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APPLICATION NOTE

Evaluation of MALDI-TOF/TOF Mass Spectrometry Approach for Quantitative Determination of Aspartate Residue Isomerization in the Amyloid-β Peptide

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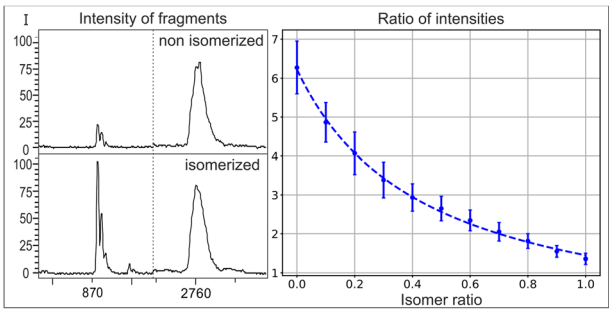

Abstract. Immunoprecipitation (IP) combined with MALDI-TOF mass spectrometry is a powerful instrument for peptide and protein identification in biological samples. In this study, the analytical capabilities of MALDI-TOF/TOF mass spectrometry for relative quantitation of isoAsp7 in Aβ(1-42) and Aβ(1-16) were investigated. The possibility of quantitative determination of isoAsp7 in Aβ(1-42) with the detection limit as low as 2 pmol has been demonstrated. The same approach was applied for a shorter peptide Aβ(1-16) and resulted in enhanced accuracy (± 3.2%), and lower detection limit (50 fmol). Pilot experiments with artificial cerebrospinal fluid and mouse brain tissue were performed and showed that the proposed IP-MALDI-TOF/TOF approach could be applied for measuring isoAβ content in biological fluids and tissues. Additionally, it was shown that 6E10 anti-amyloid antibodies might affect the accuracy of the amyloid-β quantitation in the presence of the isomerized peptide.

Keywords: Amyloid-β, Isomerization, MALDI, Immunoprecipitation

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Introduction

Detection of low amounts of peptides and proteins in biological samples is an important task for modern biochemistry. One of the most effective ways for such investigations is a combined approach based on immunoprecipitation (IP) followed by MALDI-TOF analysis. The IP-MALDI-TOF approach was recently applied to reveal the correlations between alterations of relative concentrations of different forms of amyloid-β peptide (Aβ) in human plasma with the amyloid-β brain burden and cognitive impairment in patients [1]. Similar methods were also applied to detect the presence of Aβ post-translational modifications (PTMs) [2–4]. One of such modifications of interest is the isomerization of aspartic acid [5–7] due to its possible role in aggregation and toxicity of Aβ, the mechanisms and peculiarities of which are still under study.

Formation of β-aspartic acid residues (isoAsp) through a succinimide rearrangement is a usual non-enzymatic process in peptides and proteins both in vitro and in vivo. There are many approaches for qualitative and quantitative determination of peptide isomerization based on mass spectrometry (MS) [8–10],

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liquid chromatography (LC) [11], ion mobility spectrometry (IMS) [12], electrophoresis [13], immunology [14], protein isoaspartyl methyltransferase (PIMT) activity [15], and their combinations. LC, as well as capillary electrophoresis, is widely used to directly separate native and isomerized peptides or after peptide modification using PIMT [16]. Isotopic labeling [17, 18] of products of specific proteolysis or digestion is also useful for HPLC-MS or stand-alone MS analysis of isomerized peptides as well as application of isoAsp-specific antibodies [19]. Direct separation of native and isoAsp-containing peptides is also possible using time-consuming covalent chiral derivatized LC or ultrahigh-resolution IMS [11–13].

As an alternative, tandem (ETD or ECD) mass spectrometry allows to detect isomerization without prior separation [8, 20–23]. Tandem mass spectrometry methods based on collision-induced dissociation (CID), while do not generate fragments specific for isomerized peptides, still provide differences in MS/MS fragment intensities [9, 10, 24]. MALDI MS is widely used for rapid peptide identification [19, 25] and could be applied for detection of isoAsp in peptides [10, 26, 27]. MALDI-ISD (In-Source Decay) could also be used to generate characteristic ions for isoAsp determination [25, 28] but this approach is less sensitive then MALDI-CID [29].

