

## Partial Identification of Amyloid- $\beta$ Degrading Activity in Human Serum

Ryuta Mikawa<sup>1,4</sup>, Alato Okuno<sup>1</sup>, Tatsuya Yoshimi<sup>1</sup>, Atsushi Watanabe<sup>3</sup>,  
Mitsuo Maruyama<sup>2,4</sup>, and Osamu Takikawa<sup>1</sup>

<sup>1</sup>Laboratory of Radiation Safety, Research Institute, National Center for Geriatrics and Gerontology, Obu, Japan

<sup>2</sup>Department of Mechanism of Aging, Research Institute, National Center for Geriatrics and Gerontology, Obu, Japan

<sup>3</sup>Laboratory of Research Advancement, Research Institute, National Center for Geriatrics and Gerontology, Obu, Japan

<sup>4</sup>Department of Aging Research, Program in Integrated Medicine, Graduate School of Medicine, Nagoya University, Nagoya, Japan

### ABSTRACT

The major hallmarks of Alzheimer's disease (AD) are the extracellular accumulation of pathological amyloid beta ( $A\beta$ ) in the brain parenchyma and  $A\beta$  deposition in cerebral blood walls (cerebral amyloid angiopathy; CAA). Although CAA occurs in more than 80% of AD patients, the mechanisms of  $A\beta$  deposition and clearance around the vessel walls are unknown. We found  $A\beta$ -degrading activity in human serum during analysis of the regulatory mechanism of  $A\beta$  production in human endothelial cells. To elucidate the metabolic dynamics of  $A\beta$  surrounding the brain microvessels, we identified  $A\beta$ -degrading activity in human serum (blood  $A\beta$ -degrading activity: BADA) by column chromatography and LC/MS. BADA exhibited characteristics of an acidic protein, pI 4.3, which had two different protein surface charges (low and high affinity cations). Both BADA fractions had a relative molecular mass of greater than 400 kDa. Furthermore, BADA in the low affinity cation fraction was inhibited by the serine protease inhibitor 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). We clarified alpha-2-macroglobulin (a2M) and several serine proteases from this BADA by LC-MS. Moreover, we demonstrated that BADA is increased by approximately 5000-fold in human serum by column chromatography. Therefore, BADA may play an important role in the circulation and metabolism of  $A\beta$  in human brain microvessels.

Keywords: Alzheimer's disease, amyloid-beta-degrading activity, human serum, brain microvessel

This is an Open Access article distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License. To view the details of this license, please visit (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

### INTRODUCTION

The major hallmarks of Alzheimer's disease (AD) are amyloid plaques, which are formed by the accumulation of amyloid beta ( $A\beta$ ) peptides, and neurofibrillary tangles caused by aggregation of hyper-phosphorylated tau, leading to cognitive dysfunction with aging.<sup>1,2</sup> The two basic types of AD are familial and sporadic. Familial AD (FAD), known as early-onset, is associated with muta-

Received: April 16, 2018; accepted: August 9, 2018

Corresponding Author: Ryuta Mikawa, MS

Laboratory of Radiation Safety, Research Institute, National Center for Geriatrics and Gerontology, 7-430, Morioka-cho, Obu city, Aichi 474-8511, Japan

Tel: +81-56-246-2311, Fax: +81-56-244-6591, E-mail: mikawa@ncgg.go.jp

tions of several genes and is a very rare form of the disease affecting 10% of those diagnosed with AD.<sup>3</sup> In contrast, sporadic AD, known as late-onset, is the most common form affecting approximately 90% of patients.<sup>3</sup> However, most research has been performed using transgenic mouse models harboring the FAD mutation.<sup>4</sup> Although there has been marked progress in our understanding of the pathogenesis of AD over the past several decades, the exact mechanism of AD remains unknown.

Based on an autopsy series, the prevalence of cerebral amyloid angiopathy (CAA) is significantly higher (approximately 80%) in AD brains, and ranges from 10% to 57% in the general population.<sup>5</sup> CAA is defined as the deposition of A $\beta$  in the meningeal and intracerebral vessels. Although the source of A $\beta$  in AD and CAA is considered to mainly originate from neurons in vessel walls, the detailed mechanism is unclear. Recently, it was reported that A $\beta$  is generated from component cells in brain microvessels such as endothelial cells, astrocytes and pericytes.<sup>6,7</sup> Regarding pericytes, amyloid precursor protein was confirmed to be the precursor molecule whose proteolysis generates A $\beta$ .<sup>8</sup> These cells, including neurons, have the potential to cause accumulation of A $\beta$  in blood vessel walls. Thus, AD and CAA may be caused by the disruption of A $\beta$  metabolism in cerebral vessels.

