EPIMERIC PEPTIDES IN ALZHEIMER'S DISEASE: IMPLICATIONS FOR LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY AND IMMUNOTHERAPIES

by

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ABSTRACT

EPIMERIC PEPTIDES IN ALZHEIMER'S DISEASE: IMPLICATIONS FOR LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY AND IMMUNOTHERAPIES

Elizabeth R. Readel, Ph.D. The University of Texas at Arlington, 2023

Supervising Professor: Daniel W. Armstrong

Evidence has shown that the extracellular deposition of amyloid-beta (Aβ) peptide is a contributing factor of Alzheimer's disease. Further, modifications to Aβ peptides may enhance the deposition of Aβ plaques and/or contribute to the neurodegeneration in AD patients. These aberrations include the isomerization and epimerization of L-Asp and D-Ser residues to form D-Asp, L/D-iso-Asp, and D-Ser residues, respectively. Therefore, considerable effort has been expended to create effective methods to distinguish such aberrant Aβ peptides from wild type. A useful technique to extract aberrant Aβ peptides in AD patients is by immunotherapies, or antibodies. A few immunotherapies are thought to provide some benefit. It is possible that a contributing factor to the responses of such therapies may be the presence of modified, or aberrant, Aβ peptides found in AD patients. Modifications to Aβ peptides may enhance the deposition of Aβ plaques and/or contribute to the neurodegeneration in AD patients and may alter the binding affinity to antibodies. Herein, we have developed two high-throughput chromatographic identification methods using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The first method profiles all 20 isobaric Aβ peptide epimers containing Asp, iso-Asp, and Ser isomers using modified Q-Shell and NicoShell chiral stationary phases. The second method uses a chromatographic retention U-shaped curve to investigate the

hydrophobicity of Aβ 1-38, 1-40, 1-42 and fourteen aberrant Aβ 1-42 peptides. The U-shaped curve helped us develop an efficient, selective and comprehensive method to detect both aberrant and wild type Aβ peptides simultaneously. The U-shaped curve also provides insight of the hydrophilic characteristics of D-Ser, D-Asp, and iso-Asp modified peptides. Additionally, we used immunoprecipitation to examine the binding affinity for four antibodies against 18 epimeric and/or isomeric Aβ peptides. Tandem mass spectrometry was used as a detection method, which also was found to produce highly variable results for epimeric and/or isomeric Aβ. These analytical strategies allow the direct detection and identification of all possible Asp, iso-Asp, and Ser stereoisomers in Aβ.

DEDICATION

I dedicate this dissertation to my close mentors including Professors Dr. Dwight Stoll and Dr. Amanda Nienow from Gustavus Adolphus College, and Matt Koval from Aspen Research Corporation. My journey into graduate school and during my graduate studies was exponentially enhanced by your support and guidance.

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Chapter 1

INTRODUCTION

1.1 L- Amyloid-beta Peptides in Alzheimer's Disease

1.1.1 Presence of Amyloid-beta

Alzheimer's Disease (AD) is a progressive and degenerative disease that is hallmarked by the presence of amyloid-beta (Aβ) peptide.[1–4] Aβ peptides are derived from amyloid precursor protein (APP), which is a transmembrane protein.[5,6] APP can be processed by two pathways: the alpha-secretase or the β-secretase pathway. In the alpha-secretase pathway, APP is cleaved by alpha-secretase within the amyloid beta domain, and then cleaved by gamma secretase at the C-terminus. This pathway does not generate amyloid beta. However, when APP is cleaved by β-secretase and gamma secretase, it results in amyloid beta peptide that is released into the extracellular matrix.[5,6] Typically, Aβ peptides are 37-42 amino acids in length with 42 amino acids being the predominant form in AD brains.[2] Aβ peptides can accumulate into large, insoluble extracellular plaques, which are made up of fibrils of Aβ peptides. These plaques disrupt cell function. Aβ peptides can also aggregate into smaller oligomeric species, which can disrupt cell-to-cell communications at synapse locations. Both forms of Aβ peptide aggregation lead to cell death.[7]

1.1.2 Aberrations of Amyloid-beta

Aberrant types of Aβ peptides have been detected in brain tissue extracted from AD patients. These aberrations include point mutations at various residues in addition to racemization and/or isomerization of Asp and Ser residues.[8–10] Asp residues readily form

cyclic succinimide intermediates which result in L/D-Asp or L/D-iso-Asp.[11] This racemization and/or isomerization is an intrinsic quality of Asp residues and has been documented to occur in α/β-crystallin and Aβ peptides.[12,13] Research shows an increase in Asp-antipodes in older subjects relative to younger subjects. This suggests that epimerization and/or isomerization of Asp is a time dependent hallmark of aging.[14] In fact, it was found that modifications of Asp have been found at each Asp location in the Aβ peptide. This includes 20% L/D-iso-Asp at Asp1 and 75% L/D-iso-Asp at Asp7 compared to 6% L/D-iso-Asp at Asp7 for vascular AB samples.[14] Note that in this study, the L-iso-Asp and D-iso-Asp epimers were not separated and therefore indistinguishable. Furthermore, 4-9% of Ser extracted from brain tissue of AD patients is D-Ser.[10] However, the total percentage of epimerization and/or isomerization of Asp and Ser in Aβ in both healthy individuals and AD patients is unknown. It has been suggested that unique modifications change the spatial conformations of Aβ in the brain, making them more resistant to enzymatic degradation.[10] Unmodified Aβ can be degraded by aminopeptidases such as neprilysin and Inulin-degrading enzyme.[15] However, aminopeptidases cannot degrade peptides with D-amino acids.[16] The resistance to proteolytic degradation further results in the accumulation of aberrant peptides as plaques and small oligomers thereby enhancing aggregation and neuronal toxicity.[17]

1.2 Extraction and Analysis of Amyloid-beta

1.2.1 Separation Methods to Identify Aberrations in Amyloid-beta

Liquid chromatography has been used extensively for the chromatographic separation of aberrant Aβ peptides with point mutations.[18] Point mutations can drastically change peptide solubility and on-column secondary structure, making them ideal to

separate using reversed-phase chromatography (RPLC).[19] However, epimerized and/or isomerized Aβ peptides can be more subtle in their changes to Aβ characteristics. Mass spectrometry is a common technique to identify and quantitate peptides, which is effective at distinguishing Aβ with point mutations. However, epimeric and/or isomeric peptides have the same mass and are indistinguishable by MS, making their characterization difficult. Presently, Asp and Ser modified amyloid beta peptides are typically identified by a series of preparative steps including: trypsin digestion, HCL hydrolysis, chiral derivatization, and a chromatographic separation using a reversed-phase stationary phase.[18,20,21] This methodology is tedious and time consuming, but more importantly, induces isomerization of iso-Asp residues to Asp. Therefore, a more effective method could achieve identifications of peptide epimers with minimal preparative steps. Recently, Smith's group have designed a new ion mobility spectrometry (IMS) coupled with MS called structures for lossless ion manipulations (SLIM), which enables long IMS pathlength to achieve the separation of fragmented AB peptides containing L/D-Asp and L/D-iso-Asp.[22] Indeed, this method is fast, however it has only been used to analyze the isomerization of Asp residues in Aβ, and the separation was achieved for only four out of 20 of the potential Aβ peptide epimers.

1.2.2 Antibodies to Extract and Quantitate Amyloid-beta

Useful techniques to selectively extract and/or quantitate Aβ peptides from complex matrixes are by: immunoprecipitation (IP), immunohistochemistry or Western blotting.[23–25] Each technique utilizes the specificity of antibody-antigen binding to selectively extract peptides of interest. Immunoprecipitation is especially practical to both extract and concentrate peptides and it can be used to evaluate the binding efficacy of an antibody to an antigen.[6] The elution product from IP consists only of the antigens that are

selected for by an antibody. Thus, it can be a useful technique to compare the antibody-antigen binding between epimers and/or isomers to their natural all-L antipode. There is a large selection of commercially available antibodies that target unmodified Aβ 1-42, with antibodies 6E10 and 4G8 among the first manufactured.[26,27] However, it is not well understood if antibodies targeting unmodified Aβ can target epimeric and/or isomeric species. This lack of understanding is problematic when developing immunotherapies that target Aβ species from AD patients. In fact, there are several monoclonal antibodies, or immunotherapies, targeting Aβ peptides in Alzheimer's Disease that are in various stages of clinical trials.[28,29] These immunotherapies are highly specific for the unmodified Aβ peptide. And it is possible that their success could be limited as they might be incapable of targeting aberrant species. Therefore, it is necessary to study the antibody-antigen binding effects of isomeric and/or epimeric peptide species.

1.2.3 Secondary Structure/Spatial Conformations MS/MS

Quantification of peptides is typically done by tandem mass spectrometry, which allows for high specificity. Analyzing epimers and isomers poses a significant challenge as they are indistinguishable by mass spectrometry. It is possible that by using tandem mass spectrometry (MS/MS) some structural differences are revealed by varying fragmentation.[30,31] Isomers have been shown to have more variability in their MS/MS transitions including significant differences in their fragment abundances or entirely different fragments.[20,32,33] However, it is expected that the MS/MS fragmentation is very similar amongst epimers and/or isomers. One factor that is not typically considered in the development of methods to identify and quantitate epimers and/or isomers is their sensitivity using the same instrumental conditions. Typically, a method is optimized to an all-L peptide standard, and inherently, epimers and/or isomers are assumed to have similar

instrumental responses. However, it has been suggested that epimers and/or isomers have different secondary structure, which impacts their ionization and fragmentation during MS/MS analysis.[10,34] Thereby, their unique secondary structures impact their instrument response. The increase in exploratory efforts to quantitate isomeric and/or epimeric peptides need to consider the ramifications of this as it will affect any calculated concentrations if these modifications affect sensitivity when utilizing MS/MS.

1.3 Research Objectives and Organization of the Dissertation

This dissertation focuses on advancing quantitative techniques for AB peptide epimers and/or isomers by liquid chromatography, mass spectrometry and immunoprecipitation. Chapters 2 and 3 describe liquid chromatography techniques to analyze AB peptide epimers and/or isomers. Chapter 2 focuses on the separation of 20 epimers and/or isomers of the tryptic digest fragments of Aβ peptide. Chapter 3 focuses on the full-length Aβ peptide. Chapter 4 describes the extraction and purification of Aβ epimers and/or isomers using antibodies by immunoprecipitation. Chapter 5 investigates how Aβ epimers and/or isomers have varying MS/MS responses and how this may affect quantitative analyses.

Chapter 2

Complete Identification of All 20 Relevant Epimeric Peptides in β-amyloid: a New HPLC-MS Based Analytical Strategy for Alzheimer's Research

Abstract

Although the underlying cause of Alzheimer's disease (AD) is not known, the extracellular deposition of amyloid-beta (Aβ) is considered as a hallmark of AD brains. Evidence has shown the occurrence of D-Asp, iso-Asp, and D-Ser residues in Aβ, which may be indicative of and/or contribute to the neurodegeneration in AD patients. Herein, we have developed the first high-throughput profiling technique for all 20 isobaric Aβ peptide epimers containing Asp, iso-Asp, and Ser isomers using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). This new analytical strategy allows the direct detection and identification of all possible Asp, iso-Asp, and Ser stereoisomers in Aβ, and may contribute to a better understanding of the pathogenesis of AD.

2.1 Introduction

Alzheimer's disease (AD), the most common cause of dementia,[1] is a neurodegenerative disease characterized by progressive degeneration of brain tissue.[2,3] The exact cause of the degeneration has yet to be elucidated, but one of the prime suspects is the extracellular deposition of amyloid-beta (Aβ).[4] Aβ is a proteolytically cleaved section of a larger protein called amyloid precursor protein (APP).[5,6] It is noteworthy that there is a distinction between Aβ and amyloid plaques. Aβ is a normal peptide generated throughout life although its normal function remains unclear.[3] While amyloid plaques are insoluble accumulations of Aβ between nerve cells and are the neuropathological hallmark of AD.[3] Recent studies have shown that there is a difference in Aβ between normal elderly people and AD patients.[35] N-terminal truncation of Aβ was significantly more prevalent in AD patients. The molecular composition, rather than the amount, of Aβ was thought to be more associated with neuronal toxicity. Furthermore, racemization and isomerization of Asp and Ser have been detected in Aβ and thought to contribute to AD.[9,10,12,36–38] The amount of isomerized or racemized amino acid residues in Aβ are significantly higher in AD patients compared to the normal aging populations.[12]

Ser occurs in two positions in Aβ, Ser8 and Ser26. Little is known about the exact mechanism of D-Ser formation in Aβ. Limited studies have shown that approximately 4–9% of Ser exists as D-Ser in the HCl hydrolyzed Ab peptides from AD patients.[10] The relative ratios of L- to D-Ser at the two positions have not been determined. Moreover, the percentage values of D-Ser reported previously were obtained after HCl hydrolysis, which causes an inherent amount of racemization (an estimated error 2% of the total).[10,39] It is theorized that racemization at Ser26 is more toxic as studies have shown that Ser26 racemization leads to non-degradable Ab peptide fragments in rats.[9,37,40] Asp occurs in

three places in Aβ: Asp1, Asp7, and Asp23. There is evidence of isomerization and racemization of Asp in Aβ at every position in AD patients.[10,41] Isomerization and racemization of Asp spontaneously occurs through a cyclic succinimide intermediate resulting in L/D-Asp or L/D-iso-Asp.[42] As the racemization/isomerization of Asp leads to four possible permutations, the concurrent analysis of all isomers is desired to better characterize the brain of AD patients. The prevailing consensus is that the isomerization and/or racemization of Asp and Ser either structurally destabilizes Aβ, leading to misfolding or impairs the likelihood of proper degradation.[43] Indeed, peptides/proteins containing D-amino acids appear to be more resistant to enzymatic degradation in living systems.[44–46]

The identification and detection of isomerization and/or racemization products in Aβ is challenging for several reasons. First, isomerization and racemization do not change the mass of peptides, therefore it cannot be discriminated by a single stage of MS. Second, the isomerization or racemization of a single amino acid residue in a lengthy peptide may not significantly vary chemical properties of the peptide, thus increasing the difficulty of chromatographic separation. Finally, the low abundance of these epimeric peptides requires a sensitive detection method for both qualitative and quantitative analysis. Even with the stated challenges, many analytical and bioanalytical approaches have been proposed for the investigation of isomeric residues in Aβ.[47–50]

The conventional methods used to quantify D-amino acids involved acid hydrolysis and chiral derivatization followed by reverse-phase liquid chromatography.[10] As noted earlier, acid hydrolysis induces racemization and cannot detect the presence of iso-Asp linkages. Immunohistochemistry involving the use of tailored antibodies has also shown success at the detection of isomerized Asp and Ser, but the methods are not quantitative.[12] MS-based fragmentation techniques have been applied to identify the isomerization of Asp in peptides, including low energy collision induced dissociation,[51,52] electron

transfer/capture dissociation,[53,54] and radical-directed dissociation,[55] as they generate diagnostic fragment ions for the Asp and iso-Asp containing peptides. However, inconsistent results regarding the fragmentation pattern for peptides containing Asp isomers have been reported, which may due to the differences in peptide sequence and the use of different instruments.[53]

Combination methods using LC-MS and enzymatic reactions also show promise.[48] This method involves the use of trypsin in combination with three other enzymes: endoproteinase Asp-N (cleavage at N-terminal L-Asp), protein L-isoaspartyl methyltransferase (methylation of L-iso-Asp), and D-aspartic acid endopeptidase (cleavage at C-terminal D-Asp). The resulting peptides were then applied to LC-MS for identification. However, the identification of D-Asp was not always successful with D-aspartic acid endopeptidase as reaction conditions need further optimization for complete digestion.[48]

Ion mobility spectrometry (IMS) coupled with MS has also been investigated in the study of epimer separations.[56–59] However, peptides containing Asp and iso-Asp were not able to be resolved using conventional IMS-MS due to the low resolution. Recently, Smith's group have designed a new IMS platform structures for lossless ion manipulations (SLIM), which enables long IMS pathlength to achieve the separation of 4 Aβ peptides containing L/D-Asp and L/D-iso-Asp.[50] While the SLIM platform alone is a fast analysis method, coupling LC to SLIM is desired to increase detection sensitivity and provide better separation in complex biological samples.[60] In addition, only the isomerization of Asp residues in Aβ has been studied, and the separation was achieved for only four of the twenty potential Aβ peptide epimers.

To better characterize the presence and ratio of Asp and Ser isomers simultaneously in the brain of AD patients, better and faster analytical methods are needed.