In this study, we evaluate the analytical capabilities of MALDI-TOF/TOF mass spectrometry for relative quantitation of isoAsp7 in Aβ. The approach was further evaluated with IP and model biological samples such as artificial cerebrospinal fluid and mouse brain tissue.

Materials and Methods

Synthetic peptide standards of both native and isoAsp7-containing forms of amyloid-β 1-16 and 1-42 were obtained from BioPeptide Co., Inc. (San Diego, Ca, USA). WO2 antibodies were obtained from Millipore (Merck KGaA, Darmstadt, Germany), and 6E10 antibodies from BioLegend, Inc. (San Diego, Ca, USA).

Native and isoAsp7 forms of the Aβ peptide were mixed to obtain a set of binary mixtures, in which the percentage of the isoAsp7 form varied from 0 to 100% in 10% increments with peptide concentrations varying from 10 to 100 μg/ml (Aβ1-42) or from 0.1 to 10 μg/ml (Aβ1-16). Each mixture was deposited on a Bruker AnchorChip™ 800 MALDI target plate with DHB (Aβ1-42) or HCCA (Aβ1-16) matrix using the "dried droplet" technique. Experiments were performed on an UltrafleXtreme (Bruker Daltonik Gmbh, Bremen, Germany) MALDI-TOF/TOF mass spectrometer using laser ionization fragmentation technology (LIFT) in CID mode with ultrahigh purity (> 99.999%) helium and argon as collision gases.

Artificial cerebrospinal fluid (art-CSF) was spiked with a mixture of native and isoAsp7 Aβ (isoAβ) at a 10 ng/ml total peptide (0%, 50%, 100% wt. of isoAβ) level and equilibrated by the constant rocking for 1 h at room temperature. Two-month-old mouse (B6C3-Tg(APP695)85Dbo_Tg(PSENI)85Dbo strain) with no significant pathologies were sacrificed, decapitated, and the whole brain was fast frozen and stored at − 80 °C before further processing. The brain tissue (400 mg) was homogenized in a glass tissue homogenizer on ice in Tris-buffered saline and peptides were extracted with 70% formic acid. Aβ was immunoprecipitated from art-CSF or formic acid extract using WO2 (epitope 4-10) or 6E10 (epitope 3-8) monoclonal antibodies according to the manufacturer's protocols. Analysis of the isoform content in the isolated peptides was carried out similarly to that done for synthetic Aβ(1-42). Detailed experiment description is included in the Supplementary Information.

Results and Discussion

Relative Quantitation of isoAsp7 in Full-Sized Aβ(1-42)

To obtain quantitative data, a label-free approach has been chosen, so no internal standard was necessary. Higher efficiency of peptide bond breakage near isoAsp (as compared to Asp) during low-energy fragmentation (e.g., CID) leads to an increase in the intensities of the corresponding b- and y-ions in mass-spectra [24]. Thus, the percentage of isoAsp7 Aβ could be determined directly from the ratio of the signals corresponding to the fragment ions (Figures S-1 and S-2 in Supplementary Information). In contrast to fragmentation of multiply charged ions in ESI-CID or other MALDI-based approaches (PSD or photodissociation) [9, 24, 30], we found that increased efficiency of c-ion formation creates isobaric interferences with isoAsp characteristic $[b+H_2O]^+$ ions.

