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ORIGINAL ARTICLE

MALDI-TOF mass spectrometric properties of humanin-like peptides

Klára Novotná, Ondrej Šedo and Josef Havel

Department of Analytical Chemistry, Faculty of Science, Masaryk University, Brno, Czech Republic

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Summary
The possibilities of MALDI-TOF mass spectrometric analysis of new neuro-protective peptide [G^{14}]-humanin (HNG) and similar compounds are studied with the aim of finding optimal conditions for the determination of these peptides. Acidification and washing of HNG samples using 5% (v/v) formic acid is necessary to reach a detection limit similar to other peptides. The sensitivity of HNG determination is decreased in the oxidative environment as the peptide yields oxidation of methionine and cysteine forming several species, including a disulfide dimer. During Post-source Decay (PSD) it was found that intense cleavage between Asp and Leu in HNG reduces the possibility of detecting other fragments. Better sequence coverage is gained from shorter humanin-like peptides.

Keywords: [G^{14}]-humanin – humanin derivatives – MALDI-TOF MS – PSD – peptide oxidation

INTRODUCTION

Alzheimer`s disease (AD) is one of the most serious problems of our ageing population. During this disorder, abnormal proteins accumulate in and around neurons in the brain, resulting in their cell death (www.siumed.edu/neuro/cadrd.html). Hashimoto et al. found in 2001 (Hashimoto et al. 2001) an endogenous polypeptide termed *humanin* (HN). Humanin is a linear neuropeptide composed of twenty-four amino acids (MAPRGFSCLL LLTSEIDLPVKRRA), which is capable of protecting neurons against pathological proteins, such as amyloid precursor protein, presenilin-1 or presenilin-2 mutants. The mechanism of the HN neuro-protective function is still unknown and it is under examination in several recent papers focused on its interaction with proteins related to the AD process (Jung and Nostrand 2003, Guo et al. 2003, Zou et al. 2003, Ikonen et al. 2003). The study of the structural activity of HN indicates that the peptide is active in a form of dimer where Ser7 and Leu9 play important roles (Terashita et al. 2003, Yamagishi et al. 2003).

Matrix-Assisted Laser Desorption Ionization (MALDI) Time-of-Flight (TOF) mass spectrometry (MS) is an analytical method highly suitable for the detection and characterization of bio-compounds such as peptides and proteins. In addition, the structural information gained from MALDI-TOF can be enriched using Post-source Decay (PSD) and In-source Decay (ISD) techniques (Kaufmann et al. 1994, Chaurand et al. 1999, Godovac-Zimmermann and Brown 2001, Reiber et al. 1998).

It follows from recent findings that HN and similar peptides are very good prospects for AD treatment. The aim of this work is therefore to examine the possibilities and limitations of using MALDI-TOF MS for analysis of humanin-like peptides.

MATERIAL AND METHODS

Chemicals

Bradykinin, renin substrate tetradecapeptide, angiotensin I, adrenocorticotropic hormone (fragment 18-39), insulin (from bovine pancreas), α-cyano-4-hydroxycinnamic acid (CHCA), 3,5-dimethoxy-4-hydroxycinnamic acid (SA), 2,5-dihydroxybenzoic acid (DHB), 5-methoxy-2-hydroxybenzoic acid (5-MS) and trifluoroacetic acid (TFA) were obtained from Sigma (Steinheim, Germany). Formic acid (FA) and hydrogen peroxide was from Pliva-Lachema (Brno, Czech Republic). HPLC grade acetonitrile was from Merck (Darmstadt, Germany). All reagents except for peptides were of analytical grade purity. Humanin-like peptides used in this study were purchased from Clonestar Biotech (Brno, Czech Republic). The purity of HNG was 95%. Humanin-like peptides were obtained in a mixture of [Gly^{14}]-humanin (7%), des-Met^{1}-[Gly^{14}]-humanin (5%), des-Pro^{3}-[Gly^{14}]-humanin (7%), des-Met^{1},Pro^{3}-[Gly^{14}]-humanin (34%), des-Met^{1},Pro^{3},Ser^{7}-[Gly^{14}]-humanin (7%), des-Met^{1},Pro^{3},Ser^{7},Cys^{8}-[Gly^{14}]-humanin (19%), des-Met^{1},Pro^{3},Phe^{6},Ser^{7}-[Gly^{14}]-humanin (5%) and des-Met^{1},Pro^{3},Phe^{6},Ser^{7},Cys^{8},Leu^{9}-[Gly^{14}]-humanin (11%). Deionised water used to prepare all solutions was double distilled from a quartz apparatus from Heraeus Quartzschmelze (Hanau, Germany).

