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Synthesis of hepcidin derivatives in order to develop standards for immune adsorption method

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MeCN, acetonitrile; ECL, enhanced chemiluminescence; EDT, 1,2-ethanedithiole; HEPC12-A, rabbit anti-human hepcidin IgG, affinity purified; HEPC13-A, rabbit anti-mouse/human hepcidin IgG, affinity purified; HEPC61-P, human hepcidin-25 control/blocking synthetic peptide; HRP, horseradish peroxidase; IL-6, interleukin-6; KLH, keyhole limpet hemocyanin; LEAP, liver-expressed antimicrobial peptide; NEM, *N*-ethylmaleimide; NMP, *N*-methyl-pirrolidone; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SELDI-TOF-MS, surface-enhanced laser desorption ionization–time-of-flight mass spectrometry; TMB, tetramethylbenzidin; TNF-α, tumor necrosis factor-α **Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: hepcidin; hepcidin standards; peptide synthesis; immune adsorption method

Introduction

Hepatic bactericidal protein (Hepcidin) is an iron regulatory peptide, which has been discovered just recently. It is involved in iron metabolism, and in several iron-related disorders, such as iron deficiency anemia, inflammatory anemia, anemia of chronic diseases, and hemochromatosis. It also exhibits antimicrobial activity, and as a defensin-like peptide, it is an element of innate immune system [1–3]. Hepcidin is synthesized in the liver and in the kidney, and is present in human plasma and in urine [4]. Hepcidin acutely decreases serum iron level by inhibiting both the iron release from iron stores of reticuloendothelial cells (macrophages) and the iron adsorption from the gastrointestine (duodenum and upper jejunum) and placenta [5,6]. Hepcidin inhibits the expression of iron transporters, and hence contributes to the retention of iron in the iron stores and in the small intestine [7]. Hepcidin expression is low when body iron stores are insufficient, for example, in anemia or hypoxia. The expression and biosynthesis of hepcidin are greatly induced by iron loading and inflammation [7,8]. In clinical studies, urinary excretion of hepcidin was greatly increased in patients with iron overload, infections, or inflammatory diseases [3,9,10]. Reliable determination of hepcidin level in urine would provide further insights into regulation of iron homeostasis and iron-related disorders, and thus would have great clinical relevancy. However, currently there is no widely used assay for the routine determination of hepcidin.

Hepcidin is a cysteine-rich peptide consisting of 25 amino acid residues. It forms a short hairpin with two arms linked by four disulfide bridges in a ladder-like fashion [11]. The cDNA of the human hepcidin encodes a 84-amino acid precursor – called prohepcidin – including the functionally active 25-mer hepcidin peptide [12,13]. Human urine contains three predominant forms of hepcidin, containing 20, 22, and 25 amino acid residues, and the shorter compounds are *N*-terminally truncated versions of the 25-mer peptide [14].

Several attempts have been made to develop hepcidin assay for detection and quantification of hepcidin in serum [15–19] and in urine [19,20]. These measurements are based on SDS-PAGE and Western blot, immunodot [3,21], or on SELDI-TOF-MS [15,19,20]. Unfortunately, hepcidin antibodies used in these assays are commercially not available. In addition, these methods do not provide reliable quantitative data on hepcidin levels in a wide dynamic concentration range [15,19].

Recently, Murao and Murphy [16,17] have used LC/ESI-MS/MS for the quantitative determination of serum hepcidin in the concentration range of 1–500 ng/ml. On the basis of the measurement of the sera derived from healthy human volunteers, the authors established baseline serum hepcidin levels (e.g. median: 3.6 ng/ml) [17]. The LC/ESI-MS/MS-based hepcidin measurement was validated [16]. However, the limited concentration range [16] and the relatively low accessibility of

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sophisticated instruments limit the widespread use of LC/ESI-MS/MS-based assays. Therefore, immuno-based assays would be preferred.

Serum prohepcidin, the 84-amino-acid precursor of the hepcidin, has been measured as a surrogate marker of active peptide by the ELISA method [13]. Higher than normal prohepcidin serum levels have been described in chronic renal insufficiency [22] and in hemodialysis patients [23]. It is likely that the serum prohepcidin level is influenced by the degree of renal clearance. However, serum prohepcidin levels in healthy volunteers were not significantly different from various patient groups. Besides, serum prohepcidin levels probably do not correlate with urinary hepcidin levels [20] and with clinical outcomes [24]. It remains unclear whether prohepcidin ELISA assays measure prohepcidin or accumulating cleavage products. The validation of this approach is pending.

Despite the results obtained in the past few years, hepcidin and prohepcidin analyses are generally not available. The commercially available hepcidin standards (containing 25, 22, and 20 amino acids) contain disulfide bonds in a randomized fashion. In a synthetic oxidized human 25-mer peptide (denoted by HEPC61-P, MW 2798, Alpha Diagnostic International, San Antonio, TX, USA), cysteines were air-oxidized assuming that they were able to assume natural disulfide bridges. This was not experimentally confirmed [1,12,14,25].

Here, we report on the synthesis of the truncated and *N*-terminally biotinylated derivatives of the 25-mer hepcidin. Our data show that these truncated synthetic derivatives might be suitable representatives of the 25-mer hepcidin molecule in immune-adsorption-based assays (ELISA, immunodot analysis) using commercially available polyclonal antibody.

Materials and Methods

Reagents: Preloaded TentaGel (TG) resin: Fmoc–Thr(*t*Bu)–Trt TG R (0.17 mmol/g) was purchased from Rapp Polymere (Tübingen, Germany). N^{α}-Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland) or NovaBiochem (Läufelfingen, Switzerland). Scavengers (thioanisole, EDT), coupling agents (DIPCI, HOBt, PyBOP, DIEA), and cleavage reagents (DBU, NEM), as well as biotin and *N*-(+)-biotinyl-6-aminocaproic acid were Fluka (Buchs, Switzerland) products. All solvents for the synthesis (DMF, DCM, NMP) and purification (diethyl ether, MeCN), as well as TFA, acetic acid, and H_2SO_4 were purchased from Reanal (Budapest, Hungary). ELISA reagents (streptavidin HRP conjugate, TMB, Tween-20, H_2O_2) were obtained from Sigma-Aldrich Kft (Budapest, Hungary). PVDF membrane was obtained from Millipore (Billerica, MA, USA). ELISA plate was Costar (San Diego, CA, USA), rabbit anti-human hepcidin IgG (HEPC12-A, HEPC13-A), and synthetic 25-mer hepcidin (HEPC61-P) was Alpha Diagnostic International (ADI; San San Antonio, TX, USA) and ECL Advanced Chemiluminescent Reagent was Amersham (Uppsala, Sweden) products.

Synthesis of Linear Peptides

The peptides were synthesized manually by solid phase methodology on preloaded TG resin (Fmoc–Thr(*t*Bu)–Trt TG R; 0.17 mmol/g). All amino acids were coupled as N^{α}-fluorenylmethyloxycarbonyl (Fmoc) derivatives. The following side-chain-protected amino acid derivatives were used: Tyr(^{t}Bu), Ser(^{t}Bu), Asp(O^{t}Bu), His(Trt), Arg(Pbf), Cys(Trt), and Lys(Boc). The synthetic protocol was the following: (i) DMF washing (3 × 1 min), (ii) deprotection with 2% DBU, 2% piperidine in DMF (4 times, 2 + 2 + 5 + 10 min), (iii) DMF washing (6 × 1 min), (iv) coupling of three equivalents of Fmoc-amino-acid derivative with DIPCI/HOBt *in situ* activation methodology in DMF (50 min), (v) DMF washing (3 × 1 min). The success of the coupling was monitored by ninhydrin or isatin test [26,27]. After completion of the synthesis, the peptides were cleaved from the resin using TFA : water : EDT : thioanisole : phenol = 82.5 : 5.0 : 2.5 : 5.0 : 5.0 (v/v/v/v/m) for 2.5 h at room temperature. After cleavage, the crude product was precipitated with cold *tert*-butyl-methyl-ether, washed three times with *tert*-butyl-methyl-ether and dissolved in eluent A (0.1% TFA in water (v/v)) before freeze drying. The crude products were purified by RP-HPLC.