We observed A $\beta$ -degrading activity in pooled normal human serum during our analysis on the regulatory mechanism for the production of A $\beta$  in endothelial cells. The activity, which we termed BADA (blood A $\beta$ -degrading activity), was characterized by the combination of ammonium sulfate precipitation, ion-exchange chromatography, isoelectric focusing, gel filtration and hydroxyapatite chromatography. The results demonstrated that the partially purified fraction of BADA was significantly activated by hydroxyapatite chromatography. This fraction of BADA was eluted as a high molecular weight protein (greater than 400 kDa) by gel filtration, and its isoelectric point was an acidic protein of pH 4.3 by IEF. We found that BADA exhibited different affinities to cations by hydroxyapatite and ion-exchange chromatography. Furthermore, it was inactivated by heat treatment and suppressed by serine protease inhibitors. We identified one of the major BADA proteins as alpha-2-macroglobulin (a2M) and several proteases using LC/MS analysis. A2M forms a complex with several proteases to rapidly degrade A $\beta$ .<sup>9</sup> A2M tightly binds to A $\beta$ , attenuates fibrillogenesis and the neurotoxicity of A $\beta$ , and mediates A $\beta$  degradation.<sup>9-11</sup> Moreover, a2M is genetically associated with AD.<sup>12</sup> Therefore, the a2M-serine protease complex may play an important role for the circulating A $\beta$  and metabolic microenvironment in the neurovasculature. The A $\beta$ -degrading activity of the a2M-serine protease complex in blood should be investigated as a valuable biomarker and possible target for AD in the future.

## MATERIALS AND METHODS

### *Production of A $\beta$ from HUVECs*

Human umbilical vein endothelial cells (HUVECs) from Cell Applications, Inc. were cultured in endothelial cell basal medium (ECBM) with endothelial cell growth supplement containing fetal bovine serum in 35-mm dishes ( $3 \times 10^5$  cells/dish). To measure A $\beta$ 40 in the medium after seeding for 24 h, the culture medium was changed to ECBM containing 3% (v/v) heat-inactivated pooled human serum purchased from Innovative Research, Inc., 5 ng/ml of rhFGF-basic, 5 ng/ml of rhEGF, 0.75 U/ml of heparin and 0.1  $\mu$ g/ml of hydrocortisone. Cells were incubated at 37°C in 5% CO<sub>2</sub>. Culture supernatant from HUVECs was harvested at 24, 48 and 72 h after changing the medium containing pooled normal human serum. The production of A $\beta$ 40 from HUVECs was measured using the Human/Rat beta-Amyloid (40) ELISA kit (Wako pure chemicals, co, ltd.) according to the manufacturer's protocol.

*Assay of A $\beta$ -degrading activity in human serum*

A $\beta$ -degrading activity was based on the remaining A $\beta$ 40 measured by ELISA after incubation with 100 pM synthetic human A $\beta$ 40 (Peptide Institute, Inc.) in human serum or 10–100  $\mu$ l of purified proteins from the human serum fraction at 37°C. To precipitate the protein, trichloroacetic acid (TCA) at a final concentration of 5% (w/v) was added and placed on ice for 1 h. The supernatant was removed after centrifugation at 18,000 x g for 10 min at 4°C. The pellet was washed with cold acetone and centrifuged at 18,000 x g for 10 min at 4°C. The wash step was repeated to remove all of the TCA and acetone, and the pellet was then dried at room temperature. Next, the pellet was mixed with 70% formic acid. It was completely dissolved and neutralized with a 20-fold volume of 1 M Tris-base. The solution was immediately dispensed into an ELISA well. One unit was defined as the amount of enzyme required to digest 50% of 100 pM A $\beta$ 40 in 72 h at 37°C.

