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Structural and functional characterization of hBD-1(Ser35), a peptide deduced from a DEFB1 polymorphism

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Abstract

β-Defensins are mammalian antimicrobial peptides that share a unique disulfide-bonding motif of six conserved cysteines. An intragenic polymorphism of the DEFB1 gene that changes a highly conserved Cys to Ser in the peptide coding region has recently been described. The deduced peptide cannot form three disulfide bonds, as one of the cysteines is unpaired. We have determined the cysteine connectivities of a corresponding synthetic hBD-1(Ser35) peptide, investigated the structure by circular dichroism spectroscopy, and assayed the in vitro antimicrobial activity. Despite a different arrangement of the disulfides, hBD-1(Ser35) proved as active as hBD-1 against the microorganisms tested. This activity likely depends on the ability of hBD-1(Ser35) to adopt an amphipathic conformation in hydrophobic environment, similar to the wild type peptide, as suggested by CD spectroscopy.

Keywords: Antimicrobial peptide; hBD-1; Polymorphic variant; Cysteine connectivities; Circular dichroism spectroscopy

Defensins are mammalian antimicrobial peptides present in phagocytes and epithelial cells of various animal species and are characterized by a broad-spectrum antimicrobial activity [1]. Despite some differences in length and sequence, members of the defensin family all show a characteristic three-dimensional structure, stabilized by three disulfide bonds formed by six Cys residues. The cysteines and a few other residues form a highly conserved motif and are regarded as important structural constraints for these peptides. The defensins are divided into two subgroups, the α and β defensins, based on different spacings of the six Cys residues and arrangement of the disulfide bonds [1]. A 1–6/2–4/3–5 disulfide bond pattern has been described for α-defensins [2–4] and a 1–5/2–4/3–6 motif for β-defensins [5]. This difference does not affect the overall three-dimensional folding, which is similar in both subgroups and consists of three antiparallel β-strands stabilized by three disulfide linkages [6]. Both the crystal and the solution structures of the human and mouse β-defensins have been extensively investigated [7–11]. The results indicate that the human and murine β-defensins share a highly conserved three-dimensional structure despite little sequence similarity and that the three disulfides are the principal structural constraints [7].

Among the human β-defensins (hBDs), hBD-1 is constitutively expressed at mucosal surfaces of the airways, the gastrointestinal tract, and the urogenital tissues, where this peptide is thought to provide protection against microbes [12]. The hBD-1 gene DEFB1 maps to the 8p23 region [13]. Several intragenic polymorphisms of DEFB1 have been reported [14,15]. One of these includes a T to A transversion at position 1741 of the gene in the peptide coding region of the DEFB1 gene. This substitution changes a highly conserved Cys to Ser at position 35 of the sequence, thus abolishing one

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intramolecular disulfide bond. The question thus arises on how the inability of the peptide to form all three disulfide bonds could affect the structure and the antimicrobial properties of hBD-1.

To address the question, we have chemically synthesized a hBD-1 peptide corresponding to the variant hBD-1(Ser35) sequence and investigated its structural properties by circular dichroism spectroscopy and mass spectrometric analysis of the disulfide pattern and its antimicrobial properties in in vitro assays.