For evaluation of analytical capabilities of relative quantitation of isoAβ, experiments with binary mixtures of synthetic Aβ(1-42) were carried out. The possible point-to-point variations of isoAsp concentration caused by uneven oligomerization and crystallization of native and isoAβ were overcome by randomly changing the laser irradiation position on the sample spot after every 100 shots. Isolation of the $[M+H]^+$ ion followed by CID was applied to distinguish the isoforms of the peptide. By analyzing the fragmentation spectra, three major fragments (b_7, b_{23}, y_{35}; *m/z* 871, 2758, 3642 respectively) were identified, and it was observed that the ratio of intensities of fragments b_7 and b_{23} is inverse to the percentage of the isomerized form of the peptide in the mixture. Evaluation of corresponding peak areas (including peaks of the isotopic cluster) allows to plot the calibration curves representing the dependence of the area ratio of selected ions from the percentage of isoAβ in the mixture (Figure S-3 in Supplementary Information). The overall accuracy of determination has been estimated as ± 14% (abs.) throughout the full range of isomerization ratios and concentrations, while the detection limit was determined as low as 2 pmol of Aβ per spot.

Application of Method to Model Samples

To prove the compatibility of the proposed method with analysis of biological samples, model experiments with artificial

cerebrospinal fluid (Art-CSF) and mouse brain tissue were carried out.

Art-CSF was spiked with native and isoAβ as well as their binary mixtures, then the Aβ extraction procedure using WO2 antibodies (epitope 4-10) was carried out, and the isoAsp7 form of the peptide was quantified. The results fit into the confidence interval of the method. Experiments show that this combined IP-MALDI method could be applied for the determination of isoAβ percentage in biological fluids (Figure 1) using similar Aβ extraction procedures applied earlier for Aβ detection in biological samples [1, 30, 31].

These experiments were also carried out with the 6E10 antibodies (epitope 3-8), which are most commonly used for IP of Aβ peptides. It has been shown that 6E10 antibodies are highly selective for native Aβ(1-42) and no isoAsp7 Aβ(1-42) was detected in these experiments (as well as in western blot experiments Figure S-4 in Supplementary Information). The exact reason for such selectivity requires further investigation, but it has to be noted that investigation of biological samples requires a careful choice of antibodies for IP and WB experiments in order to avoid an underestimation of peptide concentrations in the presence of the isomerized form.

Brain tissue obtained from a 2-month-old transgenic mouse that expressed human Aβ peptides was subjected to homogenization followed by Aβ immunoprecipitation (Figure S-5 in Supplementary Information). The amount of peptide that could be isolated from this biological sample using WO2 antibodies proved to be sufficient to perform isoAβ quantitation (absolute Aβ concentration in the sample was not measured). No Aβ isomerization was observed in the brain tissue in this experiment due to the relatively young age of the sacrificed animal.

Relative Quantitation of isoAsp7 in Aβ(1-16)

Since the accuracy of quantitation of isoAsp7 in full-sized Aβ is limited due to the high mass of the analyzed peptide, a similar approach was applied to a shorter peptide, which yields fragmentation spectra with a higher S/N ratio. In the case of Aβ, fragment 1-16 was chosen as such shorter peptide, since this peptide could be obtained by enzymatic cleavage of most forms of full-sized β-amyloid, and thus the overall percentage of Asp7 isomerization in all forms of Aβ in the sample could be determined. A similar set of binary mixtures of synthetic Aβ(1-16) was analyzed and rich and intensive fragmentation spectra were obtained. More than 20 fragments were identified, and their behavior was determined (Figures S-6 and S-7 in Supplementary Information). It was assumed that intensities of fragments formed due to bond breakage further from the modification site should be less affected by the changes in the isoform concentrations, than those located closer [24, 26]. Among them, fragments b_{11}, y_4, and y_{13} were chosen for screening of the dependency of fragment ion intensities from isoAβ percentage in the mixture (changes in the intensity of each identified fragment was evaluated relatively to the intensity of the selected ions). Fragments b_5, b_6, b_7, and y_{10} demonstrated a high dependence of the signal from the concentration of isoAβ in the mixture, high intensity, and S/N ratio in spectra. Simultaneously, the behavior of ions b_{11}, y_3, and y_{11} was the most independent of the isoAsp7 percentage among other intense fragments. As a result, calibration curves for relative quantitation of isoAsp7 in the Aβ(1-16) were plotted for the following ratios of peak areas: b_6/b_{11}, y_{10}/y_{11}, b_5/y_3, b_7/y_{11} (Figure 2). Fragmentation using He or Ar did not show any significant differences in ion behavior, S/N ratio, or calibration curves obtained (Figure S-8 in Supplementary Information). The overall accuracy of determination (using peak area ratios of fragments y_{10} and y_{11}, which give the highest accuracy of determination) has been estimated as ± 3.2% (abs.) throughout the full range of concentrations. The detection limit was estimated to make up 50 fmol of total peptide per spot.