*Autoradiography and Western blotting*

The purified protein fraction from human serum (Innovative Research Inc.) by DEAE chromatography with 2 nM A $\beta$ 40, including 30,000 cpm (3.5 pM) of <sup>125</sup>I-A $\beta$ 40 (PerkinElmer, Inc.), was incubated at 37°C for 72 h. The protein was precipitated by the TCA/acetone method described above. Sample buffer was added to the fraction, which was then boiled at 95°C for 5 min. The fraction was separated on a 4–20% gradient tricine gel (Cosmo Bio Co., Ltd.). Proteins were then transferred onto a PVDF membrane. Phosphate buffer saline at pH 7.4 (PBS) was added to the membrane in a hybrid bag, and assessed by phosphor imaging to detect <sup>125</sup>I-A $\beta$ 40 by Typhoon FLA 9500 (GE Healthcare Life Sciences Inc.). The membrane with PBS in the hybrid bag was boiled for 5 min and left at room temperature. The membrane was blocked for 1 h at room temperature using Blocking One solution (Nacalai Tesque, Inc.). The membrane was washed three times with TBST for 10 min. The membrane was incubated with the primary antibody, 6E10 (1/10,000; Covance, Inc.), in Can Get Signal Solution 1 (Toyobo Co.) at 4°C overnight. The membrane was then washed three times with TBST for 10 min. Next, it was incubated with the diluted secondary antibody, Mouse IgG-HRP (1/10,000; Jackson ImmunoResearch Laboratories, Inc.), in Can Get Signal Solution 2 (Toyobo) for 1 h at room temperature. The membrane was similarly washed, and proteins were detected using ECL Prime (GE Healthcare Life Sciences, Inc.), followed by exposure to Kodak BioMax MR film (Sigma-Aldrich, Inc.).

*Purified fractions of BADA*

Ammonium sulfate (AS) was slowly added to 10 ml of pooled human serum (Innovative Research Inc.) until 30% saturation, and allowed to precipitate for 30 min at 4°C with stirring. After centrifuging at 10,000 x g for 15 min at 4°C, ammonium sulfate was slowly added to the supernatant until 40% saturation for 30 min at 4°C with stirring. The pellet was then dissolved in PBS (0–30% fraction). After centrifuging at 10,000 x g for 15 min at 4°C, the supernatant was collected (30–40% fraction), and the pellet was again dissolved in PBS (40–100% fraction). Each fraction was dialyzed against PBS at 4°C. The protein concentration of each fraction after dialysis was measured using the BCA assay. A $\beta$ 40 was added to each fraction. After incubation for 72 h at 37°C, the amount of remaining A $\beta$ 40 was measured by ELISA. The isoelectric points of the 30–40% AS fraction were determined using a pH gradient electrophoresis system (Nihoneido Co.). A linear sucrose gradient from 50 to 25% was loaded onto a 90-ml electrophoresis column in the presence of 1% pharmalyte (GE Healthcare Life Sciences, Inc.). After the protein sample containing 25% sucrose and 1% pharmalyte was loaded into the top of the column, a linear sucrose gradient from 25 to 0% was loaded into the same column. Electrophoresis was performed at a constant voltage of 400 V for 48 h at 6°C. After electrophoresis, samples were fractionated

every 1.7 ml. Each fraction was neutralized by 1 M potassium phosphate buffer (KPB, pH 7.5) immediately after the pH value of each fraction was measured on ice with a pH meter (Horiba, Co. Ltd). The protein concentration of each fraction was measured by its absorbance at 280 nm. Purification was performed on an FPLC system (GE Healthcare Life Sciences, Inc.) using each column. Pooled fractions at pH 4.3 were exchanged into 5 mM potassium phosphate buffer (KPB) at pH 7.5 using the Amicon Ultra-15 centrifuge device (10,000 NMWL; Merck Millipore Inc.). After concentrating, BADA fractions were applied to a hydroxyapatite column (Bio-Rad Inc.) equilibrated with 5 mM KPB (pH 7.5). BADA was eluted with a linear gradient from 5 mM to 250 mM KPB (pH 7.5), and subsequently eluted with 500 mM KPB (pH 7.5). The absorbance at 280 nm was measured with 0.5 ml of each collected fraction. Ten microliters of each fraction was mixed with 100 pM A $\beta$ 40, and after incubation for 3 h at 37°C, the amount of remaining A $\beta$ 40 was measured by ELISA. Eluted BADA with 150 mM KPB (pH 7.5) as a low affinity cation (LAC-BADA) was exchanged into PBS and concentrated. Similarly, eluted BADA with 500 mM KPB (pH 7.5) as a high affinity cation (HAC-BADA) was similarly exchanged. Each sample was applied onto a gel filtration column (Superose12; GE healthcare Life Sciences, Inc.) equilibrated with PBS at pH 7.4. The concentration of each eluted fraction with PBS was measured by its absorbance at 280 nm. Twenty microliters of each fraction was mixed with 100 pM A $\beta$ 40, and after incubation for 3 h at 37°C, the amount of remaining A $\beta$ 40 was measured by ELISA. The molecular weight of the proteins was calculated using a gel filtration calibration kit HMW (GE healthcare Life Sciences, Inc.). Each BADA fraction purified by gel filtration was exchanged into 20 mM Tris-HCl (pH 8.0) and concentrated. Each sample was applied to an equilibrated DEAE column (GE Healthcare Life Sciences, Inc.) with 20 mM Tris-HCl (pH 8.0). BADA was eluted with a linear gradient from 0 mM to 250 mM NaCl in 20 mM Tris-HCl (pH 8.0), and subsequently eluted with 500 mM NaCl in 20 mM Tris-HCl (pH 8.0). Then, 10  $\mu$ l of each fraction was mixed with 100 pM A $\beta$ 40, and after incubation for 12 h at 37°C, the amount of remaining A $\beta$ 40 was measured by ELISA. The protein concentration of all purified samples was measured by the BCA assay.