Biotinylation of Synthetic Peptides

Four hepcidin-derived linear peptides corresponding to ^{1}DTHFPIC^{7} (**1a**); ^{1}DTHFPI^{6}S (**1aS**), (Gly)$_5$-^{1}DTHFPIC^{7} (**1c**), ^{13}CCHRSKCGMCCKT^{25} (**2a**); ^{1}DTHFPICIFCCGCCHRSKCGMCCKT^{25} (**3a**) regions were prepared using solid phase synthesis as described above. These peptides were modified with biotin or *N*-(+)-biotinyl-6-aminocaproic acid after *N*-terminal Fmoc deprotection [2% DBU, 2% piperidine in DMF (2 + 2 + 5 + 10 min)]. The resin was washed three times with DMF followed by washing twice with DMSO : DMF 3 : 1 v/v mixture in the case of *N*-(+)-biotinyl-6-aminocaproic acid. The biotin solution was prepared by DMF resulting in a concentration of 10 mg/ml, whereas the *N*-(+)-biotinyl-6-aminocaproic acid was dissolved in DMSO/DMF 3 : 1 v/v mixture. It was necessary to vortex the mixtures for several minutes to dissolve the biotin or *N*-(+)-biotinyl-6-aminocaproic acid completely. The biotin or *N*-(+)-biotinyl-6-aminocaproic acid solution was treated with a mixture of HBTU/HOBt in DMF followed by DIEA. The activated biotinylating solutions were prepared shortly before use. The coupling was carried out with HBTU/HOBt/DIEA 0.9 : 1.0 : 6.0 mol/mol (4 M excess calculated to resin capacity) reagents on solid support overnight at room temperature. The efficacy of the coupling was monitored by ninhydrin test [26]. The resin was washed with DMSO/DMF 3 : 1 v/v and DMSO/DMF 1 : 1 v/v mixtures followed by DMF and DCM. After the resin was thoroughly dried, the labeled peptides were cleaved as described above.

Purification and Characterization of Synthetic Peptides

Crude synthetic products were purified by RP-HPLC (Knauer system, Bad Homburg, Germany) using a Phenomenex Jupiter (Torrance, CA, USA) (10 mm × 250 mm) C_{18} semi-preparative column packed with 10-µm particle and 300 Å pore size. Eluent A was 0.1% TFA in water and eluent B was MeCN in water (80 : 20, v/v %) containing 0.1% TFA. Samples were dissolved in eluent A at 5 mg/ml concentration and 2 ml of solution was injected. Linear gradient elution at 4 ml/min was performed from 10% to 80% eluent B from 5 to 50 min. Peaks were detected by UV at $\lambda = 214$ nm. Separated products were identified by ESI-MS.

Analytical RP-HPLC separation was performed using Phenomenex Synergi (Torrance, CA, USA) C_{12} column (4.6 mm × 250 mm); packed with spherical 4-µm particle of 80 Å pore size. Flow rate was 1 ml/min and separation was performed using the same gradient as for the semipreparative purification. Samples were dissolved in eluent A and 20 µl of solution was injected. Peaks were detected by UV at $\lambda = 220$ nm.

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Reaction of Free Thiol Group with NEM Followed by RP-HPLC Analysis

All peptides were dissolved at the concentration of 1 mg/ml in PBS buffer (pH = 7.4) and incubated for 1 h. NEM was dissolved in absolute ethanol to obtain a stock solution (c = 0.1 mg/ml), freshly prepared daily. 1.1 M equivalent of NEM to 1 equivalent free thiol group was added to the peptide solution. The mixture was sonicated at room temperature for 5 min. There were no significant differences in peak areas of the succinimide derivatives when samples were incubated at room temperature from 5 to 60 min. Peptide solutions (in PBS, pH = 7.4) and the reaction mixtures were analyzed by RP-HPLC using a laboratory-assembled Knauer HPLC system (Bad Homburg, Germany) with an Eurospher-100 C-18 column (4 mm × 250 mm I.D., 5-µm particle size, 300Å pore size) (Knauer, Bad Homburg, Germany). The gradient elution system consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in MeCN/water = 80/20 (v/v) (eluent B). The flow rate was 1 ml/min at room temperature. Twenty microliters of samples were injected and peaks were detected at $\lambda = 214$ nm.

Reaction of Free Thiol Group with NEM Prior to ZipTipC4 Preparation Followed by ESI-MS Analysis

All peptides were dissolved at the concentration of 1 mg/ml in PBS buffer (pH = 7.4) mimicking ELISA and immunodot conditions and incubated for 1 h as described above. NEM was dissolved in absolute ethanol to obtain a freshly prepared stock solution (c = 0.1 mg/ml). 1.1 equivalent of NEM to 1 equivalent free thiol group was added to the peptide solution. The mixture was incubated at room temperature for 5 min before desalting by ZipTipC4 P10.

Sample Desalting by Solid Phase Extraction/Pipet-tip Chromatography

Peptides after the incubation in PBS (pH = 7.4, 1 h) as well as after the reaction with NEM were desalted using ZipTipC_4 P10 pipet tips (Millipore, Billerica, MA, USA). The following solutions were used during sample desalting procedure: wetting solution: MeCN; equilibration and washing solution: 1% acetic acid in high-purity water or 5% methanol in 0.1% TFA/high-purity water; sample preparation solution: 0.1% acetic acid in 50% MeCN/high-purity water or 1% formic acid/50% methanol. Desalted samples were analyzed by ESI-MS.

ESI-MS

ESI-MS experiments for the characterization of purified peptides and stability studies were performed on a Bruker Esquire 3000+ ion trap MS, equipped with ESI and operating in continuous sample injection using a syringe pump in a flow rate of 10 µl/min. Spray voltage was set to 4.0 kV, and 40.0 V orifice voltage was applied. The instrument was used in positive mode in the range of 50–3000 *m/z* with 13 000 *m/z*/s scan resolution. Samples were dissolved in a mixture of MeCN/water = 1/1 (v/v) containing 0.1% acetic acid.

Amino Acid Analysis

Amino acid analysis was performed on Sykam Amino Acid S433H analyzer (Eresing, Germany) equipped with an ion-exchange separation column, and postcolumn derivatization with ninhydrine was applied. Before analysis, samples were hydrolyzed with 6 N HCl in sealed and evacuated tubes at 110 °C for 24 h.

Urine Samples Collection and Storage

Urine samples were collected from healthy volunteers. Urines were dotted or purified immediately after sampling or were stored at −20 °C until measurement.

Direct ELISA Assay

The ELISA assay was carried out on 96-well high binding plates (Corning Costar, Schiphol-Rijk, Netherlands) using rabbit anti-human hepcidin IgG antibodies (HEPC12-A, HEPC13-A). The plates were coated with rabbit anti-human hepcidin IgG antibody (HEPC12-A or HEPC13-A) (0.05 µg/well) in PBS and incubated at 37 °C for 1 h. Plates were washed four times with PBS/0.1% Tween-20 washing buffer. The synthetic peptides derivatized with *N*-(+)-biotinyl-6-aminocaproic acid: biotin-Acp-1–7 (**1b**); biotin-Acp-13–25 (**2b**), biotin-Acp-1–25 (**3b**) or with biotin-$(Gly)_5$-1–7 (**1d**) on their *N*-terminus were added to wells in different concentrations (Figure 4). PBS was used alone as negative control. The plates were incubated for 1 h at 37 °C. The recognition was visualized using a streptavidin HRP conjugate and chromogenic substrate reaction (TMB, H_2O_2). The plate was incubated on the streptavidin HRP, after washing as above, in 1 : 4000 dilution at 37 °C for 1 h. After the washing step, 100 µl of peroxidase substrate was added to each well and the plate was incubated in the dark at room temperature. The reaction was stopped after 7 min with 1 M H_2SO_4 (50 µl/well) and absorbance values were measured at 450/620 nm using an ELISA reader (Sanofi Diagnostic, Pasteur, France).

