Journal of Chromatography, 604 (1992) 125–132
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 203

Synthetic metal-binding protein surface domains for metal ion-dependent interaction chromatography

I. Analysis of bound metal ions by matrix-assisted UV laser desorption time-of-flight mass spectrometry

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ABSTRACT

To extend the analytical capabilities of immobilized metal ion affinity chromatography (IMAC) for evaluation of biologically relevant peptide–metal ion interactions, we have prepared synthetic peptides representing metal-binding protein surface domains from the human plasma metal transport protein known as histidine-rich glycoprotein (HRG). Three synthetic peptides, representing multiples of a 5-residue repeat sequence (Gly–His–His–Pro–His) from within the histidine- and proline-rich region of the C-terminal domain were prepared. Prior to immobilization, the synthetic peptides were evaluated for identity and sample homogeneity by matrix-assisted UV laser desorption time-of-flight mass spectrometry (LDTOF-MS), a method developed recently for the mass determination of high-molecular-mass biopolymers. 2,5-Dihydroxybenzoic acid was evaluated as a matrix to facilitate the laser desorption and ionization of intact peptides and was found to be ideally suited for determinations of mass within the low-mass region of interest (641.7 to 1772.8 dalton). We observed minimal chemical noise from photochemically generated peptide-matrix adduct signals, clustering, and multiply-charged peptide species. Peptides with bound sodium and potassium ions were observed; however, these signal intensities were reduced by immersion of the sample probe tip in water. Mixtures of the three different synthetic peptides were also evaluated by LDTOF-MS after their elution through a special immobilized peptide-metal ion column designed to investigate metal ion transfer. We found LDTOF-MS to be a useful new method to verify the presence of peptide-bound metal ions. Thus, LDTOF-MS is ideally suited for the rapid (3–5 min), sensitive (< 1 pmol), accurate (±0.05%), and relatively high resolution ($m/\Delta m$ = 300–500, full width at half maximum, where m = mass) evaluation of synthetic peptides. Further, LDTOF-MS was found to be an important tool for the characterization of peptide mixtures and peptide–metal ion interactions.

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0021-9673/92/$05.00

INTRODUCTION

We have investigated the preparation of synthetic peptides for the purpose of building bioactive model surfaces which may mimic naturally occurring metal-binding domains identified on the surface of known metal transport proteins. Histidine-rich glycoprotein (HRG) is a 75 000-dalton Zn(II)- and Cu(II)-binding plasma protein with unique arrangements of repeating His (11–13 mol%) and Pro (14–16 mol%) residues in the C-terminal region [1–7]. A 5-residue primary sequence of GHHPH is found in tandem repeats of up to 25 residues [6]. We have synthesized HRG peptides of the type $(GHHPH)_nG$, where $n = 1$–3, to investigate the affinity of this protein surface metal-binding domain for free metal ions and to evaluate metal ion-dependent macromolecular recognition of the native peptide.

Matrix-assisted UV laser desorption time-of-flight mass spectrometry (LDTOF-MS) has been introduced recently [8–13] as a means for the mass determination of high-molecular-mass biomolecules. One purpose of this communication is to discuss LDTOF-MS as a routine method for monitoring the accuracy and completeness of automated peptide synthesizers. More importantly, we have found LDTOF-MS to be a new method to evaluate directly the interaction of transition metal ions with synthetic peptides representing metal-binding protein surface domains (see also refs. 14–17 and Note added in proof).

EXPERIMENTAL

Synthesis of HRG metal-binding peptides (GHHPH tandem repeats)

