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Synthesis and characterization of human α-defensins 4-6

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Abstract: Human α-defensins are small, Cys-rich, cationic proteins expressed predominantly in neutrophils and intestinal epithelia. They play important roles in innate and adaptive immunity against infection. Progress in studying these molecules can be accelerated by access to large quantities of high-quality materials, which have been obtained mainly from natural sources. Here, we report total synthesis of human α-defensins 4, 5, and 6, also known as HNP4, HD5, and HD6, using the optimized *N,N*-diisopropylethylamine (DIEA) in situ neutralization/2-(1 H-benzotriazolyl)-1,1,3, 3-tetramethyluroniumhexafluorophosphate (HBTU) activation protocol for solid-phase Boc chemistry. Oxidative folding/disulfide formation was achieved directly using crude peptides, resulting in an overall synthetic yield of 10–16% with high purity. Antimicrobial activity assays were performed with *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, using colony-counting methods, and the results demonstrated differential activity against these strains. Our report describes a highly efficient synthetic approach that enables thorough structural and functional studies of these three important immunologic molecules.

Introduction

Defensins, a group of small cationic proteins featuring a β-sheet-rich fold and a framework of three disulfide bonds, have attracted considerable interest in recent years (1,2). Defensins function not only in the innate immune system against infectious microbes, but also in adaptive immunity by activating certain types of immune cells (3–6). In

humans, two classes of defensins, termed α- and β-defensins, have been identified, which differ with respect to their tissue specificities, amino acid composition and linkage pattern of six conserved cysteine residues.

To date, six human α-defensins have been identified, comprising 29–35 amino acid residues with the disulfide connectivity Cys^1-Cys^6, Cys^2-Cys^4, and Cys^3-Cys^5. The amino acid sequences of six human α-defensins are shown below.

HNP1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP2	CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP4	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRV
HD5	ATCYCRHGRCATRESLSGVCEISGRLYRLCCR
HD6	AFTCHCRR-SCYSTEYSYGTCTVMGINHRFCCL

The first four members of the human α-defensin family, also termed human neutrophil peptide 1–4 (HNP1–4), were originally isolated from azurophilic granules of circulating neutrophils (7–10). Of these, HNP1–3, accounting for 5–7% of the total neutrophil protein, are two orders of magnitude more abundant than HNP4. The other two human α-defensins (HD5–6) were initially found in Paneth cells in the small intestine and secreted into the gut lumen in response to bacterial stimulation (11,12). Recent studies show that human α-defensins are also expressed in a variety of other cells and tissues involved in the host defense against infection (13–15). Notably, HNP1–3 differ only at the N-terminal residue. HNP4, HD5, and HD6, on the other hand, show less sequence similarity with HNP1–3 or each other.

The biological functions of human α-defensins are diverse. Members of the family exhibit broad antimicrobial activities at micromolar concentrations against bacteria, fungi, and certain enveloped viruses. HNP1–3 have been reported to suppress HIV-1 infection in vitro (16–18). HNP4 has anti-adrenocorticotropic hormone (ACTH) activity at nanomolar concentrations (10). Recently, it was reported that an HD5 transgene expressed in mouse Paneth cells conferred resistance in mice to intestinal infection with *Salmonella typhimurium* (19). Thus far, little is known about HD6.

Many studies to date have utilized human α-defensins purified directly from various cells and tissues, but low yield makes extensive structural and functional studies costly and time-consuming. Solid-phase peptide synthesis is an efficient method to produce highly pure proteins of small size. Access to abundant, high-quality defensins through total chemical synthesis would accelerate progress in the defensin research. Syntheses of human defensins and analogs through chemical approaches have been explored in recent years, including HNP1–3 and human β-defensins (20–26). Although HNP1–3 have been extensively characterized, there are few studies of the structure or function of HNP4, HD5, and HD6, partly because of the absence of an efficient method to produce large quantities of pure materials. The development of such methods would narrow the gap in our understanding of HNP1–3 relative to the other three human α-defensins, facilitating comparative studies of all six human α-defensins. This report describes for the first time high-yield synthesis of HNP4, HD5 and HD6, using the optimized *N*,*N*-diisopropylethylamine (DIEA) in situ neutralization/2-(1 H-benzotriazolyl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) activation protocol developed by Kent and colleagues for solid-phase Boc chemistry (27,28).

