Comparative studies for amyloid beta degradation: “Neprilysin vs insulysin”, “monomeric vs aggregate”, and “whole Aβ40 vs its peptide fragments”

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ABSTRACT

Amyloid beta (Aβ) proteins are produced from amyloid precursor protein cleaved by β- and γ-secretases, and are the main components of senile plaques pathologically found in Alzheimer’s disease (AD) patient brains. Therefore, the relationship between AD and Aβ has been well studied for both therapeutic and diagnostic purposes. Several enzymes have been reported to degrade Aβs in vivo, with neprilysin (NEP) and insulysin (insulin-degrading enzyme, IDE) being the most prominent. In this article, we describe the mass spectrometric characterization of peptide fragments generated using NEP and IDE, and clarify the differences in digestion specificities between these two enzymes for non-aggregated Aβ40, aggregated Aβ40, and Aβ40 peptide fragments, including Aβ16. Our results allowed identification of all the peptide fragments from non-aggregated Aβ40 NEP, 23 peptide fragments consisting of 2–11 amino-acid residues, 17 cleavage sites; IDE, 23 peptide fragments consisting of 6–33 amino-acid residues, 15 cleavage sites. Also, we confirmed that IDE can digest only whole Aβ40, whereas NEP can digest both Aβ40 and partial structures such as Aβ16 and peptide fragments generated by the digestion of Aβ40 by IDE. Furthermore, we confirmed that IDE and NEP are unable to digest aggregated Aβ40.

1. Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disease that causes memory and cognitive impairment and accounts for about 50–56% of dementia cases worldwide [1,2]. The pathological features of AD include senile plaques and neurofibrillary tangles consisting of aggregated amyloid β (Aβ) proteins [1,2] and hyperphosphorylated tau proteins [3], respectively. Of these, the deposition of Aβ is recognized as the central event in the etiology of AD because it is the primary event in the disease process [4].

Aβs are produced by cleavage of an integral membrane amyloid precursor protein (APP) at the N-terminus by β-secretase, followed by cleavage at the C-terminus by γ-secretase [5]. Variations in the position cleaved by γ-secretase generates several homologs with different lengths. Aβ40 and Aβ42 are the major homologs generated and are 40 and 42 amino-acid residues long, respectively. Aβ monomers can easily aggregate under physiological conditions to form fibrils via soluble oligomers [6]. Neurotoxicity of the soluble oligomers [7] and synaptic plasticity/memory impairment caused by the dimer [8] have also been reported.

Almost twenty enzymes are known to contribute to Aβ degradation, including neprilysin (NEP), insulysin (insulin-degrading enzyme, IDE), angiotensin-converting enzyme, and cathepsin B. The therapeutic utility of these enzymes in AD has been studied [9–12], including extensive studies of NEP and IDE in AD patients [13,14]. Furthermore, fluorescence assays for quantifying the enzyme activities of NEP and IDE have also been reported [15].

NEP is a zinc-dependent metalloprotease present in, for example, the brain, heart, peripheral vasculature, adrenal gland and lungs. NEP degrades not only Aβ but also a variety of bioactive peptides such as angiotensins and enkephalins [16]. In control mice, infusion of an NEP inhibitor (thiorphan) has been reported to induce Aβ40 and Aβ42 deposition and fibrillization [17], and in APP transgenic mice, the
overexpression of NPY has been reported to reduce senile plaques [18].

IDE is also a zinc-dependent metalloprotease that is present in the liver, kidney, and brain [19] and degrades not only insulin and glucagon, but also Aβ to regulate Aβ in vivo [20]. In IDE-deficient mice, the degradation of Aβ was reduced by more than 50% concomitantly with the increase in the amount of Aβ in the brain [20], suggesting that IDE may be a link between AD and type 2 diabetes mellitus (which is a risk factor for AD) [21]. Therefore, Aβ-degrading enzymes such as NEP and IDE are recognized as key enzymes for AD therapy and as links between AD and its risk factors.

Generally, there are two major diagnostic strategies for AD: PET imaging using Pittsburgh Compound B (2-(4-aminophenyl)-6-hydroxybenzothiazole) for Aβ plaques and neurofibrillary tangles in brain [22], and the quantification of Aβ40, Aβ42, and tau in cerebrospinal fluid (CSF) using an immunosensor assay or liquid chromatography (LC)-mass spectrometry (MS) [23]. However, there are few reported attempts to analyze specific Aβ peptide fragments generated by degrading enzymes, such as the N-terminal [24] and C-terminal [25] Aβ peptide fragments in CSF. Therefore, the potential use of specific Aβ peptide fragments generated by Aβ-degrading enzymes as clearance markers requires clarification of their detailed specificities, including details of the cleavage sites, the effect of combining several degrading enzymes, and the activities of the various components involved in the aggregation process (monomer, oligomer, and fibril).