Our approach for the separation and identification of Aβ peptide epimers uses trypsin digestion in combination with chiral HPLC stationary phases and MS/MS as the detection method. Unlike previously reported methods, the detection and quantification of L/D-Ser, L/D-Asp and L/D-iso-Asp isomers for all positions in Aβ are possible. This method does not use HCl digestion, thus avoids possible racemization and leaves iso-Asp linkages intact. Moreover, HPLC-MS/MS is a simple yet quantitative platform that is easily accessible. The digestion of Aβ by trypsin produces four peptide groups, and the amino acid sequence of the potential tryptic Aβ peptide epimers containing Asp and Ser are shown in Table 2-1 (Group D was not studied as it does not contain Asp or Ser residues). In total, there are 20 possible peptide combinations containing Asp and Ser isomers. This is the first report of the complete separation of all 20 Aβ epimers containing isomeric Asp and Ser residues at every position.

Table 2-1. Amino acid sequence of the three groups of Aβ peptide epimers with Asp and Ser isomeric residues

Aβ (1-5) **Group A**	A1: {L-Asp}AEFR	A2: {D-Asp}AEFR
	A3: {L-isoAsp}AEFR	A4: {D-isoAsp}AEFR
Aβ (6-16) **Group B**	B1: H{L-Asp}{L-Ser}GYEVHHQK	B2: H{D-Asp}{L-Ser}GYEVHHQK
	B3: H{L-Asp}{D-Ser}GYEVHHQK	B4: H{D-Asp}{D-Ser}GYEVHHQK
	B5: H{L-isoAsp}{L-Ser}GYEVHHQK	B6: H{L-isoAsp}{D-Ser}GYEVHHQK
	B7: H{D-isoAsp}{L-Ser}GYEVHHQK	B8: H{D-isoAsp}{D-Ser}GYEVHHQK
Aβ (17-28) **Group C**	C1: LVFFAE{L-Asp}VG{L-Ser}NK	C2: LVFFAE{D-Asp}VG{L-Ser}NK
	C3: LVFFAE{L-Asp}VG{D-Ser}NK	C4: LVFFAE{D-Asp}VG{D-Ser}NK
	C5: LVFFAE{L-isoAsp}VG{L-Ser}NK	C6: LVFFAE{L-isoAsp}VG{D-Ser}NK
	C7: LVFFAE{D-isoAsp}VG{L-Ser}NK	C8: LVFFAE{D-isoAsp}VG{D-Ser}NK

2.2 Experimental

2.2.1 Materials and Chemicals

All tryptic amyloid-beta (Aβ) peptide standards were purchased from Peptide 2.0 (Chantilly, VA, USA) at > 98% purity. Ammonium formate and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-MS grade methanol and water were purchased from Sigma-Aldrich and ultrapure water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All the peptide standards were prepared in methonal and water (50:50) at the concentration of 1 mg/mL.

2.2.2 Instruments and stationary phases

Initial screening work for the separation and characterization of Aβ peptide standards was performed on a 1220 Infinity II HPLC instrument (Agilent Technologies, Santa Clara, CA, US). Among all the stationary phases screened, three chiral stationary phases have shown promising results: modified Q-Shell-1, modified Q-Shell-2, and NicoShell chiral stationary phases which were provided by AZYP, LLC (Arlington, TX, USA). Thus, the separation conditions of Aβ peptide epimers were further optimized on these chiral stationary phases.

HPLC-MS/MS analysis was performed on LCMS-8060 (Shimadzu Scientific Instruments, Columbia, MD, USA), triple quadrupole spectrometer with electrospray ionization (ESI). The drying gas and nebulizing gas flow rate were 10 L/min and 2 L/min, respectively; the desolvation line temperature and heat block temperature were 275 °C and 400 °C, respectively. HPLC-MS/MS was operated in multiple reaction monitoring (MRM) mode with positive ESI source. Peptide fragmentation ions were further confirmed on LCMS-IT-TOF (Shimadzu Scientific Instruments), ion trap and time-of-flight mass

spectrometer with ESI. Collision energies and MRM transitions were optimized for each peptide group. Shimadzu LabSolution software was used for data acquisition.

2.3 Results and Discussion

Peptide epimers are diastereomers and can be separated on reverse phase columns, however, better separations of peptide epimers have been achieved on chiral stationary phases.[61,62] The chiral stationary phases used in this study are modified Q-Shell[63] and NicoShell[64], which were provided by AZYP, LLC (Arlington, TX, USA). Modified Q-Shell-1 is a combination of terbutyl-derivatized and non-derivatized quinine. Modified Q-Shell-2 is the commercial Q-Shell with excess (3-mercaptopropyl)- triethoxysilane linker. The optimized data treated separations for the trypsin digest Ab peptide epimers are shown in Figures 1–3.[65] All separations were achieved on chiral HPLC stationary phases using MS compatible mobile phases. It is important to note that the separation windows do not overlap when the three peptide groups are analyzed simultaneously. For example, the separation of Ab (1–5) Group A peptide epimers was achieved on modified Q-Shell chiral stationary phase within 7 min. Under the same separation condition, Group B peptides elute at the void volume, while Group C peptides retain longer than 7 min. Thus, there is no interference between the three groups during analysis.

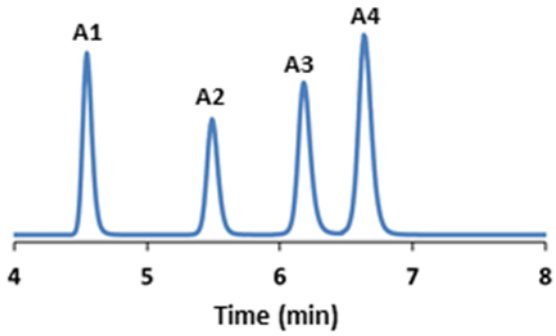

Figure 2-1. Separation of Aβ (1-5) peptide epimers on modified Q-Shell chiral stationary phase (3 x 150 mm, 2.7 µm). Condition: 5/95 methanol/5 mM ammonium formate (pH 3.0), 0.3 mL/min, 23 °C. Group B peptides elute at dead volume while Group C peptides are retained for much longer compared to Group A.

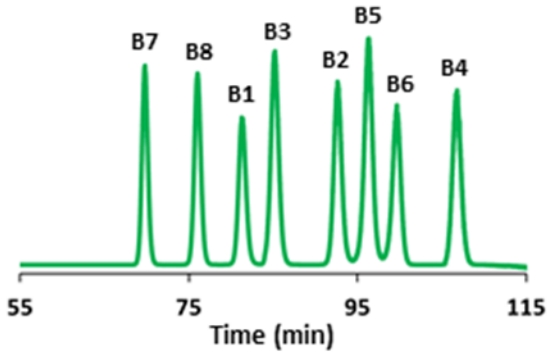

Figure 2-2. Separation of Aβ (6-16) peptide epimers on NicoShell chiral stationary phase (3 x 150 mm, 2.7 µm). Condition: 35/65 acetonitrile/10 mM ammonium formate (pH 4.5), 0.2 mL/min, 45 °C. Group A and C peptides elute before Group B peptides.

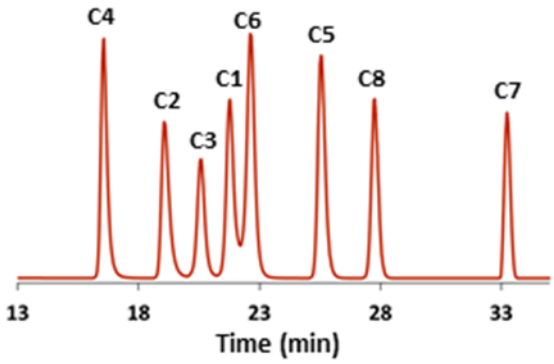

Figure 2-3. Separation of Aβ (7-28) peptide epimers on modified Q-Shell chiral stationary phase (3 x 150 mm, 2.7 µm). Condition: 85/15 methanol/5 mM ammonium formate (pH 3.5), 0.2 mL/min, 10 °C. Group A and B peptides elute around dead volume.

The HPLC-UV method has been successfully transferred to HPLC-MS/MS to improve detection sensitivity. HPLC-MS/MS was operated in multiple reaction monitoring (MRM) mode with positive ESI source. Improved limit of detections for the Aβ peptides are in the range of 40 to 250 pg. Collision energies and MRM transitions were optimized for each peptide group, results are summarized in Table 2-2.

Table 2-2. Results of MRM optimization and LODs for tryptic Aβ peptides on LCMS-8060

	Precursor (m/z)	**Product (m/z)**	**Q1 (V)**	**CE**	**Q3 (V)**	**LODs (pg)**
Group A	637.5 (M^{+1})	322.2(y_2 ion)	-32	32	-15	40
Aβ (1-5)	637.5 (M^{+1})	522.0 (y_4 ion)	-32	31	-26	-
Group B	669 (M^{+2})	110.2 (H ion)	-32	48	-19	250
Aβ (6-16)	669 (M^{+2})	253. 3 (b_2 ion)	-32	27	-24	-
Group C	663.5 (M^{+2})	1113.3 (y_{10} ion)	-32	24	-32	55
Aβ (17-28)	663.5 (M^{+2})	185.4 (a_2 ion)	-32	23	-18	-

2.4 Conclusions

In conclusion, we have developed the first comprehensive analytical platform that allows for the separation and quantification of all 20 possible Aβ peptide epimers containing isomeric Asp and Ser residues. The ability to fully resolve 20 of the Aβ peptide epimers is extremely valuable for characterizing the Asp and Ser isomerization/racemization in Aβ from biological samples. This method can be used to answer questions about which position(s) is/are more abundant in D-Ser, or D-Asp, or D- and L-iso-Asp in Aβ. Such investigations have not been feasible, but now may be. In addition, there are no reports on whether isomerization/racemization of one position enhances the likelihood of alterations at other positions. Our method allows investigations of such correlations between the isomerization and racemization of different positions when studying AD tissues. In the future, we will examine brain tissues and plasma samples from AD patients using this simple and high-throughput analytical platform. We expect that characterization of these Asp and Ser isomers in Ab from AD patients will contribute to a better understanding of the etiology of the disease.

Chapter 3

Rapid and Selective Separation of Amyloid Beta from its Different Stereoisomeric Point Mutations Implicated in Neurodegenerative Alzheimer's Disease

Abstract

Extracellular deposition of amyloid-beta (Aβ) peptide is a hallmark of Alzheimer's disease. The isomerization and epimerization of Aβ peptides have been linked to the enhanced deposition of Aβ plaques. Therefore, consider effort has been expended to create effective methods to distinguish such aberrant Aβ peptides from wild type. Herein, we have developed chromatographic retention U-shaped curves to investigate the hydrophobicity of Aβ 1-38, 1-40, 1-42 and fourteen aberrant Aβ 1-42 peptides. Using this information, we developed the first selective and comprehensive method that can easily detect both aberrant and wild type Aβ peptides simultaneously using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS). We show for the first time show that D-Ser modifications to Aβ cause the peptide to be more hydrophilic, as does D-Asp and iso-Asp.

3.1 Introduction

The deposition and aggregation of amyloid-beta (Aβ) peptides are central to the pathology of Alzheimer's disease (AD) as they are predictive biological markers for the diagnosis of AD in patients.[66] The most common Aβ peptides are Aβ 1-38, Aβ 1-40, and Aβ 1-42. The primary peptide comprising Aβ aggregates in the brain is Aβ 1-42.[2] However, aberrant types of Aβ have been detected. Aberrations include epimerization and/or isomerization of Asp and Ser residues and point mutations at various residues.[8–10] It has been suggested that such variations in Aβ contribute to AD and increase neuronal toxicity.[10,12] Aberrant types of Aβ are more prone to aggregation and are commonly found in Aβ plaques of AD patients.[7] Thus, it would be highly beneficial to AD research to be able to easily detect aberrant Aβ 1-42 in the presence of wild type Aβ.

Racemization and/or isomerization is intrinsic to Asp as it readily forms a cyclic succinimide intermediate which results in L/D-Asp or L/D-iso-Asp.[11] This phenomenon has been well documented to occur in α/B-crystallin and Aβ.[12,13] Research has shown that certain proteins of older subjects had more L-iso-Asp, D-iso-Asp and D-Asp compared to younger subjects. This suggested that epimerization and/or isomerization of Asp is time dependent and occurs naturally as a hallmark of aging.[14] There is evidence that racemization and/or isomerization of Asp and Ser residues can occur at every position of Aβ found in AD patients.[10] These locations include Asp1, Asp7, Asp23, Ser8, and Ser26. Studies have also shown that Aβ peptides extracted from AD patient's brains contain more isomerization and/or epimerization of Asp compared to non-AD patient's brains.[12] This includes 20% L/D-iso-Asp at Asp1 and 75% L/D-iso-Asp at Asp7 for senile plaque Aβ samples compared to 6% L/D-iso-Asp at Asp1 for vascular Aβ samples.[6] Note that in this study the L-iso Asp and D-iso-Asp epimers were not separated. Furthermore,

approximately 4-9% of Ser residues in AD patient's brains are D-Ser.[10] However, the total percentage of epimerization and/or isomerization of Asp or Ser in Aβ in both healthy and ill patients is unknown. It is thought that these modifications directly affect the folding and interactions of Aβ peptides which then results in extracellular deposition of Aβ peptides.[10] Unmodified Aβ can be degraded by aminopeptidases such as neprilysin and Insulin-degrading enzyme.[15] However, it has been shown that aminopeptidases cannot degrade peptides with D-amino acids.[16] The resistance to proteolytic degradation further results in the accumulation of aberrant peptides as plaques thereby enhancing aggregation and plaque formation.[17] Therefore, it is paramount to have selective and comprehensive methods that can easily detect both aberrant and unmodified Aβ peptides.

Presently, liquid chromatography has been used extensively for the chromatographic separation of aberrant Aβ. However, most work concerns Aβ peptide with point mutations.[18] Point mutations can drastically change peptide, solubility, and on-column secondary structure making them ideal to separate using reverse phase liquid chromatography (RPLC).[19] However, epimerization and/or isomerization can be more subtle in their changes to Aβ characteristics. Such changes are undetectable by mass spectrometry and can make traditional separations and characterizations difficult. Previous work for the detection of epimerization and/or isomerization of Aβ involved degradation of Aβ to single amino acids or the use of trypsin to cut the peptide into characteristic fragments.[18,21,51] As less work has been dedicated to creating practical methods for the separation of intact epimerized and/or isomerized Aβ peptides along with unmodified Aβ peptides, the focus of this work is to directly and rapidly separate these compounds.

The use of RPLC is often coupled with mass spectrometry as it offers increased sensitivity. However, mass spectrometry cannot easily identify epimerization/isomerization as these modifications do not change the mass of the peptide. Thus, it is important to have

a high selectivity method to detect stereoisomeric modifications. Previously, several methods were developed to separate Aβ 1-38, Aβ 1-40, and Aβ 1-42, however these methods did not consider the possibility of isomerization/epimerization of Asp and Ser.[50,60,67] To examine the differences, we first created a highly selective separation of Aβ 1-38, Aβ 1-40, and Aβ 1-42, then further applied this method to study 14 aberrant peptides (Figure 3-1). These aberrations include isomeric and/or epimeric modifications of L-iso, D-iso, and D-Asp at Asp1, Asp7 and Asp23, as well as the epimeric D-Ser modification at Ser8 and Ser26, of Aβ 1-42. In addition, three peptides with two epimeric modifications at Asp23 and Ser26 also were studied to investigate the combination effect.

Aβ (1-38,-40,-42) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

1a: DAEFRH[D-Asp]SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
1b: DAEFRH[L-iso-Asp]SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
1c: DAEFRH[D-iso-Asp]SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
2a: DAEFRHD[D-Asp]GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
2b: DAEFRHD[L-iso-Asp]GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
2c: DAEFRHD[D-iso-Asp]GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
2d: DAEFRHD[D-Ser]GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
3a: DAEFRHDSGYEVHHQKLVFFAE[D-Asp]VGSNKGAIIGLMVGGVVIA
3b: DAEFRHDSGYEVHHQKLVFFAE[L-iso-Asp]VGSNKGAIIGLMVGGVVIA
3c: DAEFRHDSGYEVHHQKLVFFAE[D-iso-Asp]VGSNKGAIIGLMVGGVVIA
3d: DAEFRHDSGYEVHHQKLVFFAEDVG[D-Ser]NKGAIIGLMVGGVVIA
4a: DAEFRHDSGYEVHHQKLVFFAE[D-Asp]VG[D-Ser]NKGAIIGLMVGGVVIA
4b: DAEFRHDSGYEVHHQKLVFFAE[L-iso-Asp]VG[D-Ser]NKGAIIGLMVGGVVIA
4c: DAEFRHDSGYEVHHQKLVFFAE[D-iso-Asp]VG[D-Ser]NKGAIIGLMVGGVVIA

Figure 3-1. List of Aβ peptide epimers assessed in this study. Above, complete peptide sequence of Aβ 1-38 (end indicated by two Gly residues in blue), Aβ 1-40 (end indicated by two Val residues in purple) and Aβ 1-42 (end indicated in Ile and Ala residues in green). Note that the first 38 amino acid residues are the same for all three peptides. Below, Aβ 1-42 peptide sequences with varying isomerization and/or racemization modifications of Asp and Ser residues at positions 1, 7, and 23, and positions 8 and 26, respectively. The Aβ are labeled arbitrarily by a number followed by a letter. The bracketed, colored text indicates the position and type of modification.