Materials and methods

Peptide synthesis, cleavage, and purification. A synthetic peptide corresponding to the native hBD-1 was purchased by Peptide International (Louisville, Kentucky, USA). hBD-1(Ser35) was synthesized on a Milligen 9050 automated synthesizer (Applied Biosystems, Foster City, USA) by the solid-phase technique using the Fmoc chemistry. Side chain protecting groups are as follows: Trityl (Trt) for Cys, Gln, Asn, His, *t*-butyl (*t*Bu) for Tyr, Thr, Ser, Asp, *t*-butyloxycarbonyl (Boc) for Lys, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. The starting resin was Fmoc-L-Lys(Boc)-PEG-PS (0.15 meq/g) and the synthesis was carried out at 45 °C in *N*,*N*-dimethylformamide (DMF) with a 6-fold excess of Fmoc-amino acids, activated in situ by an equivalent amount of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), in the presence of 0.6 N *N*-methylmorpholine. The cysteine derivatives were used as preformed OPfp-esters in the presence of 5% *N*-hydroxybenzotriazole (HOBt) in DMF to reduce racemization. Fmoc-deprotection was carried out with a solution containing piperidine, DMF, and *N*-methyl-2-pyrrolidone (NMP) (1:2:2), supplemented with 0.7% (v/v) 1,8-diazabicyclo (5.4.0)-undec-7-ene (DBU). This reagent was omitted in the last coupling to prevent dehydration of the Asp side chain. To improve the yield, difficult couplings (as predicted by the Peptide Companion software—CoshiSoft) were performed with an 8-fold amino acid excess, using an equimolar amount of *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as coupling agent instead of TBTU, followed by double coupling and capping with 0.33 M *N*-acetylimidazole in DMF. Immediately before each coupling step, the reaction column was saturated with the "magic mixture", containing dichloromethane (DCM)/NMP/DMF (1:1:1, v/v), with 2 M ethylene carbonate and 1% Triton X-100. The peptide cleavage from the resin was carried out using a mixture of trifluoroacetic acid/water/thioanisole/phenol/ethanedithiol/triisopropylsilane (82.5:5:4:4:2.5:2, v/v) for 2 h at room temperature. The crude peptide was repeatedly extracted with methyl butyl ether and purified by reversed phase high performance liquid chromatography (RP-HPLC) on a preparative (19 × 300 mm) C18 Delta-Pak column (Waters, Bedford, MA) using an appropriate 0–80% water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid.

Oxidation of Cys residues. After RP-HPLC purification of the peptide, the oxidation reaction is carried out as follows: several aliquots (0.3 mg) of hBD-1(Ser35) were dissolved in 0.4 ml of 0.1 M Tris–HCl buffer, pH 8.5, and left at room temperature for 8 h or longer. In all cases, no precipitates were observed and the reaction mixtures were then immediately frozen. The oxidized peptide was purified by RP-HPLC on a preparative Delta-Pak C18 (7.8 × 300 mm, Waters, Bedford, MA).

Proteolytic digestion. To establish the cysteine pairings, the peptide (100 μg) was digested using 5 μg human neutrophil elastase (ratio 20:1) in 0.1 M ammonium acetate, pH 5.5, at 37 °C for 8 h. The resultant fragments were separated by RP-HPLC on an analytical (4.6 × 50 mm) C18 Symmetry column (Waters, Bedford, MA) and analyzed by mass spectrometry.

Analytical assays. The molecular mass of hBD-1(Ser35) and of proteolytic fragments of this molecule was determined using an API I ion spray mass spectrometer (PE SCIEX, Toronto, Canada). Peptide concentration was determined based on the presence of three tyrosine residues, using a molar extinction coefficient at 280 nm of 1280 $cm^{-1} M^{-1}$ for each tyrosine residue [16]. Determination of free sulfhydryl groups was performed with Ellman's reagent [17].

Circular dichroism spectroscopy was carried out on a Jasco J-600 spectropolarimeter (Jasco, Tokyo, Japan) with the cell path length of 1 mm. Peptide samples (10–20 μM) were dissolved in 10 mM sodium phosphate buffer, pH 7.5, in the absence or presence of 30 mM SDS.