In this article, we describe comparative studies on Aβ degradation conducted using two degrading enzymes (NEP vs. IDE), various sizes of Aβ (whole Aβ40 vs. its peptide fragments), and two aggregation states of Aβ (non-aggregated Aβ40 vs. aggregated Aβ40).

2. Materials and methods

2.1. Reagents and materials

Specific reagents and materials were purchased as follows: Aβ40 (human, DAEFRHDSGY EVHHQKLFF AEDVGSNKGA IGLMLVGGVV) and Aβ16 (human, DAEFRHDSGY EVHHQKLFF) (Anaspec, Inc., Fremont, CA, USA); NEP (human recombinant, solution in Tris, NaCl and ZnCl2) (R&D Systems, Inc., Minneapolis, MN, USA); IDE (human recombinant, solution in Tris and NaCl) (Bon Opus Biosciences, LLC., Millburn, NJ, USA); sequencing grade modified trypsin (Promega Co., Madison, WI, USA); acetonitrile (MeCN), formic acid (FA), and tetrahydrofuran (THF) (Sigma-Aldrich, Inc., St. Louis, MO, USA); 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); OptiPlate-384, White Opake 384-well MicroPlate (384 well plate) (PerkinElmer, Waltham, MA, USA); AMPLISeal (plate seal) (Greiner Bio-One, Baden-Württemberg, Germany); and Protein LoBind® tubes, 0.5 mL (Eppendorf, Hamburg, Germany). All other general chemicals, vials, and gases were of the highest grade available and were obtained from local providers.

2.2. Reagent solutions

All stock solutions were stored in Protein LoBind® tubes to minimize the adsorption of Aβs.

Preparation and storage of Aβ40 (0.25 mM) solution: Lyophilized Aβ40 in its original vial was dissolved and monomerized in a corresponding amount of HFIP [26] to give a 1 mg/mL solution. This solution was sonicated for 8 min, incubated for 1 h, re-sonicated for 8 min at room temperature (r.t.), and evaporated to dryness under a N2 stream. The residue was dissolved in a corresponding amount of 0.1% (v/v) aq. NH4OH/MeCN (4:1, v/v) [27] to give a 0.25 mM solution. After 12 min sonication, the solution was divided into aliquots (30 μL) and stored at –80 °C. Prior to use, the solutions were thawed, mixed by pipetting repeatedly (10 μL × 10), then an aliquot was transferred into a tube (0.5 mL) and evaporated under a N2 stream. The residue was re-dissolved in H2O/MeCN (4:1, v/v) by sonication for 3 min to give a 0.25 mM solution.

Preparation and storage of Aβ16 (0.55 mM) solution: Lyophilized Aβ16 in its original vial was dissolved in a corresponding amount of H2O/MeCN (4:1, v/v) and sonicated for 12 min to give a 0.55 mM solution. The solution was divided into aliquots (30 μL) and stored at –80 °C. Prior to use, the solution was thawed and used as-is.

Preparation and storage of ThT (0.125 mM) solution: ThT was dissolved in a corresponding amount of H2O/MeCN (4:1, v/v) to give a 0.125 mM solution. The solution was divided into aliquots (50 μL) and stored at –30 °C. Prior to use, the solution was thawed and diluted with PBS to give a 0.125 mM solution.

Preparation and storage of NEP solution: A commercially provided solution of NEP (0.44 μg/μL, 22.7 μL) was divided into aliquots (2.27 μL) and stored as stock solutions at –30 °C. Prior to use, the solution was thawed and diluted with 25 mM Tris-HCl buffer (containing 200 mM NaCl and 5 μM ZnCl2) to give a 0.1 μg/μL solution. This was further diluted with 10 mM PBS to give a 14 ng/μL solution.

Preparation and storage of IDE solution: A commercially provided solution of IDE (0.6 μg/μL, 16.7 μL) was diluted with 20 mM Tris-HCl buffer (containing 150 mM NaCl) to give a 0.1 μg/μL solution.
solution was divided into aliquots (10 μL) and stored as stock solutions at −80 °C. Prior to use, the solution was thawed and diluted with 10 mM PBS to give a 14 ng/μL solution.

Preparation and storage of trypsin solution: Lyophilized trypsin (20 μg) was dissolved in the supplied trypsin resuspension buffer (50 mM aq. acetic acid) to give a 0.1 mg/μL solution. The solution was divided into aliquots (10 μL) and stored as stock solutions at −80 °C. Prior to use, the solution was thawed and used as-is.

Control solution to monitor Aβ aggregation: A mixture of Aβ40 (0.25 mM, 15 μL), PBS (200 μL) and ThT (0.125 mM, 40 μL) was vortex-mixed for 30 s. The solution was mixed by repeated pipetting (75 μL × 5), transferred to 3 wells (75 μL each) of a 384 well plate, and covered with a plate seal for use in ThT assays [28]. The blank sample was prepared with H2O/MeCN (4:1, v/v, 10 μL) instead of Aβ40 (0.25 mM, 10 μL) and transferred to 3 wells (75 μL each).