3.2 Methods

3.2.1 Chemicals and Sample Preparation

Amyloid beta (Aβ) 1-40 and 1-42 standards were purchased from Genscript (Piscataway, NJ, USA) at > 95% purity. Aβ 1-38 standard was purchased from rPeptide (Watkinsville, GA, USA) at > 95% purity. All modified Aβ peptide standards were purchased from Peptide 2.0 (Chantilly, VA, USA) at > 95% purity. Boric acid, sodium hydroxide and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-MS grade acetonitrile was purchased from Sigma-Aldrich and ultrapure water was obtained from a Milli-Q-water system (Millipore, Bedford, MA, USA). All peptide standards were prepared in 10 mM borate buffered to pH 9.2 with sodium hydroxide, at a concentration of 1 mg mL-1. Samples were diluted to 100 ug mL-1 for injection into the LC-MS.

3.2.2 LC-MS

Screening and optimization of all epimer standards were performed on an LCMS-8040 (Shimadzu Scientific Instruments, Columbia, MD, USA), triple quadrupole spectrometer equipped with electrospray ionization (ESI) ion source. The MS was operated under single ion mode (SIM) with a positive ESI source and instrument conditions: drying gas and nebulizing gas flow rate of 15 L/min and 2 L/min, respectively; desolvation line temperature and heat black temperature of 275 °C and 400 °C, respectively. Aβ 1-38 was analyzed at both 1378.2 m/z and 1033.9 m/z to confirm peak, 1-40 was analyzed at both 1444.2 m/z and 1083.4 m/z to confirm peak, and 1-42 was analyzed at both 1505.8 m/z and 1129.6 m/z to confirm peak. The epimers were separated using BIOshell™ IgG 1000Å C4 column (4.6 mm i.d x 10 cm length, pore size 1000Å, particle size 2.7 um; Supelco, Bellefonte, PA, USA). The optimized separation conditions include an isocratic hold of 27%

mobile phase B (99.9% acetonitrile, 0.1% formic acid) from 0 to 5 minutes followed by a step gradient to 50% mobile phase B at 5.01 minutes and an isocratic hold at 50% mobile phase B until 10 minutes.

3.2.3 Data Analysis

Shimadzu LabSolutions software was used for data acquisition. Retention factor (k) and selectivity (α) were calculated for all epimers. Retention factor was calculated using $k = (t_R-t_0)/t_0$, where t_R is the retention time of the peak of interest and t_0 is the dead time of the column. Selectivity was calculated using $\alpha=k_2/k_1$, where k_1 and k_2 refer to the retention factors of the first and second peaks, respectively. Final data (n=2) in Figures 3-3 to 3-5 were treated using the power law method.

3.3 Results and Discussion

Initially, peptide hydrophobicity and solubility was investigated by constructing chromatographic retention curves for all unmodified and aberrant Aβ peptides (Figure 3-2). Each peptide's retention was analyzed at various isocratic solvent ratios (water:acetonitrile). Peptides retain longer at higher and lower solvent ratios of acetonitrile. The retention factors of Aβ 1-38, 1-40 and 1-42 are distinct at 28% acetonitrile with retention factors of 0.27, 1.09, and 2.75, respectively, and at 75% acetonitrile with retention factors of 0.42, 0.36, and 0.39, respectively. The retention times of Aβ 1-38, 1-40 and 1-42 are identical at 50% acetonitrile with retention factors of 0.02, 0.03, and 0.03, respectively. This phenomenon results in a definitive U-shape curve when plotted over a wide range of solvent ratios. Further, the aberrant Aβ peptides adopted the same U-shape retention behavior (Figure 3-2).

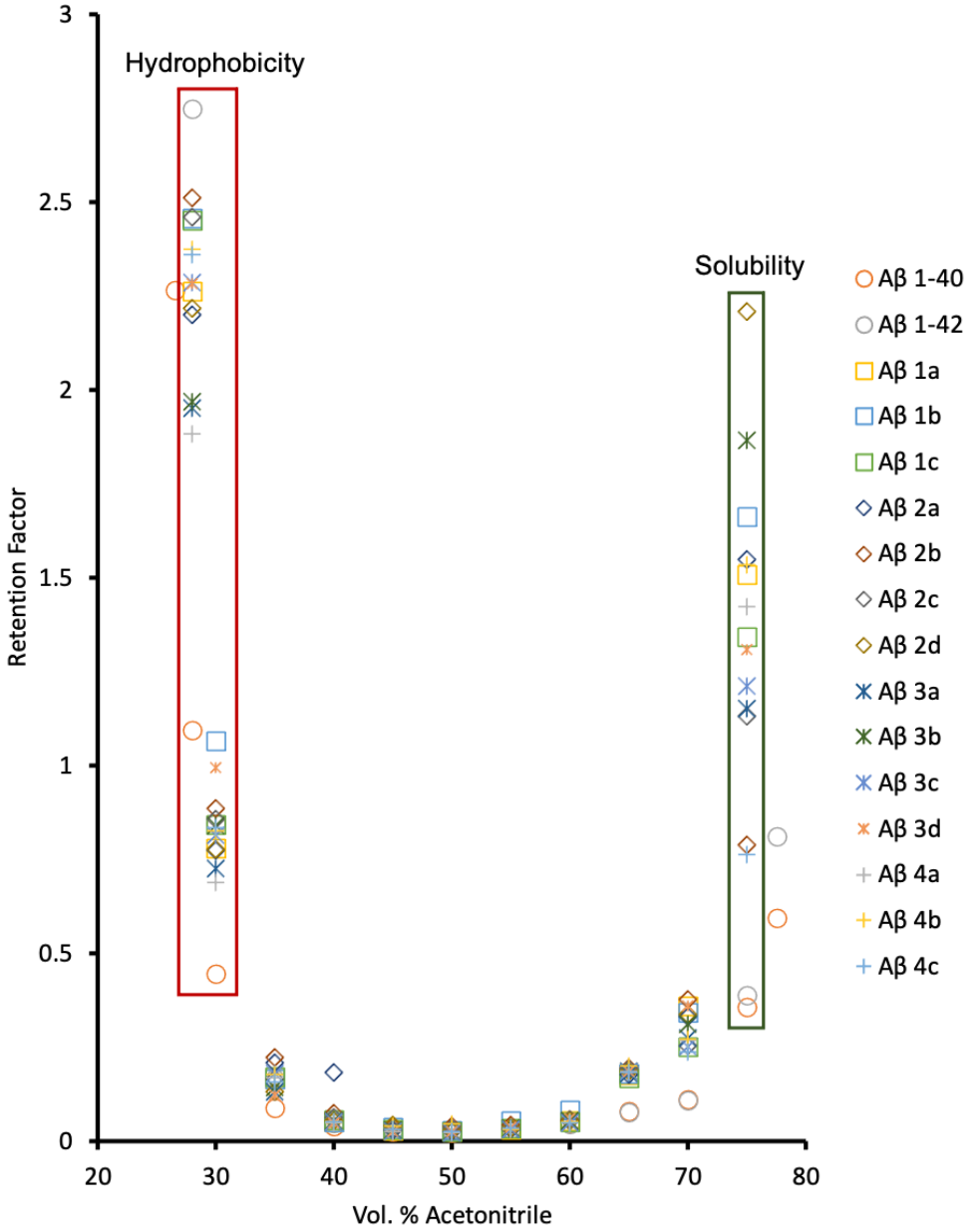

Figure 3-2. Retention factor of all Aβ peptides at varying concentrations of water and acetonitrile. Please refer to fig. 1 for the descriptions of abbreviated Aβ peptides located in the key. Peptides eluting at lower vol. % acetonitrile indicate hydrophobicity differences while peptides eluting at higher vol. % acetonitrile indicate solubility differences. Chromatographic conditions include: BIOshell™ IgG 1000 Å C4 (100 x 4.6 mm, 2.7 μm), varying concentrations of water/0.1% formic acid and acetonitrile/0.1% formic acid, 0.4 mL/min, 25 °C.

The retention factor for all modified Aβ peptides was in between the retention factors of unmodified Aβ 1-40 and 1-42 at 28% acetonitrile (Figure 3-2). At 75% acetonitrile the retention of the modified Aβ was greater than both unmodified Aβ 1-40 and 1-42. Between 35% to 70% acetonitrile there is less distinction between aberrant Aβ peptides and Aβ 1-42. However, once a threshold ratio of water/acetonitrile is reached, on column selectivity significantly increases resulting in distinct retention factors. Thus, our results show that Aβ peptides are extremely sensitive to mobile phase conditions, as even epimerization of a single residue can significantly affect the retention factor. To conclude, interactions between the peptide and the column at higher percentages of water indicate hydrophobicity differences in the peptide whereas interactions at higher percentages of acetonitrile indicate solubility differences.[68]

Aβ peptides had increasingly worse peak shapes as the peptide increased in length. Aβ 1-38 had good peak shape regardless of solvent ratio, however, Aβ 1-40 and 1-42 suffered from peak tailing at all concentrations of acetonitrile except between 44-55% acetonitrile under isocratic conditions. The excessive peak tailing makes the isocratic method unsuitable for the analysis of Aβ epimers as excessive tailing can mask lower concentration Aβ epimer peaks which mass spectrometry detection will not be able to distinguish from other Aβ peptides. However, using a standard gradient method either resulted in coeluting peaks and/or excessive peak tailing. Therefore, a combination of isocratic and gradient methods were screened to best optimize selectivity and peak shape. It was clear that the optimal solvent ratios to separate the Aβ peptides were between solvent percentages 25% to 30% acetonitrile (Figure 3-2). It should be noted that while there was selectivity at 75% acetonitrile, the peak tailing was significantly worse and therefore not pursued. Using this data, a separation was created with exceptional selectivity and peak shape by implementing a 5-minute isocratic hold at 28% acetonitrile

followed by a step ramp to 50% acetonitrile (Figure 3-3). The resolution between Aβ 1-40 and 1-42 for this method is much greater compared to previous reports.[69]

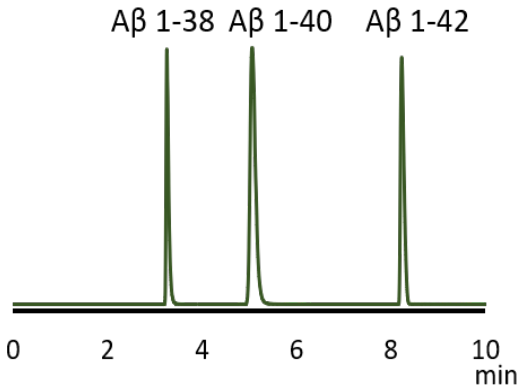

Figure 3-3. Chromatographic separation of Aβ peptides 1-38, -40, and -42 using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). Chromatographic conditions include: BIOshell™ IgG 1000 Å C4 (100 x 4.6 mm, 2.7 μm), 5 min isocratic hold starting conditions: 28/72/0.1 acetonitrile/water/formic acid followed by a step ramp to 50/50/0.1 acetonitrile/water/formic acid, 0.4 mL/min, 25 °C.

It should be noted that the peptide retention (and thus its elution) was sensitive to minute changes in the acetonitrile:water solvent ratio during the initial 5 minute isocratic hold . The separation of Aβ 1-38, 1-40, and 1-42 drastically differed when the acetonitrile:water solvent ratio during the initial 5 min isocratic hold was altered by even 1%. At 29% and 30% acetonitrile, there was still significant peak tailing for Aβ 1-42. While resolution between all wild type Aβ peptides at 29% acetonitrile was baseline, more resolution was needed between Aβ 1-40 and 1-42 to accommodate the modified Aβ 1-42 epimers. The ideal separation occurs at 28% acetonitrile, where resolution of all Aβ peaks is retained but there is a significant resolution increase between Aβ 1-40 and 1-42. If the solvent ratio was changed to 27% acetonitrile during the initial isocratic hold, the resolution between Aβ 1-40 and 1-42 was not baseline. Given these results, extra care must be taken to prepare exact mobile phase compositions for the separation of Aβ wild type peptides and epimers.

The starting ratio of water to acetonitrile was crucial to the final separation of not only Aβ 1-38, 1-40, and 1-42, but the separation of these compounds from aberrant Aβ. Changing the starting ratio by 1% significantly impacts the retention of unmodified Aβ (Figure 3-4). Therefore, much care should be put into preparing solvents prior to analysis. For reference, a complete table of all retention factors for each peptide at the optimal separation conditions and several isocratic conditions is included (Table 3-1).

This method was applied to the epimeric and isobaric Aβ peptides to investigate the effect of epimerization/isomerization on peptide hydrophobicity in reverse phase conditions. Interestingly, all of the aberrant/modified Aβ peptides eluted between Aβ 1-40 and 1-42 peaks (Figure 3-4). Reverse phase chromatographic separations give valuable information about peptide characteristics such as changes in hydrophobicity. Previous investigations of iso-Asp modifications in α-crystallin showed that L-iso-Asp, D-Asp, and D-iso-Asp modifications were more hydrophilic compared to the naturally occurring L-Asp containing peptides.[9] Our results indicate that the same trend occurs in Aβ 1-42. All Asp modified Aβ 1-42 peptides investigated in this study eluted faster compared to the unmodified peptide (Figure 3-5). Previous work also found that the D-Asp containing epimer was the most hydrophilic modification, which is further confirmed by this data (Figure 3-5).

Table 3-1. Retention times for each peptide following chromatographic conditions: BIOshell™ IgG 1000 Å C4 (100 x 4.6 mm, 2.7 μm), varying concentrations of water/0.1% formic acid and acetonitrile/0.1% formic acid, 0.4 mL/min, 25 °C.

	Retention Factor[a]	Retention Factor[b]	Retention Factor[c]	Retention Factor[d]
Aβ 1-38	0.27	0.38	0.02	0.42
Aβ 1-40	1.09	1.10	0.03	0.36
Aβ 1-42	2.75	2.75	0.03	0.39
Aβ 1a	1.91	2.26	0.03	1.51
Aβ 1b	2.21	2.46	0.03	1.66
Aβ 1c	2.13	2.45	0.03	1.34
Aβ 1x	1.35	1.30	0.03	0.88
Aβ 2a	1.66	2.20	0.03	1.55
Aβ 2b	2.03	2.51	0.04	0.79
Aβ 2c	2.03	2.46	0.04	1.13
Aβ 2d	1.90	2.22	0.03	2.21
Aβ 3a	1.55	1.95	0.03	1.15
Aβ 3b	1.86	1.97	0.03	1.87
Aβ 3c	2.00	2.29	0.03	1.21
Aβ 3d	1.81	2.28	0.03	1.31
Aβ 3A	1.51	1.88	0.03	1.42
Aβ 3B	1.87	2.37	0.05	1.53
Aβ 3C	2.08	2.36	0.03	0.76

[a]Isocratic 28% Acetonitrile for 5 minutes, then step gradient to 50% Acetonitrile for 5 minutes
[b]Isocratic 28% Acetonitrile for 10 minutes
[c]Isocratic 50% Acetonitrile for 10 minutes
[d]Isocratic 75% Acetonitrile for 10 minutes

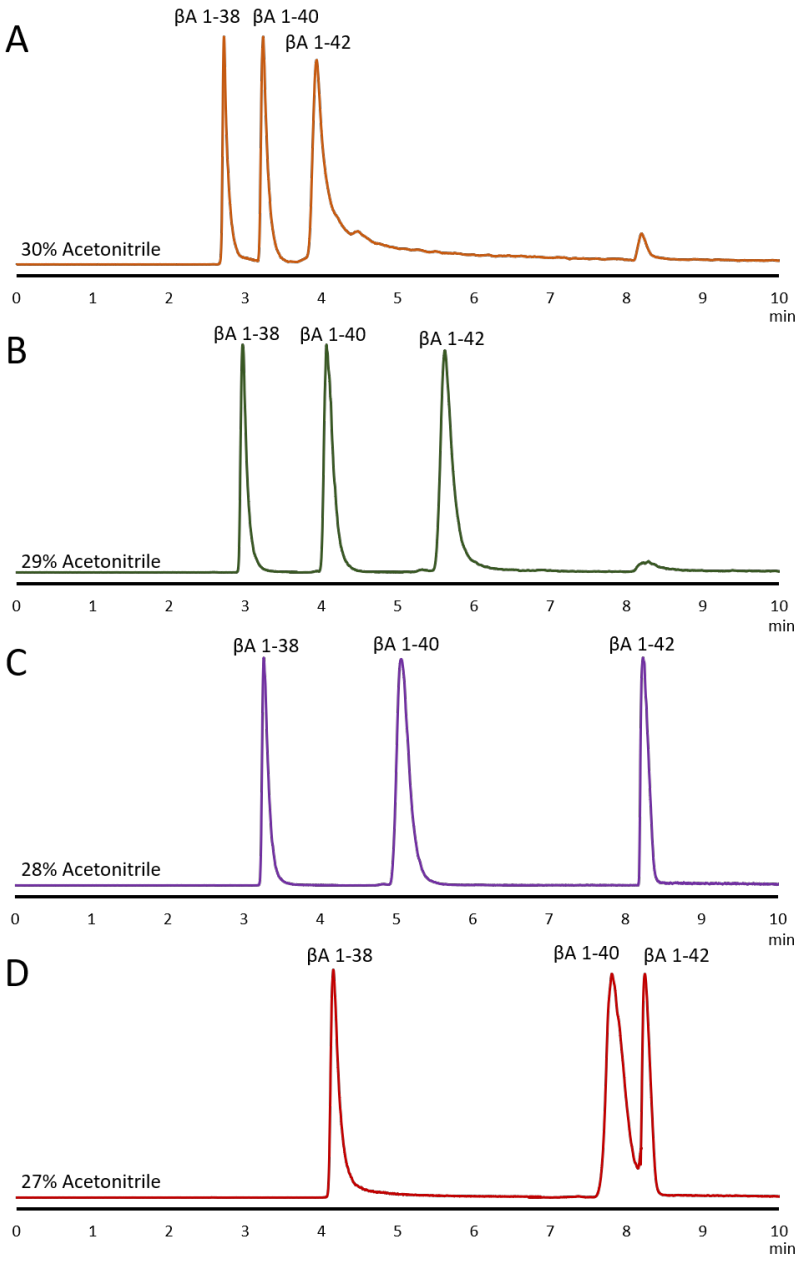

Figure 3-4. Chromatogram of Aβ 1-38, 1-40 and 1-42 peptides at varying concentrations of water to acetonitrile starting conditions. (A) 70/30 (B) 71/29 (C) 72/28 and (D) 73/27 water/acetonitrile/0.1% formic acid isocratic hold for 5 minutes followed by step ramp to 50/50 water/acetonitrile at 5.01 minutes. Chromatographic conditions include: BIOshell™ IgG 1000 Å C4 (100 x 4.6 mm, 2.7 μm), 0.4 mL/min, 25 °C.