The 6-residue HRG peptide $(GHHPH)_1G$ (1-mer), the 11-residue HRG peptide $(GHHPH)_2G$ (2-mer), and the 16-residue HRG peptide $(GHHPH)_3G$ (3-mer) were synthesized on an Applied Biosystems Model 430A automated peptide synthesizer using 9-fluorenylmethyloxycarbonyl (Fmoc)–N-methylpyrrolidone (NMP) chemistry (FastMoc, Applied Biosystems). C-Terminal residues were attached onto *p*-hydroxymethylphenoxymethyl (HMP) resin using standard dicyclohexylcarbodiimide (DCC) procedures. The remaining active hydroxyl groups on the resin were capped with benzoic anhydride before initiation of peptide synthesis. Coupling of amino acid derivatives to the C-terminal residue was achieved stepwise by removal of the N-terminus Fmoc group (with piperidine) followed by coupling of the next amino acid using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the coupling reagent. Upon completion, the N-terminus Fmoc protecting group was removed with piperidine. Deprotection of side chain residues and decoupling from resin was achieved by incubation for 1–2 h at room temperature in 95% trifluoroacetic acid. Peptide purity was verified by reversed-phase high-performance liquid chromatography on a Waters RCM Delta-Pak C_{18} Prep Pak cartridge (100 mm × 25 mm I.D.; 15-μm particle size; 30-nm pore size). Peptide amino acid sequences were verified by Edman (see ref. 18) degradation with an Applied Biosystems Model 473A automated peptide sequence analyzer. The calculated mass of each synthetic peptide was verified by UV LDTOF-MS [14,17] and by electrospray ionization mass spectrometry [17]. The calculated mass values were based upon IUPAC average atomic masses for C (12.011), N (14.007), O (15.999), H (1.008), S (32.060) and P (30.974).

Preparation of synthetic peptides for LDTOF-MS

Synthetic peptides were deblocked and cleaved from the resin and either lyophylized directly or purified by reversed-phase high-perforance liquid chromatography. Saturated solutions of 2,5-dihydroxybenzoic acid ($M_r = 154.12$) were prepared fresh for each experiment in Milli-Q water. Typically, 1–2 μl of a 1–100 nmol/ml peptide solution (in water, buffer or 70% acetonitrile in water was mixed with 1–2 μl of 2,5-dihydroxybenzoic acid; 2 μl of this mixture were applied to the 2-mm diameter stainless-steel probe tip and air-dried at room temperature (25°C). Where indicated, the dried peptide-matrix deposit on the probe tip was washed in Milli-Q water (25°C) and redried. The probe was then inserted through a vacuum lock into the mass spectrometer. Vacuum pump down time averaged 2–4 min.

UV laser desorption time-of-flight mass spectrometry

MS was performed on a Vestec Model 2000 laser

TABLE I

VERIFICATION OF AMINO ACID SEQUENCE AND MASS FOR SYNTHETIC HRG PEPTIDES

Code	Peptide sequence	Molecular mass (M_r)	$[M+H]^+$	
			Calculated	Observed
1-mer	GHHPHG	640.7	641.7	641.6
2-mer	GHHPHGHHPHG	1206.3	1207.3	1207.5
3-mer	GHHPHGHHPHGHHPHG	1771.8	1772.8	1772.5

desorption linear time-of-flight mass spectrometer. Frequency-tripled output from a Q-switched neodymium–yttrium aluminum garnet (Nd-YAG) pulsed laser (355 nm, 5 ns pulse, Lumonics HY400) or a pulsed nitrogen laser (337 nm, 3 ns pulse, Laser Science) was focused (12-in. focal length) through a fused-silica window to irradiate a spot (*ca.* 150 μm $\times$ 300 μm) of the insertion probe tip on which the matrix/analyte solution had been dried. A variable attenuator was used to maintain the laser power density at the optimal threshold for desorption/ionization of the analyte species (*ca.* $1–2 \cdot 10^6$ W/cm). Ions desorbed by pulsed laser irradiation were accelerated to 30 keV and allowed to drift along a 2-m flight path (maintained at 30 μPa) to a 20-stage focused mesh electron multiplier. A Lecroy model TR8828D transient recorder (5-ns time resolution) and LeCroy 6010 MAGIC controller were used with software permitting real-time signal averaging of multiple laser shots (all spectra presented are the signal average of 100 laser shots). Data were transferred from the MAGIC controller to a Compaq 386/33 personal computer. The resulting mass spectrum, calculation of peak centroids, and data reduction were performed using PC-based software (LabCalc, Vestec).