2.3. Proteolysis of Aβ40 or Aβ16

All the reactions were performed in Protein LoBind® tubes except for the ThT assay. Digestion studies were performed using a ratio of Aβ (substrate):enzyme = 40:1 (w/w).

Digestion of non-aggregated Aβ40 by NEP or IDE: A mixture of Aβ40 (0.25 mM, 15 μL), PBS (105 μL), and NEP or IDE (14 ng/μL 30 μL) was incubated at 37 °C for 1, 3, and 7 days.

Digestion of non-aggregated Aβ40 by both NEP and IDE: A mixture of Aβ40 (0.25 mM, 35 μL), PBS (105 μL), NEP (14 ng/μL, 15 μL) and IDE (14 ng/μL, 15 μL) was incubated at 37 °C for 3 and 7 days.

Digestion of non-aggregated Aβ40 by IDE followed by NEP: A mixture of Aβ40 (0.25 mM, 30 μL), PBS (210 μL) and IDE (14 ng/μL, 60 μL) was incubated at 37 °C for 7 days. An aliquot of the solution (100 μL) and NEP (14 ng/μL, 20 μL) was mixed and incubated at 37 °C for 7 days. As a control, an aliquot of the solution (100 μL) and PBS (20 μL) was incubated at 37 °C for 3 days.

Digestion of Aβ40 by NEP or IDE: A mixture of Aβ40 (0.55 mM, 10 μL), PBS (70 μL), and NEP or IDE (14 ng/μL, 20 μL) was incubated at 37 °C for 3 days.

Digestion of aggregated Aβ40 by IDE, NEP, or trypsin: A mixture of Aβ40 (0.25 mM, 20 μL) and PBS (480 μL) was vortex-mixed for 30 s. The solution was mixed by repeated pipetting (75 μL × 5), transferred to wells (75 μL each) of a 384 well plate, and covered with a plate seal. After incubation at 37 °C for 24 h in a Gemini XPS microplate spectrofluorometer (Molecular Devices, LLC, San Jose, CA, USA), IDE, NEP, or trypsin (0.1 μg/μL, 0.81 μL) was added and the mixture was incubated at 37 °C for 3 and 7 days. Aggregation was confirmed by the ThT assay using the above control solution to monitor Aβ40 aggregation.

2.4. Conditions for the ThT assay

A Gemini XPS microplate spectrofluorometer was used in kinetic mode with the following parameters: temperature, 37 °C; read mode, top read; wavelength, ex. 456, em. 489 nm; sensitivity, 12 readings PMT sensitivity Medium; run time, 24 h; interval, 15 min; automix, before first read, 15 s; between reads, 300 s; autocalibrate, on; assay plate type, 384 well standard clrbtm; column priority; carriage speed, normal; and auto read, off. SoftMax Pro Software (version 5.4.1, Molecular Devices) was used for data analysis. The data were organized using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) to calculate the average, relative error. The fluorescence intensities were used after subtracting the corresponding controls.

2.5. LC conditions

An Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a 1100 G1312A binary pump, 1100 G1379A degasser, 1100 G1367A autosampler, 1100 G1316A column heater, and a 1100 G1315B photodiode array was used for LC systems 1–4 with the following common chromatographic conditions: Mobile phases (A) 0.1% (v/v) FA in H2O, (B) 0.1% (v/v) FA in MeCN; flow rate 0.2 mL/min; and column temperature, 40 °C.

LC system 1 (for hydrophobic peptide fragments from non-aggregated Aβ40): A Cosmosil® 5C18-AR-II (octadeylsilyl, ODS) column (150 × 2.0 mm i.d., 5 μm; Nacalai Tesque, Inc.) was used with the following linear gradient: 0 min, 0%; B: 140 min, 42%; B: 141 min, 65%; B: 155 min, 65%; B: 165 min, 45%; B: 166 min, 20%; B: 175 min, 45%; B: 176 min, 20%; B: 185 min, 45%; B: 186 min, 20%; B: 195 min, 45%; B: 196 min, 20%; B: 205 min, 45%; B: 206 min, 20%; B: 215 min, 45%; B: 216 min, 0%; B: and 240 min, 0%. An aliquot of the solution (20 μL) was injected into the system. The eluate obtained between 3–140 min was introduced into the MS system.

LC system 2 (for polar peptide fragments from non-aggregated Aβ40 and Aβ16): A Hypercarb® porous graphitic carbon (PGC) column [29] (100 × 2.1 mm i.d., 5 μm; Thermo Fischer Scientific Inc., Waltham, MA, USA) was used with the following linear gradient: 0 min, 0%; B: 100 min, 50%; B: 101 min, 65%; B: 115 min, 65%; B: 116 min, 0%; B: and 140 min, 0%. An aliquot of the solution (20 μL) was injected into the system. The eluate obtained between 3–100 min was introduced into the MS system.

