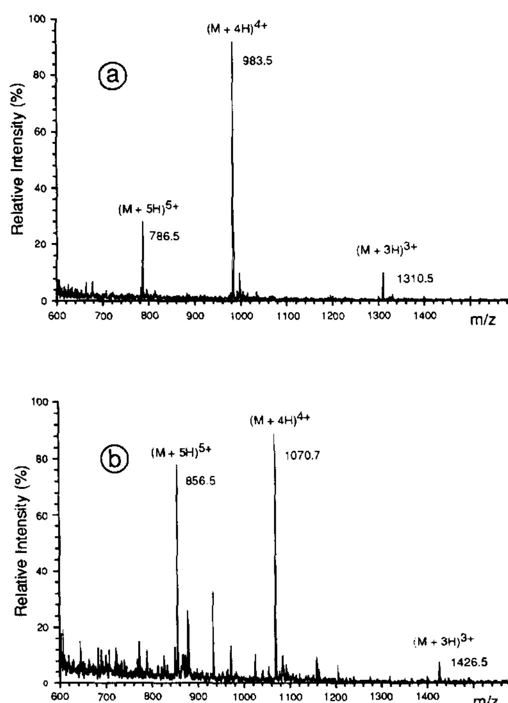

Fig. 3. Mass spectra of the (a) native and (b) reduced and alkylated peptide. The molecular masses were calculated from multiple charged ions as 3928.0 ± 0.5 Da from 786.5 Da, 983.5 Da, and 1310.5 Da (a) and as 4276.5 ± 0.6 Da from 856.5 Da, 1070.7 Da, and 1426.5 Da (b), respectively.

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                     1                 10                  20                  30          36
hBD-1                D H Y N C V S S G G Q C L Y S A C P I F T K I Q G T C Y R G K A K C C K

TAP                  N P V S C V R N K G I C V P I R C P G S M K Q I G T C V G R A V K C C R K K

Gal 1α             G R K S D C F R K N G F C A F L K C P Y L T L I S G K C S R F H L - C C K R I W
Gal 1              G R K S D C F R K S G F C A F L K C P S L T L I S G K C S R F Y L - C C K R I W
Gal 2                    L F C - - K N G S C H F G G C P S H L I K V G S C F G F R S - C C K W P W N A

BNBD-1               D F A S C H T N G G I C L P N R C P G H M I Q I G I C F R P R V K C C R S W
BNBD-2           V R N H V T C R I N R G F C V P I R C P G R T R Q I G T C F G P R I K C C R S W
BNBD-3      pE G V R N H V T C R I N R G F C V P I R C P G R T R Q I G T C F G P R I K C C R S W
BNBD-4      pE R V R N P Q S C R W N M G V C I P F L C R V G M R Q I G T C F G P R V P C C R R
BNBD-5      pE V V R N P Q S C R W N M G V C I P I S C P G N M R Q I G T C F G P R V P C C R
BNBD-6      pE G V R N H V T C R I Y G G F C V P I R C P G R T R Q I G T C F G R P V K C C R R W
BNBD-7      pE G V R N F V T C R I N R G F C V P I R C P G H R R Q I G T C L G P R I K C C R
BNBD-8           V R N F V T C R I N R G F C V P I R C P G H R R Q I G T C L G P Q I K C C R
BNBD-9      pE G V R N F V T C R I N R G F C V P I R C P G H R R Q I G T C L G P Q I K C C R
BNBD-10     pE G V R S Y L S C W G N R G I C L L N R C P G R M R Q I G T C L A P R V K C C R
BNBD-11              G P L S C R R N G G V C I P I R C P G P M R Q I G T C F G R P V K C C R S W
BNBD-12              G P L S C G R N G G V C I P I R C P V P M R Q I G T C F G R P V K C C R S W
BNBD-13      S G I S G P L S C G R N G G V C I P I R C P V P M R Q I G T C F G R P V K C C R S W
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Fig. 4. Sequence comparison of hBD-1 with tracheal antimicrobial peptide (TAP), gallinacins (G al 1α, Gal 1 and Gal 2) and bovine neutrophil β-defensins 1–13 (BNBD-1 to BNBD-13). Bold type letters represent identical amino acids compared to hBD-1. Highly conserved amino acids are boxed. Spacers are inserted for optimal alignment.