Experimental Procedures

Materials

Boc-L-amino acids were purchased from Peptides International (Louisville, KY, USA). HBTU was purchased from Qbiogen, Inc. (Carlsbad, CA, USA). Boc-Val-OCH_2-PAM, Boc-Leu-OCH_2-PAM and Boc-Arg(Tos)-OCH_2-PAM resins as well as DIEA were obtained from Applied Biosystems, Inc. (Foster City, CA, USA). Trifluoroacetic acid (TFA) was purchased from Halocarbon, Inc. (River Edge, NJ, USA), hydrogen fluoride (HF) from Matheson Trigas (Bridgeport, NJ, USA). Dimethylformamide (DMF; sequencing grade), dichloromethane (DCM; synthesis grade) and acetonitrile [high-performance liquid chromatography (HPLC) grade] were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Solid-phase peptide synthesis

All three peptides were synthesized on an Applied Biosystems 433A synthesizer using the published DIEA in situ neutralization/HBTU activation protocol for Boc solid-phase peptide synthesis (27,28). Specifically, Boc-amino acids (2.2 mmol) were activated in DMF for 3 min by 2.0 mmol of HBTU in the presence of 20% DIEA (v/v), and coupled in stepwise fashion for 10 min on 0.25 mmol of

appropriate PAM resin. TFA (100%) was used for removal of *N*-Boc groups; DMF and DCM were used for batch washes throughout the entire synthesis. The following side chain protections were used: Arg(Tosyl), Asn(Xanthyl), Asp(OcHxl), Cys(4MeBzl), Glu(OcHxl), His(Bom), Lys(2-ClZ), Ser(Bzl), Thr(Bzl), Tyr(BrZ). After chain assembly, the peptides were deprotected and cleaved by anhydrous HF in the presence of 5% *p*-cresol/thiocresol (1 : 1) at 0 °C for 1 h, followed by precipitation with cold ether.

Protein oxidative folding

Oxidative folding/disulfide formation of synthetic defensins was achieved through thiol-disulfide shuffling in the presence of reduced (3 mM) and oxidized (0.3 mM) glutathione, and different concentrations of GuHCl. Specifically, crude peptides were first dissolved in 8 M GuHCl prepared in water, containing appropriate concentrations of reduced and oxidized glutathione, followed by a rapid dilution with 0.25 M $NaHCO_3$, resulting in a final pH value of 8.3. For HNP4, the final concentrations of crude peptide and GuHCl were 0.25 mg/mL and 4 M, respectively. In the case of HD5 and HD6, the final concentrations of crude peptide and GuHCl were 0.5 mg/mL and 2 M, respectively. The folding proceeded overnight at room temperature. The desired product was purified by preparative reversed phase (RP) HPLC and subsequently lyophilized.

RP-HPLC and ESI-MS

Analytical RP-HPLC was performed on an Agilent 1100 series system (Palo Alto, CA, USA) or a Waters 2690 Alliance system equipped with a Waters Symmetry 300™ analytical C18 column (5 μm, 4.6 × 150 mm). Preparative RP-HPLC was carried out on a Waters Delta Prep 600 system (Milford, MA, USA) equipped with a Vydac preparative C18 column (15–20 μm, 50 × 250 mm). Solvents used for HPLC purification were water containing 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). Electrospray ionization mass spectrometry (ESI-MS) analyses were carried out on a Micromass ZQ-4000 single quadruple mass spectrometer (Milford, MA, USA). Samples were suspended in methanol/water/acetic acid (49 : 49 : 2) and infused by a syringe pump at 10 μL/min. De-convolution of data was performed using the Micromass MaxEnt software.

Antimicrobial assays

Four human α-defensins, HNP1, HNP4, HD5, and HD6 were tested for antibacterial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. Assays for antimicrobial activity were essentially the same as previously described for defensins (29). The bacteria were grown to mid-logarithmic phase in tryptic soy broth, and then diluted to 1×10^6 cfu/mL in 10 mM potassium phosphate, 1% tryptic soy broth, pH 7.4. 100 μL of cells were incubated in the presence of different concentrations of peptides for 3 h at 37 °C. The cells were then diluted serially in the same buffer, plated on Luria Broth plates, and incubated for 18 h at 33 °C, and the colonies were counted. Bactericidal activity was expressed as the ratio of colonies counted to the number of colonies on a control plate. LD_{50}, LD_{90}, and minimum bacterial concentraion (MBC) are defined as the minimum tested concentration of peptide at which at least 50%, 90%, and 99.9% of the viable cells are killed, respectively. All assays were carried out in triplicate. The results are shown in Table 1.