LC system 3 (for hydrophobic peptide fragments from Aβ16): A Cosmosil® 5C18-AR-II column (150 × 2.0 mm i.d., 5 μm, 120 Å; Nacalai Tesque, Inc.) was used with the following linear gradient: 0 min, 0%; B: 140 min, 42%; B: 141 min, 65%; B: 155 min, 65%; B: 156 min, 0%; and 180 min, 0%. An aliquot of the solution (15 μL) was injected into the system. The eluate obtained between 3–140 min was introduced into the MS system.

LC system 4 (for hydrophobic peptide fragments from aggregated Aβ40): A Cosmosil® 5C18-AR-II column (150 × 2.0 mm i.d., 5 μm, 120 Å; Nacalai Tesque, Inc.) was used with the following linear gradient: 0 min, 0%; B: 140 min, 42%; B: 141 min, 65%; B: 155 min, 65%; B: 156 min, 20%; B: 165 min, 45%; B: 166 min, 20%; B: 175 min, 45%; B: 176 min, 20%; B: 185 min, 45%; B: 186 min, 20%; B: 195 min, 45%; B: 196 min, 20%; B: 205 min, 45%; B: 206 min, 20%; B: 215 min, 45%; B: 216 min, 0%; B: and 240 min, 0%. THF (50 μL) was injected tenth at 4-min intervals from 160 min to prevent carryover. An aliquot of the solution (30 μL) was injected into the system. The eluate obtained between 3–140 min was introduced into the MS system.

2.6. MS conditions

An LCQ-DECA ion-trap mass spectrometer (Thermo Fischer Scientific Inc.) equipped with an electrospray ionization (ESI) source was used in positive ion mode with the following parameters: spray voltage, 4.5 kV; capillary temperature, 300 °C; sheath gas flow rate, 85.0 arb; and auxiliary gas flow rate, 15.0 arb. The parameters for data-dependent MS/MS were as follows: Full scan range, m/z 100–2000 (for LC systems 1, 3, and 4) or 100–1000 (for LC system 2); precursor, top 3 ions; default charge state, 2; default isolation width, 2; normalized collision energy (CE), 45%; activation Q, 0.25; activation time, 30 ms; minimum MS signal required, 100,000; and minimum MS2 signal required, 5000. Xcalibur™ (version 2.0 SR2) was used for the data analyses.

2.7. Criteria for identification of peptide fragments

Proteome Discoverer (version 1. 3) (Thermo Fischer Scientific Inc.) was used to identify peptide fragments using the following parameters: Minimum precursor mass, 100 Da; maximum precursor mass, 5000 Da; enzyme, no-enzyme (unspecific); precursor mass tolerance, 2 Da;
fragment mass tolerance, 0.8 Da; dynamic modification, oxidation (methionine); target false discovery rate (strict), 0.01; and target false discovery rate (relaxed), 0.05. All the identified peptide fragments were confirmed by checking the MS/MS spectra.

3. Results and discussion

3.1. General experimental setting

Aβs are challenging proteins to handle and analyze because they tend to adsorb and aggregate, and their concentration often changes because they adsorb on tubes and proteins [30]. Furthermore, significant carryover from previous injections occurs during HPLC analyses [31]. Therefore, Aβ40 was initially monomerized using HFIP [26] and stored as a 0.1% (v/v) aq. NH4OH/MeCN (4:1, v/v) solution [27]. The ODS column was washed using a quick zigzag gradient alternating between 20% B and 45% B before column equilibration (LC systems 1 and 4). Multiple injections of THF were made for LC system 4 (for aggregated Aβ40). Short peptides are generally too polar (hydrophilic) to be retained on versatile ODS columns. Therefore, we concomitantly used a PGC column, which is good for hydrophilic peptides [29], to cover all the peptide fragments.

3.2. Non-aggregated Aβ40 incubated with NEP

Aβ40 was digested into 23 peptide fragments (2–11 amino-acid residues, 17 cleavage sites) (Fig. 1) without residual intact Aβ40 after 3 days. Interestingly, the intensity patterns of several peptide peaks changed during prolonged incubation up to 7 days (Fig. 1): the peak corresponding to F20AEDVGSNK30 (m/z 1094.43, retention time (tR) = 28.30 min on ODS) decreased as the intensities of the peaks corresponding to F20AEDVGSNK30 (m/z 966.33, tR = 25.74 min on ODS) and F20AEDVG25 (m/z 637.12, tR = 33.65 min on ODS) increased, suggesting that long peptide fragments can be further digested by NEP. Each amino acid sequence and its tR identified by LC-MS with LC systems 1 and 2 is summarized in Table 1.

3.3. Non-aggregated Aβ40 incubated with IDE

Aβ40 was digested into 23 peptide fragments. The intensity of each peak gradually increased in a time-dependent manner on the decrease in residual Aβ40. However, some Aβ40 remained even after 7 days, suggesting that IDE is less active than NEP in degrading Aβ40 in our experimental setting (Fig. 2). The peptide fragments tended to be longer (5–33 amino-acid residues, 15 cleavage sites), and most of the peptide fragments were longer than 12 amino-acid residues, except for G20AIIG35, M25VGGV39, and L34MVGGV40. In contrast, the longest peptide fragment obtained using NEP consisted of 11 amino-acid residues. Each amino acid sequence and its tR identified by LC-MS with LC systems 1 and 2 is summarized in Table 2.