Antimicrobial assays. The antimicrobial activity was determined using published methods [18,19] against the following strains: *Pseudomonas aeruginosa* ATCC 27853 and a mucoid strain isolated from a cystic fibrosis patient, a methicillin-resistant clinical isolate of *Staphylococcus aureus* (MRSA), and clinical isolates of *Cryptococcus neoformans* and *Candida albicans*, provided by Dr. P. De Paoli (Centro di Riferimento Oncologico of Aviano, PN, Italy). Bacteria were maintained on Müller–Hinton agar plates (BD Biosciences). Single colonies were cultured overnight in Müller–Hinton Broth (MHB) at 37 °C, then diluted with fresh MHB (1:25), and allowed to grow to mid-log phase at 37 °C. Bacteria were pelleted by centrifugation at 1000*g* for 10 min, washed once in 0.9% NaCl, and resuspended in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. Working dilutions were prepared at 0.2×10^7 CFU/ml (assessed by turbidity) in 10 mM phosphate buffer (pH 7.4) containing 1% MHB. Bacteria (0.2×10^6 CFU/ml, final concentration) were incubated with various dilutions of peptides, prepared in sterile 0.01% acetic acid. The incubation was performed in 10 mM phosphate buffer (pH 7.4) containing 1% MHB, in a final volume of 100 μl. After 3 h of incubation at 37 °C, bacterial suspensions were serially diluted in cold 0.9% NaCl, plated on Müller–Hinton agar plates, and then incubated for 12–16 h at 37 °C to allow colony counts. When required, the incubation medium was supplemented with 150 mM NaCl. Sabouraud's medium was used in place of MHB and the incubation was performed at 30 °C, when the antifungal activity was tested. Fungal strains (*C. albicans* and *C. neoformans*) were maintained on Sabouraud's agar plates. Colonies were scraped and resuspended in sterile 0.9% NaCl and the number of CFU/ml was assessed by measuring the optical density at 600 nm. Fungal suspensions were then diluted in 10 mM phosphate buffer containing 1% Sabouraud at a final concentration of $1 \times 10^4 ml^{-1}$ to determine the antifungal activity.

Results

Peptide synthesis and oxidation

The sequences of native hBD-1 and hBD-1(Ser35) are reported in Fig. 1. The disulfide connectivities of hBD-1 are reported according to [8,9,5]. hBD-1(Ser35) was synthesized with an overall yield of crude peptide of 63%

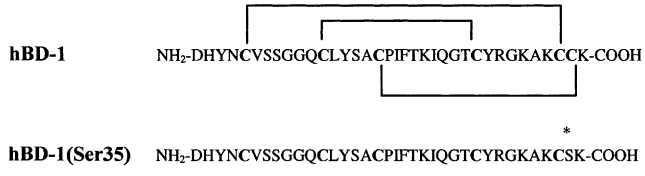

Fig. 1. Sequence of hBD-1 and hBD-1(Ser35). Unbroken lines indicate the disulfide connectivities of hBD-1. The asterisk indicates the substitution of Cys35 with Ser in hBD-1(Ser35).

after cleavage from the resin and the peak corresponding to hBD-1(Ser35) after RP-HPLC purification was 15% of the total. (Fig. 2A). Mass spectrometric analysis of the purified, reduced hBD-1(Ser35) indicated a mass of 3918.6 Da versus a theoretical mass of 3918.5 Da. The peptide was subjected to air-oxidation and purified by RP-HPLC (Fig. 2B) leading to a yield of oxidized peptide of approximately 7.5% of the crude product. Mass determination of this material indicated a molecular mass of 3915.0 Da, in good agreement with a mass of 3914.5 Da as expected for the presence of two disulfide bridges.