Results and Discussion

Synthesis of HNP4

HNP4 was first discovered in 1988 as an anti-ACTH molecule (10). It was independently isolated from neutrophils as an antibiotic peptide 100-fold less abundant than its previously described predecessors, HNP1–3 (7–9). Despite intense interest over the years in the properties of HNP1–3, studies of the fourth member of the neutrophil α-defensin family, since its identification, have remained largely nonexistent in the literature. Further, in contrast to HNP1–3, chemical synthesis of HNP4 has not yet been reported. Motivated by the conspicuous absence of studies of HNP4, we embarked on the total chemical synthesis of this antimicrobial peptide, overcoming a variety of unexpected technical pitfalls associated with the synthesis.

Crude HNP4, after HF cleavage and ether precipitation, gave rise to a molecular mass of 3715.3 Da, in agreement with the expected value of 3715.5 Da calculated on the basis of the average isotopic compositions of reduced HNP4. However, while the crude peptide exhibited an acceptable purity as indicated by ESI-MS analysis (data not shown), it displayed an extremely broad and strongly tailing peak of apparent heterogeneity when analyzed on

Table 1. **Antibacterial activity of four synthetic human α-defensins against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 as determined by colony counting**

Defensin	Strain	LD_{50}			LD_{90}			MBC		
HNP1	*E. coli*	3.1	6.3	6.3	12.5	6.3	6.3	25	25	25
	S. aureus	6.3	3.1	3.1	50	25	25	>100	100	100
HNP4	*E. coli*	6.3	6.3	6.3	25	12.5	12.5	50	50	25
	S. aureus	ND	ND	ND	ND	ND	ND	ND	ND	ND
HD5	*E. coli*	6.3	0.8	1.6	6.3	1.6	3.1	12.5	25	6.3
	S. aureus	1.6	1.6	1.6	25	50	50	100	100	100
HD6	*E. coli*	>100	>100	>100	>100	>100	>100	>100	>100	>100
	S. aureus	>100	>100	>100	>100	>100	>100	>100	>100	>100

LD_{50}, LD_{90}, and MBC are reported as the lowest concentration (μg/mL) resulting in at least 50%, 90%, or 99.9% killing, respectively. Triplicate results are ordered (e.g. leftmost LD_{50} corresponds to leftmost MBC, and so forth).

analytical C18 RP-HPLC (Fig. 1A). We observed similar behavior when an analytical C4 RP column was used (data not shown). Therefore, instead of pursuing purification of HNP4, we decided to fold the crude peptide, directly, in the presence of reduced and oxidized glutathione and GuHCl. We found that a moderately high concentration of GuHCl (4 M) was required in order to effectively suppress massive HNP4 aggregation during disulfide formation. In addition, 8 M GuHCl was needed to completely dissolve crude materials prior to oxidative folding. Notably, after overnight folding, a chromatographically homogeneous and separable species with a shortened retention time on C18 RP-HPLC emerged out of the crude preparation (Fig. 1B). ESI-MS analysis of the major species indicated a loss of six mass units compared with the reduced form, suggesting formation of three disulfide bridges in the molecule. The folded species was purified by preparative C18 RP-HPLC using a linear gradient of 20–40% B at a flow rate of 40 mL/min over 120 min. On a 0.25-mmol scale of synthesis, approximately 150 mg of folded and purified HNP4 were obtained, representing an overall synthetic yield of 16%. Shown in Fig. 2 is the final product analyzed by C18 RP-HPLC and ESI-MS. The observed molecular mass of 3709.3 ± 0.5 Da is within experimental accuracy of the expected value of 3709.5 Da calculated from the average isotopic compositions of folded HNP4.