3.4. Non-aggregated Aβ40 incubated with both NEP and IDE

Since multiple proteases are involved in Aβ elimination in vivo, Aβ40 was incubated with both NEP and IDE (Fig. 3). The total ion current chromatogram (TICC) and the identified peptide fragments were almost identical to those obtained using NEP only (Fig. 1). In addition, the long peptide fragments listed in Table 2 (12~ amino-acid residues) were not detected, similar to the results obtained by digestion with both NEP and IDE (Fig. 3), confirming that the long fragment peptide obtained using IDE were

3.5. Non-aggregated Aβ40 incubated with IDE followed by NEP

Since the experiment above suggested that the long fragment peptide obtained using IDE could be further digested by NEP, Aβ40 was incubated with IDE, followed by digestion using NEP. The TICC pattern of the peptide fragments (Fig. 4) was identical to the one obtained using NEP only (Fig. 1). In addition, the long peptide fragments obtained by digestion with IDE (12~ amino acid residues) were not detected, similar to the results obtained by digestion with both NEP and IDE (Fig. 3), confirming that the long fragment peptides obtained using IDE were
**Table 1**

Peptide fragments found from non-aggregated Aβ40 incubated with NEP.

| Sequence number | Sequence   | Charge | m/z     | $t_R$ (min) | LC     |
|-----------------|------------|--------|---------|-------------|--------|
| 12 - 17         | VHHQKL     | 1      | 761.37  | 3.70        |        |
| 4 - 9           | FRDOSG     | 1      | 718.33  | 4.29        |        |
| 10 - 11         | YE         | 1      | 311.02  | 5.21        |        |
| 39 - 40         | VV         | 1      | 216.95  | 6.03        |        |
| 21 - 29         | AEDVGSNKG  | 1      | 876.27  | 10.44       |        |
| 31 - 33         | TIG        | 1      | 301.97  | 17.54       |        |
| 20 - 22         | FAE        | 1      | 365.93  | 18.68       |        |
| 34 - 35         | LM         | 1      | 262.99  | 19.13       |        |
| 10 - 17         | YEHHQKL    | 4      | 264.98  | 23.45       |        |
| 20 - 28         | FAEDVGSNK  | 1      | 966.33  | 25.74       |        |
| 20 - 29         | FAEDVGSNKG | 1      | 1023.39 | 25.85       |        |
| 20 - 30         | FAEDVGSNKGA| 1      | 1094.43 | 28.30       |        |
| 34 - 38         | LMVGG      | 1      | 476.03  | 33.08       | ODS    |
| 20 - 25         | FAEDVG     | 1      | 637.12  | 33.65       | PGC    |
| 17 - 19         | LVF        | 1      | 378.00  | 51.90       |        |
| 31 - 34         | IGL        | 1      | 415.07  | 54.11       |        |
| 34 - 40         | LMVGGVV    | 1      | 674.24  | 68.28       |        |
| 39 - 40         | VV         | 1      | 217.00  | 5.44        |        |
| 36 - 38         | VGG        | 1      | 232.00  | 7.06        |        |
| 1 - 3           | DAE        | 1      | 333.99  | 14.59       |        |
| 34 - 35         | LM         | 1      | 263.02  | 15.19       |        |
| 31 - 33         | TIG        | 1      | 301.99  | 16.95       |        |
| 23 - 28         | DVGSDK     | 1      | 619.51  | 19.55       |        |
| 23 - 29         | DVGSDK     | 1      | 676.31  | 24.47       |        |
| 18 - 19         | VF         | 1      | 265.08  | 25.83       |        |
| 21 - 29         | AEDVGSNK   | 1      | 876.35  | 28.38       |        |
| 34 - 38         | LMVGG      | 1      | 476.11  | 29.56       |        |
| 4 - 5           | FR         | 1      | 322.18  | 33.15       |        |
| 10 - 11         | YE         | 1      | 311.07  | 33.94       |        |
| 20 - 22         | FAE        | 1      | 365.97  | 38.29       |        |
| 31 - 34         | IGL        | 1      | 415.13  | 38.53       |        |
| 17 - 19         | LVF        | 1      | 378.02  | 41.31       |        |
| 20 - 28         | FAEDVGSNK  | 1      | 966.37  | 46.25       |        |
| 20 - 25         | FAEDVG     | 1      | 637.10  | 49.44       |        |

**Fig. 2.** TICCs of non-aggregated Aβ40 incubated with IDE for 1 day (top), 3 days (middle), and 7 days (bottom). (A) LC system 1 (ODS for hydrophobic peptides), (B) LC system 2 (PGC for polar peptides). Identified peptides are shown in Table 2, together with the charge state, precursor MS (m/z), and $t_R$ (min).
further digested by NEP. Each amino acid sequence and its identified by LC-MS with LC system 1 is summarized in Table 4.