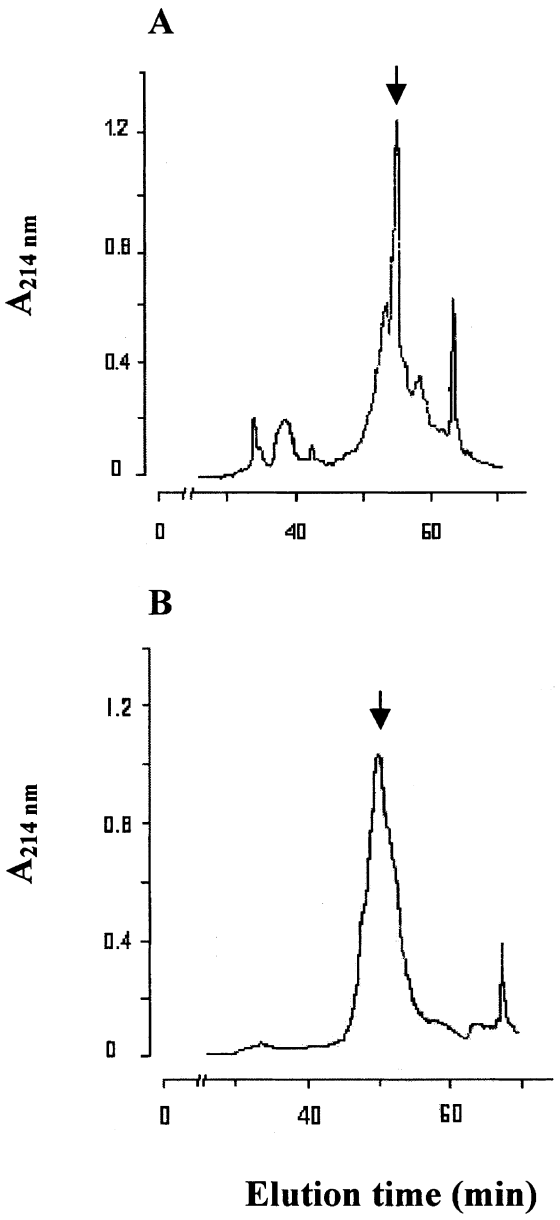

Fig. 2. RP-HPLC purification profiles of crude reduced hBD-1(Ser35) (A) and of the oxidized peptide (B). The arrow indicates the peak corresponding to the peptide with the correct molecular mass.

Circular dichroism studies

To investigate the secondary structure of hBD-1(Ser35) and compare this structure to that of the native hBD-1, CD spectra were recorded in the absence or presence of micellar SDS, used as a model of the negatively charged bacterial membranes (Fig. 3). The native peptide displayed similar spectra in 10 mM sodium phosphate buffer, pH 7.4 and in the presence of 30 mM SDS, consisting of a broad negative band at around 205–210 nm, and a small negative shoulder at around 220 nm. This pattern, which is the sum of the spectral contributions from all the secondary structural elements, indicated only minor spectral differences upon addition of the detergent, confirming that the folding of hBD-1 is mainly stabilized by the presence of the three disulfide bonds [7,9]. Conversely, the mutant peptide displayed a spectrum typical of a random coil in aqueous solution and underwent a marked conformational transition upon addition of 30 mM SDS (Fig. 3). The resulting spectrum was very similar to that of the native peptide under identical conditions. The observed con-