To understand the anomalous chromatographic behavior of crude HNP4 on RP-HPLC, we reduced a sample of the folded and purified material using excess amounts of dithiothreitol (DTT) in the presence of 6 M GuHCl at pH 7.5, and subsequently analyzed it on C18 RP-HPLC. As demonstrated in Fig. 3, the reduction procedure essentially replicated the chromatogram previously seen with the crude, reduced HNP4, suggesting that an inherent chemical property of the reduced peptide, regardless of its purity, is

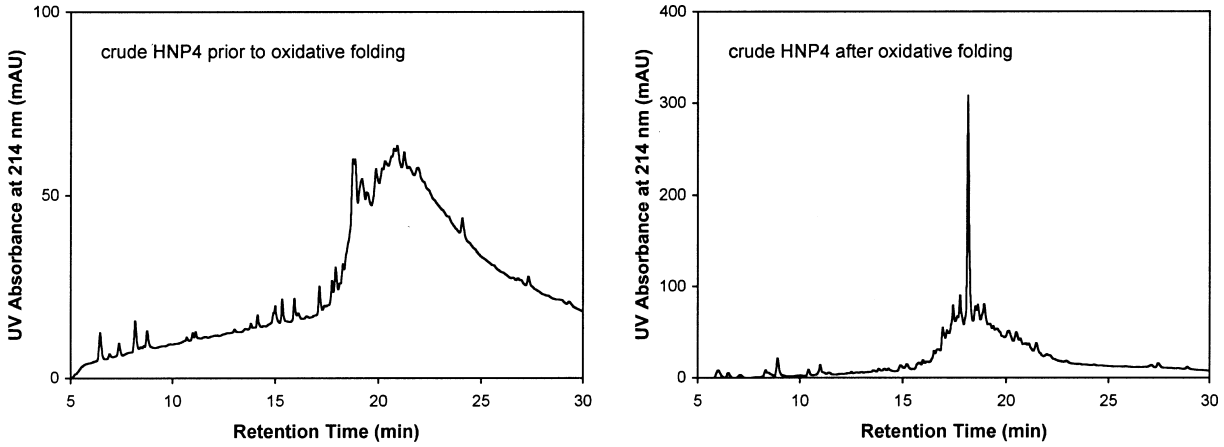

Figure 1. Crude HNP4 analyzed by reversed phase high-performance liquid chromatography (RP-HPLC), before and after oxidative folding/disulfide formation. The chromatographic data were obtained at 40 °C using a linear gradient of 5–65% B at a flow rate of 1 mL/min over 30 min. Note that minor precipitation was observed during folding, and the quantities of peptide injected before and after folding were not equal.

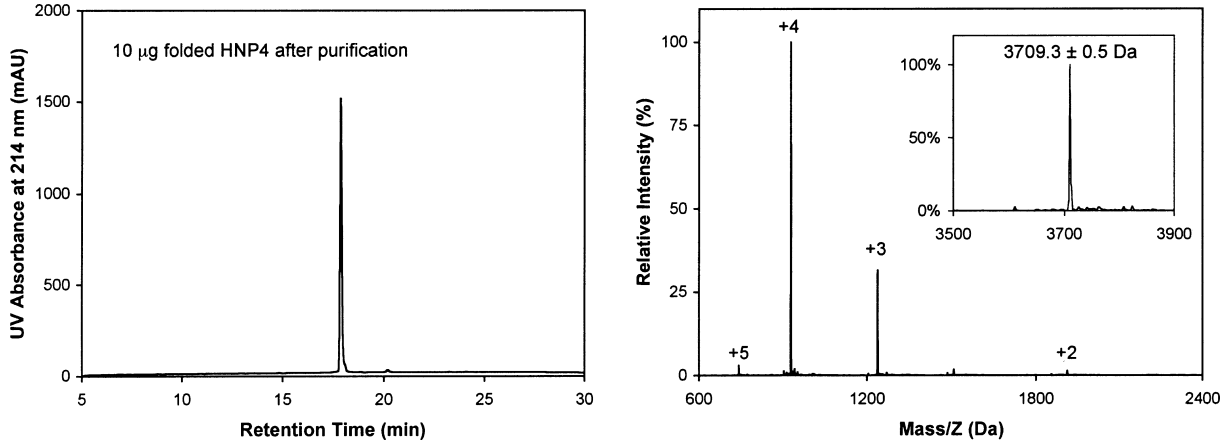

Figure 2. Folded and purified HNP4 analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS). The HPLC analysis was performed under the same conditions as previously described in Fig. 1. The determined molecular mass of HNP4 by ESI-MS, 3709.3 ± 0.5 Da, is within experimental error of the expected value of 3709.5 Da calculated on the basis of average isotopic compositions of folded HNP4.

responsible for such chromatographic anomaly. It is plausible that sequence-dependent hydrophobic interactions between reduced HNP4 and the column matrix contribute, although the precise nature of this interaction at the molecular level remains obscure.