Competitive ELISA Assay

The competitive ELISA assay was carried out on 96-well high binding ELISA plates using rabbit anti-human hepcidin IgG antibodies (HEPC12-A, HEPC13-A). The plates were coated with rabbit anti-human hepcidin IgG antibody (HEPC12-A or HEPC13-A) (0.05 µg/well) in PBS (pH = 7.04) and incubated at 37 °C for 1 h. Plates were washed four times with PBS/0.1% Tween-20. The biotinylated peptides **1b,1d,2b**, or **3b** were added to the wells at fixed concentrations (50.0, 25.0, or 12.5 ng/µl) and the peptide without biotin **1a, 1aS** (^{1}DTHFPI^{6}S), **2a** or **3a** at different concentration (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 ng/µl – and PBS was used alone as negative control). The plates were incubated for 1 h at 37 °C. The recognition was visualized using a streptavidin HRP conjugate and chromogenic substrate reaction (TMB, H_2O_2). The plate was incubated on the streptavidin HRP, after washing as above, in 1 : 4000 dilution at 37 °C for 1 h. After washing, 100 µl of peroxidase substrate was added to each well and the plate was incubated in the dark at room temperature. The reaction was stopped after 7 min with 50 µl/well 1 M H_2SO_4 and absorbance values were obtained at 450/620 nm in an ELISA reader.

Immunodot Assay

The assay was carried out on Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA). Before use, the membrane was rinsed with methanol and PBS (pH = 7.4). Four microliters of standard and sample were dotted in PBS/0.01% acetic acid. The membrane was fixed using 0.05% glutaraldehyde in PBS (pH = 7.4), for 20 min at room temperature. The membrane was blocked in 3% milk powder in PBS. The membrane was incubated with rabbit anti-human hepcidin IgG antibody (HEPC12-A or HEPC13-A) 1 : 1000 v/v in the 1 : 3 mixture of 3% milk powder in PBS and Tween PBS

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(0.1% Tween 20) at room temperature, overnight. After washing in 0.1% milk powder in PBS/0.1% Tween-20 (pH = 5.0), 8 × 5 min, at room temperature, the membranes were incubated in 3% milk powder in PBS for 10 min at room temperature. After the previous step, the membranes were incubated in goat anti-rabbit-HRP antibody 1 : 10 000 dilution at room temperature, for 1 h. After the washing steps, ECL advanced chemiluminescent reagent was used as developer. The membrane was exposed for 10 min, and the image was visualized using Gel Logic Imaging System (Kodak, Budapest, Hungary).

Results and Discussion

Human hepcidin is produced from a precursor molecule containing 84 amino acid residues, including a 24-amino-acid-long putative signal peptide. The secreted forms of hepcidin in blood and in urine consist of the *C*-terminal 20, 22, or 25 amino acid residues [12,13] of the precursor molecule. In humans, the 20- and 25-amino-acid-containing versions appear to be the major secreted peptides. The three alternatively spliced peptides differ only at the *N*-terminus [14]. The 13–25 sequence is conserved in all three human hepcidin peptides.

The commercially available polyclonal, affinity purified HEPC12-A antibody was generated in rabbits using KLH-conjugated 13–25 synthetic peptide. This antibody is able to detect the three different secreted forms of human hepcidin comprised of 20, 22, and 25 amino acids. The HEPC13-A polyclonal, affinity-purified antibody was also generated in rabbits. In the case of this product, the KLH-conjugated *N*-terminal 7-mer peptide corresponding to the *N*-terminus of mature human and mouse 25-amino-acid hepcidin was used as antigen. The 1–7 peptide overlaps with the 20-amino-acid-major-secreted hepcidin only in two amino acid residues; therefore, it is understandable that the HEPC13-A does not have significant reactivity with the 20-amino-acid-secreted peptide.

Here, we report on the synthesis of linear, fully reduced 25-mer hepcidin (peptide **3a**), its 7-mer- (peptides **1a, 1aS, 1c**) and 13-mer (peptide **2a**)-truncated versions for immune-adsorption-based assays (ELISA, immunodot analysis, according to the available rabbit polyclonal antibodies) (Table 1). We have studied the influence of truncation of hepcidin sequence on the antibody recognition. We have compared the binding properties of the

Table 1. Sequence and analytical data of peptides corresponding to the 1–25 hepcidin

Code	Peptides	Amino acid analysis[a] Measured/[Calculated]	RP-HPLC retention time[b] (min)	$M_{monoisotopic}$ (measured)[c]	$M_{monoisotopic}$ (calculated)
1a	[1]DTHFPIC[7]	D 0.97 [1]; T 1.00 [1]; P 1.10 [1]; C 0.95 [1]; I 0.97 [1]; F 1.01 [1]; H 0.95 [1]	32.0	831.3	831.4
1aS	[1]DTHFPI[6]S	D 0.97 [1]; T 1.00 [1]; S 0.98 [1]; P 1.10 [1]; I 0.97 [1]; F 1.01 [1]; H 0.95 [1]	31.0	677.3	677.3
1b	Biotin-Acp-[1]DTHFPIC[7]	D 0.99 [1]; T 1.02 [1]; P 0.98 [1]; C 0.95 [1]; I 0.97 [1]; F 1.01 [1]; Acp 0.93 [1]; H 0.95 [1]	36.0	1169.5	1169.7
1c	$(Gly)_5$-[1]DTHFPIC[7]	D 0.97 [1]; T 1.00 [1]; P 1.10 [1]; G 5.70 [5]; C 0.95 [1]; I 0.97 [1]; F 1.01 [1]; H 0.95 [1]	34.0	1115.4	1115.4
1d	biotinyl-$(Gly)_5$-[1]DTHFPIC[7]	D 0.99 [1]; T 0.98 [1]; P 1.05 [1]; G 5.81 [5]; C 0.95 [1]; I 0.99 [1]; F 1.01 [1]; H 0.98 [1]	35.0	1341.7	1341.0
2a	[13]CCHRSKCGMCCKT[25]	T 1.00 [1]; S 0.98 [1]; G 1.05 [1]; C 2.45 [3]; M 0.86 [1]; K 1.89 [2]; R 0.95 [1]	34.5	1458.7^+	1458.6^+
2b	Biotin-Acp-[13]CCHRSKCGMCCKT[25]	T 1.00 [1]; S 0.99 [1]; G 0.99 [1]; C 2.38 [3]; M 0.86 [1]; Acp 0.92 [1]; K 1.85 [2]; R 0.95 [1]	37.0	1798.8^+	1798.8^+
3a	[1]DTHFPICIFCCGCCHRSKCGMCCKT[25]	D 0.97 [1]; T 2.02 [2]; S 0.99 [1]; P 0.98 [1]; G 1.89 [2]; C 7.35 [8]; M 0.85 [1]; I 1.97 [2]; F 2.01 [2]; H 1.95 [2]; K 1.88 [2]; R 0.97 [1]	36.0	2797.0^+	2797.2^+
3b	Biotin-Acp-[1]DTHFPICIFCCGCCHRSKCGMCCKT[25]	D 0.99 [1]; T 1.98 [2]; S 0.99 [1]; P 0.98 [1]; G 1.89 [2]; C 7.35 [8]; M 0.86 [1]; I 1.97 [2]; F 1.99 [2]; Acp 0.94 [1]; H 1.90 [2]; K 1.89 [2]; R 0.98 [1]	41.0	3136.2^+	3136.0^+

Biotin-Acp, *N*-(+)-biotinyl-6-aminohexanoic-acid; Acp, 6-aminohexanoic-acid.
[a] Acid hydrolysis (6 M HCl, 110 °C, 18–24 h).
[b] Conditions: Phenomenex Jupiter C_{18} column (4.6 mm × 250 mm) with 5 μm silica (300 Å pore size), linear gradient elution: 10–70% B in 60 min, eluents: 0.1% TFA in water (eluent A) and 0.1% TFA in MeCN/water = 80/20 v/v (eluent B), flow rate = 1.0 ml/min, λ = 214, 280 nm.
[c] Determined by ESI-MS; $^+M_{average}$ (measured/calculated).

