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Short communication

Identification of peptide products from enzymatic degradation of amyloid beta

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ABSTRACT

Deposition of insoluble amyloid plaques is one of the known hallmarks of Alzheimer's disease. Amyloid beta 1-42 is the main component in these plaques, and the soluble oligomers of this peptide are believed to contribute to synaptic degradation and dementia. Enzymatic hydrolysis of amyloid beta is important to keep its tissue concentration low to avoid oligomerization. We have employed four enzymes involved in in vivo degradation of amyloid beta, to identify amyloid beta 1-42 hydrolysis products in vitro. Liquid chromatography coupled to (high resolution) mass spectrometry was used to identify the proteolysis products. Novel cleavage sites were discovered for all four enzymes. For each enzyme, the peptide was incubated for several different periods from 0.5 to 210 min, and the proteolysis products from each period were characterized. Thus, both the initial cleavage sites and the full degradation profiles were revealed. Knowledge about the fate of amyloid beta is important to better understand the mechanism underlying Alzheimer's disease, and the reported proteolysis products can be used as targets in future investigations on amyloid beta clearance.

1. Introduction

One of the characteristics of Alzheimer's disease (AD) is the deposition of amyloid plaques in the brain. Amyloid beta (Aβ) peptides are derived from cleavage of the trans-membrane amyloid precursor protein by β- and γ-secretase [1]. The concentration of Aβ in vivo is dependent on both the formation and elimination by several Aβ-degrading proteases [2]. While the formation of Aβ is relatively constant through life, a reduction in the rate of degradation has received an increased attention as an explanation for elevated levels of Aβ in the brain of patients suffering from AD [3]. High concentrations of amyloid beta induce a faster Aβ oligomerization, i.e. Aβ peptides tightly adhered into repeating beta sheets [4–6]. There is evidence suggesting that the soluble oligomers have a higher neurotoxicity than monomers and are responsible for synaptic degradation [7–9]. Several enzymes that are known to hydrolyze amyloid beta have also been shown to regulate the endogenous amyloid beta concentration, such as neprilysin (NEP) [10,11], endothelin converting enzyme (ECE)-1 [12,13] and insulin degrading enzyme (IDE) [14,15].

The Aβ degradation has been determined by sandwich enzyme-linked immunosorbent assay (ELISA) or western blot methods, in which the decline in $A\beta_{1-42}$ and/or $A\beta_{1-40}$ concentration over time is measured [14,16]. Although these methods monitor the overall change in intact peptide concentrations, the identification of the specific Aβ hydrolysis products from the different enzymes is important to understand the molecular pathogenesis of AD. Various peptide mapping experiments have been performed for several amyloid beta degrading enzymes including collecting fractions from liquid chromatography (LC) followed by off-line mass spectrometry (MS) [17–20] and LC-MS [21]. In addition, the digestion methods varies between enzymes with incubation from only 5 s [22] aiming only to reveal the initial cleavage sites to six hours for a full cleavage site identification [20].

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; Cat D, cathepsin D; ECE-1, endothelin converting enzyme; FA, formic acid; IDE, insulin degrading enzyme; LC, liquid chromatography; MS, mass spectrometry; NEP, neprilysin.

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In this study $A\beta_{1\text{-}42}$, the most neurotoxic of the Aβ species, was digested with four enzymes known to be involved in intracellular and extracellular Aβ degradation; ECE-1, IDE and Cathepsin D (Cat D), which are involved in intracellular degradation; and IDE and NEP, which is responsible for extracellular degradation (e.g. cerebrospinal fluid). The peptide hydrolysis products were identified by LC-MS. The enzymatic digestion products were characterized after different incubation times, thus both the initial sites of cleavage and a full overview of all peptides produced at longer digestion times were identified.