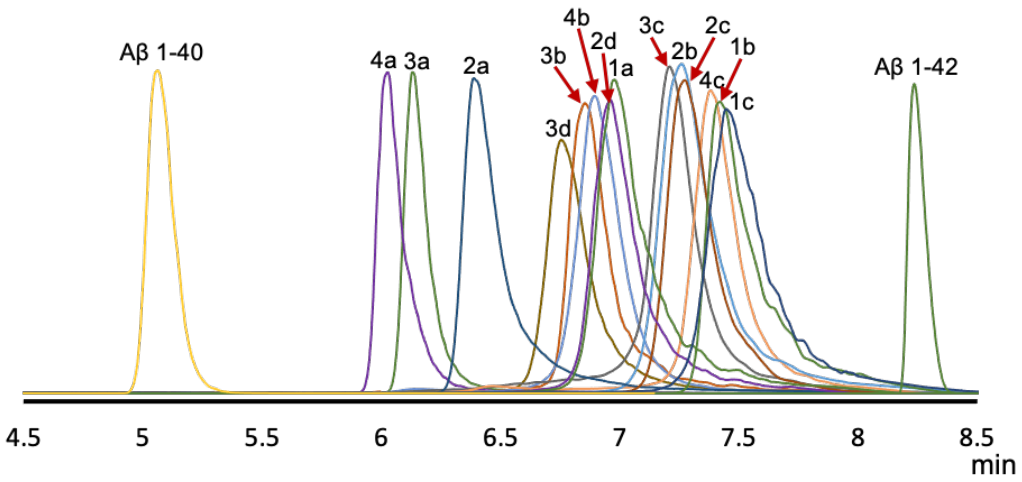

Figure 3-5. Chromatograms of all Aβ peptides analyzed in this study. Note that all aberrant Aβ peptides elute between Aβ 1-40 and Aβ 1-42. See Fig. 1 for peak identity. Chromatographic conditions: BIOshell™ IgG 1000 Å C4 (100 x 4.6 mm, 2.7 μm), 5 min isocratic hold starting conditions: 28/72/0.1 acetonitrile/water/formic acid followed by a step ramp to 50/50/0.1 acetonitrile/water/formic acid, 0.4 mL/min, 25 °C.

It is important to note that the elution order of the modified peptides was not random. Modifications which were closer to the C-terminus retained longer compared to those near the N-terminus for a given modification. For example, the shortest retained L-iso-Asp modified Aβ occurred when the modification was at D23 followed by identical modifications at D7 and D1, respectively. This trend of modifications closer to the C-terminus was consistent for all Asp epimerization/isomerization modifications (Table 3-1). Previous work has indicated that the 17-42 segment of Aβ was the most hydrophobic region of the peptide.[22] It may be suggested that this segment is the primary interaction site with the hydrophobic stationary phase.[25] The current data supports this as the modifications nearer to the C-terminus produce greater changes in retention (Figure 3-6). Thus, chromatographic retention differences for Asp and Ser modifications to Aβ, particularly those in the 17-42 segment, are not unexpected.[9,10,22,26,27]

Epimerization/isomerization can distort the peptide backbone. The location of this distortion uniquely affects both Aβ secondary structure and chromatographic retention. This trend also was observed when L-Ser was replaced by D-Ser in Aβ. Aβ with D-Ser at S30 (Aβ 3d) eluted prior to Aβ with D-Ser at S8 (Aβ 2d). These local configurational differences result in altered secondary structure. Epimeric and isomeric modifications nearer to the C-terminus appear to affect hydrophobic association more than the same modifications near the N-terminus. This is further elucidated as both the S30 and D23 positions were modified to observe hydrophobicity changes with two modifications. Keeping S30 constant as D-Ser, the elution order for modified Asp was D-Asp, D-iso-Asp, L-iso-Asp with retention factors of 1.51, 1.87, and 2.08, respectively (Table 3-1). In addition, it was shown that D-Ser epimers of Aβ 1-42 are more hydrophilic compared to L-Ser. When Aβ 1-42 was modified at the S8 and S26 positions, the S26 Aβ eluted first, indicating more hydrophilicity. This is the first time that the D-Ser modifications of Aβ 1-42 have been characterized.

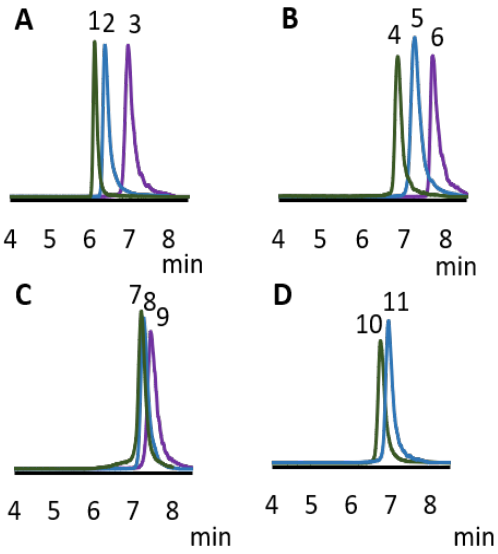

Figure 3-6. Total ion Chromatograms for each type of single modification at every Asp or Ser. Retention peaks of (A) D-Asp modification at positions (1)D23, (2)D7, and (3)D1 (B) L-iso-Asp modification at positions (4)D23, (5)D7, and (6)D1; (C) D-iso-Asp modification at positions (7)D23, (8)D7, and (9)D1 ; (D) retention peaks of D-Ser modification at positions (10)S26 and (11)S8. Chromatographic conditions: BIOshell™ IgG 1000 Å C4 (100 x 4.6 mm, 2.7 μm), 5 min isocratic hold starting conditions: 28/72/0.1 acetonitrile/water/formic acid followed by a step ramp to 50/50/0.1 acetonitrile/water/formic acid, 0.4 mL/min, 25 °C.

Given the correlation of hydrophobicity to elution order on reverse phase columns, it can be concluded that modification to Asp residues in Aβ to D-Asp, D-iso-Asp or L-iso-Asp decrease the hydrophobicity of Aβ as their retention times are shorter. Circular dichroism studies have shown that differences in on-column retention also relate to differences in secondary structure, to dimer/tetramer aggregation, and proteolytic sensitivity.[13,69–73] Iso-Asp has been shown to generate large structural changes in other proteins.[74] It has also been shown that certain modified Aβ leads to acute aggregation and plaque development in mouse models.[75,76] The stable aggregation of Aβ peptides which contain D-amino acids are more resistant to proteolytic degradation compared to the wild-type peptide.[17,65,76] Such resistance to proteolytic degradation allows for long term accumulation thereby enhancing associated deleterious effects.[77] Additionally, recent

evidence has shown that hydrophobic aggregated is cooperative in nature and which would be further exacerbated from the accumulation of non-degradable Aβ peptides.[78]

While it may be tempting to directly correlate an increase in hydrophobicity to increased peptide aggregation, there does not seem to be such a direct correlation. Indeed, structural changes caused by alteration of Aβ peptides through mutation, isomerization, or epimerization are thought to play a large role in the propensity of Aβ peptides to aggregate.[79,80]

3.4 Conclusions

This is the most comprehensive study of the separation of Asp and Ser epimerization/isomerization modifications in Aβ 1-42. This method can not only separate unmodified Aβ 1-38, Aβ 1-40, and Aβ 1-42 with high selectivity but also separates all stereoisomeric modifications of Aβ 1-42 involving Asp or Ser in a sequence that is characteristic of the epimeric peptide. Further, the hydrophobicity/hydrophilicity of the peptide is directly affected by the location of the aberrant amino acid within the peptide, i.e., the hydrophilicity of the aberrant peptide increases when the modification is nearer to the C-terminus. The ability to separate aberrant isomeric and epimeric Aβ 1-42 peptides from wild type Aβ 1-42 peptide is extremely valuable as a significant amounts of Aβ fibrils in AD patients have aberrant Aβ 1-42 present. Also, this method is likely applicable to the separation of other point-mutation Aβ peptides. In the future, this method will be applied to examine Aβ peptides extracted from AD brain tissues.

Chapter 4

Antibody Binding of Amyloid Beta Peptide Epimers/Isomers: Ramifications for Immunotherapies and Drug Development

Abstract

Evidence has shown that the extracellular deposition of amyloid-beta (Aβ) peptide is a contributing factor of Alzheimer's disease (AD). Considerable effort has been expended to create effective antibodies, or immunotherapies, targeting Aβ peptides. A few immunotherapies are thought to provide some benefit. It is possible that a contributing factor to the responses of such therapies may be the presence of modified, or aberrant, Aβ peptides found in AD patients. These aberrations include the isomerization and epimerization of L-Asp and D-Ser residues to form D-Asp, L/D-iso-Asp, and D-Ser residues, respectively. Modifications to Aβ peptides may enhance the deposition of Aβ plaques and/or contribute to the neurodegeneration in AD patients and may alter the binding affinity to antibodies. Herein, we used immunoprecipitation to examine the binding affinity for four antibodies against 18 epimeric and/or isomeric Aβ peptides. Tandem mass spectrometry was used as a detection method, which also was found to produce highly variable results for epimeric and/or isomeric Aβ.

4.1 Introduction

The misfolding and deposition of amyloid-beta (Aβ) peptides into aggregates is associated with the progression and diagnosis of Alzheimer's disease (AD).[2,3,81] Aβ peptides are commonly 37-42 amino acids in length, with Aβ 1-42 as the predominant form in the brain.[2] However, aberrant types of Aβ peptides have been detected that may include point mutations at various residues in addition to racemization and/or isomerization of Asp and Ser residues.[82] It has been suggested that such variations contribute to and enhance neuronal toxicity as unique modifications change the spatial conformation and also hinder enzymatic degradation of Aβ.[10,12,36–38] Further, Aβ peptides can form varying higher order structures such as insoluble extracellular plaques or soluble oligomeric species.[83] These higher order structures compromise targeting efforts, such as immunotherapies which rely on antibody binding efficacy to extract Aβ from AD brain.[84] Note that antibodies target peptide regions, known as epitopes, that are specific linear segments of amino acids. However, some antibodies specifically target spatial conformations, or higher order structures of a peptide. Rarely do antibodies have epitope regions that select for both a specific, linear segment and a higher ordered spatial conformation.[85] Thus, the combination of aberrant Aβ species, and their subsequent unique solubilities and spatial conformations, imposes further challenges to developing effective antibodies targeting all Aβ peptide sequences.

Commercial antibodies targeting Aβ, and thus immunotherapies, exclusively target unmodified Aβ peptides (i.e. the wild-type (WT) Aβ peptide).[84,86] It is assumed that WT Aβ contains no isomeric and/or epimeric centers. No published work has considered whether these antibodies also target epimeric and/or isomeric Aβ peptides. Interestingly, there are a few studies that have shown a negative effect on antigen binding when an iso-Asp

residue is located in, or near, the binding region of certain antibodies.[87–89] Thus, one should consider altered antibody binding affinities to antigens or peptides that have isomerized and/or racemized amino acids. Further, these aberrations may have consequences when developing immunotherapies.[90] Evidence shows that Aβ peptide can easily isomerize and/or epimerize in at least five locations including Asp1, Asp7, Asp23, Ser8 and Ser26 in AD brain. It has been reported that at least 20% of Asp1 and 75% of Asp7 of Aβ are L/D-iso-Asp in AD brain samples as compared to 6% of Asp1 in non-AD Aβ brain samples.[12] Additionally, 4-9% of Ser residues in AD brain are D-Ser.[10] Research has shown elevated levels of L-iso-Asp, D-iso-Asp and D-Asp in older subjects as compared to younger subjects, suggesting that the conversion of L-Asp to its succinimide antipodes is a time-dependent hallmark of aging.[12] Therefore, to better target the diverse catalogue of possible epimeric and/or isomeric Aβ peptides, it is critical to understand how antibody binding efficacies may be disrupted, or possibly enhanced, for modified/aberrant peptides.

Immunoprecipitation (IP) is typically used to purify and concentrate peptides from challenging matrices and can be used to evaluate the binding efficacy of an antibody to epimeric and/or isomeric proteins.[91] IP relies on the binding efficacy of an antibody-antigen to an agarose stationary phase. The elution product will consist only of the antigens that are selected for by the antibody.[92] Thus, the epimers and/or isomers can be compared to their natural all-L antipode. Antibodies 6E10 and 4G8 are among the first monoclonal antibodies commercially available that target Aβ 1-42.[27,93] They are commonly used in immunoprecipitation, immunohistochemistry, and Western blotting to measure or identify Aβ from biological matrices.[94–96] A high-resolution mapping technique identified the epitope region for 6E10 to Aβ residues 4-10 and for 4G8 to Aβ residues 18-23 (Figure 4-1).[97] Unlike antibodies 4G8 and 6E10, which recognize linear segments of Aβ 1-42, OC-type monoclonal antibodies which recognize conformational epitopes and prefer amyloid

aggregates over monomers.[85] Antibodies mOC98 and mOC23 have N-terminal epitope binding regions (Figure 4-1). While they are classified as having conformational epitopes, they also are known to be reactive to monomeric Aβ 1-42.[98] It should be noted that the aforementioned antibodies were developed using the WT Aβ peptide as it was assumed to be the only naturally occurring form.

Figure 4-1. Epitope binding region of the four antibodies used in this study, with names indicated on the right in orange. The epitope binding region is highlighted on the Aβ 1-42 sequence for each antibody. The aberrant aspartic acid residues are in blue while the aberrant serine residues are orange.

An additional consideration for the analysis of epimeric and/or isomeric peptides are their detection by MS/MS. Epimeric and/or isomeric peptides can have unique solubilities and spatial conformations in solution which may affect the instrument response when analyzed by MS/MS.[99] In addition, epimers and/or isomers may have preferred ionization charge states and fragmentation pathways, thereby affecting their MS/MS intensities.[100–102] Effectively, this makes the quantitative analysis of epimeric and isomeric peptides difficult to achieve when using the same MS/MS conditions.

The goal of this study was to investigate antibody binding of Aβ 1-42 versus its epimers and/or isomers and to determine the effect, if any, of such stereochemical aberrations. To accomplish this, 18 epimers and/or isomers with single- and double-point mutations were screened against four antibodies. These 18 epimers/isomers had aberrations including L-iso-Asp, D-iso-Asp, D-Asp, and D-Ser modifications at positions Asp1, Asp7, Asp23, Ser8 and Ser26, respectively. The four antibodies screened include 4G8, 6E10, mOC98, and mOC23.