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new synthetic-truncated peptides and peptide HEPC-61. We have synthesized the *N*-terminal biotinylated derivatives of **1a, 2a**, and **3a** peptides. As biotinylating agents, biotin (peptide **1d**) or *N*-(+)-biotinyl-6-aminocaproic acid was used (peptides **1b,2b,3b**) (Table 1). The conjugated biotin moiety binds one molecule of streptavidin (carrying reporter groups, e.g. HRP) resulting in signal amplification. The binding of biotin and streptavidin is one of the strongest noncovalent interactions found in nature. Because the binding sites for biotin are buried deep inside streptavidine (about 9 ´Å below the protein surface), spacers improve both the accessibility and the reaction rate of biotinylated compounds with respect to streptavidine, often enhancing the sensitivity of assay systems [28]. The most common spacer group is 6-aminocaproic acid (Acp), which increases the distance of the side arm by about 9 Å. In the case of *N*-(+)-biotinyl-6-aminocaproic acid compound, there is a C6-spacer between the biotin and the peptide molecule; hence, it reduces the steric hindrance and increases accessibility for streptavidin. In the case of the labeling with biotin molecule

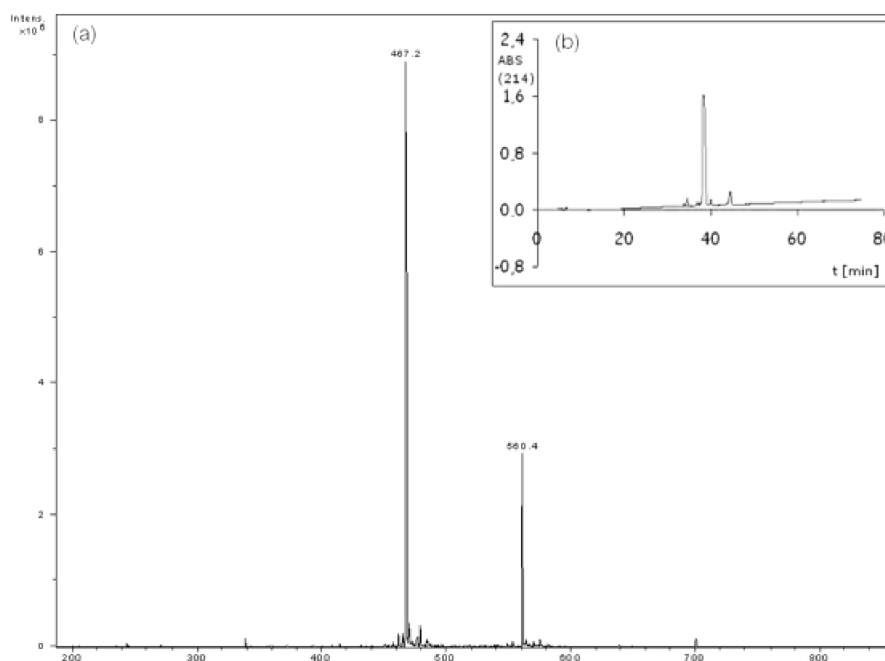

Figure 1. ESI-MS spectrum (a) and RP-HPLC chromatogram (b) of the peptide **3a**, measured molecular ion $[M+6H]^{6+} = 467.2$, $M_{calculated}$ (monoisitopic) = 2797.2.

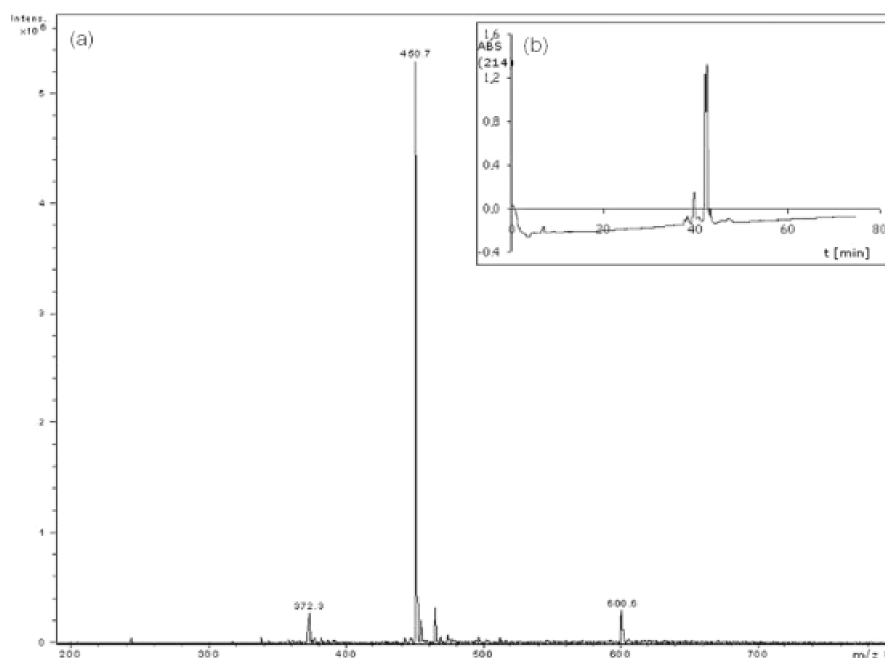

Figure 2. ESI-MS spectrum (a) and RP-HPLC chromatogram (b) of the peptide **2b**, measured molecular ion $[M+4H]^{4+} = 450.7$, $M_{calculated}$ (monoisitopic) = 1789.8.

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(without Acp spacer), a pentaglycine-elongated version (**1d**) of the peptide **1a** was used to increase the accessibility of the biotin moiety. The pentaglycin spacer was used in our previous studies to increase the speed of conjugation (to improve the accessibility) and to decrease the possibility of dimer formation [29]. The application of pentaglycin sequence as spacer arm is commonly used to create a 'one bead, one peptide' combinatorial library [30]. The interaction between biotinylated peptides and biotin-binding streptavidin–HRP was used in competitive ELISA assays.

Synthesis and Chemical Characterization

Three hepcidin-derived linear peptides corresponding to 1–7 (**1a**), 13–25 (**2a**), and 1–25 (**3a**) regions and an *N*-terminally pentaglycine-elongated peptide (**1c**) were synthesized using Fmoc/*t*Bu chemistry on preloaded TG resin with DIPCI/HOBt coupling method. These linear peptides were modified on solid support with biotin (**1d**) or *N*-(+)-biotinyl-6-aminocaproic acid (**1b, 2b,** and **3b**) on their *N*-terminus using HBTU/HOBt/DIEA coupling method for ELISA studies. After cleavage, the crude linear peptides and the *N*-terminally modified derivatives were completely soluble in eluent A (0.1% TFA in water) and could easily be purified by preparative RP-HPLC method. The homogeneity and the primary structure of the peptides and their biotinylated derivatives were identified by analytical RP-HPLC, amino acid analysis, and ESI-MS analysis (Table 1). The peptides were obtained as linear products of good quality according to the RP-HPLC chromatograms (data not shown), amino acid analysis, and ESI-MS data (Table 1, Figures 1, 2, and 3(a), (b)).

Stability of the lyophilized peptides and peptide derivatives kept at 4 °C was controlled by RP-HPLC and ESI-MS in every month for 6 months. In the case of peptides **1a, 1c, 1b**, and **1d**, no dimers were observed, the shelf stability of these compounds was extremely good. No intermolecular disulfide bond formation was observed for 18 months (data not shown). Despite of this observation, we have synthesized the serine-containing peptide **1aS** to replace cysteine-containing peptide **1a** in ELISA and dot blot assays.

Application of Peptides in Immune-adsorption-based Assays

In order to study the applicability of the synthetic peptides described above in immunoassays, we have used these compounds in ELISA as well as in immunodot arrangements. For comparison, we have used synthetic peptide HEPC61-P as control (Alpha Diagnostic International).

Direct ELISA was carried out using peptide **3b, 2b, 1b**, and peptide **1d** (Table 1 and Figure 4(a)–(d)). The plates were coated with HEPC12-A or HEPC13-A (Figure 4(a)–(d), respectively).