2. Materials and methods

2.1. Materials

Human $A\beta_{1\text{-}42}$ was purchased from Polypeptide group (Strasbourg, France), dissolved in dimethyl sulfoxide (DMSO) to a concentration of 4 mg/mL, aliquoted, and stored at −20 °C until use. Cat D (human liver, product number C8696, Sigma–Aldrich, Saint Louis, MO, USA) was dissolved in cold 100 mM formic acid (FA)/sodium formate buffer (pH 3.3) to a concentration of 6.25 U/mL, aliquoted, and stored at −80 °C until use. Aqueous solutions of 0.08 mg/mL NEP (human, recombinant, amino acid 53-750, catalog number BML-SE532-0010, Enzo Life Sciences, Lausen, Switzerland), 0.55 mg/mL IDE (rat, recombinant, amino acid 43-1019, catalog number I8601-04) and 0.50 mg/mL ECE-1 (mouse, recombinant, amino acid 89-769, catalog number E3650-02) both purchased from USBiological (MA, USA) were stored at −80 °C until use. In accordance with the manufacturers' recommendation, the recombinant enzymes were not purified prior to use, and negative control experiments were not conducted. $ZnCl_2$ (0.1 M), aqueous NH_3 25% (v/v), and LC-MS quality water and acetonitrile containing 0.1% FA was obtained from Sigma–Aldrich. For buffer preparation and pH adjustment, NaCl, HCl and MES hemisodium salt was purchased from Sigma–Aldrich while NaOH and tris(hydroxymethyl)aminomethane (tris) was obtained from Merck (Darmstadt, Germany).

2.2. Enzymatic digestion

$A\beta_{1\text{-}42}$ at a final concentration of 50 ng/μL was digested with each of the enzymes to determine the peptide degradation pattern. The enzyme to substrate ratio was 1:50 for Cat D and 1:25 for IDE, ECE-1, and NEP, and the total volume was 800 μL for all experiments. A MES buffer (50 mM MES, 0.1 M NaCl, pH 6.0) was used for ECE-1 hydrolysis, a tris buffer (50 mM tris·HCl, 0.1 M NaCl, pH 7.5) for IDE and a tris buffer (50 mM tris, pH 9.0) for NEP, and Cat D digestion was performed in a 100 mM sodium formate/FA buffer (pH 3.3), all according to manufacturer's recommendations. The incubations were conducted at 37 °C for various times (0.5, 10, 30, 60, 90 120, 150, 180 and 210 min). $ZnCl_2$ was added to IDE and ECE-1 digestions (54 and 73 pmol, respectively) to ensure high enzyme activity. After the desired time, the enzymatic reactions were quenched by the addition of aqueous NH_3 (25% v/v) ammonium hydroxide solution for Cat D and concentrated FA for ECE-1, IDE and NEP to a final concentration of 1% (v/v).

2.3. Liquid chromatography-mass spectrometry of $A\beta_{1\text{-}42}$ proteolysis products

All LC-MS equipment, including the columns, was obtained from Thermo Scientific (Waltham, MA, USA). The LC separation was performed with an Accela 1250 UHPLC pump and a PAL autosampler connected with a C4 Accucore column (2.1 mm inner diameter (i.d.) × 150 mm length (*L*), 2.6 μm particles) for peptides obtained from IDE, ECE-1 and NEP proteolysis, whereas a BioBasic C18 (2.1 mm i.d. × 50 mm *L*, 5 μm particles) was used for peptides proteolyzed with Cat D. A flow rate of 300 μL/min was used for all analyses and injection volume was set to 20 μL. A solvent gradient running from 1 to 45% acetonitrile in water with 0.1% formic acid (FA) in 20 min was applied for peptide separation on the Accucore column, and a methanol gradient running from 2 to 70% methanol water (0.1% FA) in 27.5 min was used for the BioBasic column. Solvent was diverted to waste for 0.5 and 2.5 min for the BioBasic and Accucore columns, respectively.

Mass spectrometry was performed on a Q-Exactive Orbitrap with a heated electrospray ionization source, operated at +3.2 kV. Data were recorded by data-dependent MS/MS, using a resolution of 70,000 in MS and 17,500 in MS/MS, a scan range from *m/z* 400 to 1500, and an AGC target of 3e6 (MS) and 2e5 (MS/MS). Normalized collision energy was set from 25 to 35% and underfill ratio was set to 1%. A dynamic exclusion of 30 s and exclusion of peptides with charge of +1 and ≥+6 was applied. Monoprotonated peptides (charge of +1) were analyzed separately using a targeted MS/MS method for positive identification (Fig. S1, supplementary material).

3. Results and discussion

Enzymatic degradation of Aβ has gained increasing attention as degrading proteases regulate its tissue concentrations. The enzymatic degradation of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ is often measured as the reduction of the intact peptides, however, to fully understand the clearance process of Aβ in vivo, its proteolytic products should be identified. Therefore, we aimed to characterize the proteolysis products as complete as possible by incubating $A\beta_{1\text{-}42}$ with enzymes related to both intracellular and extracellular degradation.