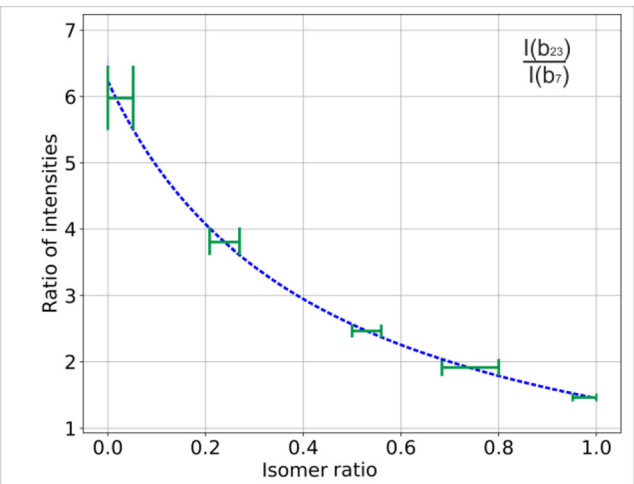

Figure 1. Results of the analysis of model samples placed on the calibration curve. Fragmentation with Ar as the collision gas

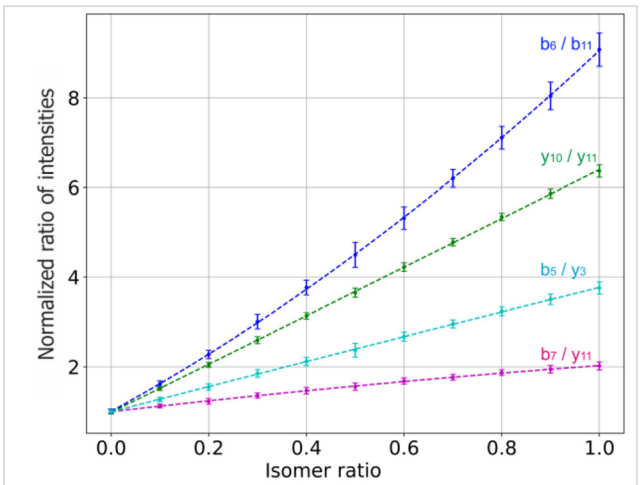

Figure 2. Calibration curves plotted for fragment 1-16 of the Aβ peptide using ratios of relative intensities: b_6 (*m/z* 756.3)/b_{11} (*m/z* 1307.5), blue; y_{10} (*m/z* 1199.5)/y_{11} (*m/z* 1336.6), green; b_5 (*m/z* 619.3)/y_3 (*m/z* 412.2), cyan; b_7 (*m/z* 871.4)/y_{11} (*m/z* 1336.6), magenta. Fragmentation with Ar as the collision gas

Conclusions

A MALDI-TOF/TOF approach for rapid and sensitive label-free quantification of aspartic acid isomerization was developed and tested on model samples. The proposed approach was successfully combined with immunoprecipitation to be used for investigation of Asp7 isomerization of the amyloid-β 1-42 peptide in biological specimens. The overall Aβ isomerization ratio in the sample could be determined by supplementary enzymatic cleavage, which also improves the quantification performance. It was demonstrated that WO2 antibodies are preferable for amyloid-β isolation in case of potential presence of isoAsp7 peptide since 6E10 antibodies are specific only to the native peptide form what leads to an underestimation of the total peptide concentration in the presence of the isomerized form.

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