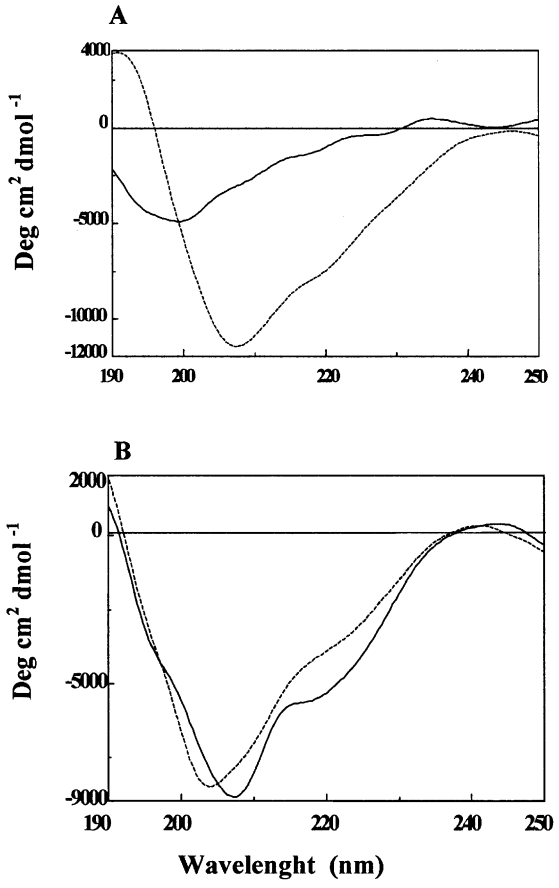

Fig. 3. Circular dichroism spectra of hBD-1(Ser35) (A) and hBD-1 (B) in the presence (------) or absence (——) of 30 mM SDS in 10 mM sodium phosphate buffer, pH 7.5.

formational transition suggests that hBD-1(Ser35) has a less constrained structure than the native hBD-1 peptide due to the absence of a disulfide bridge, which however appears to fold correctly in the presence of an anisotropic environment.

Characterization of the disulfide arrangement

To determine the arrangement of the disulfide bonds, the oxidized hBD-1(Ser35) was subjected to limited proteolysis followed by mass determination of the peptide fragments. The digestion was carried out using elastase, as inspection of the peptide sequence indicated several neutral residues suitable for cleavage by this enzyme. An acidic pH was chosen to minimize disulfide shuffling. The reaction mixture was subjected to HPLC purification after digestion and the fractions were collected and analyzed by mass spectrometry. Molecular masses of the peptide fragments and their assignments are listed in Table 1. In some cases, two explanations were possible for the same experimental mass. These were taken into account when supported by the identification of complementary fragments. Overall, the molecular weights of the fragments identified by mass spectrometry are consistent with Cys5–Cys12 + Cys17–Cys34 connectivities and unpaired Cys27. Only one fragment could also correspond to a 17–27 disulfide bond and unpaired Cys34, but, in the absence of any other fragments derived from this arrangement, it was not taken into consideration.

Antimicrobial activity

We examined the antimicrobial activity of hBD-1(Ser35) against bacterial and fungal strains, such as *P. aeruginosa*, *S. aureus*, *C. neoformans*, and *C. albicans* which often cause chronic infections in immunocompromised patients and in cystic fibrosis patients and compared the activities with those of native hBD-1. In Fig. 4 the results of assays performed using *P. aeruginosa* ATCC 27853 (Fig. 4A) and *P. aeruginosa* M, a mucoid strain isolated from a cystic fibrosis patient (Fig. 4B), are reported. Both strains were susceptible to hBD-1(Ser35) in the 1–20 μM concentration range. After 3 h of incubation at 37 °C, the variant peptide caused a reduction in the number of viable colonies of the reference strain by 2.7 and 3.2 log at 5 and 20 μM peptide concentrations, respectively, as compared with control

Table 1
hBD-1(Ser35) proteolytic fragments

Measured mass[a]	Calculated mass	Δ	Deduced sequence	Disulfide bridges
793.8	793.92	0.12	20 26 FTKIQGT	
	794	0.2	17 19 33 36 CPI KCSK	Cys17–Cys34
1273.09 ± 0.13	1273.4	0.31	1 6 11 14 DHYNCV QCLY	Cys5–Cys12
	1273.48	0.39	20 30 FTKIQGTCYRG	Cys27–H
1282.99 ± 0.01	1283.63	0.64	17 23 33 36 CPIFTKI KCSK	Cys17–Cys34
1630.69 ± 0.13	1630.74	0.05	1 15 DHYNCVSSGGQCLYS	Cys5–Cys12
	1630.98	0.29	15 19 27 36 SACPI CYRGKAKCSK	Cys17–Cys34/Cys27–H or Cys17–Cys27/Cys34–H
1648.59 ± 0.4	1648.74	0.15	1 10 11 15 DHYNCVSSGG QCLYS	Cys5–Cys12
1668.88 ± 0.42	1668.07	0.81	17 25 31 36 CPIFTKIQG KAKCSK	Cys17–Cys34

[a] Experimental mass measured by API I ion spray mass spectrometer; Δ—difference between measured and calculated mass.