To verify that HNP4 is correctly folded, we determined the disulfide connectivity in the molecule using the previously published LC-MS protocol to map peptide fragments generated by proteolysis and manual Edman degradation (20–22). Our results unequivocally demonstrated a connection of Cys^1-Cys^6, Cys^2-Cys^4, and Cys^3-Cys^5 (data not shown), consistent with the conserved disulfide topology found in known mammalian α-defensins. Recently, the crystal structure of synthetic HNP4, similar to the known crystal structure of HNP3 (30), has been solved at 1.8 Å resolution (Szyk, A., Wu, Z., Tucker, K., Yang, D., Lu, W. and Lubkowski, J., manuscript in preparation), further supporting the correct folding of HNP4.

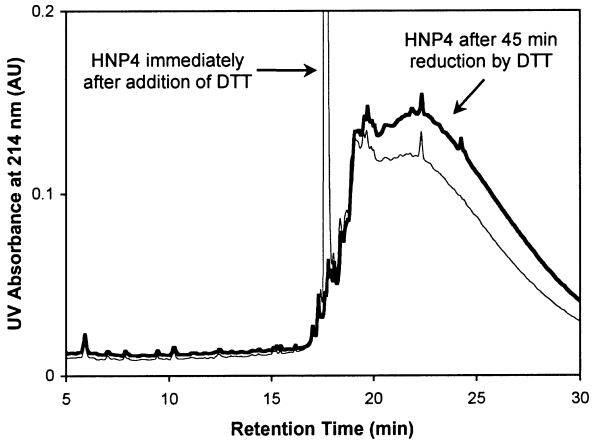

Figure 3. Reduction of folded and purified HNP4 by DTT, monitored by reversed phase high-performance liquid chromatography (RP-HPLC) (5–65% B over 30 min at 1 mL/min and 40 °C). The data were collected immediately after addition of DTT (thin line) and 45 min after DTT reduction (thick line).

Synthesis of HD5 and HD6

The two intestinal α-defensins, HD5 and HD6, secreted in response to bacterial stimulation of human Paneth cells, were first identified in 1992 (11,12). As is the case with HNP4, however, synthetic production of both defensins has not yet been reported in the literature. Encouraged largely by the high-yield synthesis of HNP4, we folded both HD5 and HD6 using crude materials without prior HPLC purification. Shown in Fig. 4 are HPLC traces of crude HD5 and HD6 before and after oxidative folding/disulfide formation performed under similar conditions in the presence of 2 M GuHCl. Shifting of the retention time of the populated species is consistent with burial of hydrophobic residues into a folded structure stabilized by disulfide bonding. It became apparent that the oxidative folding step alone significantly reduced chromatographic heterogeneity seen with the reduced materials, in particular, with HD5. After preparative C18 RP-HPLC purification, about 90 mg of defensin each were obtained, accounting for an overall yield of 10% on a 0.25-mmol synthetic scale.

Folded and purified HD5 and HD6 were analyzed on C18 RP-HPLC and ESI-MS, and data are shown in Fig. 5. For both proteins, the observed molecular masses 3581.8 ± 0.5 Da (HD5) and 3708.1 ± 0.3 Da (HD6) are in agreement with the calculated average isotopic masses of