RESULTS AND DISCUSSION

The accurate mass determination of synthetic peptides has been recognized as a valuable tool in preparation of high-quality peptides [19,20]. It has been reported [21] that as many as 50% of the synthetic peptide preparations characterized by MS at protein chemistry core laboratories contained peptides with incorrect masses; others were not homogeneous. For analytical (*i.e.*, quantitative) affinity chromatography, the accuracy with which synthetic ligands are prepared is essential to avoid ambiguities in data reduction and interpretation.

The amino acid sequence of each of the three synthetic peptides was verified by sequential Edman degradation. Each of the synthetic peptides was also evaluated by LDTOF-MS. We found excellent agreement between the calculated and observed mass values; there was no evidence of incomplete or intermediate products resulting from peptide synthesis. These results are summarized in Table I. Representative mass spectra around the parent molecular ions are shown for the case of the synthetic HRG peptide $(GHHPH)_2G$.

Fig. 1. shows the LDTOF mass spectrum obtained for the synthetic HRG peptide $(GHHPH)_2G$ (2-mer). In this case, the peptide sample [prepared in 40% acetonitrile–0.1% trifluoroacetic acid (TFA)] was mixed with an equal volume of 2,5-dihydroxybenzoic acid matrix, applied to the probe tip, and allowed to air-dry at room temperature. Analysis by LDTOF-MS revealed an M_r of 1207.5 for the protonated molecule $[M+H]^+$. The smaller peak observed at an M_r of 1229.1 (Fig. 1) represents the mass of the sodium adduct $[M+Na]^+$. The relative quantities of free peptide and the peptide-sodium adduct were found to vary with the solvent concentration of sodium ions. This is an important consideration for the routine evaluation of peptides after affinity chromatography in high salt buffers and, therefore, was investigated further.

The full effects of added buffer salts on the LDTOF mass spectrum obtained for the synthetic HRG peptide $(GHHPH)_2G$ (2-mer) is illustrated in Fig. 2. The peptide sample was prepared in a commonly used buffer (20 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.0), applied to the probe tip with 2,5-dihydroxybenzoic acid (in water) as the matrix, allowed to air-dry, and analyzed by LDTOF-MS.

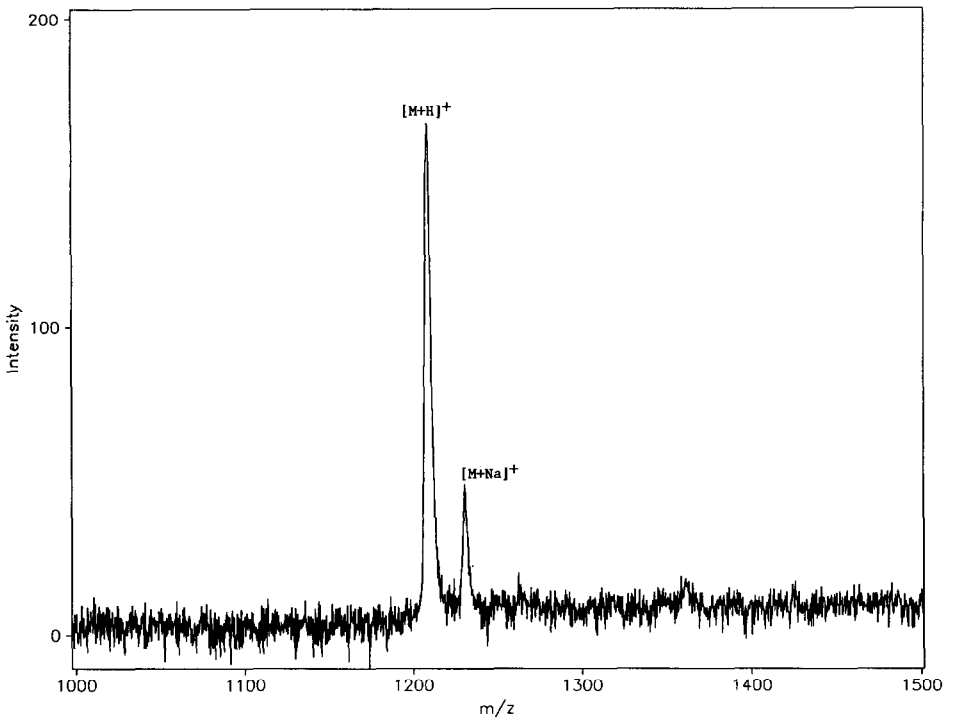

Fig. 1. LDTOF mass spectrum obtained for the synthetic HRG peptide $(GHHPH)_2G$ with 2,5-dihydroxybenzoic acid as the matrix. The peptide sample (in acetonitrile–TFA) was applied to the probe tip, air-dried, washed gently with Milli-Q water and analyzed.