4.2 Experimental

4.2.1 Reagents and Materials

WT Aβ 1-42 peptide was purchased from Genscript (Piscataway, NJ, USA) and the aberrant Aβ 1-42 synthetic peptides (> 95% purity) were purchased from Peptide 2.0 (Chantilly, VA, USA). Stock solutions of each Aβ standard were prepared in 10 mM borate buffered to pH 9.1 with sodium hydroxide. Boric acid, sodium hydroxide, formic acid and trifluoroacetic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Peptide concentrations were standardized using a Pierce™ BCA Protein Assay kit from Thermo Fisher Scientific (Waltham, MA, USA).

Purified anti-β-Amyloids 17-24 Antibody (4G8) and 1-16 Antibody (6E10) were purchased from BioLegend (San Diego, CA, USA) while mOC-type antibodies recombinant anti-beta amyloid 1-42 mOC98 and mOC23 were purchased from Abcam (Cambridge, UK). Antibody-antigen binding was measured using Pierce Classic IP Kit by Thermo Fisher Scientific (Waltham, MA, USA). Per IP experiment, 11 pmol Aβ peptide (2 µL of 25 ug/mL stock solution solution) and 13.2 pmol of antibody were added to 300 µL IP lysis/wash buffer and allowed to bind to form antigen-antibody complexes. Protein A/G plus agarose resin was added to couple with antibody-antigen complexes. Complexes were washed in

IP lysis/wash buffer followed by conditioning buffer. Aβ peptides were immunoprecipitated using elution buffer. Elution buffer was evaporated and samples were reconstituted in 20 µL of 0.1 M borate buffered to pH 9.1 and analyzed by LC-MS/MS. All samples were prepared in triplicate.

HPLC-MS grade acetonitrile was purchased from Sigma-Aldrich and ultrapure water was obtained from a Milli-Q-water system (Millipore, Bedford, MA, USA). Samples were analyzed using an LCMS/MS-8050 (Shimadzu Scientific Instruments, Columbia, MD, USA), triple quadrupole spectrometer equipped with a positive ESI source and instrument conditions: drying gas and nebulizing gas flow rate of 15 L/min and 2 L/min, respectively; desolvation line temperature and heat block temperature of 275 °C and 400 °C, respectively. All-L Aβ 1-42 and aberrant Aβ 1-42 immunoprecipitated standards were quantitated using the MS/MS transition 903 m/z to 886 m/z. The peptides were eluted using BIOshell™ IgG 1000Å C4 column (4.6 mm i.d x 10 cm length, pore size 1000Å, particle size 2.7 um; Supelco, Bellefonte, PA, USA). The elution conditions include a ramp from 5% mobile phase B (99.9% acetonitrile, 0.1% formic acid) and 95% mobile phase A (99.9% water, 0.1% formic acid) to 50% mobile phase B and 50% mobile phase A from 0 to 5 minutes, followed by an isocratic hold at 50% mobile phase B and 50% mobile phase A from 6 to 8 minutes, then a wash in 100% mobile phase C (50.0% water, 49.9% acetonitrile, 0.1% trifluoracetic acid) for 10 minutes, followed by a column reconditioning at 5% mobile phase B and 95% mobile phase A for 10 minutes. Shimadzu LabSolutions software was used to integrate the peak areas.

4.3 Results

A selection of antibodies was chosen to examine the effect of antigen binding across several epitope regions of the all-L and aberrant Aβ 1-42. As can be seen, the antibody epitopes specifically targets Asp and Ser positions, and/or directly adjacent regions (Figure 4-1). These antibodies include 4G8, 6E10, mOC98, and mOC23. Note that antibody mOC98 has two epitope binding regions which accommodate amino acids Asp7, Asp23, and Ser26. Antibody mOC23 does not overlap with any Asp or Ser residues, but is encompassed by Asp1, Asp7 and Ser8. The four antibodies were screened against all Aβ peptides listed in Figure 4-2. The aberrant Aβ 1-42 peptides are listed below the all-L peptide. To address whether the antibody epitope region was specific to the configuration of Asp or Ser residues, peptides with single mutations for all possible Asp modifications and Ser were screened. These peptides correspond to Aβ1 - Aβ11 (see Figure 4-2). It is likely that more than one modification will be present on Aβ 1-42 extracted from AD brain. To address this possibility, a selection of double modified peptides was analyzed. Note that the double modifications are at positions Asp23 and Ser26 or Asp1 and Asp7. Double modifications at positions Asp23 and Ser26 were chosen as these amino acids are in the core of Aβ 1-42. The amino acids in the middle of Aβ 1-42 contribute to higher order structures.[103] In contrast, double modifications at positions Asp1 and Asp7 were selected as these amino acids are in the region that binds many commercial antibodies. In addition, Aβ 1-42 extracted from AD brain has found significant modification at positions Asp1 and Asp7. These “aberrant” peptides correspond to Aβ12 - Aβ18 (Figure 4-2).

Wild-Type (all-L)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ1. D-(D23):	DAEFRHDSGYEVHHQKLVFFAE{D-Asp}VGSNKGAIIGLMVGGVVIA
Aβ2. D-(D7):	DAEFRH{D-Asp}SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ3. D-(D1):	{D-Asp}AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ4. D-(isoD23):	DAEFRHDSGYEVHHQKLVFFAE{D-iso-Asp}VGSNKGAIIGLMVGGVVIA
Aβ5. D-(isoD7):	{D-iso-Asp}AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ6. D-(isoD1):	{D-iso-Asp}AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ7. L-(isoD23):	DAEFRHDSGYEVHHQKLVFFAE{L-iso-Asp}VGSNKGAIIGLMVGGVVIA
Aβ8. L-(isoD7):	DAEFRH{L-iso-Asp}SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ9. L-(isoD1):	{L-iso-Asp}AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ10. D-(S26):	DAEFRHDSGYEVHHQKLVFFAEDVG{D-Ser}NKGAIIGLMVGGVVIA
Aβ11. D-(S8):	DAEFRHD{D-Ser}GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ12. D-(D23),D-(S26):	DAEFRHDSGYEVHHQKLVFFAE{D-Asp}VG{D-Ser}NKGAIIGLMVGGVVIA
Aβ13. L-(isoD23),D-(S26):	DAEFRHDSGYEVHHQKLVFFAE{L-iso-Asp}VG{D-Ser}NKGAIIGLMVGGVVIA
Aβ14. D-(isoD23),D-(S26):	DAEFRHDSGYEVHHQKLVFFAE{D-iso-Asp}VG{D-Ser}NKGAIIGLMVGGVVIA
Aβ15. L-(isoD1),L-(isoD7):	{L-iso-Asp}AEFRH{L-iso-Asp}SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ16. D-(D1),L-(isoD7):	{D-Asp}AEFRH{L-iso-Asp}SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ17. L-(isoD1),D-(D7):	{L-iso-Asp}AEFRH{D-Asp}SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ18. D-(D1),D-(D7):	{D-Asp} AEFRH{D-Asp}SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

Figure 4-2. List of Aβ peptide isomers and/or epimers assessed in this study, with the aberrations indicated in brackets. The peptides are labeled with the first letter indicating the stereochemical configuration (i.e., L,D) (in purple) and then the amino acid residue and position in parenthesis. The aberrant aspartic acid residues are in blue while the aberrant serine residues are in orange.

Antibody binding efficacy was determined by an immunoprecipitation procedure followed by reconstitution in borate buffer and analyzed by liquid chromatography tandem mass spectrometry (see Methods). The subsequent MS/MS peak areas were integrated, and their intensity areas were plotted as seen in Figure 4-3A. Note that these antibodies were developed using the WT Aβ peptide. Further, the MS/MS detection also was optimized

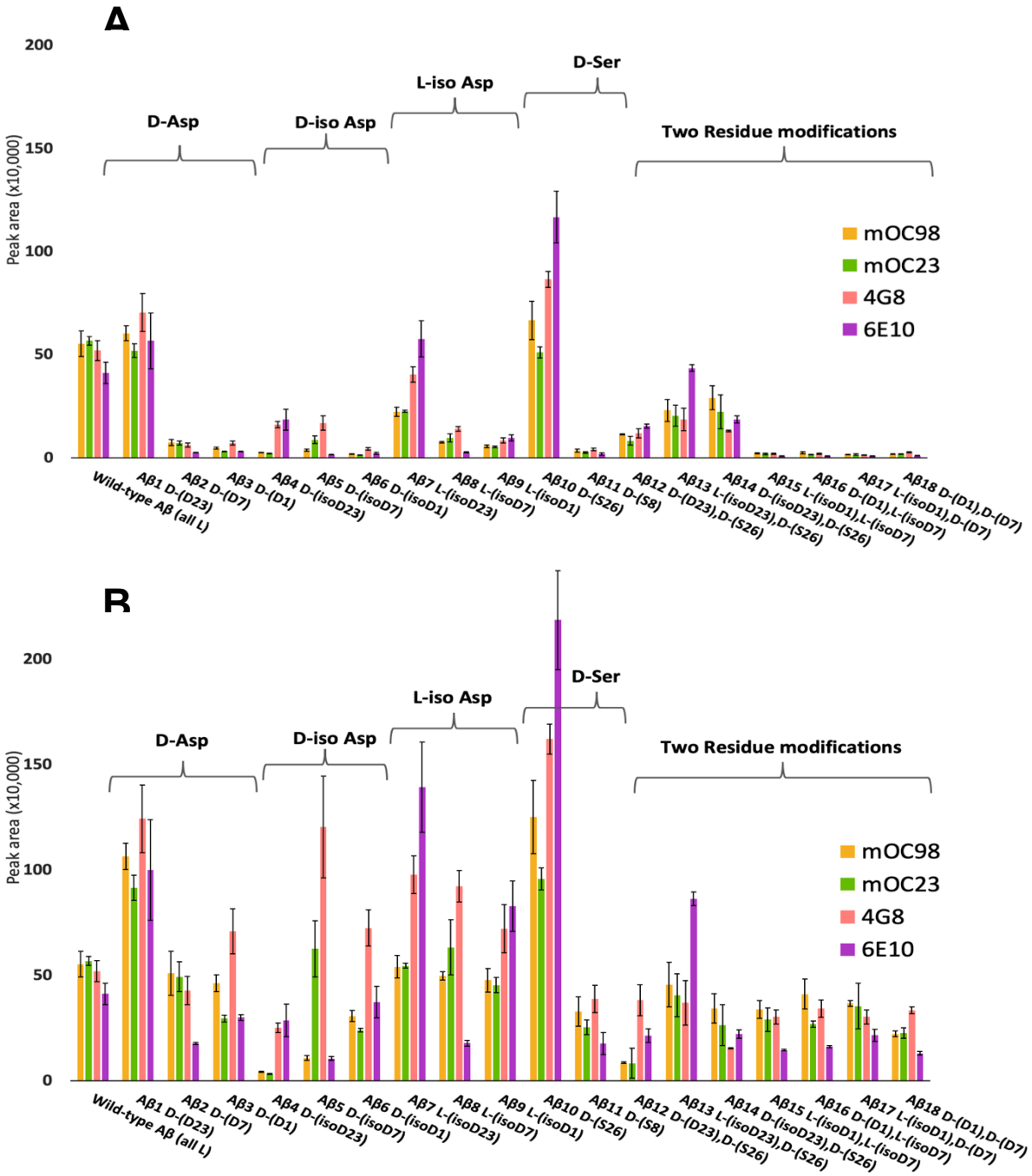

Figure 4-3. 4A and 4B are resulting peak areas of immunoprecipitation extraction of 18 peptides with four antibodies (see above figures for details). Figure 4-3B shows the data after correction for sensitivity of MS/MS detector of each peptide. The peptides are labeled according to Figure 4-2.

using the WT Aβ peptide. The antigen binding for the WT Aβ was was similar for all antibodies (4G8, 6E10, mOC98 and mOC23) which was not unexpected. However, it also is apparent (Figure 4-3A) that the signal intensities for all but two of the “aberrant” peptides (i.e. Aβ1 D-(D23) and Aβ10 D-(S26)) are far lower than those of the WT Aβ peptide. Indeed, the peptides with epimerizations or isomerization near the N-terminus of the Aβ usually had signal intensities approximately 10-50 times less than the WT Aβ peptide. The cause of these diminished signals could be due to the altered binding between the antibody and the “aberrant” forms of Aβ, differences in the MS/MS of the “aberrant” Aβ or some combination of these two effects.

Further, calibration curves for each aberrant peptide were created using the MS/MS conditions optimized for the WT Aβ. The slopes for each aberrant peptide varied from the slope of the WT Aβ when using the same instrument conditions, and in some cases, quite significantly. Figure 4-3A data was corrected with the individual calibration curves to reveal the correct binding affinities as shown in Figure 4-3B. Among the peptides with single modifications, a few have binding affinities that are higher than the WT Aβ 1-42. For Aβ1 D-(D23) and Aβ 10 (D-(S26), every respective antibody extraction is higher than the WT Aβ antibodies. In contrast, Aβ2 D-(D7), Aβ4 D-(isoD23) and Aβ11 D-(S8) have lower binding affinities for all screened antibodies. In most cases, two or three of the four screened antibodies extracted single aberration peptides comparably to the WT Aβ. Additionally, in all but three cases (i.e. Aβ1 D-(D23), Aβ10 D-(S26) and Aβ7 L-(isoD23)) between one to four antibodies extracted significantly less of the aberrant Aβ peptides.

There is no pattern that alludes to the success of antibody binding based off the type of modification or the modification location, either within or near an epitope region. General, antibody 4G8 consistently reported having the highest, or second highest, binding efficacy, except for Aβ2 D-(D7). All peptides with double modifications, had binding

affinities that were lower than the WT Aβ, except for Aβ13 L-(isoD23), D-(S26), in which only antibody 6E10 comparably extracted this epimer. For the peptides with double modifications at the N-terminus, peptides Aβ15 - Aβ18, antibody 6E10 consistently had the lowest binding efficacy. In contrast, peptides with double modifications nearer the middle of Aβ, at Asp23 and Ser26, did not show this trend. Overall, peptides with single modifications tended to have higher binding affinities than peptides with double modifications.

Data trends in Figures 4-3A and 4-3B contrast because Figure 4-3A data had not been corrected to consider how MS/MS specificity may be affected by aberrations in peptides. To further highlight the differences in MS/MS specificity, the integrated peak area for the 20 μg/mL standard of each peptide is plotted in Figure 4-4. Each type of Asp and Ser modifications are individually plotted against their positions in the Aβ peptide. The peak area, or sensitivity, decreases as the modification proceeds from the core region of Aβ to the N-terminus of Aβ. The sensitivity does not appear to depend on the type of modification. This includes the peptides with double modifications. Double modifications that occur in the core region of Aβ have more MS/MS sensitivity than peptides with double modifications at the N-terminus under same instrument conditions.