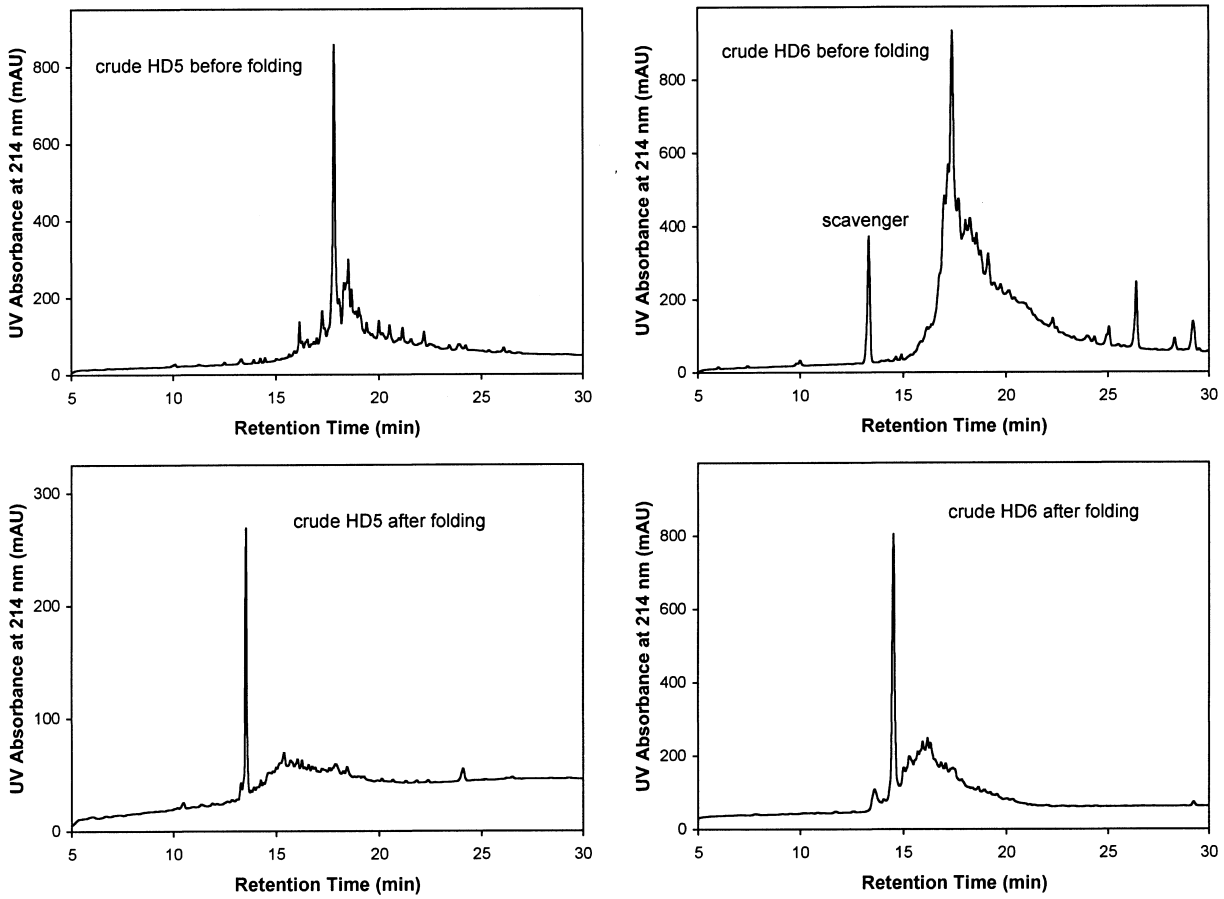

Figure 4. Crude HD5 and HD6 analyzed by reversed phase high-performance liquid chromatography (RP-HPLC), before and after oxidative folding/disulfide formation. HPLC analyses were carried out at 40 °C using a linear gradient of 5–65% B at a flow rate of 1 mL/min over 30 min. Note that minor precipitation was observed during folding, and the quantities of peptide injected before and after folding were not equal.