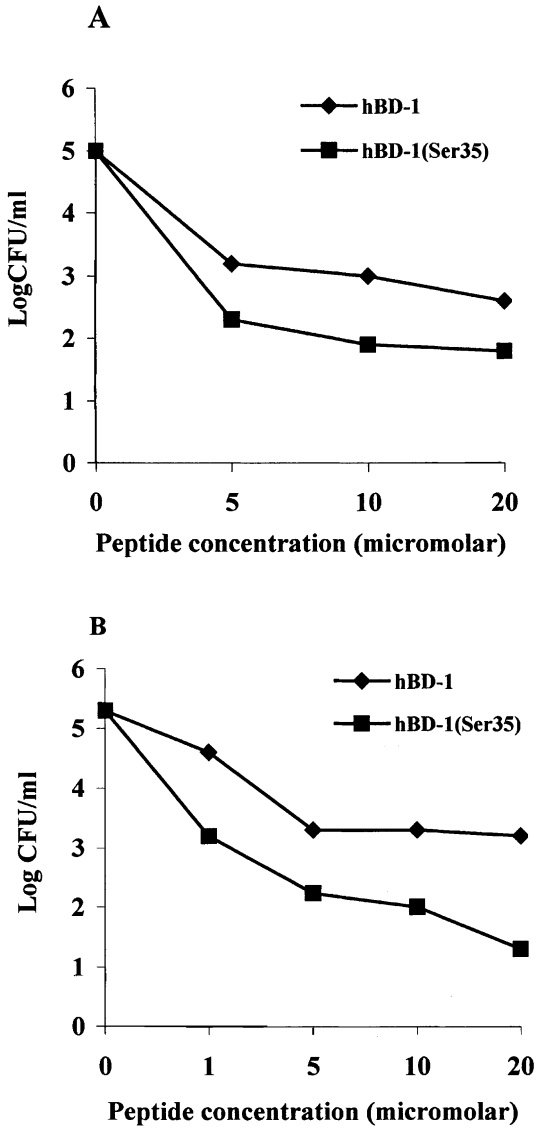

Fig. 4. Antibacterial activities of hBD-1 and hBD-1(Ser35). (A) *P. aeruginosa* ATCC 27853 (1.8×10^5 CFU/ml) and (B) *P. aeruginosa* M (2.1×10^5 CFU/ml) were incubated with the indicated amounts of hBD-1 or hBD-1(Ser35) for 3 h at 37 °C in 10 mM sodium phosphate buffer containing 1% Müller–Hinton broth. The samples were serially diluted in 0.9% NaCl, plated on Müller–Hinton agar, and incubated overnight at 37 °C to allow colony counts. Data are the means of two experiments performed in duplicate.

sample at the same incubation time, while a 2.4 log reduction was observed with the native peptide at 20 μM. The variant peptide caused a 4 log reduction of the M strain of *P. aeruginosa* at the highest peptide concentration used, whereas the native peptide was slightly less active (Fig. 4B). Neither peptide affected the growth of *S. aureus* MRSA at 40 μM concentration (data not shown). hBD-1(Ser35), at the same concentration, proved more effective than the native hBD-1 against *C. neoformans* (80% vs. 25% decrease in colony counts, Fig. 5). Both peptides were inactive against *C. albicans* up to 40 μM peptide concentration (not shown). Finally,