The synthetic peptides modified with *N*-(+)-biotinyl-6-aminocaproic acid (peptide **3b, 2b,** and **1b**) or with biotin (**1d**) were added to wells in the concentration range of 0.5–32 ng/μl and 0.5–12 ng/μl concentrations (Figure 4(a)–(d), respectively). According to our data using the polyclonal affinity-purified rabbit anti-human hepcidin antibody HEPC12-A, the truncated peptide **2b** exhibited less binding activity than the peptide **3b** (Figure 4(a), (b)). The HEPC13-A antibody recognition of peptides **1b** and **1d** was compared. In these peptides, two different spacers similar in function (namely, the pentaglycine and the aminocaproic acid residues) were applied between the biotin residue and the peptide 1–7. The peptide **1d** showed less activity than peptide **1b** as summarized in Figure 4(c), (d).

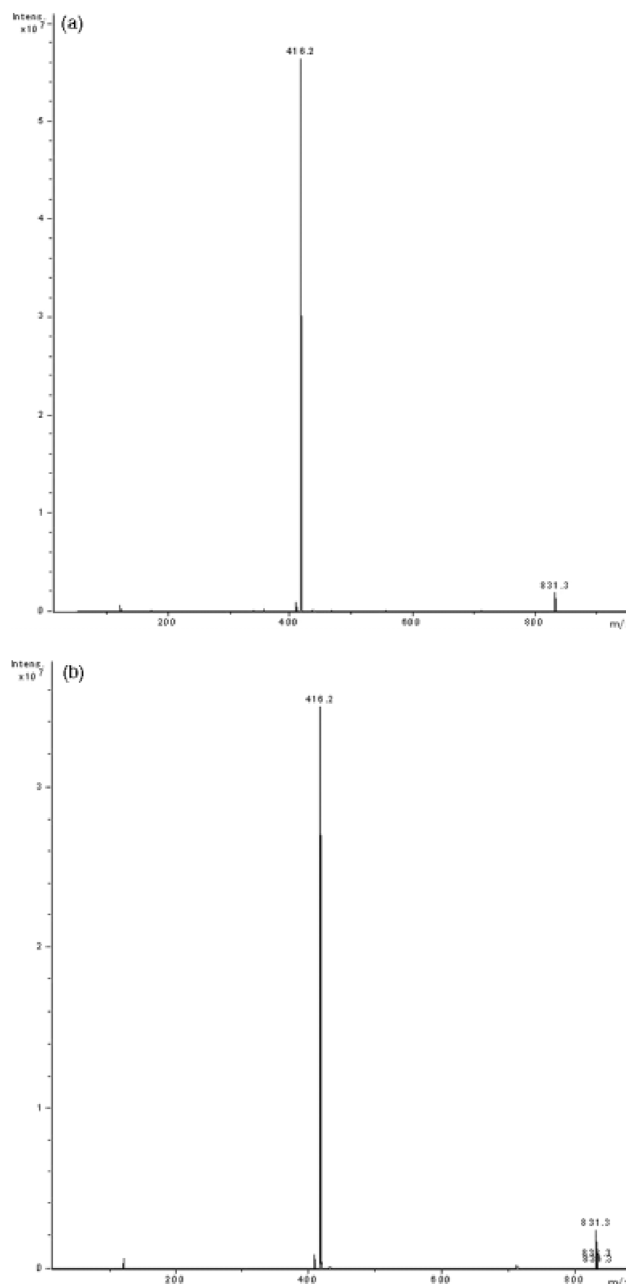

Figure 3. ESI-MS spectra of linear synthetic peptide **1a**, dissolved in water (a) first day and (b) third day, measured molecular ion $[M + 2H]^{2+} = 416.2$, $M_{calculated}$ (monoisitopic) = 830.4.

The data of competitive ELISA assay with peptides **1a** and **1aS** versus peptide **1b** are summarized on Figure 5. According to our data, the pentaglycine-spacer-containing compound, peptide **1c** showed the least activity (data not shown). The addition of the ε-aminocaproyl spacer arm gave better conditions (possibly more flexibility, etc.) to increase the accessibility of the biotin moiety. This improvement was detectable in ELISA experiments where steric hindrance is important. The effectivity of peptide **1aS** was comparable with peptide **1a**.

In our immunodot assay, we applied the peptide **3a** and its truncated version: peptide **1a**. Peptide HEPC61-P was used as control. We applied urine samples from healthy volunteers (Figure 6). The data of the immunodot blot showed that the peptides **3a** and **1a**

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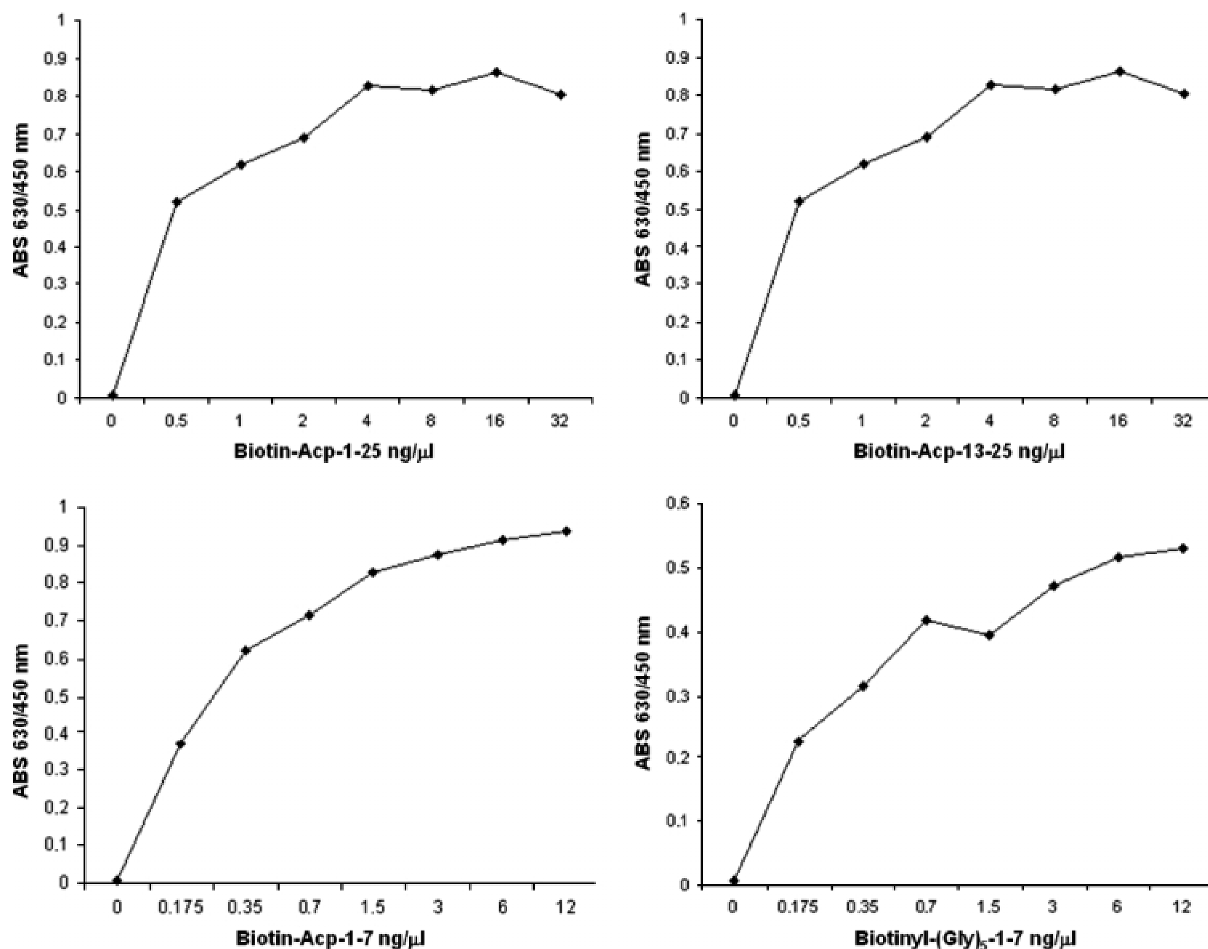

Figure 4. Antibody recognition (rabbit anti-human hepcidin IgG, HEPC12-A (a, b) and HEPC13-A (c, d), respectively) of the synthetic linear peptides (a) **3b**, (b) **2b**, (c) **1b**, and (d) **1d**.