The protonated molecule (M_r 1207.5) observed originally (Fig. 1) was absent. Instead, two peaks were observed. The first peak represents the peptide with one sodium adduct (M_r 1229.1). The second peak represents the peptide with two sodium adducts (M_r 1251.7). This particular example represents one of the more extreme cases we have observed. The effects of buffer salts on the mass of the synthetic HRG peptide (*i.e.*, the presence of sodium and potassium adducts), however, could be reduced or eliminated. When the dried peptide sample on the probe tip was washed with Milli-Q water (by immersion of the inverted probe tip into a reservoir), subsequent analysis by LDTOF-MS revealed the protonated molecule and one additional peak, $[M+K]^+$ (Fig. 3). A summary of molecular ions observed for the synthetic 2-mer peptide is presented in Table II.

We have also explored the use of LDTOF-MS for the direct evaluation of transition metal ion interactions with known metal-binding peptides and even with mixtures of these peptides. The results shown in Fig. 4 reveal the LDTOF mass spectrum observed for a mixture of all three synthetic HRG peptides (1-mer, 2-mer and 3-mer) after receiving Cu(II) ions by metal ion transfer from a stationary phase of immobilized metal ions (see Table III). In this example, the mixture of three HRG peptides had been passed through a column of the immobilized HRG peptide $(GHHPH)_2G$ loaded with Cu(II) ions. The immobilized HRG peptide had been coupled at a low ligand density (3 μmol metal ion bound/ml gel) to a commercial preparation of activated agarose (Affi-10, Bio-Rad). The eluted HRG peptide $(GHHPH)_1G$ (1-mer) was observed as a protonated molecule free of bound metal ions (peak labeled 1.0; M_r 641.6), with one bound Cu(II) ion (peak labeled 1.1; M_r 703.0), and with two bound Cu(II) ions (peak labeled 1.2; M_r 764.5). The middle set of peaks revealed the mass of the $(GHHPH)_2G$ peptide free of bound metal ions (peak labeled 2.0; M_r 1207.7), with one bound Cu(II) ion (peak labeled 2.1; M_r 1269.9), with two bound Cu(II) ions (peak labeled 2.2; M_r 1332.1),