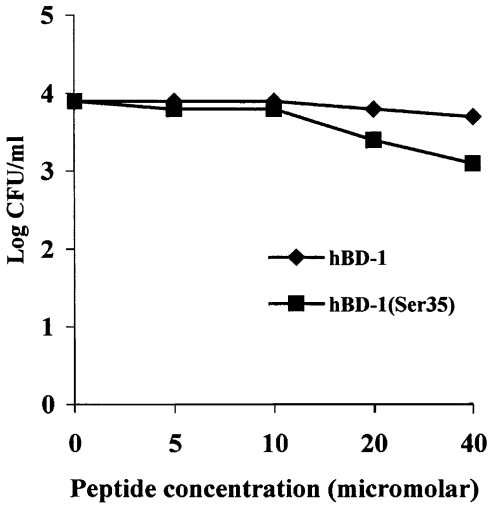

Fig. 5. Antifungal activities of hBD-1 and hBD-1(Ser35) against a clinical isolate of *C. neoformans*. *C. neoformans* (1×10^4 CFU/ml) was incubated with the indicated amounts of hBD-1 or hBD-1(Ser35) for 3 h at 30 °C in 10 mM sodium phosphate buffer containing 1% Sabouraud's broth. After dilution in 0.9% NaCl, samples were plated on Sabouraud's agar and incubated for 48 h at 30 °C, to allow colony counts. Data are the means of two experiments performed in duplicate.

the antibacterial and antifungal activities of both peptides were completely abolished by addition of 150 mM NaCl to the incubation medium (not shown).

Discussion

A polymorphic variant of the human DEFB1 gene was recently identified [15]. The gene showed a T to A substitution that resulted in the replacement of a strictly conserved Cys with Ser at position 35 of the peptide sequence (Fig. 1). Several studies on the structure of defensins [2,6,20] indicate that the six cysteine residues are important structural constraints for the three-dimensional folding of these peptides and this has also been confirmed by the recently solved crystal and solution structures of hBD-2 [8,10] and hBD-1 [7,9]. In principle, the lack of one of the disulfide bridges should have important consequences on the three-dimensional folding and on the antimicrobial activity of hBD-1. The present study aimed at investigating the structural and functional properties of this polymorphic variant, so that a synthetic peptide, hBD-1(Ser35), was prepared, purified, and allowed to fold spontaneously in the presence of air-oxygen, to favor natural propensity to form disulfide bonds. The oxidized molecule showed the correct molecular mass for a two disulfide peptide. The appearance of a dimeric hBD-1(Ser35) was observed after a prolonged (24 h) oxidation time. The in vitro activity of this peptide was also tested and found to be comparable to that of the monomer (data not shown).

Interesting data were obtained from CD spectra. hBD-1(Ser35) exhibited an unordered structure in phosphate buffer and underwent a conformational transition upon addition of micellar SDS, which mimicks the negatively charged bacterial membranes. The CD pattern, with a minimum at around 205–210 nm, and a small shoulder at around 220 nm, was very similar to that of hBD-1 (Fig. 3). Both peptides appeared to contain a similar extent of secondary structure. As only a few circular dichroism studies of defensin peptides have been reported, despite a huge body of data on their three-dimensional structures, the CD spectra were also interpreted in the light of the recently resolved crystal and solution structures of hBD-1 [7,9]. The spectra are slightly different from those reported for NP-1, a rabbit α-defensin [21], and from those reported for the cathelicidin peptide protegrin PG-1 [22]. Rabbit NP-1 can be considered representative of "classical" α-defensins, which are characterized by a triple-stranded antiparallel β-sheet [3,6,23]. Protegrins contain two disulfide bonds and are characterized by a two-stranded antiparallel β-sheet conformation [24]. The CD spectra reported for NP-1 and PG-1 show a negative band at around 205–210 nm, similar to hBD-1 and hBD-1(Ser35), and a positive band in the 225–230 nm region [21,22], which is absent in hBD-1 and its variant. The negative band at 205–210 nm indicates a β-sheet conformation in both hBD-1 and hBD-1(Ser35).