### 3.6. Aβ16 incubated with NEP or IDE

To further compare the digestion patterns between NEP and IDE, a shorter Aβ40 homologue (Aβ16) was incubated with IDE or NEP. The amino acid sequence of Aβ16 contains 4 and 5 cleavage sites identified in experiments using Aβ40 digested by NEP (Table 1) and IDE (Table 2), respectively. The cleavage sites, shown as slashes, are: NEP, DAE/FR/HDSG/YE/VHHQK; IDE, D/AEFRHD/SGYEV/H/Q/K. Thus, Aβ16 was digested by NEP into the five peptides D1AE3, F4R5, H9DSG9, Y10E11, and V12HHQ16 (Fig. 5AB), and the cleavage sites were identical to those in Aβ40. In contrast, Aβ16 was not digested by IDE (Fig. 5CD). These results suggest that IDE digests Aβ40 by recognizing the whole structure, whereas NEP digests Aβ40 by recognizing the partial structure.

### 3.7. Aggregated Aβ40 incubated with NEP, IDE or trypsin

The Aβ sequence has an amphipathic character because the N-terminal segment is hydrophilic whereas the C-terminal segment is hydrophobic. Monomeric Aβ prefers to adopt random coil or α-helix

#### Table 2
Peptide fragments found from non-aggregated Aβ40 incubated with IDE.

| Sequence number | Sequence | Charge | m/z  | \( t_R \) (min) | LC |
|-----------------|---------|--------|------|----------------|-----|
| 2 - 14          | DAEFRIDSGYEVHII | 2      | 792.32 | 23.57          | ODS |
| 1 - 14          | DAEFRIDSGYEVHII | 2      | 850.04 | 27.25          |     |
| 1 - 13          | DAEFRIDSGYEVHII | 2      | 781.36 | 31.27          |     |
| 1 - 18          | DAEFRIDSGYEVHQQKLV | 3   | 723.21 | 35.99          |     |
| 35 - 40         | MVGGV      | 1      | 561.01 | 41.26          |     |
| 1 - 19          | DAEFRIDSGYEVHQQKLVF | 3 | 772.38 | 45.92          |     |
| 14 - 28         | HQKLVFPAEDVGSNK | 2      | 860.15 | 50.69          |     |
| 1 - 28          | DAEFRIDSGYEVHQQKLVFPAEDVGSNK | 3 | 1088.09 | 53.85          |     |
| 1 - 20          | DAEFRIDSGYEVHQQKLVF | 3    | 821.44 | 55.83          |     |
| 16 - 28         | KLVFFPAEDVGSNK | 2      | 727.43 | 56.64          |     |
| 34 - 40         | MVGGV      | 1      | 674.19 | 58.04          |     |
| 1 - 33          | DAEFRIDSGYEVHQQKLVFPAEDVGSNKGAIIG | 3 | 1225.24 | 62.50          |     |
| 17 - 28         | LVFFPAEDVGSNK | 1      | 1325.35 | 64.37          |     |
| 17 - 31         | LVFFPAEDVGSNK | 2      | 783.29 | 71.49          |     |
| 28 - 40         | KGATIGLMVGGV | 2      | 607.36 | 77.43          |     |
| 27 - 40         | KGATIGLMVGGV | 2      | 664.45 | 78.14          |     |
| 21 - 40         | AEVGSNGATIGLMVGGV | 2 | 943.40 | 83.34          |     |
| 20 - 40         | AEVGSNGATIGLMVGGV | 2 | 1017.42 | 87.25          |     |
| 14 - 40         | HQKLVFPAEDVGSNKGATIGLMVGGV | 3 | 929.37 | 88.29          |     |
| 29 - 40         | GATIGLMVGGV | 1      | 1085.38 | 90.13          |     |
| 19 - 40         | FPAEDVGSNKGATIGLMVGGV | 2 | 1090.75 | 93.61          |     |
| 8 - 31          | SYEVHQQKLVFPAEDVGSNKGAT | 2 | 1316.89 | 106.11         |     |
| 29 - 33         | GAIIG      | 1      | 430.00 | 24.24          | PGC |
| 35 - 40         | MVGGV      | 1      | 561.09 | 34.41          |     |
| 34 - 40         | MVGGV      | 1      | 674.19 | 43.83          |     |

![Fig. 3. TICCs of non-aggregated Aβ40 incubated with both NEP and IDE for 3 days (top) and 7 days (bottom). (A) LC system 1 (ODS for hydrophobic peptides), (B) LC system 2 (PGC for polar peptides). Identified peptides are shown in Table 3, together with the charge state, precursor MS (m/z), and \( t_R \) (min).](image-url)
Table 3
Peptide fragments found from non-aggregated Aβ40 incubated with both NEP and IDE.