Apparatus

All measurements of mass spectra were performed on AXIMA CFR (Kratos Analytical, Manchester, United Kingdom) mass spectrometer, equipped with a nitrogen laser wavelength of 337 nm. The energy of the laser was changed in relative units, where 180 units correspond to maximum power of 6 mW and 0 units to 0 mW. External calibration was carried out with bradykinin, renin substrate tetradecapeptide, angiotensin I, adrenocorticotropic hormone (fragment 18-39) and bovine insulin. All analyses were performed using 200 laser shots and, in the case of PSD, using 500 laser shots. Laser power for PSD and ISD measurements was increased 50% above the peptide $[M+H]^+$ threshold. Nested PSD calibration was from the manufacturer. PSD spectra were smoothed by an average of 10 channels. Each PSD analysis was performed at least three times.

Sample preparation

A solution of peptides in 5% FA, 1 µl, was dropped on the target, then immediately, 1 µl of matrix solution (10 mg/ml or in the case of CHCA saturated solution in 0.1% TFA:acetonitrile 1:1, v/v) was added to the sample and mixed by pipetting up and down directly onto the target. The samples were dried by an air stream at room temperature and then washed using a drop of 5% FA. Then, the sample plate was introduced into the mass spectrometer and analysed when the vacuum reached approximately 10^{-4} Pa.

RESULTS AND DISCUSSION

MALDI-TOF mass spectrometry of HNG

The first step in the mass spectrometric analysis was to find the most suitable matrix which would yield good ionisation, and thus the most intense signal, preferably without alkali metal adducts and, in addition, the best resolution. We studied the use of several matrices that are generally used for the ionisation of peptides (CHCA, SA, DHB, DHB:5-MS 1:1 (w/w)). The intensities of $[M+H]^+$ and alcali metal adducts differ very significantly from one matrix to another. The most suitable was found to be CHCA, yielding the highest intensity of peptide peaks and acceptable signal-to-noise ratio. The detection limit we accomplished in linear positive ion mode (conditions described in *Sample preparation* part) was estimated to be 40 fmol. In an aqueous solution (without FA), the detection limit is 10-fold higher. The HNG peptide molecular ion can also be observed in the negative mode (with CHCA as a matrix), but the detection limit is about 3 orders higher.

In PSD spectra of HNG measured in positive mode, we found only a few peaks of y_n ions, their deaminated forms and b_n ions as well as internal fragments (Fig 1A). There is an intense peak, designated as b_{17} that corresponds to cleavage between Asp^{17} and Leu^{18}. In negative PSD spectrum we observed approximately ten peaks of different a_n, b_n, c_n, y_n ions and internal fragments. Using ISD technique does not facilitate the peptide sequence determination, as mainly complex internal fragments are formed. Peptides missing two or more amino acids close to the N-terminus yield much "richer" PSD spectra (Fig 1B) showing several b_n and y_n fragments, which can then be used to confirm the sequence. *De novo* peptide sequence determination from such PSD spectra would be rather difficult. The performance of PSD technique is improved after quantitative oxidation of humanin-like peptides (Šedo et al. 2004).