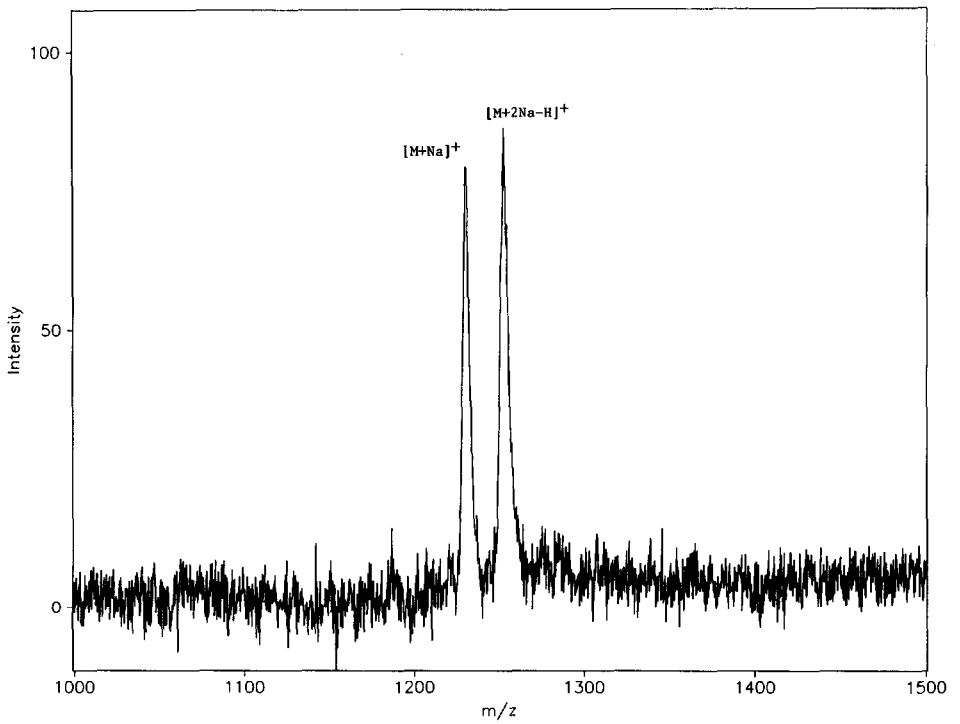

Fig. 2. Effects of buffer salts on the LDTOF mass spectrum obtained for the synthetic HRG peptide $(GHHPH)_2G$. The peptide sample was applied to the probe tip with 2,5-dihydroxybenzoic acid as the matrix, allowed to air-dry and analyzed.

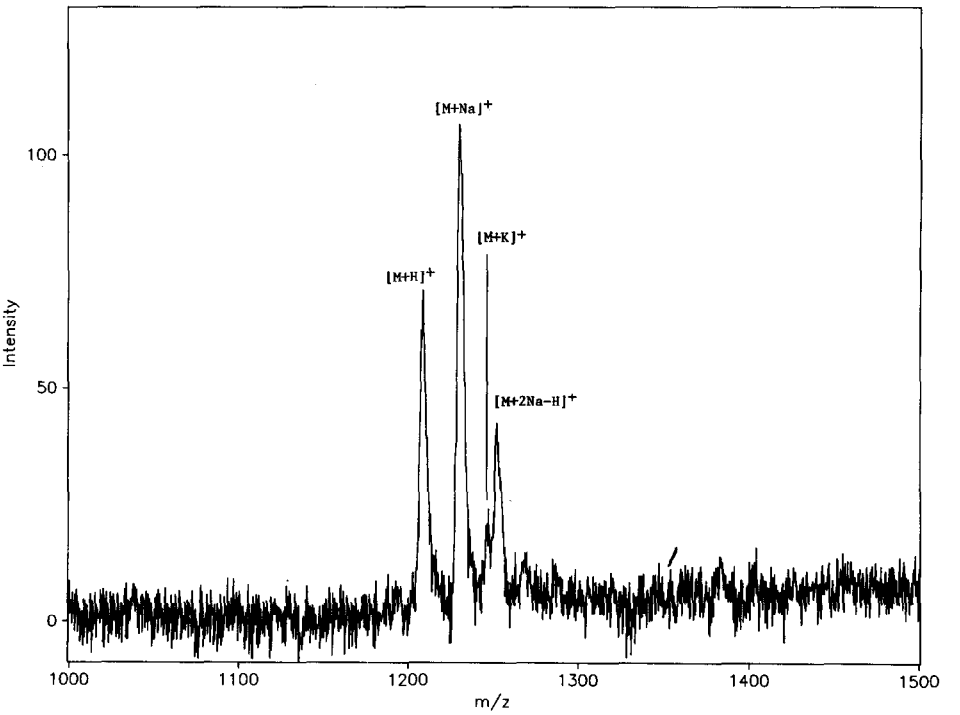

Fig. 3. Removing the effects of buffer salts on the mass of the synthetic HRG peptide $(GHHPH)_2G$. The peptide sample was applied to the probe tip with 2,5-dihydroxybenzoic acid as the matrix, allowed to air-dry, washed briefly with Milli-Q water and analyzed by LDTOF-MS.

TABLE II

MOLECULAR ION MASS ASSIGNMENTS FOR PEAKS OBSERVED IN FIGS. 1 3

Synthetic HRG peptide: GHHPHGHHPHG (2-mer).