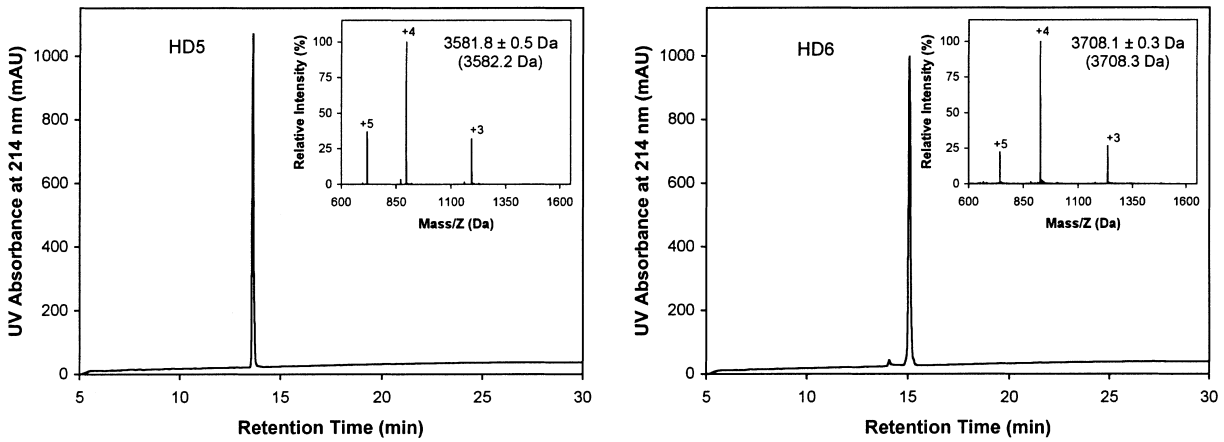

Figure 5. Folded and purified HD5 and HD6 analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS). The chromatographic conditions were the same as described for previous figures. The expected molecular masses of folded HD5 and HD6, calculated from their average isotopic compositions, are in parentheses.

3582.2 and 3708.3 Da, respectively. It is worth pointing out that folded HD5 and HD6 are noticeably less hydrophobic (more polar) than HNP4, as indicated by their relative retention on C18 RP-HPLC. We did not determine the disulfide connectivity in HD5 and HD6. However, in addition to our HPLC and ESI-MS results consistent with the correct disulfide formation, several lines of evidence support that HD5 is properly folded. First, synthetic HD5 showed a carbohydrate/glycoprotein-binding property identical to a characterized recombinant form (R. Lehrer, UCLA, personal

communication). Secondly, preliminary two-dimensional nuclear magnetic resonance (NMR) spectroscopic studies of synthetic HD5 yielded well-dispersed Total Correlation Spectroscopy (TOCSY) expected for a folded protein (H.-C. Siebert, Utrecht University, the Netherlands, personal communication). Thirdly, HD5 and HD6 have recently been crystallized and diffracted to high resolution, and the structure determination is under way (J. Lubkowski, personal communication).

Functional characterization of synthetic HNP4, HD5, and HD6

Since their discovery, the characterization of human defensin antimicrobial activity has been a frequently pursued avenue of research. In contrast to HNP1–3 and the human β-defensins, however, reports describing antimicrobial properties of HNP4, as well as intestinal defensins, HD5 and HD6, are sparse, perhaps due to the absence of a practical method to purify or synthesize sufficient quantities of high-quality material. In fact, no functional data has been published for HD6. Wilde *et al.* studied the in vitro bactericidal activity against *E. coli* K12 strain MC4100, *Streptococcus faecalis* ATCC 29212, and a clinical isolate of *Candida albicans* (9). Interestingly, they reported an LD_{50} of HNP4 117-fold lower than that of HNP1 against the *E. coli* strain studied. To assess the antibacterial properties of synthetic α-defensins, we subjected them to a bactericidal assay against two different bacterial strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. In our tests, we also included synthetic HNP1, previously characterized structurally, biochemically and functionally (21,22). As shown in Table 1, HNP4 and HNP1 gave the same LD_{50} values against *E. coli* ATCC 25922, in contrast with the results of Wilde et al. obtained using a different assay protocol with a different *E. coli* strain. Our synthetic preparation of HNP4 was highly pure (see Fig. 2), and its correct fold was recently confirmed through the solution of its crystal structure (results to be published elsewhere). Bactericidal assay results were also obtained for the two intestinal defensins. Whereas HD5 was quite potent against both bacteria tested, HD6 showed activity against neither. Although both molecules are of similar mass and comparable isoelectric points, their amino acid sequence identity is only 34%. Results of the structural studies for recently crystallized synthetic HD5 and HD6 should shed light on the structural basis for this difference in activity. Further tests are necessary to ascribe a function to HD6 and determine what, if any, is its contribution to immunity.

Conclusion

Human α-defensins are important immunologic proteins involved in the host defense against infection. Studies of the structure/function relationships for these proteins and their mechanisms of action, while largely unexplored, will clearly benefit from a convenient access to large quantities of quality materials. This report describes high-yield chemical synthesis of highly pure HNP4, HD5, and HD6, which was achieved through productive folding/disulfide formation, directly, of crude materials. Our work provides a practical and robust approach to synthetic HNP4, HD5, and HD6 that should accelerate multifaceted investigations of these multifunctional proteins at the molecular level.

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