| Sequence number | Sequence | Charge | m/z  | t_R (min) |
|-----------------|----------|--------|------|-----------|
| 12 - 17         | VHHQL    | 1      | 761.02 | 3.69      |
| 4 - 9           | FRHDG    | 1      | 718.00 | 4.28      |
| 10 - 11         | YE       | 1      | 310.75 | 5.18      |
| 39 - 40         | VV       | 1      | 216.67 | 6.04      |
| 21 - 28         | AEDVSNK  | 2      | 410.02 | 8.35      |
| 21 - 29         | AEDVSNK  | 2      | 438.53 | 10.79     |
| 35 - 38         | MVGG     | 1      | 362.69 | 14.15     |
| 31 - 33         | IIIG     | 1      | 301.66 | 18.28     |
| 20 - 22         | FAE      | 1      | 365.69 | 19.53     |
| 34 - 35         | LM       | 1      | 262.69 | 19.80     |
| 10 - 17         | YEVHQL   | 4      | 264.66 | 24.17     |
| 20 - 28         | FAEDVSNK | 2      | 483.66 | 26.36     |
| 20 - 29         | FAEDVSNK | 2      | 512.20 | 26.55     |
| 20 - 30         | FAEDVSNK | 2      | 547.70 | 29.00     |
| 34 - 38         | LMVGG    | 1      | 475.71 | 33.50     |
| 20 - 25         | FAEDVG   | 1      | 636.78 | 34.26     |
| 17 - 19         | LVF      | 1      | 377.68 | 52.14     |
| 31 - 34         | IIGL     | 1      | 414.70 | 54.25     |
| 18 - 20         | VFF      | 1      | 411.71 | 57.15     |
| 34 - 40         | LMVGGV   | 1      | 673.88 | 68.11     |
| 39 - 40         | VV       | 1      | 216.74 | 5.52      |
| 36 - 38         | VGG      | 1      | 231.68 | 7.10      |
| 1 - 3           | DAE      | 1      | 333.68 | 14.30     |
| 34 - 35         | LM       | 1      | 262.76 | 15.03     |
| 31 - 33         | IIIG     | 1      | 301.73 | 16.85     |
| 35 - 38         | MVGG     | 1      | 362.71 | 18.54     |
| 21 - 25         | AEDV     | 1      | 489.71 | 22.40     |
| 18 - 19         | VF       | 1      | 264.75 | 25.59     |
| 21 - 29         | AEDVSNK  | 2      | 438.47 | 28.27     |
| 34 - 38         | LMVGG    | 1      | 475.77 | 29.29     |
| 10 - 11         | YE       | 1      | 310.74 | 33.29     |
| 20 - 22         | FAE      | 1      | 365.68 | 37.74     |
| 31 - 34         | IIGL     | 1      | 414.76 | 38.35     |
| 17 - 19         | LVF      | 1      | 377.02 | 41.01     |
| 20 - 28         | FAEDVSNK | 2      | 483.69 | 46.67     |
| 20 - 29         | FAEDVSNK | 2      | 512.01 | 48.62     |
| 20 - 25         | FAEDVG   | 1      | 636.85 | 48.93     |

Fig. 4. TICCs of non-aggregated Aβ40 incubated with IDE, followed by NEP. (A) Control (incubated with IDE only), (B) experiment (incubated with IDE, followed by NEP). Identified peptides are shown in Table 4, together with the charge state, precursor MS (m/z), and t_R (min).
structures, but gradually changes to a β-sheet structure during the aggregation process [6]. Aggregated Aβ40 was prepared by monitoring using the ThT assay, which is a β-sheet-specific fluorescence assay used as the "gold standard" for selectively identifying amyloid fibrils [28].

The fluorescence intensity was maximum after 24 h incubation and then remained essentially unchanged. After confirming aggregation, a control solution prepared without ThT (no ThT in the digestion samples) was mixed with NEP, IDE, or trypsin (positive control, cleavage at the C-terminus of R and K), and incubated at 37°C. Aggregated Aβ40 was not digested by either NEP or IDE by prolonged incubation up to 7 days (Fig. 6AB), suggesting that Aβ40 gained resistance against digestion by both NEP and IDE. In contrast, aggregated Aβ40 was digested by trypsin to form four peptide fragments: D1AEFR5, H6DSGYEVHHQK16, L17VFFAEDVGSNK28, and G29AIIGLMVGGVV40 (Fig. 6C).