Cat D is an aspartyl protease with a reported cleaving selectivity between two adjacent hydrophobic residues [23,24], although Ile or Val cannot be at the N-terminal side of the cleavage site (often referred to as position P_1) [23]. The ion chromatogram of identified proteolysis peptides formed after incubation of $A\beta_{1\text{-}42}$ with Cat D for 210 min is shown in Fig 1a. Nine peptides were identified, originating from four cleavage sites at Leu17/Val18, Phe19/Phe20, Phe20/Ala21 and Leu34/Met35 (Table 1). In accordance with the reported enzyme selectivity, no peptide products were identified with Ile at position P_1 [23]. By processing of a C-terminal fragment of human amyloid precursor protein known as C100, Sadik et al. found the latter three cleavage sites within the $A\beta_{1\text{-}42}$ sequence [19]. The peptide $A\beta_{1\text{-}17}$ in Fig. 1a (in addition to a low abundant $A\beta_{18\text{-}34}$ peptide, see supplementary Table S1) suggests a low cleavage frequency between Leu17/Val18. Based on the observation of $A\beta_{1\text{-}34}$, $A\beta_{35\text{-}42}$ and a lower intensity $A\beta_{1\text{-}19}$ after incubation for 30 s, the initial site of cleavage was identified as Leu34/Met35 and Phe19/Phe20 (see Fig S1 in supplementary material for time dependent degradation).

IDE is a zinc metalloprotease with selectivity towards smaller peptides and proteins with a size limit of about 75 amino acids due to the size of the cavity at the enzyme active site [25,26], which explain why IDE can degrade $A\beta_{1\text{-}42}$ as monomer but not amyloid fibrils. The ion chromatogram in Fig. 1b shows the most abundant peptides identified after incubation of $A\beta_{1\text{-}42}$ with IDE for 210 min. In total, 31 peptides originating from 14 cleavage sites were observed. An additional six proteolytic sites was found based on low abundant peptides (Table 1). Previously, only eight cleavage sites have been reported for $A\beta_{1\text{-}40}$ [18,21,22]. The high number of proteolytic sites can be expected as the enzyme is known for its selectivity from substrate binding rather than at catalysis [27]. Even after incubation for 30 s, nine sites of proteolysis were observed.

ECE-1 is a zinc metallopeptidase selective towards hydrophobic amino acids at position P_1' [28]. An ion chromatogram of