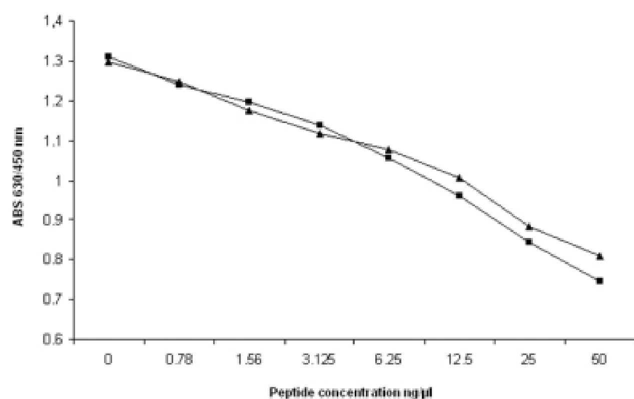

Figure 5. Comparison of the standard curves of the competitive ELISA using peptide **1b** versus peptide **1a** (■) or **1aS** (▲). The concentration of peptide **1b** was 6.25 ng/μl.

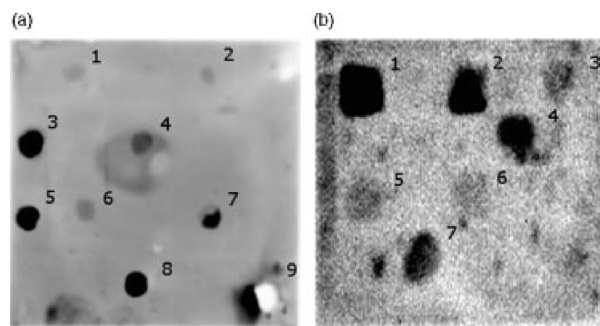

Figure 6. Results of immunodot assay with peptides **1a** and **3a**, peptide HEPC61-P and urine samples (#10, #11 and #12) using polyclonal antibodies HEPC13-A (a) and HEPC12-A (b). (a) **1** peptide **1a** ($c = 50$ ng/ml), **2** peptide **1a** ($c = 100$ ng/ml), **3** peptide **1a** ($c = 250$ ng/ml), **4** peptide **3a** ($c = 100$ ng/ml), **5** peptide **3a** ($c = 250$ ng/ml), **6** peptide **3a** ($c = 50$ ng/ml), **7** urine sample #11, **8** urine sample #12, **9** positive control (rabbit sera 1 : 1000 v/v in PBS, pH = 7.4). (b) **1** peptide **3a** ($c = 250$ ng/ml), **2** peptide **3a** ($c = 100$ ng/ml), **3** peptide **3a** ($c = 50$ ng/ml), **4** peptide HEPC61-P ($c = 100$ ng/ml), **5** peptide HEPC61-P ($c = 50$ ng/ml), **6** urine sample #10, **7** positive control (rabbit sera 1 : 1000 v/v in PBS, pH = 7.4).

are appropriate standards with rabbit antibody HEPC13-A and the peptide **3a** appropriate standard with rabbit antibody HEPC12-A. Namely, their binding property to the polyclonal rabbit hepcidin antibodies was comparable with activity of peptide HEPC61-P. The presence of hepcidin species was detected qualitatively in healthy individuals' urine samples. Our dot blot was usable in nanogram per milliliter concentration range of hepcidin derivatives.

Stability/Redox Status Studies with Peptide Derivatives Using NEM

In order to study the stability of the synthetic peptides during ELISA and immunodot assays, we have used two different methods:

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(i) RP-HPLC analysis of the peptide solutions and (ii) direct ESI-MS analysis of the peptide solutions followed by ZipTipC4 separation. We have checked the solutions of peptides directly after 1-h incubation in PBS (pH = 7.4) followed by ZipTipC4 separation. Our other approach was to determine the redox status of cysteine in our synthetic peptides using NEM as blocking moiety of the free sulfhydryl groups before ZipTipC4 separation. By this new method recently developed in our laboratory [31], it is possible to determine the cysteine content in the intact peptide. The reaction with NEM is simple, in neutral or slightly basic medium (as PBS pH = 7.4), it takes place in a few minutes. The product, the *S*-(*N*-ethylsuccinimido)-cysteinyl-peptide can be detected by RP-HPLC and ESI-MS. In the case of peptides **1a, 1b, 1c**, and **1d**, there were no dimers observed in PBS buffer (pH = 7.4).

We summarized the results of the direct RP-HPLC analysis in Figure 7(a), (b) in the case of peptide **1a** and **1b**. The peptides on RP-HPLC chromatogram were homogeneous (Figure 7(a), (b), solid lines) after 1-h incubation in PBS (pH = 7.4), the dimer forms of the peptides were not detected. The reaction with NEM (excess NEM is a new peak, R_t = 15.1 min, Figure 7(a), (b), broken lines) resulted in the *S*-(*N*-ethylsuccinimido)-cysteinyl-peptides (R_t = 25.3 min, Figure 7(a); R_t = 27.2 min, Figure 7(b), respectively) and their structures were confirmed by ESI-MS (data not shown). There were no additional peaks on the chromatograms, dimer forms of peptides were not detected.

ESI-MS spectra of peptide **1a** were obtained before and after incubation (Figure 8(a)–(d), respectively). All the pictures show the same isotopic pattern. In Figure 8(a), (c), the *m/z* spacing between the adjacent peaks is 0.5 indicating the presence of doubly charged, $[M+2H]^{2+}$ ions, as well as Figure 8(b), (d), the *m/z* spacing between the adjacent peaks is 1.0 indicating the presence of singly charged, $[M+H]^{1+}$ ions. Considering these spectra, we proved that peptide **1a** does not form dimers under ELISA and immunodot circumstances. The reaction with NEM has proved our previous observations in PBS (Figure 9(b)). The reaction with NEM resulted in the succinimide derivative of peptide **1a**, the structure was confirmed with doubly charged $[M+2H]^{2+}$ = 478.9 ions and single charged $[M+H]^{1+}$ = 956.6 ions.

We have demonstrated the stability of peptide **1d** in PBS buffer using ESI-MS after ZipTipC4 desalting. The doubly charged $[M+2H]^{2+}$ = 672.0 and the singly charged $[M+H]^{1+}$ = 1342.7 ions were detected in both stages (0 min and after 1-h incubation, Figure 10(a), (c), respectively). We also used NEM reagent to determine the redox status of the *C*-terminal cysteine of peptide **1d** (before and after 1-h incubation, Figure 10(b), (d) respectively).