### 4. Conclusion

In this article, we described the mass spectrometric characterization of the digestion specificities of NEP and IDE for non-aggregated Aβ40, aggregated Aβ40, and Aβ40 peptide fragments, including Aβ16. Howell et al. [32] and Leissring et al. [33] reported similar degradation studies but overlooked several polar peptide fragments and did not examine the relationship between NEP and IDE. We identified all the peptide fragments from non-aggregated Aβ40, as follows (Fig. 7): NEP, 23 peptide fragments consisting of 2–11 amino-acid residues (17 cleavage sites), and for IDE, 23 peptide fragments consisting of 6–33 amino-acid residues (15 cleavage sites). Our use of a PGC column [29] made it possible to retain polar peptide fragments and to identify novel cleavage sites: for example, a recent review suggested that Aβ40 is cleaved at only 10 and 5 sites by NEP and IDE, respectively [12]. Also, we confirmed that IDE can digest only whole Aβ40, in contrast with NEP, which can digest whole Aβ40, partial structures such as Aβ16, and peptide fragments generated.

### Table 4

Peptide fragments found from non-aggregated Aβ40 incubated with IDE followed by NEP.

| Sequence number | Sequence       | Charge | m/z   | t₁₀ (min) | LC  |
|-----------------|----------------|--------|-------|-----------|-----|
| 12 - 17         | VHQQKL         | 1      | 761.13| 3.62      | ODS |
| 4 - 9           | FRHDSG         | 1      | 718.18| 4.31      |     |
| 10 - 11         | Y2             | 1      | 310.84| 5.22      |     |
| 39 - 40         | VV             | 1      | 216.81| 6.04      |     |
| 21 - 28         | AEDVGSNK       | 1      | 819.11| 8.08      |     |
| 21 - 29         | AEDVGSNKX      | 2      | 438.50| 10.35     |     |
| 35 - 38         | MVGG           | 1      | 362.82| 13.55     |     |
| 31 - 33         | IIG            | 1      | 301.79| 17.76     |     |
| 20 - 22         | FAE            | 1      | 365.86| 18.96     |     |
| 34 - 35         | LM             | 1      | 262.79| 19.32     |     |
| 10 - 17         | YEVQQKL        | 4      | 264.83| 23.62     |     |
| 20 - 28         | FAEDVGSNK      | 2      | 483.66| 25.99     |     |
| 20 - 29         | FAEDVGSNKG     | 2      | 512.27| 26.30     |     |
| 20 - 30         | FAEDVGSNKX     | 2      | 547.76| 28.67     |     |
| 34 - 38         | LMVGG          | 1      | 475.90| 33.15     |     |
| 20 - 25         | FAEDVG         | 1      | 636.94| 33.90     |     |
| 19 - 28         | FFAEDVGSNK     | 2      | 557.35| 41.71     |     |
| 14 - 18         | HQKL           | 2      | 312.84| 42.63     |     |
| 17 - 19         | LVF            | 1      | 377.84| 51.95     |     |
| 31 - 34         | IIGL           | 1      | 414.90| 54.09     |     |
| 18 - 20         | VFF            | 1      | 411.84| 57.00     |     |
| 34 - 40         | LMMGTVV        | 1      | 674.01| 68.18     |     |

**Fig. 5.** TICCs of non-aggregated Aβ16 incubated with NEP (A and B) or IDE (C and D). LC system 3 (ODS for hydrophobic peptides) was used for (A) and (C). LC system 2 (PGC for polar peptides) was used for B and D.
from Aβ40 by IDE digestion. Furthermore, we confirmed that IDE and NEP cannot digest aggregated Aβ40, although trypsin can.

Several previously reported articles support our results. Shen et al. reported the detailed substrate recognition and catalytic mechanism of IDE by digesting insulin, Aβ40, amylin, and glucagon with IDE [34]. The N-terminal and C-terminal domains of IDE form a pocket to encapsulate substrates [34], and the change from the open conformation to the closed conformation results in digestion [35]. Therefore, large oligomers and fibril Aβ40 are too big to enter the catalytic site, in contrast to small monomeric Aβ40 in a random coil or α-helix conformation. This can also explain why APP is not digested by IDE [32]. It is interesting that several peptides containing the turn region (E22DVGS26) were found in non-aggregated Aβ40 digested by IDE: insulin contains a turn region that is the degradation target of IDE [34]. NEP utilizes a different catalytic mechanism because it is more flexible regarding substrate size. We found that NEP digested smaller peptide fragments generated from Aβ40, including Aβ16. Kanemitsu et al. [36] reported that NEP can degrade the dimer of Aβ40, and the dimer, trimer and tetramer of Aβ42. They did not examine the degradation of aggregated Aβ. However, these findings suggest that the flexibility of NEP regarding substrate size can cover not only Aβ peptide fragments but also small Aβ oligomers.

The therapeutic utility of Aβ-degrading enzymes in AD has been studied [9–12], and thus the resulting peptide fragments in plasma and cerebrospinal fluid should be analyzed to estimate their usefulness as Aβ clearance markers. Moreover, Kallikrein-related peptidase 7 [37] and synthetic peptides derived from the Box A region of Tob 1 protein [38] were recently reported to degrade even aggregated Aβ. Therefore, if there are specific Aβ peptide fragments derived from aggregated Aβ in senile plaques, they could be used as therapeutic markers.
The authors declare that there is no conflict of interest.

Data availability

Data will be made available on request.

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