Molecular ion	M_r	
	Calculated	Observed
$[M+H]^+$	1207.3	1207.5
$[M+Na]^+$	1229.3	1229.1
$[M+K]^+$	1245.3	1246.1
$[M+2Na-H]^+$	1250.3	1251.7

with three bound Cu(II) ions (peak labeled 2.3; M_r 1394.3), and some with four bound Cu(II) ions (peak labeled 2.4; M_r 1456.8). The last group of peaks on the right represents the mass of the $(GHHPH)_3G$ (3-mer) peptide free of any bound metal ions (peak labeled 3.0; M_r 1772.5), with one bound Cu(II) ion (peak labeled 3.1; M_r 1835.0), with two bound Cu(II) ion (peak labeled 3.2; M_r 1897.3), with three bound Cu(II) ions (peak labeled 3.3; M_r 1959.5), and with four bound Cu(II) ions (peak labeled 3.4; M_r 2022.4). The small peaks marked by an asterisk indicate the presence of an additional sodium adduct. The incremental differences of approximately 62.5 dalton (as opposed to the 63.5 dalton difference expected) observed for the molecular ions with multiple bound Cu(II) ions suggests that, in all cases, the addition of each metal ion results in the displacement of one proton; this effect is under further investigation (see Note added in proof). The near baseline resolution of these peaks within a narrow mass range, and the apparent absence of multiply-charged molecular ions, illustrates the analytical potential of LDTOF-MS.

These results suggest that LDTOF-MS, aside from being a rapid and efficient method for the routine verification of peptide mass, may be an effective tool to investigate directly the interaction of peptides with various metal ions. On the basis of our

TABLE III

MOLECULAR ION MASS ASSIGNMENTS FOR PEAKS OBSERVED IN FIG. 4

The small intermediate peaks in Fig. 4 marked with an asterisk (*e.g.*, between peaks 1.0 and 1.1 and between peaks 2.0 and 2.1) represent a sodium adduct, for example $[M+Na]^+$.

Peak No.	Molecular ion[a]	Calculated[b]	Observed	Difference in mass
1-mer $(GHHPH)_1G$				
1.0	$[M+H]^+$	641.7	641.6	0
1.1	$[M+Cu]^+$	704.2	703.0	61.4
1.2	$[M+2Cu]^+$	766.7	764.5	61.5
2-mer $(GHHPH)_2G$				
2.0	$[M+H]^+$	1207.3	1207.7	0
2.1	$[M+Cu]^+$	1269.8	1269.9	62.2
2.2	$[M+2Cu]^+$	1332.3	1332.1	62.2
2.3	$[M+3Cu]^+$	1393.8	1394.3	62.2
2.4	$[M+4Cu]^+$	1456.3	1456.8	62.5
3-mer $(GHHPH)_3G$				
3.0	$[M+H]^+$	1772.8	1772.5	0
3.1	$[M+Cu]^+$	1835.3	1835.0	61.5
3.2	$[M+2Cu]^+$	1897.8	1897.3	62.3
3.3	$[M+3Cu]^+$	1959.3	1959.5	62.2
3.4	$[M+4Cu]^+$	2021.8	2022.4	62.9

[a] The simplified representation of molecular ion species does not reflect the probable loss of one proton (H^+) for each additional bound Cu(II) ion.

[b] The increase in peptide (molecular ion) mass associated with the presence of multiple bound Cu(II) ions was calculated using the major Cu isotope (63.0 a.m.u.).