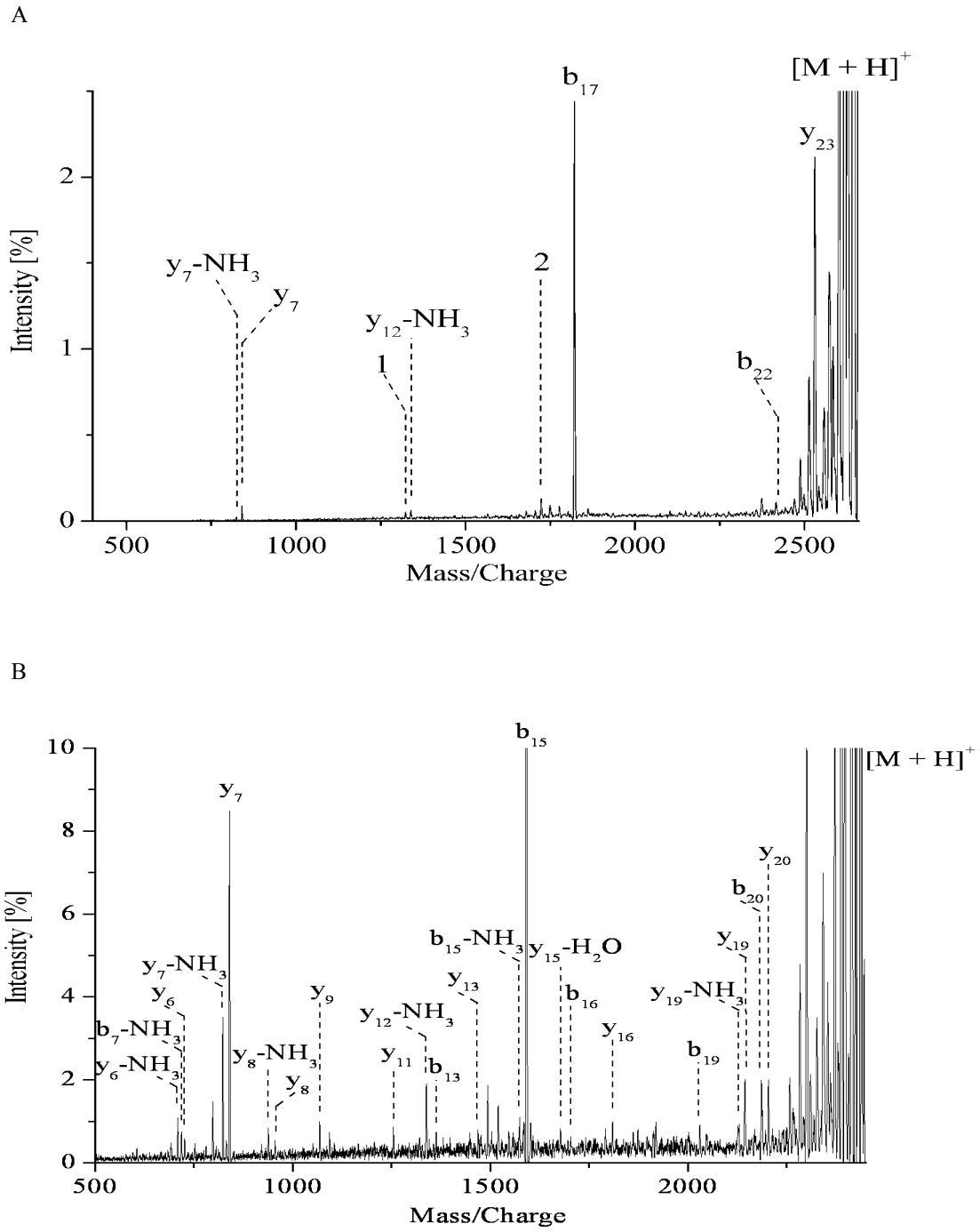

Fig. 1. **PSD spectra of HNG (A) and des-Met[1] Pro[3]-[Gly[14]]-humanin (B)**. The spectra were obtained with ion gate set to the interval 2630–2685 (A) and 2420–2450 (B). Fragments denoted by a number: 1, LLTGEIDLPVKR-NH_3; 2, SCLLLLTGEIDLPVKR-28 (The difference of 28 Da corresponds to the loss of neutral CO molecule).