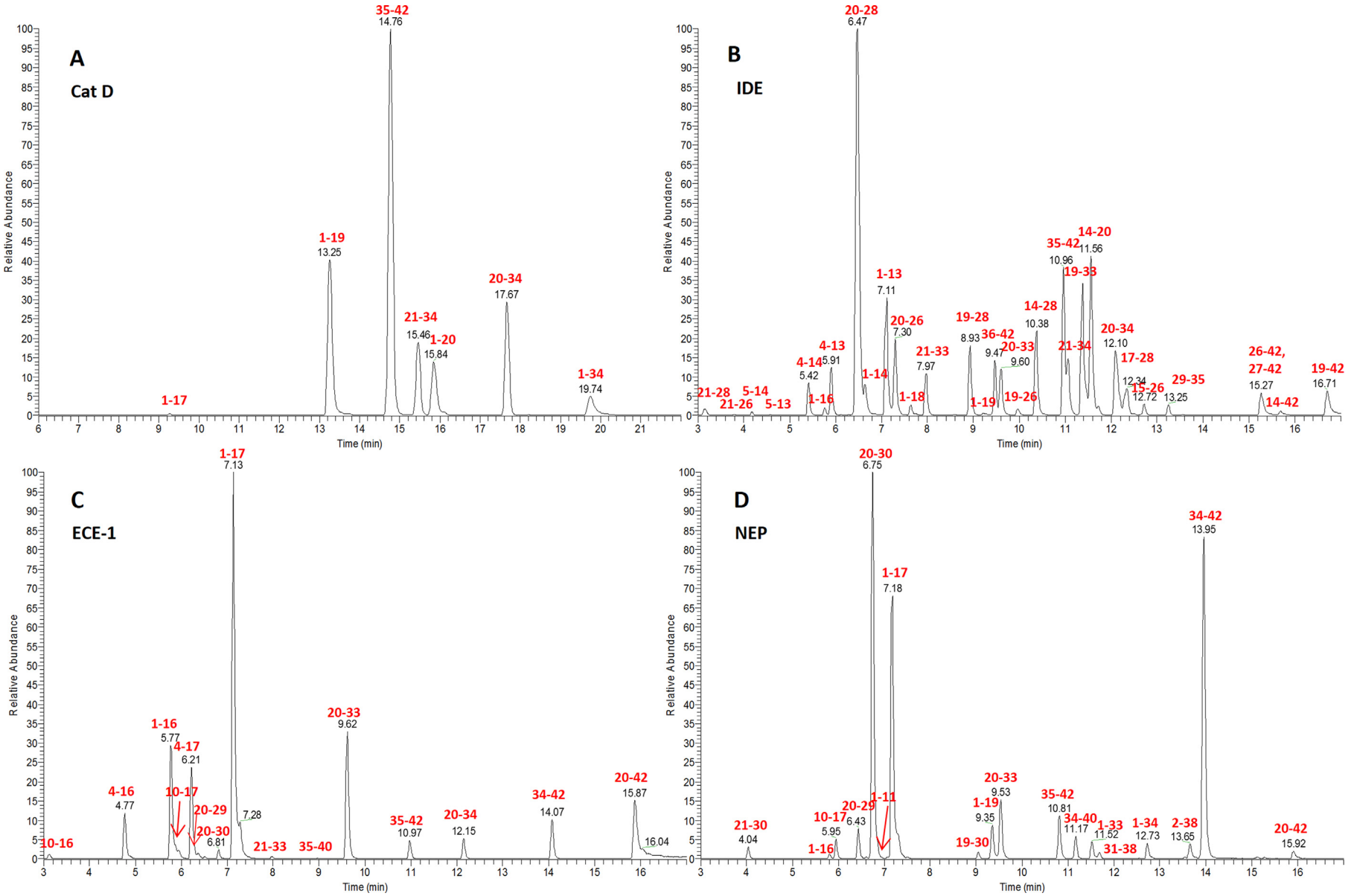

Fig. 1. Extracted ion chromatograms from reversed phase separations of peptide products identified from amyloid beta 1-42 digested for 210 min with four different enzymes. The enzymes used were (a) Cat D, (b) IDE, (c) ECE-1 and (d) NEP. Peptides were annotated according to the sequence of intact $A\beta_{1-42}$.

Table 1
Summary of cleavage sites identified at 210 min and 30 s incubation times of $A\beta_{1-42}$ in this study and previously reported cleavage sites for $A\beta_{1-40}$ and a C-terminal fragment of amyloid precursor protein (C100). Cleavage sites yielding low abundant proteolysis products from IDE incubation are marked with asterisk (*) (see also Table S1 in supplementary material).

Cat D	210 min	30 s	[19][a]
	17/18		
	19/20	19/20	19/20
	20/21		20/21
	34/35	34/35	34/35

ECE-1	210 min	30 s	[20][b]
	3/4		
	9/10		
	16/17	16/17	16/17
	17/18	17/18	17/18
	19/20	19/20	19/20
	29/30		
	33/34		
	34/35	34/35	

IDE	210 min	30 s	[18][b]	[22][b]	[21][b]
	3/4				
	4/5				
	5/6*				
	7/8*				
	13/14	13/14	13/14	13/15	13/14
	14/15	14/15	14/15	14/15	14/15
	15/16*				15/16
	16/17	16/17			
	18/19	18/19	18/19	18/19	
	19/20	19/20	19/20	19/20	19/20
	20/21		20/21	20/21	20/21
					21/22
	23/24*				
	25/26				
	26/27				
	27/28*	27/28			
	28/29	28/29	28/29	28/29	28/29
	33/34				
	34/35	34/35			
	35/36	35/36			
	38/39*				

NEP	210 min	30 s	[17][b]	[21][b]
	1/2			
				2/3
			3/4	
				5/6
	9/10		9/10	9/10
	11/12			
				13/14
	16/17			
	17/18			
	18/19			
	19/20	19/20	19/20	19/20
	20/21			
				28/29
	29/30			29/30
	30/31	30/31	30/31	30/31
				32/33
	33/34	33/34	33/34	33/34
	34/35	34/35		
	38/39			
	40/41			