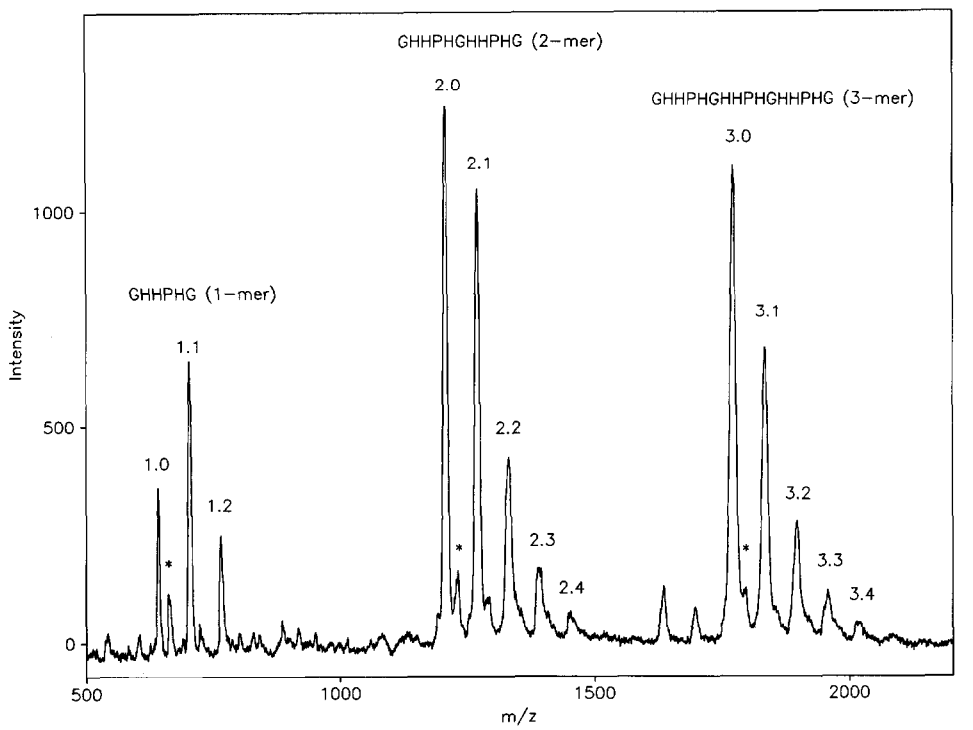

Fig. 4. LDTOF mass spectra of a mixture of all three synthetic HRG peptides (1-mer, 2-mer and 3-mer) after elution from a column of immobilized $(GHHPH)_2G$ (2-mer) loaded with Cu(II) ions. The peptide affinity column used for metal ion transfer was prepared by coupling $(GHHPH)_2G$ to Affi-10 (Bio-Rad). Cu(II) ions were loaded as described in ref. 20. The column was equilibrated with 20 m*M* sodium phosphate buffer (pH 7.0) with 0.5 *M* NaCl. An equimolar mixture of the three different synthetic peptides (free of bound metal ions) was passed through the column unretained. Flow-through fractions were analyzed directly by LDTOF-MS. The peptide sample was applied to the probe tip with 2,5-dihydroxybenzoic acid as the matrix, allowed to air-dry, and analyzed by LDTOF-MS. The metal ion-free HRG peptides $(GHHPH)_1G$ (1-mer peak 1.0), $(GHHPH)_2G$ (2-mer peak 2.0), and $(GHHPH)_3G$ (3-mer peak 3.0) are observed along with peptides with 1 or more bound Cu(II) ions. The small peaks marked by an asterisk indicate the presence of a quasi-molecular sodium ion. A detailed description of the peaks observed in this spectrum is provided in the text.

more recent work, published after the initial presentation of this paper (see Note added in proof) we now know that LDTOF-MS presents the opportunity to investigate several biopolymer–metal ion interaction parameters, such as stoichiometric distributions of bound metal ions [14,15,17], metal ion-dependent molecular recognition events, metal ion transfer, and the detection and mapping of metal ion-binding peptides in samples derived from enzymatic digestion [16].

ACKNOWLEDGEMENTS

We thank Dr. Marvin Vestal at Vestec Corporation for helpful discussions and critical evaluation of this manuscript.

This project has been funded, in part, with federal funds from the US Department of Agriculture, Agricultural Research Service under Cooperative Agreement number 58-6250-1-003. The contents of this publication do not necessarily reflect the views or policies of the US Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

NOTE ADDED IN PROOF

Since the original presentation of results outlined in this paper, several additional manuscripts have been published (see refs. 14–17). More recently, specific peptide–metal ion interaction chemistries in-

volving both N and S ligands have been investigated by electrospray ionization mass spectrometry [22,23].

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