Based on mass spectrometric analysis of the proteolytic fragments (Table 1) the cysteine bridges in the variant peptide are as follows: Cys5–Cys12/Cys17–Cys34 with unpaired Cys27. This arrangement is supported by the following observations: (i) the Cys5–Cys12 linkage was found in the fragments 1–6 + 11–14 (1273.4 Da), 1–15 (1630.74 Da), and 1–10 + 11–15 (1648.74 Da); (ii) Cys27 was found reduced in the fragment 20–30 (1273.48 Da); (iii) the Cys17–Cys34 bridge was deduced from fragments 17–19 + 33–36 (794 Da), 17–23 + 33–36 (1283.63 Da), and 17–25 + 31–36 (1668.07 Da). It is noteworthy that in those cases where the experimental mass could be assigned to more than one putative fragment, all the interpretations were equally acceptable, as they differed from the experimental values by a standard deviation of $\leqslant$0.39 Da. However, only the proteolytic fragments deriving from the 5–12 and 17–34 Cys pairings were complementary to each other, mutually supporting their identification. The deduced arrangement 5–12/27–H/17–34 can be discussed in the light of the recently resolved crystal structures of hBD-1 and hBD-2 [8,9] that show the presence of 5–34/12–27/17–35 cysteine connectivities. All the secondary structural elements, i.e., the short N-terminal α-helix and the three central β-strands, which form a compact globular core, are held together by these linkages. In hBD-1(Ser35), Cys35 is missing and Cys17 is bound to Cys34, nearest to the missing Cys35. In turn, Cys5 can no longer be engaged in a linkage with Cys34 and forms a bridge with Cys12, leaving Cys27 unpaired. Based on circular dichroism measurements, the variant molecule appears to be more flexible than hBD-1. The Cys5–Cys12 linkage may contribute to this flexibility, as the region corresponding to the β1-strand of hBD-1 is not tightly connected in hBD-1(Ser35) to the regions corresponding to β2- and β3-strands. In addition, Cys27 is not engaged in disulfides and this further contributes to the observed flexibility. The region encompassing the three β-strands and the loop region between β2- and β3-strands in hBD-1 are likely unordered in hBD-1(Ser35). It is interesting to note that, based on CD spectra, the secondary structure of hBD-1(Ser35) is similar to that displayed by hBD-1 in an anisotropic environment. This implies that an amphipathic conformation can also be adopted in the absence of one of the three disulfides.

We found that hBD-1(Ser35) exhibits antibacterial and antifungal activities against strains susceptible to hBD-1 (*P. aeruginosa* and *C. neoformans*), in the same concentration range (5–40 μM) and at comparable time intervals (3 h). Furthermore, the activities of both peptides are abolished in the presence of 150 mM NaCl. It is reasonable to correlate the activity of hBD-1(Ser35) with the adoption of an amphipathic conformation, as suggested by CD spectra, when the molecule reaches the bacterial surface, which in turn allows the molecule to perturb the cytoplasmic bacterial membrane. Although the exact mechanism of bacterial killing has not yet been fully understood, many studies indicate that an amphipathic conformation is required for membrane perturbation, which is responsible for the subsequent bacterial death [24–27].

It is somewhat surprising that the activity of hBD-1(Ser35) is not decreased, compared to hBD-1. In fact, SAR studies on other cysteine-containing antimicrobial peptides, i.e., porcine protegrins [24], which contain two disulfide bridges, indicate that the disulfide-bridged β-sheet is a key requirement to the antibacterial activity and stability of these peptides. Similar correlations have been recently investigated for hBD2 [8], demonstrating a complete loss of activity when the peptide was reduced and carboxyamidomethylated. By analogy, a somewhat decreased antimicrobial effect of hBD-1(Ser35), which lacks one of the three disulfides, might be expected. Conversely, the results of the antimicrobial assays indicate that the remaining disulfide bridges are sufficient to stabilize the three-dimensional conformation required for activity and suggest that the protective role of the peptide in individuals carrying this mutation may not be impaired.

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