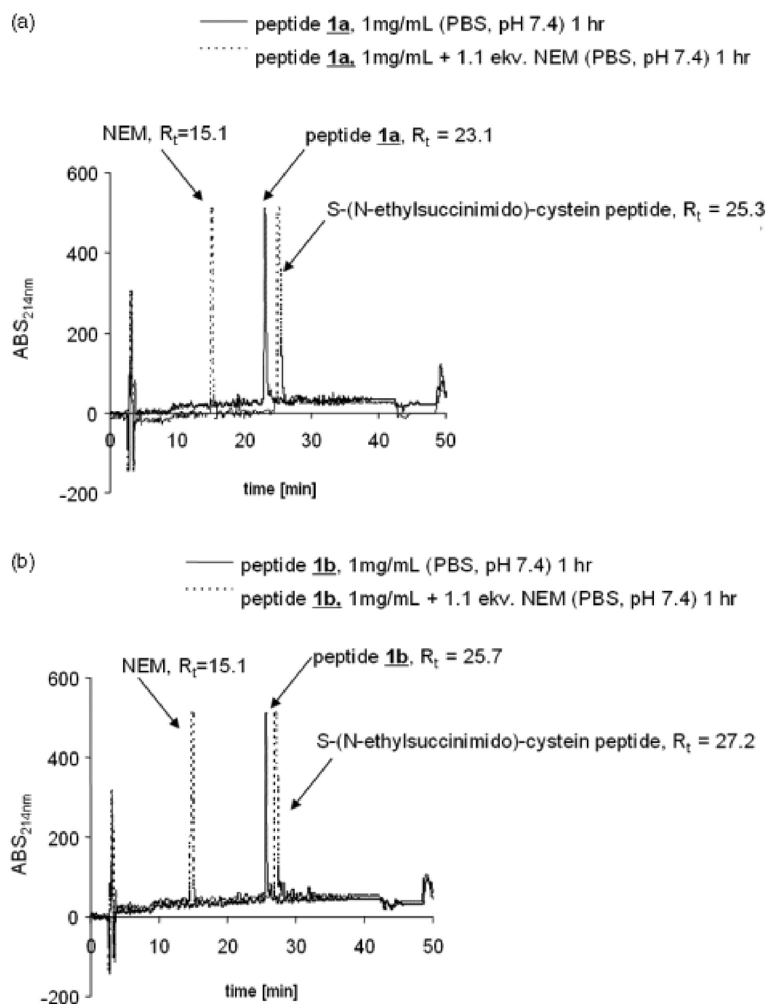

Figure 7. Analysis of peptide **1a** (a) and **1b** (b) by analytical RP-HPLC after 1-h incubation in PBS (pH = 7.4) (solid line) and after reaction with NEM (broken line). The eluent B content was from 5% to 60% in 35 min. The reaction with NEM (R_t = 15.1 min) resulted in the succinimide derivative [R_t = 25.3 min (a), R_t = 27.2 (b)]; the structure was confirmed by ESI-MS.

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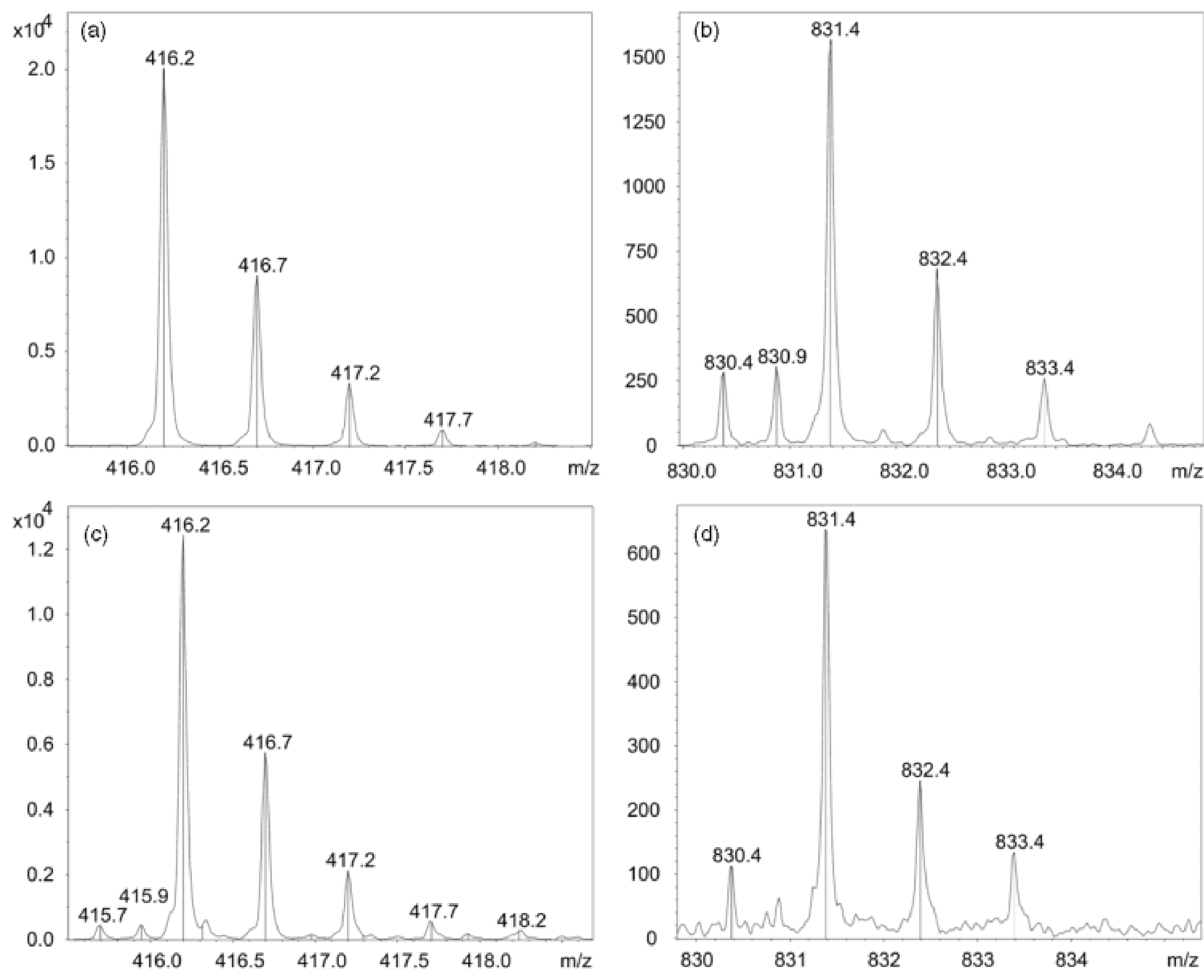

Figure 8. ESI-MS spectra of peptide **1a** dissolved in PBS buffer (pH = 7.4). The spectra were recorded after ZipTipC_4 separation, measured molecular ions $[M + 2H]^{2+} = 416.2$ and $[M + H]^{1+} = 831.4$, $M_{calculated}$ (monoisitopic) = 830.4. (a) and (b): 0 min; (c) and (d): after 1-h incubation in PBS.

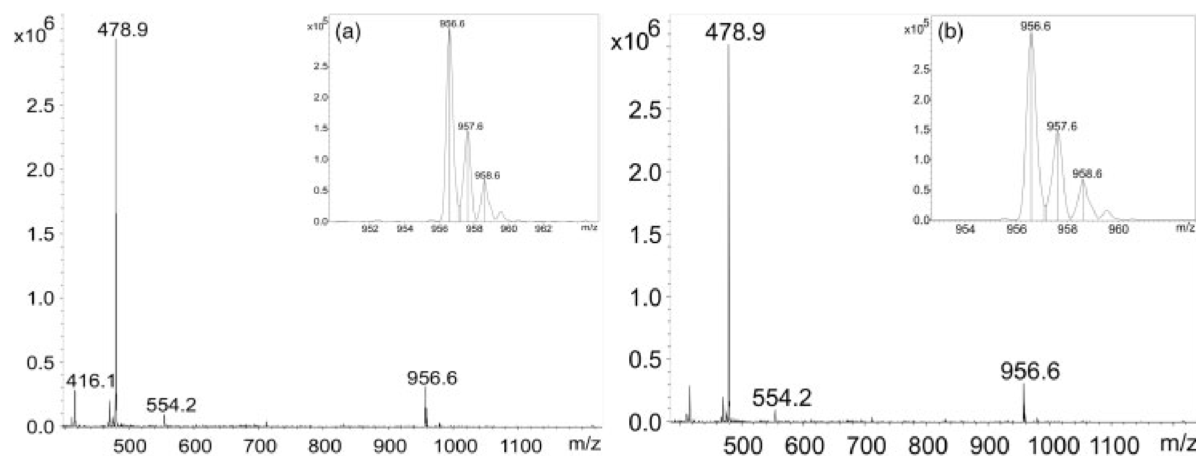

Figure 9. ESI-MS spectra of peptide **1a** and its succinimide derivative after the reaction with NEM and followed ZipTipC_4 separation. Measured molecular ions $[M + 2H]^{2+} = 478.9$; $[M + H]^{1+} = 956.6$; $M_{calculated}$ (monoisitopic) = 955.6.

The doubly charged $[M + 2H]^{2+} = 734.5$ and the singly charged $[M + H]^{1+} = 1467.8$ ions were observed.