Oxidation of humanin-like peptides

As HNG contains methionine and free cysteine, it can be easily oxidised. After one hour of exposition to ambient air at room temperature, the peptide solution contains oxidised peptides with molecular peaks of $[M+n*16+H]^+$ (n=1–5), resulting from partial methionine and cysteine oxidation. In addition, a peak with m/z=5312.35 Da can be observed in HNG MALDI-TOF mass spectra after oxidation. This peak corresponds to a dimer formed *via* a disulfide bridge between two cysteine residues. It was further proved that after adding diluted hydrogen peroxide (0.01%) to a fresh solution of HNG (3.76 μM), the dimeric peak can be observed instantaneously. The spectrum related to the dimer

formation of HNG is demonstrated in Fig. 2. Oxidated species decrease the sensitivity of HNG determination and moreover, make significantly more complicated the spectra interpretation in the case of analysis of a mixture of more humanin-like peptides.

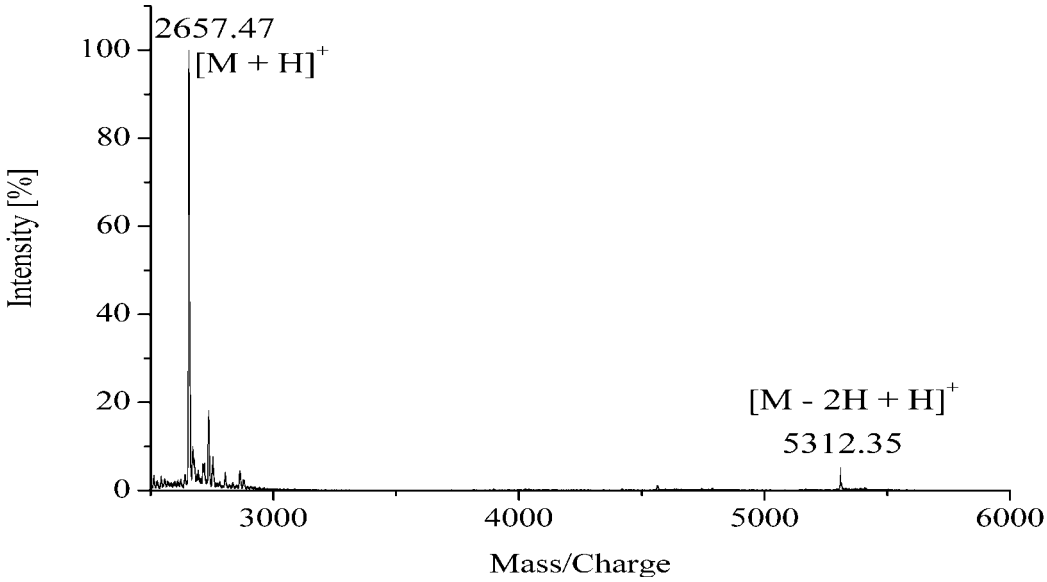

Fig. 2. **Mass spectrum of HNG in linear positive ion mode.** 1 μl of sample solution (37.6 pmol) was mixed by pipetting up and down (directly on the target) with 1 μl of H_2O_2 (0.01%) and then with 1μl of SA matrix solution. Matrix SA gave a better signal-to-noise ratio for HNG dimer than CHCA.

CONCLUSIONS

It was observed that humanin-like peptides can be ionised both in positive and negative modes. The positive ion mode offers a more intense signal than the negative mode, allowing the detection of 40 fmol of HNG. Sequencing HNG by PSD technique results in poor spectra with a main peak that corresponds to cleavage between Asp and Leu. Decrease in HNG length results in the possibility of detecting more fragments. Humanin-like peptides easily yield oxidative processes that may result in mass spectra complexity and lower sensitivity in the determination.

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✉ **Address:**
Josef Havel, Department of Analytical Chemistry, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic; havel@chemi.muni.cz