amino acids of hBD-1. The antisense primer Unip-2 was also used for cDNA first strand synthesis. It is complementary to the poly(A) tail of eukaryotic messenger RNA. Specific cDNA fragments for hBD-1 including the primer sequences with 218 bp length were amplified from mRNA pools from human kidney and vagina and subsequently sequenced (Fig. 2).

The DNA sequences contained an open reading frame with a 100% match for the translated amino acid sequence of hBD-1. The codon for the last amino acid Lys^{36} is followed by a stop codon (TGA). The untranslated 3′ terminal region is 107 bp in length and contains a potential polyadenylation signal (AATAAA) in the expected distance from the Unip-2 hybridization site (poly(A) tail).

A database search revealed that this novel human peptide has a high homology to bovine neutrophil β-defensins (BNBD 1–13), tracheal antimicrobial peptide (TAP) from bovine trachea and gallinacins (Gal 1α, 1 and 2) from chicken leukocytes (Fig. 4). Conserved amino acids are the six cysteines and the residues Gly^{10}, Pro^{18}, Gly^{25}, Thr^{26}, and Lys^{33}. They include a highly conserved motif of nine amino acid residues denoted as core-motif for β-defensins [8]. The homology of identical amino acids ranges from 25% to 39% for the 13 bovine neutrophil β-defensins, 36% for TAP and 28% to 36% for gallinacins. Including chemical similar amino acids the overall homology is up to 58%. The cDNA of hBD-1 has no homology to the cDNA of TAP [14], the only cDNA published for β-defensins.

β-Defensins belong to the family of cationic and cysteine-rich antimicrobial peptides. They are present in leukocytes and epithelial cells from the respiratory tract and act in host defense. The novel peptide hBD-1 was isolated from blood ultrafiltrate. About 3 mg of hBD-1 were isolated from 4800 litres of HF, so hBD-1 is present in nanomolar concentration in HF. Concentrations of small peptides in HF with molecular masses below 10 kDa are the same as in plasma, since they are readily filtered during hemofiltration [17]. Therefore it is likely that plasma concentrations of hBD-1 in patients with renal failure, from whom HF is obtained, are in nanomolar range. Plasma concentrations in normal persons remain to be determined. The source which releases hBD-1 into the blood is not known. Since the message for hBD-1 is expressed in kidney and vagina as demonstrated by the cloning from these tissues, it implies that the urogenital tract belongs to those tissues synthesizing endogenous antibiotic peptides to establish an antimicrobial barrier. Further investigations will reveal the pattern of expression and the biological effect of hBD-1 and its relation to chronic renal failure.

Table 1
Amino acid composition of hBD-1

Amino acid	n_{AAA} [a]	n_{SA} [b]	Amino acid	n_{AAA} [a]	n_{SA} [b]
Ala	2.03	2	Leu	1.06	1
Arg	1.06	1	Lys	4.26	4
Asp(x)[c]	2.38	2	Phe	1.00	1
Cys	ND[e]	6	Pro	0.97	1
Glu(x)[d]	2.06	2	Ser	2.38	3
Gly	4.12	4	Thr	1.71	2
His	0.88	1	Tyr	2.53	3
Ile	2.00	2	Val	1.03	1

[a] n_{AAA}, number of amino acids determined by amino acid analysis. All values relative to phenylalanine.
[b] n_{SA}, number of amino acids determined by sequence analysis.
[c] Asp(x), aspartic acid and asparagine.
[d] Glu(x), glutamic acid and glutamate.
[e] ND, not determined.

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