[a] Enzyme substrate was a C-terminal fragment of amyloid precursor protein (C100) and the cleavage sites in this table are cleavage sites identified within the $A\beta_{1-42}$ sequence.
[b] $A\beta_{1-40}$ was used as the enzyme substrate for the cleavage sites in the table.

degradation products identified after 210 min digestion of $A\beta_{1-42}$ with ECE-1 is shown in Fig. 1c. A total of 15 peptides corresponding to proteolysis between eight residues were identified and all sites had a hydrophobic residue at position P_1'. Eckman et al. reported three cleavage sites in $A\beta_{1-40}$, between Lys16/Leu17, Leu17/Val18 and Phe19/Phe20 [20]. Our experiments revealed the same three sites when $A\beta_{1-42}$ was incubated for 30 s, in addition to a fourth cleavage site at Leu34/Met35. At elongated incubation a total of 8 cleavage sites were identified.

NEP is a zinc metallopeptidase, selective towards hydrophobic amino acids in position P_1' [29]. Like IDE, NEP also contains a catalytic chamber that can encapsulate $\leq$5 kDa peptides, due to the size of the catalytic chamber (about 20 Å) [30], and hydrolyzes $A\beta_{1-42}$ monomers [16,31]. The 18 identified peptides from a 210 min NEP degradation of $A\beta_{1-42}$ are shown in Fig. 1d, corresponding to 14 cleavage sites. Similar to ECE-1, all the identified sites of proteolysis for NEP have a hydrophobic amino acid at position P_1'. In comparison to ECE-1, NEP cleaved at more sites, and both the 210 min and the 30 s incubation showed a tendency for cleavage closer to the C-terminal of $A\beta_{1-42}$ (Table 1).

More proteolysis products were observed in the present study than previously reported, which may be attributed to two conditions. Firstly, in previous experiments, off-line LC fraction collection followed by MALDI-TOF or LC ion trap MS has been used to identify the proteolysis products. These instruments all have shortcomings regarding sensitivity and/or mass resolution compared to the LC-high resolution MS instrument used in the present work, and the higher number of proteolysis products reported here may be attributed to improved instrumentation. This is further supported as most of the novel observed cleavage sites yielded low abundant proteolysis products. One exception from this is Aβ 1-17 produced by NEP, however, this fragment has been observed in vivo [32], although its origin is not known. Secondly, and in our opinion of less significance, is the possibility that one or more of the enzymes employed contained minute amount(s) of undesired proteases. As the used enzymes were of high purity and the identified proteolytic sites were consistent with previously reported P_1 and P_1' substrate selectivity, we consider contamination unlikely. Immunoprecipitation followed by LC-MS have been used to map amyloid beta proteolysis products in cerebrospinal fluid [33]. Considering the high amount of both C- and N-terminal truncated peptides found in the present study, several antibodies with their epitopes spread over the entire sequence would be required to precipitate and characterize the complete endogenous degradation profile. There is an increased interest in using blood based samples for the early diagnosis of AD. Recently, evidence for a reduced metalloprotease activity in serum for from AD patients compared to normal controls was provided [34]. In order to confirm if this reduced protease activity relates to reduced Aβ processing, direct measurements of Aβ degradation products should be performed. While the degradation pattern of $A\beta_{1-42}$ from blood serum has not been described, the proteolysis products reported here, may serve as a starting point for targeted analysis to add support to the anticipated reduced Aβ protease activity. Knowledge about the degradation products is also important for assessing hydrophobicity and neurotoxicity, which again is important for assessing the therapeutic potential of intervention strategies directed at amyloid beta degrading proteases [18].

4. Conclusion

A large number of cleavage sites of $A\beta_{1\text{-}42}$ are hereby reported for the enzymes Cat D, IDE, ECE-1, and NEP, many of which have previously not been reported. The knowledge about peptide products from amyloid beta degradation may be used to assess neurotoxicity or serve as potential targets to investigate $A\beta_{1\text{-}42}$ degradation in vivo. To gain further insight into the mechanisms behind Aβ clearance more research aimed to identify and measure the Aβ degradation products directly is needed, a challenge suitable for LC-MS.

Conflict of interest

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.06.023.

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