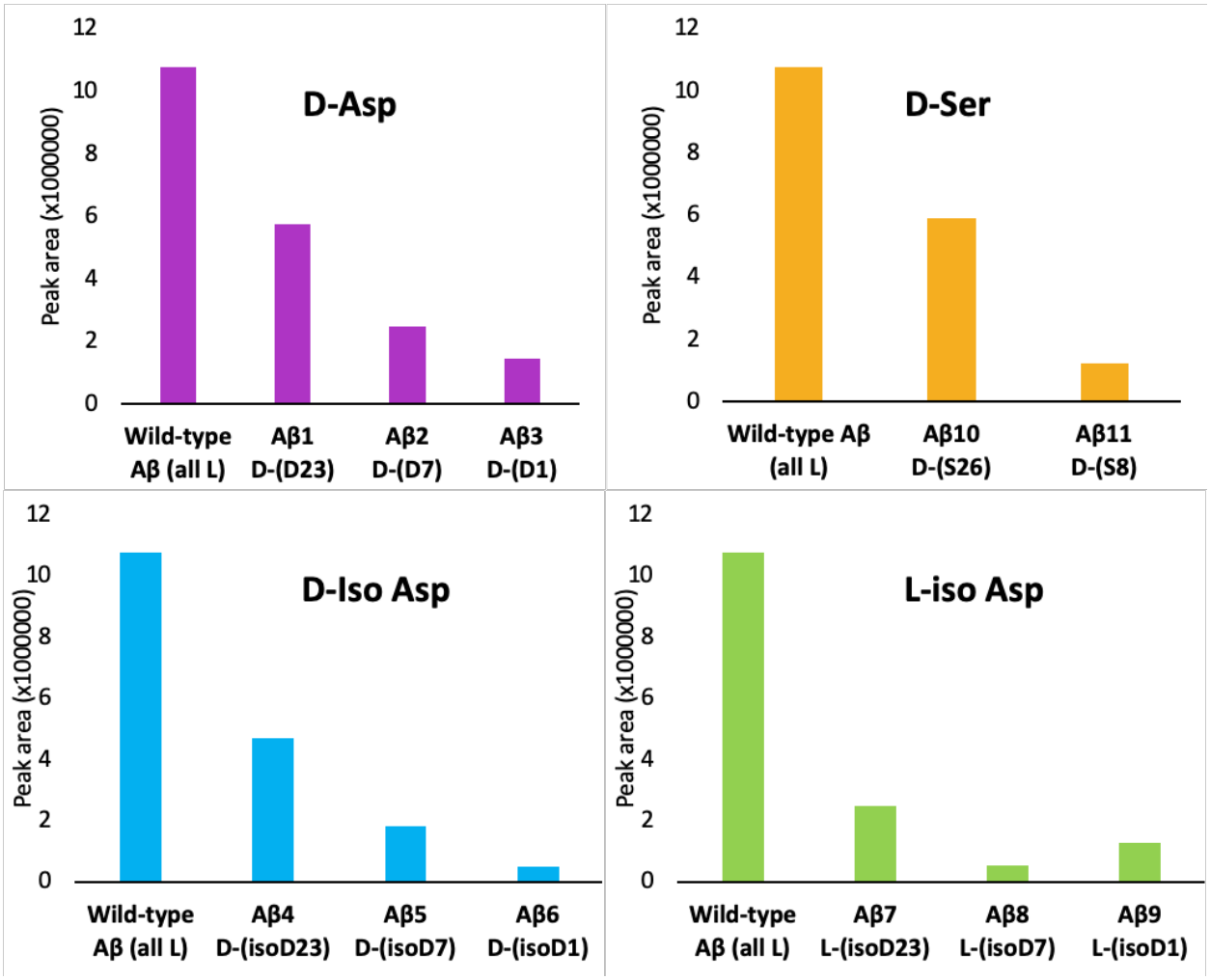

Figure 4-4. Peak areas of various peptides at 20 µg/mL concentration under the same MS/MS conditions (see Method). The wild-type peptide is plotted along with group of peptides with specified aberrations in each category (D-Asp, D-ser, D-iso Asp, L-iso Asp). Note, any epimeric or isomeric peptides is less sensitive than all L-peptide under the optimized conditions for all L-peptide.

4.4 Discussion

We have shown that isomeric and/or epimeric peptides of Aβ 1-42 have different binding affinities for antibodies 4G8, 6E10, mOC98, and mOC23 by immunoprecipitation. This selection of antibodies has diverse epitope regions which allows a better understanding of antibody – Aβ binding affinities. It expected that all four antibodies bind the unmodified WT Aβ with the same efficacy as each antibody was manufactured using an unmodified, WT Aβ peptide. For this reason, WT Aβ served as a standard reference for the epimers and/or isomers.

Figure 4-3A and 4-3B highlights the disadvantage of mass spectrometry data collection. Both figures depict the same dataset, but Figure 4-3B corrects for the effect of MS/MS variability. Our studies reveal that epimers and/or isomers have different sensitivities while using the same MS/MS conditions and that many epimeric and isomeric peptides are less sensitive. The secondary structure of Aβ monomers may be responsible for the depressed signal for epimeric and/or isomeric peptides. The secondary structures of Aβ monomers have alpha helical structures located near the core of the peptide, encompassing amino acids 8-25 to 28-38[103] (which may also inhibit or enhance antibody binding capabilities). Our group has previously shown that aberrant Aβ peptides have different retention times when chromatographically separated using reversed-phase liquid chromatography.[99] This effect is a result of unique on-column interactions due to structural differences induced by peptide modifications between epimers and/or isomers. The unique secondary structures could lead to fragmentation differences during MS/MS analysis, and thereby affect the sensitivity of the peptide epimers and/or isomers when using the same MS/MS conditions. Interestingly, as seen in Figure 4-4, the locations of the Aβ modification seems to affect the sensitivity more than the nature of the modification. Specifically,

epimeric or isomeric centers near the N-terminus of Aβ had the biggest effects. This is the first investigation of peptide epimers and/or isomers that highlights the changes in MS/MS specificity based on the aberration location within a peptide. Nevertheless, we can easily correct for MS/MS specificity caused by peptide aberrations by creating calibration curves for each aberrant peptide at the same MS/MS conditions. Hence, the dataset in Figure 4-3B best reflects the binding affinities of the antibodies to the aberrant Aβ peptides, and all further discussion of the results focuses on the Figure 4-3B data.

Antibodies 4G8 and 6E10 were typically the most successful antibodies at extracting aberrant Aβ peptides, especially with epimers and/or isomers with core-amino acid modifications. These antibodies have especially high binding affinities for Aβ7 L-(isoD23), Aβ1 D-(D23) and Aβ 10 D-(S26). For all antibodies tested, Aβ1 D-(D23) and Aβ10 D-(S26) had higher antibody binding affinities than the all-L Aβ. Note that Aβ4 D-(isoD23) had poor binding for all screened antibodies. Antibodies 4G8 and 6E10 are epitope sequence dependent while antibodies mOC98 and mOC23 are conformation dependent. It is evident that the epimers and/or isomers must have different conformations as their binding affinities for mOC98 and mOC23 vary dramatically, and rarely did an mOC-based antibody bind preferentially in comparison to 4G8 and/or 6E10. Also, there was no specific epitope that was predictive as to the success of an antibody-antigen pairing. Rather, modifications that occurred in the middle part of the peptide generally had a higher binding efficacy, independent of the antibody epitope region.

Single amino acid modifications were individually assessed for all possible forms of Asp and Ser, at all locations. There were no observable patterns regarding which antibody outperformed the others for Aβ peptides with single amino acid modifications (aberrations). However, the relative selectivity of the four tested antibodies for any Aβ with a single amino acid aberration could be 2-10 times different in most cases. Furthermore,

the location of the amino acid aberration within Aβ had a significant effect on binding effectiveness. Specifically, antibody binding affinities decreased when the epimer or isomeric amino acid was near the N-terminus of Aβ. The most dramatic example of this effect is for Aβ9 D-(S8) versus Aβ10 D-(S26). Collectively, Aβ10 D-(S26) had the highest combined selectivity for the antibodies while Aβ11 D-(S8) had a very low recovery, as was found for Aβ with double modifications. Peptides with D-Asp and L-iso-Asp also share this effect: the same modification at the core of Aβ has generally higher antibody binding than modifications located near the N-terminus. Aβ peptides with a D-iso-Asp modification are the exception to this rule. The differences in binding are attributed to structural changes induced by the modification. This could explain why several of the aberrant peptides have higher binding than the unmodified Aβ 1-42, while many have significantly lower binding. Two-residue modifications most consistently had the lowest binding affinity of all the peptides screened. The double modifications located at the core of Aβ do not necessarily have higher binding than modifications at the N-terminus, which is in contrast to with the singly modified peptides. It appears that having multiple modifications increases the possibility for unique peptide spatial conformations which, in turn, inhibits antibody binding. Thus, it was generally observed that Aβ with two amino acid modifications did will not bind as effectively as the all-L Aβ.

In a few cases, aberrant Aβ peptides may induce more favorable antibody-antigen interactions, or rather, stabilize the most complimentary spatial arrangement of an antigen. Isomeric and/or epimeric Aβ monomers can form aggregates adding to their unique spatial conformations. A combination of complimentary factors may induce antibody-antigen binding, such as unique monomer secondary structure, or formation of small oligomers. Note that the all-L Aβ peptide gradually forms oligomers, then protofibrils, and eventually fibrils. This rate dependent formation should be unique for the aberrant species, leading to

unique binding capabilities of higher order structures. It is clear from these results that the best way to guarantee a complete extraction of all Aβ peptides is to utilize a cocktail of antibodies that include amino acid sequence and conformation specific epitopes. However, this may not be a plausible solution for immunotherapies targeting Aβ peptides in Alzheimer's Disease patients.

Antibodies are being tested and used as AD drugs and a significant factor contributing to their efficacy is directly related to their binding affinities to the Aβ peptide. These immunotherapies have been in development for over a decade, and several have completed clinical trials and proceeded to marketing.[28,29] However, these immunotherapies are not a cure-all for Alzheimer's disease. In fact, they are targeted to slow the progression of AD in patients. A myriad of reasons could explain their lack of success including some failures in clinical trials occurring after AD has progressed too much. However, it is also a possibility that immunotherapies are less effective when targeting aberrant forms of Aβ peptide in AD patients. A significant amount of Aβ extracted from AD brain has aberrations located at the N-terminus – which as shown can be a challenging epitope location for antibody interactions. Indeed, we were able to achieve some binding affinities for all aberrant Aβ peptides screened, however, immunotherapies targeting Aβ peptide presumably have epitope regions that are highly selective and could be less likely to bind peptides with aberrations. Hence, it should be considered that such therapies may be less effective *in vivo* given the nature and amounts of aberrant Aβ peptides.

4.5 Conclusions

Studies targeting aberrant Aβ peptides need to evaluate epimers and/or isomers as discrete peptides from their all L-antipodes. Indeed, epimers and/or isomers can have unique spatial conformations and/or aggregational behaviors which can affect antibody binding and change MS/MS fragmentation. For these reasons, epimers and/or isomers could impose considerable challenges when developing immunotherapies. Further investigations testing binding affinities of Aβ peptides for marketed immunotherapies and those in various stages of clinical trials clarify the actions and/or shortcomings of these therapies. It is also plausible that previous analyses that used MS/MS and/or antibodies to identify aberrant peptides in AD brain likely underrepresented the population of aberrant Aβ. Clearly, additional in-depth studies on therapeutic antibodies binding to aberrant Aβ and their MS/MS behaviors are warranted. Investigations of various forms of Aβ 1-42, in addition to tryptic digest fragments, are currently underway.

Chapter 5

The MS/MS Effect on Epimers and/or Isomers: An Investigation of Amyloid-Beta Peptides

5.1 Introduction

Amino acids are the essential building blocks of proteins in all living organisms.[104] Amino acids can exist in either L- or D- form, except for glycine which lacks a chiral center. Initially, the L-amino acid was thought to be the only relevant form in higher organisms, while D-amino acids were thought to be absent. D-amino acids were eventually discovered in higher organisms, including free amino acids as agonists for N-methyl-D-aspartate (NMDA) receptors[105,106] or embedded within peptides. For example, α/B-crystallin proteins extracted from eye lenses and amyloid-beta (Aβ) peptides extracted from Alzheimer's Disease patients have indicated elevated presence of D-Asp, L-iso-Asp, D-iso-Asp and/or D-Ser.[10,107,108] This, suggests that the presence of D-amino acids play an active role in biological systems of higher organisms.

Various methods to scout for D-amino acids include capillary electrophoresis (CE), gas chromatography (GC), and high performance liquid chromatography (HPLC).[109] Of these methods, HPLC is regarded as the best approach for the analysis of free amino acids. It is quantitatively reproducible and chromatographic separations can be achieved with a diverse portfolio of chiral stationary phases and detection methods.[110] However, there are several comprehensive sample preparation steps required to prepare free-amino acids for HPLC analysis. After proteins are extracted and purified from a biological matrix, they are fragmented typically by tryptic digestion, which cleaves peptides at lysine or arginine to form short peptides. Peptide fragments undergo acid hydrolysis to form individual amino acids.[10] Acid hydrolysis can induce Asn deamidation to Asp and induce iso-Asp to form

Asp. This is problematic when sequencing and quantifying peptides extracted from diseases that are defined by the elevated presence of iso-Asp, such as Alzheimer's Disease. A different solution to detect D-amino acids in peptides and proteins would be a separation of epimers and/or isomers, thereby not requiring acid hydrolysis, nor separation by either chiral or achiral stationary phases.[111,112]

Amyloid-beta (Aβ) a 42-amino acid long protein related to the progression and diagnosis of Alzheimer's Disease (AD).[1,4] It contains three Asp and two Ser residues, at positions Asp1, Asp7, Asp23, Ser8, and Ser26. In Aβ extracted from AD patients it was found that at all Asp and Ser positions there was either the D-antipode and/or the L/D-iso-Asp.[10,41] However, quantitation and identifying the exact location of these "different" amino acids has been problematic as most approaches are inadequate to identify isomers, or used acid hydrolysis to process the protein thereby eliminating iso-aspartic and partially racemizing Asp (and all other constituent amino acids).[10,41] Recently, a method was developed that separated all 20 possible epimers and/or isomers of the Aβ tryptic digest using a combination of chiral stationary phases with HPLC.[67] In another study, a complimentary separation of AB peptides that did not undergo tryptic digest were separated using a reversed-phase achiral column.[34] Both methods are excellent solutions for identifying modified peptides extracted from AD patients. Likewise, the sensitivity was enhanced by using mass spectrometry detection.

Mass spectrometry (MS) as a tool to quantitate the presence of D-amino acids in peptides is not without caveats. Peptide epimers and/or isomers have the same exact mass, and are therefore indistinguishable by MS. Therefore, a baseline chromatographic separation and use of standards are necessary to identify possible epimers and/or isomers. If baseline

separation is achieved, it is also likely that peptide epimers and/or isomers respond to MS/MS conditions uniquely. It has been shown that amino acid enantiomers and epimeric and/or isomeric peptides can have different MS responses when analyzed using the same conditions.[30,31] Some studies have indicated that this MS/MS effect may be caused by differences in fragment ions between Asp and its antipodes.[20,32,33] However, they have not considered that differences in peptide ionizability may also contribute to the variability in instrumental specificity for peptide epimers and/or isomers.[113–117] For quantitative studies of peptide epimers and/or isomers, proper calibration curves for each entity are needed to accurately identify the amount of epimers and/or isomers. Indeed, a recent study highlighted the effect of modified Asp and Ser residues on the MS of Aβ peptides 42 amino acids in length.[ref] This was the first report that disclosed whereby the location and type of modification perturbs peptide sensitivity for longer peptides.[118]

The goal of this work was to evaluate the epimeric and/or isomeric tryptic fragments of Aβ peptide to determine if they are uniquely affected by MS/MS (Table 5-1). The post-tryptic digest fragments include three groups of peptides: group A are pentapeptides with four possible epimeric/isomeric modifications. Groups B and C contain 11 and 12 amino acids, respectively, and each have eight epimers and/or isomers, as they both contain an Asp and Ser residues. Note the location of the modifications, or “aberrations”, within these peptides. For group A, the Asp residue is located at the first position from the N-terminus while for group B the Asp residue is located at the second position from the N-terminus and for group C the Asp residue is located at the seventh position from the N-terminus. Previously it was found that the location of the modification within the full-length peptide affect the MS signals.[118] For each group, the unmodified, all-L peptide was optimized for the highest signal in selected reaction monitoring (SRM), then analyzed in SRM, selected

ion mode (SIM), and then product ion scan (PIS). SIM is a good indicator of the ionizability as the peptides are not subjected to fragmentation, which can vary between peptide epimers and/or isomers in SRM.[118] Variations in fragmentation will be elucidated in PIS by identifying the most intense fragments. If epimers and/or isomers have preferential fragmentation pathways that affect the sensitivity of MS/MS detection, this must be investigated, identified and if possible, quantified.

Table 5-1. Amino acid sequence of the three groups of Aβ peptide epimers with Asp and Ser isomeric residues

Aβ (1-5)	A1: {L-Asp}AEFR	A2: {D-Asp}AEFR
Group A	A3: {L-isoAsp}AEFR	A4: {D-isoAsp}AEFR
	B1: H{L-Asp}{L-Ser}GYEVHHQK	B2: H{D-Asp}{L-Ser}GYEVHHQK
Aβ (6-16)	B3: H{L-Asp}{D-Ser}GYEVHHQK	B4: H{D-Asp}{D-Ser}GYEVHHQK
Group B	B5: H{L-isoAsp}{L-Ser}GYEVHHQK	B6: H{L-isoAsp}{D-Ser}GYEVHHQK
	B7: H{D-isoAsp}{L-Ser}GYEVHHQK	B8: H{D-isoAsp}{D-Ser}GYEVHHQK
	C1: LVFFAE{L-Asp}VG{L-Ser}NK	C2: LVFFAE{D-Asp}VG{L-Ser}NK
Aβ (17-28)	C3: LVFFAE{L-Asp}VG{D-Ser}NK	C4: LVFFAE{D-Asp}VG{D-Ser}NK
Group C	C5: LVFFAE{L-isoAsp}VG{L-Ser}NK	C6: LVFFAE{L-isoAsp}VG{D-Ser}NK
	C7: LVFFAE{D-isoAsp}VG{L-Ser}NK	C8: LVFFAE{D-isoAsp}VG{D-Ser}NK

5.2 Materials and Methods

5.2.1 Reagents and Materials

All tryptic amyloid-beta (Aβ) peptide standards were purchased from Peptide 2.0 (Chantilly, VA, USA) at > 98% purity. Stock solutions of each Aβ standard were prepared in 10 mM borate buffered to pH 9.1 with sodium hydroxide. Boric acid, sodium hydroxide, formic acid and trifluoroacetic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Peptide concentrations were standardized using a Pierce™ BCA Protein Assay kit from Thermo Fisher Scientific (Waltham, MA, USA). Calibration curves for each tryptic Aβ peptide was created with a range of 5 ug/mL to 100 ug/mL standards. Each standard was injected in triplicate (relative standard deviation <10% for each sample) in single ion mode (SIM), single reaction monitoring (SRM), and product ion scan (PIS). For instrument conditions in each mode, refer to Table 5-2. Conditions for each mode were optimized for the all-L Aβ peptide. The linearity for each curve had an R^2 of minimum 0.990.