In the case of peptides **2a, 2b, 3a**, and **3b**, inter- or/and intramolecular disulfide bond formation was observed during the 1-h incubation in PBS buffer (data not shown). The original fully reduced, linear forms of these compounds were also detected.

Conclusions

In this paper, we describe the synthesis of truncated and *N*-terminally modified hepcidin derivatives as possible representatives of 25-mer peptides to be used with commercially available polyclonal antibodies for immune adsorption method.

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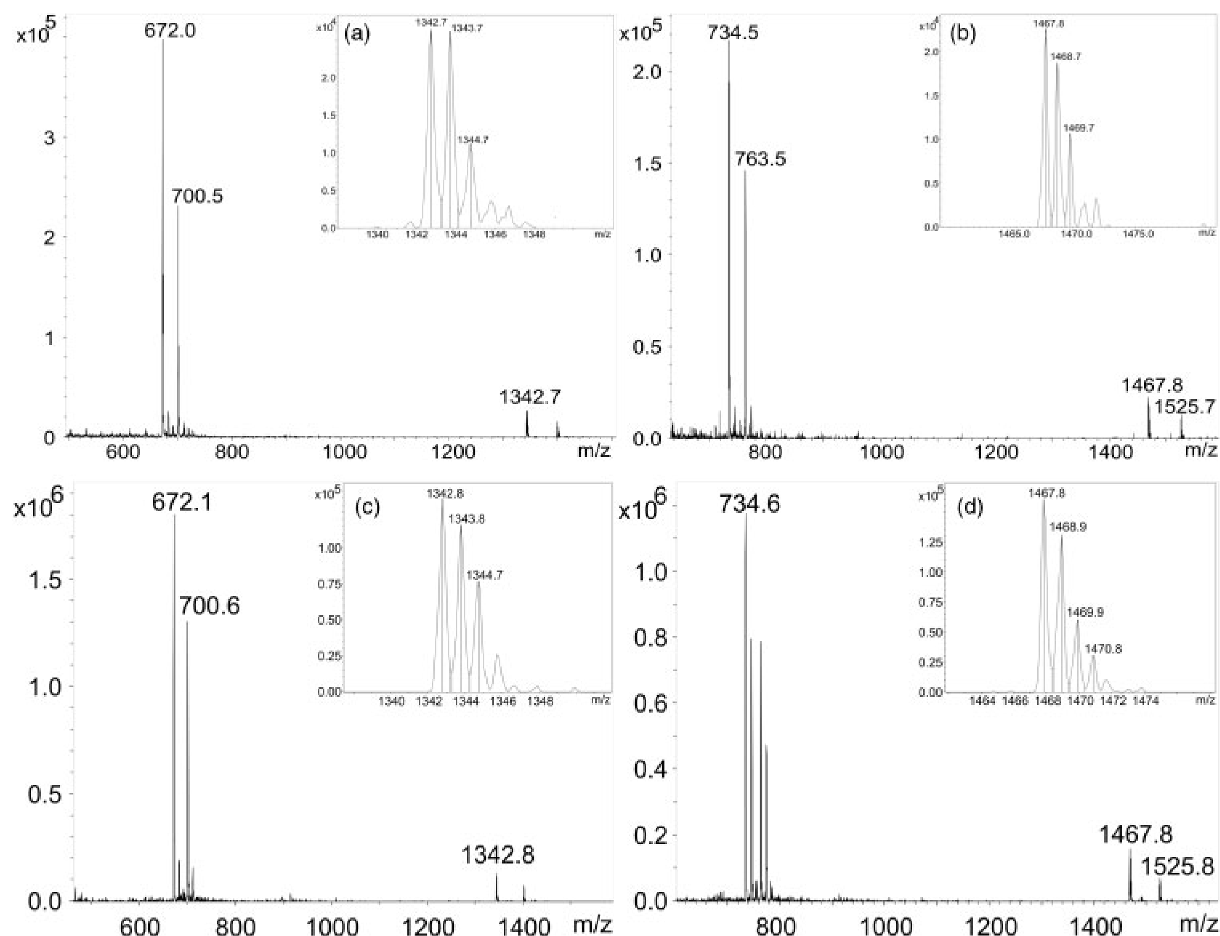

Figure 10. ESI-MS spectra of peptide **1d** (a) before and (c) after the incubation in PBS (measured molecular ions $[M+2H]^{2+} = 672.0$, $[M+H]^{+} = 1342.7$; $M_{calculated}$(monoisitopic) = 1341.7) and its succinimide derivative after the reaction with NEM (b) before and (d) after the incubation in PBS (measured molecular ions $[M+2H]^{2+} = 734.6$, $[M+H]^{+} = 1467.8$; $M_{calculated}$ (monoisitopic) = 1466.6).

The peptides **1a, 1c**, and their *N*-terminally modified derivatives **1b** and **1d** have long shelf stability and no dimerization were observed. Stability was assessed by RP-HPLC and ESI-MS in PBS buffer (pH = 7.4) mimicking ELISA or immunodot circumstances. Two different attempts have been made to investigate their stability during incubation in PBS buffer: (i) to detect the intact, cysteine-containing peptide itself and (ii) to detect the sulfhydryl–maleimide adduct of the peptide using RP-HPLC and ESI-MS analysis. By this method, it is possible to determine the cysteine content in the intact peptide. The reaction with NEM is very simple, it takes place in a few minutes. The product, the *S*-(*N*-ethylsuccinimido)-cysteinyl-peptide was measured by RP-HPLC or ESI-MS using ZipTipC4 desalting before analysis. The results confirmed that dimerisation did not occur during incubation in the case of peptides **1a, 1c, 1b**, and **1d**.

The commercially available antibodies named HEPC12-A and HEPC13-A were generated using KLH-conjugated 13–25 and 1–7 synthetic peptides, respectively. There is no available information about these peptides and conjugates beside the information above (their cystein redox status, type of the chemical linkage between the peptide and the KLH, etc., are not known). According to the given information (product information sheet from Alpha Diagnostic International), we can expect that these antibodies are also able to recognize the synthetic peptides 13–25 (**2a**) and 1–7 (**1a**) prepared by authors. We have done direct ELISA experiments using peptide HEPC-61, our synthetic peptides (peptide **1a** and its serine-containing derivatives: **1aS**) and the antibody HEP13-A. We did not detect differences between the antibody recognitions of these peptides. We found that the HEPC-13A is probably a 'core-specific' antibody, which can recognize the sequence pattern of DTHFPIC. Let us presume that the chemical linkage between the carrier KLH and the 1–7 peptide (DTHFPIC) did not affect the side chains of the amino acid residues. This might be important, because in the case of conjugation through one of the side chains, that particular residue would not participate in the peptide–antibody interaction. That side chain could not have been available for the immune system of the host animal at the time of the immunization. We have learned also from the product information that antibody HEPC-13A does not recognize the 20-mer version of secreted hepcidin. A potential explanation for this experimental phenomenon is that antibody HEPC-13A requires the presence of FPIC amino acids as minimal epitope for the antibody recognition, whereas the 20-mer does not contain the FP residues.

The peptide **1aS** (containing serine instead of cystein) was recognized by antibody HEP-13A. This result suggests that the cystein residue probably is not important for the antibody binding or it could be replaced by the isoatomic serine. We have demonstrated that the immunogenicity of peptides **1a** and **1aS** is comparable with the commercially available standard, HEPC61-P. With the replacement of the cysteine residue by serine in peptide

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1aS, we can avoid most of the disadvantages of the presence of cysteine during the synthesis.

These hepcidin-derived peptides **1a, 1aS, 1b** used in ELISA and immunodot assays with polyclonal rabbit antibody HEPC13-A are easy, quick, and cheap to produce in large quantities compared with the native form. Taken together, we can state that our peptides **1a** and **1aS** might be suitable representatives of the 25-amino-acid form of hepcidin in immune adsorption method. Our opinion is that the peptide **1a** or **1aS** might also be a positive control for assays using HEPC-13A antibody because the short DTHFPIC sequence was used as immunizing antigen during antibody production.

Acknowledgements

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