Table 5-2. Results of SIM, PrIS, and MRM optimization for tryptic Aβ peptides on TSQ Quantis Plus

	Mode	Precursor (m/z)	Product (m/z)	CE (V)
Group A AB (1-5)	SIM	637.1	-	-
	PrIS	637.1	Scan (150-700)	20
	MRM	637.1	322.1	20
Group B AB (6-16)	SIM	668.7	-	-
	PrIS	668.7	Scan (150-700)	28
	MRM	668.7	253.0	28

Group C AB (17-28)	SIM	663.2	-	-
	PrIS	663.2	Scan (150-700)	35
	MRM	663.2	185.1	35

5.2.2 HPLC-MS/MS Instrument Settings

HPLC-MS grade acetonitrile was purchased from Sigma-Aldrich and ultrapure water was obtained from a Milli-Q-water system (Millipore, Bedford, MA, USA). Samples were analyzed using a TSQ Quantis Plus (Thermo Fisher Scientific Waltham, MA, USA), triple quadrupole spectrometer equipped with a positive ESI source and instrument conditions: drying gas and nebulizing gas flow rate of 10 L/min and 1 L/min, respectively; desolvation line temperature and heat block temperature of 325 °C and 350 °C, respectively. The peptides were eluted using BIOshell™ IgG 1000Å C4 column (4.6 mm i.d x 10 cm length, pore size 1000Å, particle size 2.7 um; Supelco, Bellefonte, PA, USA). The elution conditions include an isocratic hold at 50% mobile phase B (99.9% acetonitrile, 0.1% formic acid) and 50% mobile phase A (99.9% water, 0.1% formic acid) for 5 minutes. Chromeleon software was used to integrate the peak areas.

5.3 Results and discussion

The MS/MS response for all 20 proteinogenic Aβ peptide tryptic fragment epimers and/or isomers were investigated and compared to their all-L antipode. Each peptide was analyzed in selected ion mode (SIM) and via selected reaction monitoring (SRM). Differences in peptide response in SIM indicate the ionizability of a peptide for specific charge states, while differences in peptide response in SRM indicate a combination of peptide ionizability and unique peptide fragmentation. Instrument responses for SRM and SIM are shown at 100 µg/mL (Figures 5-1 to 5-3). Note, there are three groups of peptides (Table 5-1), and the SIM and SRM instrument conditions were optimized for the native all-L isomer for each group (Table 5-2). Further, the isomers in each group were analyzed using the optimized SIM and SRM conditions for the native all-L isomer.

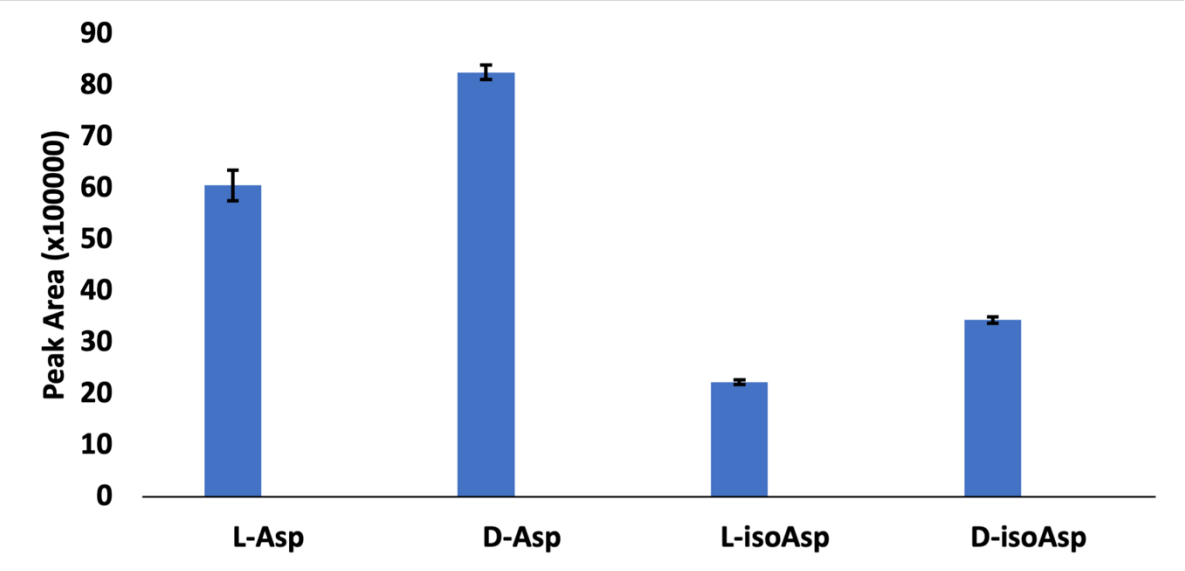

Figure 5-1. Selected ion mode response of group A peptide epimers with 100 µg/mL standards. Each fragment modification is indicated with the type of modification.

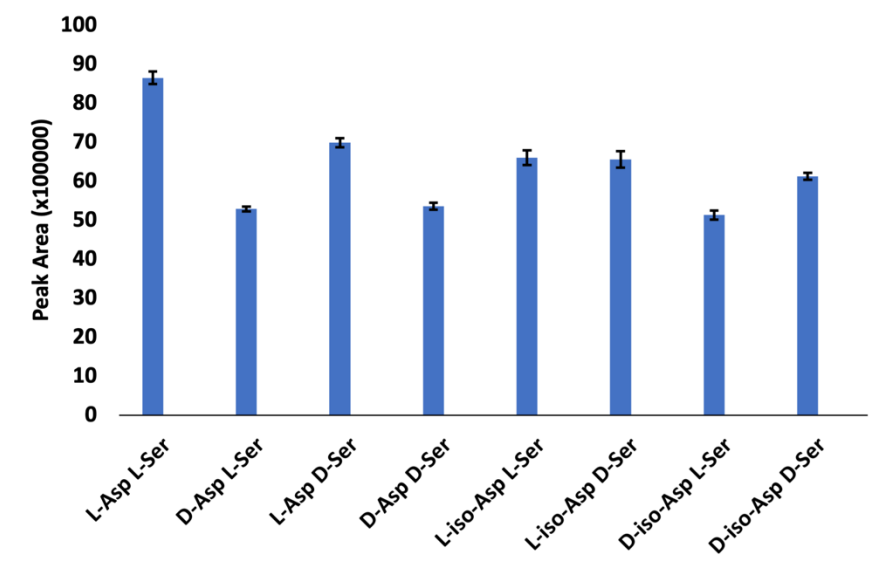

Figure 5-2. Selected ion mode response of group B peptide epimers with 100 µg/mL standards. Each fragment modification is indicated with the type of modification.

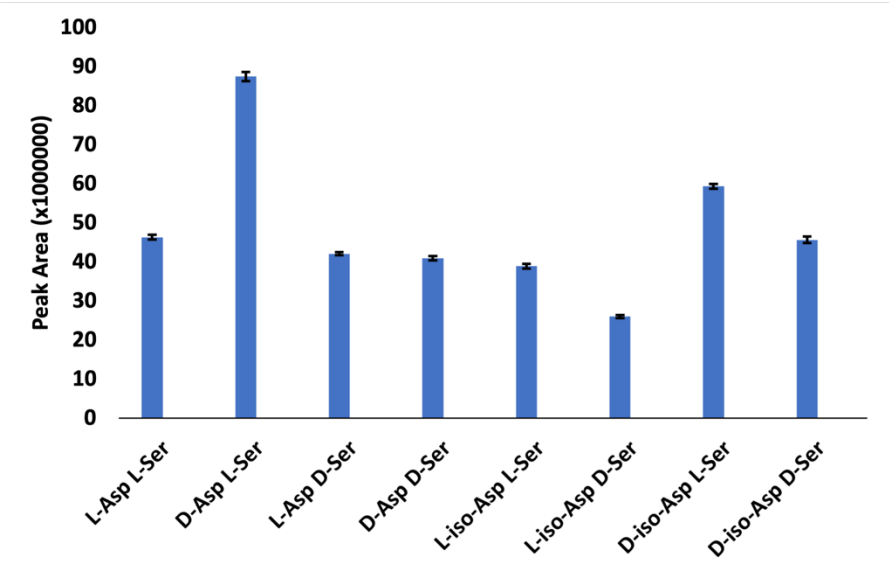

Figure 5-3. Selected ion mode response of group C peptide epimers with 100 µg/mL standards. Each fragment modification is indicated with the type of modification.

5.3.1 Trends Among Epimers and/or Isomers in Groups A, B, and C

For group A peptides, the D-Asp modified peptide had the highest instrument response in SIM (Figure 5-1). The SIM results suggest that the D-Asp containing epimer is more ionizable than the native all-L amino acid containing peptide. Both isomers containing L-iso-Asp and D-iso-Asp modifications had the lowest instrument response due to poor ionizability (Figure 5-1). Groups B and C peptides have two different modifications, including an Asp and a Ser residue, and thus have eight total epimers and/or isomers (see Table 5-1). For Group B, the native all-L peptide had the highest instrument response in SIM compared to the modified peptides (Figure 5-2). For Group C, there are several epimers and/or isomers that have higher instrumental responses than the native all-L peptide (Figure 5-3). This includes peptides with the modifications D-Asp/L-Ser, D-iso-Asp/L-Ser, and D-iso-Asp/D-Ser (see Tables 5-1 for structures). Groups A, B, and C peptides were also analyzed in SRM (data not shown). Group A and C SRM responses have the same relative ratios as the SIM for their respective peptide epimers and/or isomers, suggesting that differences in SRM response between peptide epimers and/or isomers are primarily affected by peptide ionization. The relative ratio of signal response for group B SRM responses are approximately 50% of the native all-L peptide signal, in comparison to the SIM signal which is approximately 75% of the native all-L peptide signal for the peptide isomers and/or epimers. This suggests that the SRM response is affected by both the ionization and the unique fragmentation of epimers and/or isomers. There is not a lot of literature that considers the ionizability of peptide epimers and/or isomers in electrospray ionization. In most cases, it is presumed that they ionize in similar proportions, and any resulting discrepancies in abundance is due to MS/MS analysis. Ionizability significantly depends on the secondary structure and the amino acid sequence of the peptide.

5.3.2 Peptide Epimer Fragments

The signal responses for peptide epimers and/or isomers are unique at the same concentration while using SRM. Peptide epimers and/or isomers typically fragment into the same peptide species, however, they may fragment into these species in variable amounts. Each of the 20 epimeric and/or isomeric peptides were analyzed using product ion scan (PIS) to observe the ratios between the most intense fragments of the native all-L peptides and the same fragment for the epimers and/or isomers. The fragmentation energy used for each PIS is the same fragmentation energy used in SRM. This fragmentation energy has been optimized to increase the abundance of the product ions for the native all-L peptide for each group of peptides. The fragment ions with the highest abundance for the all-L peptides are plotted with their subsequent relative abundances (Figures 5-4 to 5-6). Note, for group A peptides, the top 10 fragment ions were the same for each epimer and/or isomer (Figure 5-4). For group B peptides, approximately 80% of the top 10 fragment ions for the epimers and/or isomers were the same as the native all-L peptide (Figure 5-5). For group C peptides, approximately 90% of the top 10 fragment ions for the epimers and/or isomers were the same as the native all-L peptide (Figure 5-6). However, many epimers and/or isomers had varying ratios of their fragment ion abundances compared to the native all-L peptides. These differences are discussed in greater detail.

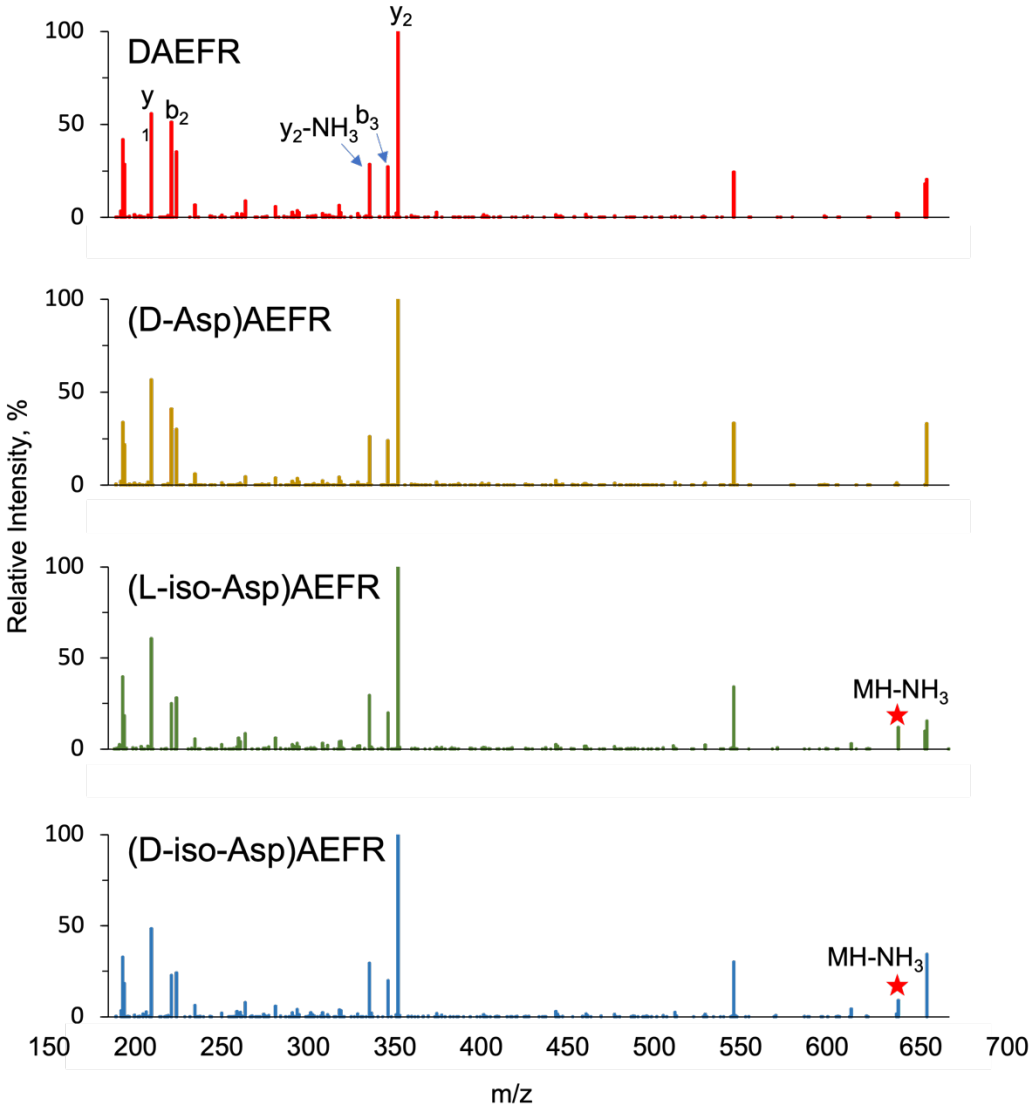

Figure 5-4. Relative abundance for group A peptides analyzed by product ion scan (PIS) for peptide standard 100 ug/mL.

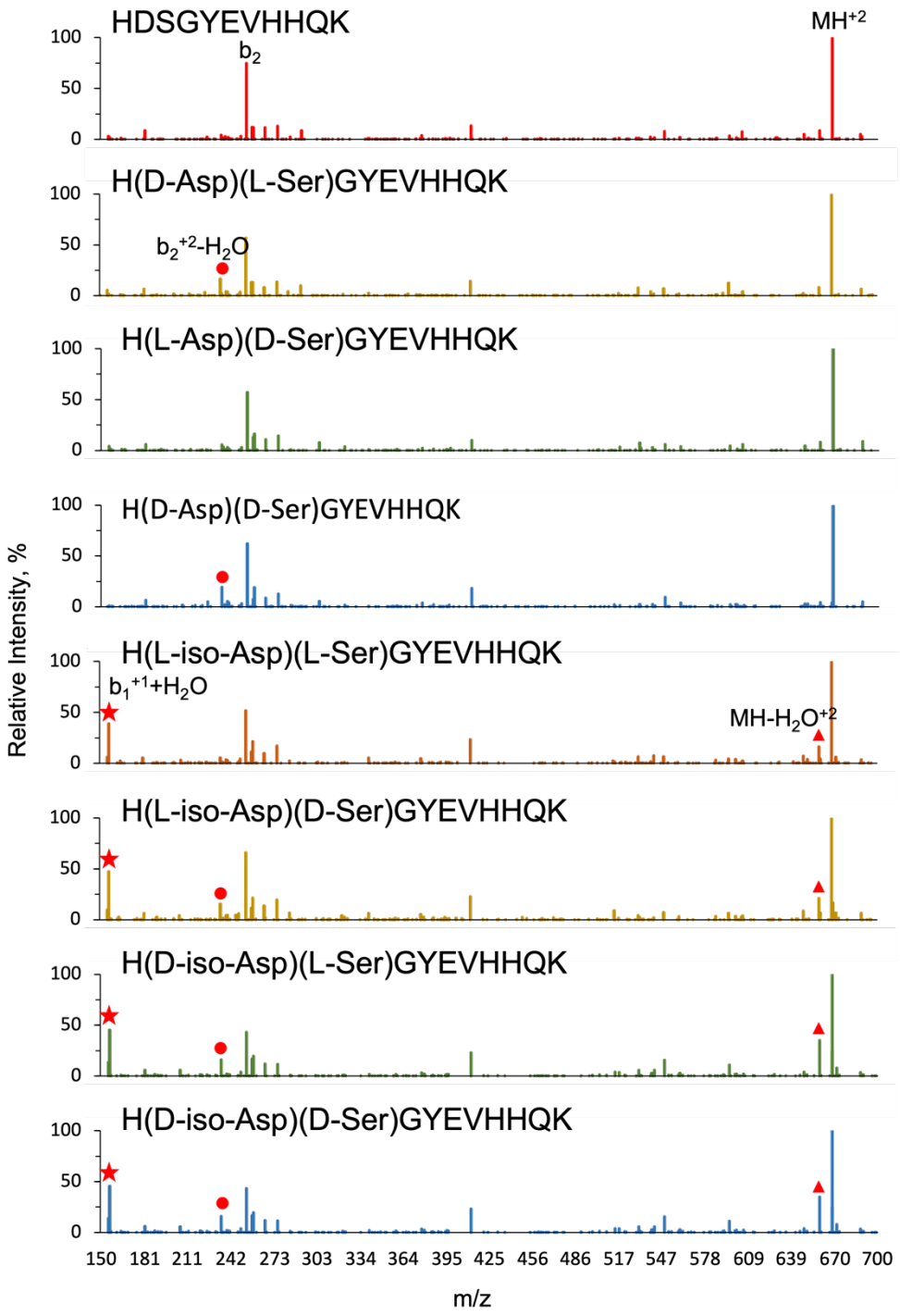

Figure 5-5. Relative abundance for group B peptides analyzed by product ion scan (PIS) for peptide standard 100 ug/mL.

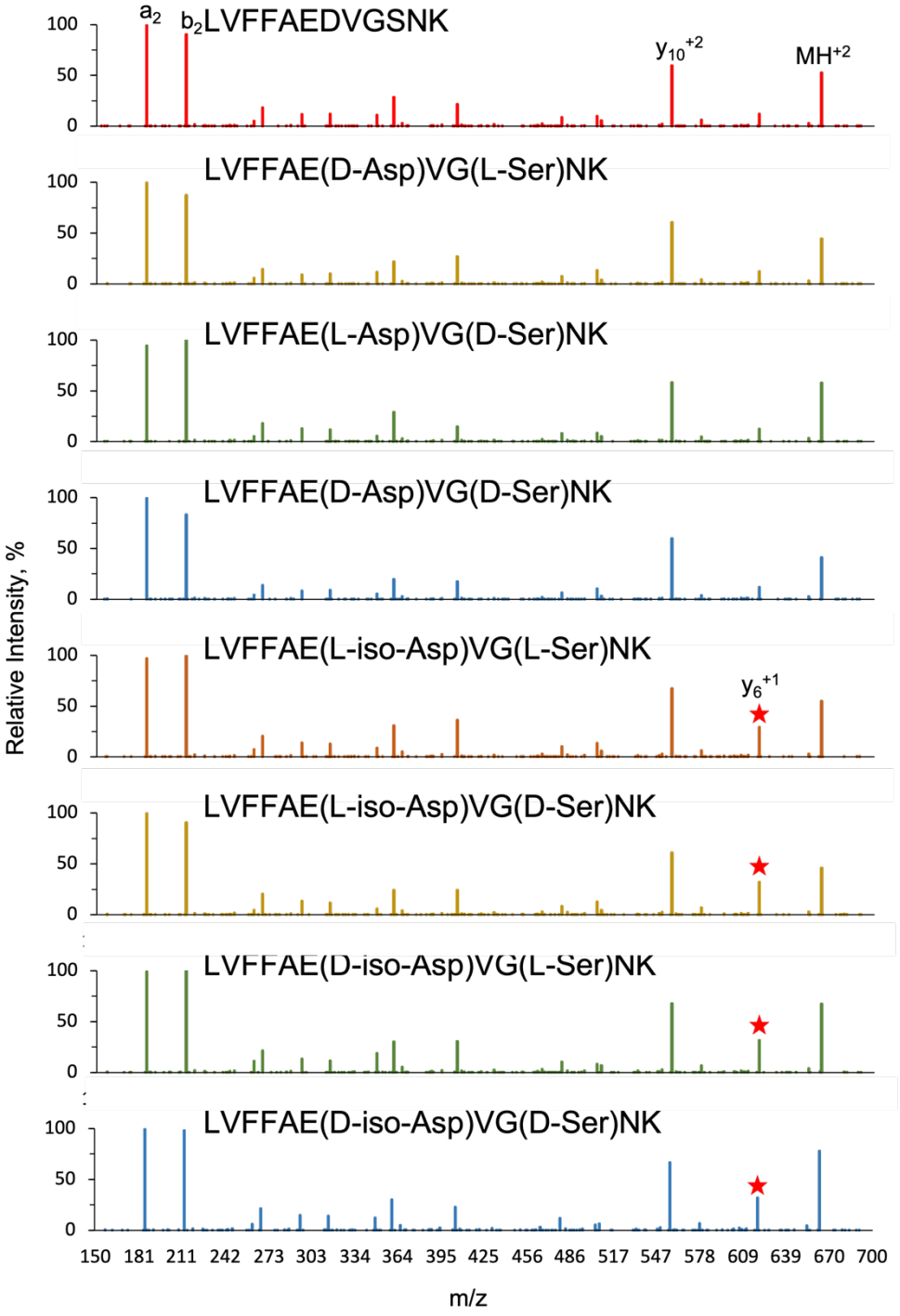

Figure 5-6. Relative abundance for group C peptides analyzed by product ion scan (PIS) for peptide standard 100 ug/mL.

Group A epimers and/or isomers yielded characteristic ammonia losses at the MH-NH_3 and y_2-NH_3 ions. An ammonia loss is common for peptides containing Arg at the C-terminus, or peptides that are products of trypsin digestion. The proposed mechanism for loss of ammonia from Arg includes tautomerization of the side chain and a carbonyl attack from the peptide backbone. This results in an ammonia loss and a cyclic formation of the Arg-NH_3 residue to the peptide backbone. Intriguingly, the relative intensity of the y_2-NH_3 ion, which includes the Phe and Arg residue located at the C-terminus, is similar amongst all Group A peptide epimers and/or isomers. However, the MH-NH_3 ion has significantly greater abundance in the L/D-iso-Asp containing peptides than the L/D-Asp containing peptides. This indicates that the isomerization of Asp might facilitate, or stabilize, the formation of MH-NH_3, even though it is located at the N-terminus. It is likely that a salt bridge forms between the alpha-Asp and Arg residue, which inhibits the Arg from forming a ring with the peptide backbone.

The iso-Asp containing epimers and/or isomers from Group B peptides yield characteristic b_1+H_2O ions. The mechanism for this ion is facilitated by a basic residue adjacent to an isomerized Asp, which can form an internal rearrangement. Note, in group B peptides the iso-Asp is located between a His and Ser residue (Table 5-1). This fragment ion is sterically unfavorable for an alpha-Asp residue as its product would contain a three membered ring. Thus, making this fragment unique to iso-Asp containing peptides. Further, the MH-H_2O^{+2} and b_2^{+2}-H_2O ions are also characteristic peaks for the modified peptides. A water loss is common in Ser-containing peptides. The peptide backbone attacks the Ser side chain to form a ring, resulting in the loss of water. The iso-Asp containing peptides have significantly larger water loss fragments for the MH^{+2} ion, suggesting that the addition of an extra carbon in the peptide backbone may make this ring formation (and loss of water) sterically more favorable. However, this is not a plausible explanation for the b_2^{+2} water

loss, as this ion does not contain a Ser residue. Another possible mechanism for the water loss could be the formation of a succinimide. It is possible for all Asp and iso-Asp residues to form this intermediate, and the ion abundance could be facilitated by the sterics of the neighboring Ser residue. Instead of a water loss from Ser, both the b_2^{+2} and the MH^{+2} ion water losses could actually be the formation of a succinimide. For group B peptides, when a peptide fragments into ions with cleavage locations near an amino acid that has racemized and/or isomerized, there will be larger variability in fragment ion abundances between peptide epimers and/or isomers.

Group C peptides lack unique fragment ion peaks and abundancies which is unlike group B, which has also an Asp and Ser residues and has many unique fragments between peptide epimers and/or isomers. Fragment ions for all peptides in Group C, including the epimers and/or isomers, are similar in mass and intensity, except the y_6 fragment ion (Figure 5-6). This fragment is most abundant in the iso-Asp containing peptide species and indicates cleavage near at the C-terminal side of the Asp residue.

The ionizability and fragmentation of each group of peptides (i.e. A, B and C in Table 5-1) are influenced by the location and type of epimeric and/or isomeric modification. Within each group of peptides, all epimers and/or isomers had the same “most abundant fragment ions”, and similar fragment ions for the next most abundant species. However, the signal responses indicated by SIM Figures 5-1 to 5-3, shows that these peptides have varying ionization in SIM which can affect the precursor fragment abundance when analyzed by SRM, thereby decreasing the sensitivity of epimers and/or isomers when analyzed using instrument conditions that have been optimized to enhance the instrument response for the native all-L peptide.

Another factor affecting sensitivity is the type of epimeric and/or isomeric modification and the location of the modification. In all cases, when the fragment ion was

indicative of peptide cleavage near an iso-Asp modified amino acid, the instrument response increased for the iso-Asp modified peptides in comparison to the native all-L peptides. This is especially apparent for the b_1+H_2O fragment ion for the group B peptides in which only the iso-Asp modified peptides have significant instrumental responses. In some cases, the instrument response also increased for the D-Asp and D-Ser modified species as well. One other study documented the instrumental response of L-Asp peptides modified to D-Asp, L-iso-Asp, and D-iso-Asp and found several unique fragments for the D-iso-Asp modified peptides.[119] However, peptide fragment ions that occurred several amino acids away from the modified amino acids had less variability in instrument response amongst all peptide epimers and/or isomers. Thus, in order to best differentiate peptide epimers and/or isomers from each other, it is best to analyze them using fragment product ions indicative of cleavage sites near the peptide modification. However, for quantification studies, it may be best to quantify peptides using fragment ions with peptide cleavage sites furthest from the modified site, as there is less variance in instrument response between peptide epimers and/or isomers.

Conclusions

In this study we have shown that peptide epimers and/or isomers from tryptic digested Aβ 1-42 have unique ionizations and fragmentations and contribute to varying instrumental responses when analyzed with MS/MS. Generally, peptides that contained iso-Asp groups had unique fragment ions: group B peptides had three unique fragment ions while group A peptides had one unique fragment ion. However, in many cases a contributing factor to the instrumental sensitivity differences between the peptide epimers and/or isomers is the peptide ionizability, of which both Groups A and B had the most variability. These factors highlight the problem of quantifying peptide epimers and/or isomers by MS/MS without correcting for sensitivity differences.

Chapter 6

General Summary

Chapter 2 describes the first comprehensive analytical platform for the separation of all 20 possible amyloid-beta peptide epimers containing L/D-Asp, L/D-iso-Asp and L/D-Ser isomers using HPLC-MS/MS. Isomerization and racemization of Asp and Ser residues have been reported in amyloid-beta peptides from Alzheimer's patients, which was suggested to contribute to Alzheimer's disease. The 20 possible epimeric peptides are divided into three fragment groups, which can be chromatographically separated using modified Q-Shell and NicoShell chiral stationary phases. All possible modifications can be determined simultaneously using this simple and high-throughput analytical method to determine their presence in the brain of Alzheimer's patients.

Chapter 3 describes the first separation method of aberrant amyloid-beta 1-42 peptide epimers containing L/D-Asp, L/D-iso-Asp and L/D-Ser isomers using HPLC-MS/MS. In total, 14 epimeric and/or isomeric peptides are analyzed. This method is also the most comprehensive separation method for amyloid-beta 1-38, 1-40, and 1-42, which all three variations are present in elevated amounts in patients with Alzheimer's Disease. All chromatographic separations are achieved using a reversed phase C4 stationary phase. In addition, U-shaped retention curves were established for all peptides analyzed in this study. The U-shaped curves show that the retention factor for each epimeric and/or isomeric peptide is unique at different acetonitirile:water concentrations in the mobile phase. This ultimately indicates that secondary structure of each epimeric and/or isomeric peptide must be unique. This analytical method is an excellent tool to quickly identify whether or not a sample matrix has aberrant amyloid-beta peptide species.

Chapter 4 shows the impact of antibody antigen binding affinities for amyloid-beta peptides to antibodies targeting various epitope regions of these peptides. Four antibodies with varying epitope binding regions were screened against 18 aberrant amyloid-beta peptides. Their binding affinities were compared to an all-L unmodified amyloid-beta 1-42. This is the first study that shows how isomerized and/or epimerized peptides affect binding abilities of antibodies and antigens, and how the location of these aberrations affect binding efficacy. Calibration curves for each aberrant peptide were created and it was found that the sensitivity for each epimeric and/or isomeric peptide was dependent on a combination of the type of modification and the location of the modification. It was determined that if these MS/MS responses are not accounted for then the binding affinities are not adequately represented. The MS/MS results further explore these affects and are described in Chapters 5 and 6.

Chapter 5 explores the impact of epimerization and/or isomerization on MS/MS sensitivity when using the same instrument conditions, which have been optimized for an all-L, unmodified peptide. Chapter 5 investigates three groups of fragments from amyloid-beta 1-42 and subsequent epimeric and/or isomeric peptides. In Chapter 5 it was found that a combination of ionization and unique fragmentation in the second quadrupole contributed to the MS/MS sensitivity for each epimeric and/or isomeric peptide analyzed. In most cases, the all-L, wild-type amyloid beta peptide had the most sensitivity using conditions optimized for its conformation.

This dissertation focuses on two chromatographic techniques for the analysis of amyloid-beta peptides and its epimers and/or isomers using HPLC-MS/MS. These established analytical methods have efficiently separated L/D-Asp, L/D-iso-Asp, and L/D-Ser containing peptide epimers and/or isomers with good sensitivity and accuracy and can be used for the future study of amyloid-beta peptides extracted from Alzheimer's Disease

brain samples. We have also explored the binding efficacy of antibodies targeting amyloid-beta and how the binding ability is affected by aberrations. Finally, we noticed the MS/MS sensitivity was highly variable between peptide epimers and/or isomers and must be accounted for when quantitating aberrant species of amyloid-beta peptides. In the future, we will examine brain tissues and plasma samples from Alzheimer's patients to characterize the amyloid-beta peptides, and anticipate that this will contribute to a better understanding of the etiology of the disease.

Appendix A

Publication Information and Contributing Authors

Chapter 2. A manuscript published in Chemical Communication. Siqi Du, Elizabeth R. Readel, Michael Wey, Daniel W. Armstrong, 2020, 56, 1537-1540. DOI: 10.1039/c9cc09080k.

Chapter 3. A manuscript published in Analytica Chimica Acta. Elizabeth R. Readel, Michael Wey, Daniel W. Armstrong, 2021, 1163:338506. DOI: 10.1016/j.aca.2021.338506.

Chapter 4. A manuscript submitted to Scientific Reports. Elizabeth R. Readel, Arzoo Patel, Joshua I. Putman, Siqi Du, Daniel W. Armstrong, 2023.

Chapter 5. A manuscript to be submitted soon. Elizabeth R. Readel, Umang Dhaubhadel, Arzoo Patel, Daniel W. Armstrong, 2023.

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