#### *Identification of BADA by LC-MS*

Purified LAC-BADA by DEAE chromatography was separated by 12.5% SDS-PAGE, and the bands were excised from gels with Oriole Fluorescent Gel Stain (Bio-Rad, Inc.). The gel slices were digested with trypsin (Promega, Inc.), and subjected to peptide-mass fingerprinting analysis using LC-MS (Thermo Fisher Scientific, Inc.) and the MASCOT search program (Matrix Science Inc.).

#### *Inhibitor of BADA*

Aliquots of 15  $\mu$ l of LAC-BADA purified by DEAE chromatography were mixed with 100 pM A $\beta$ 40 with 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) or 1 mM ethylenediaminetetraacetic acid (EDTA), a serine protease and metalloprotease protease inhibitor, respectively. To examine the effects of heat treatment, LAC-BADA was incubated for 5 min at 95°C and A $\beta$ 40 was added to the solution. After incubation for 16 h at 37°C, the amount of remaining A $\beta$ 40 was measured by ELISA.

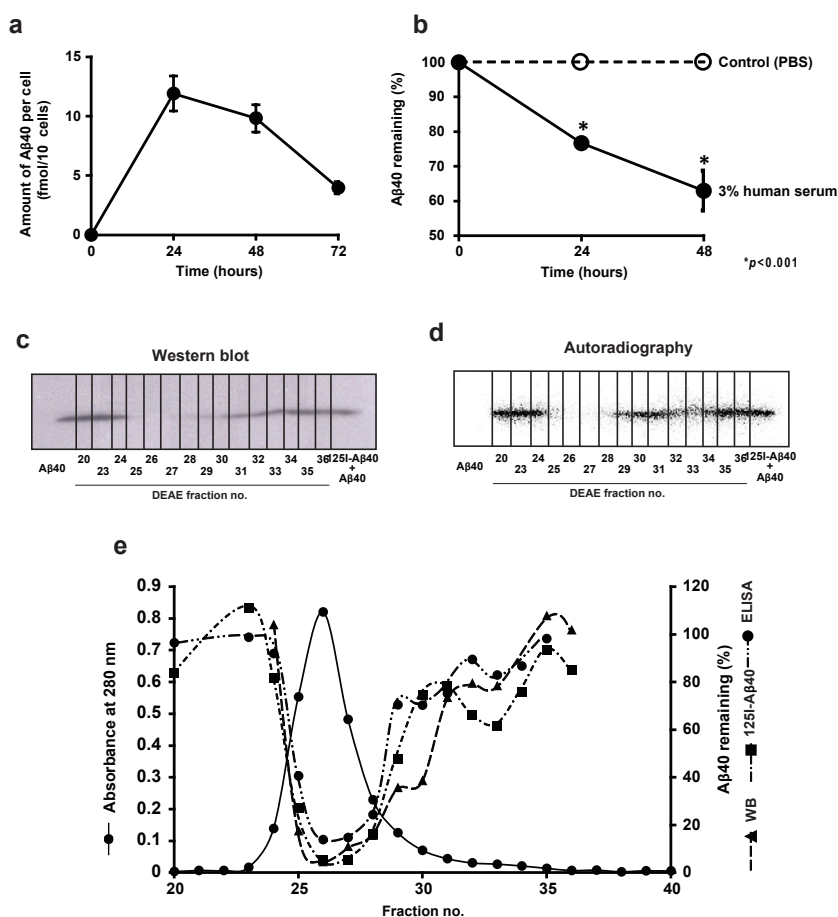
## RESULTS

#### *Amyloid $\beta$ peptide-degrading activity in human serum*

To investigate the time-dependent production of A $\beta$  in endothelial cells, we measured

Identification of Blood Amyloid- $\beta$  Degrading Activity

endogenous A $\beta$ 40 in the medium using HUVECs by ELISA at 24, 48 and 72 h. Although the production of A $\beta$ 40 increased for 24 h and plateaued at 48 h, A $\beta$ 40 decreased at 72 h in the medium (Fig. 1a). To examine whether the decreased A $\beta$  was due to A $\beta$ -degrading activity, we added synthesized A $\beta$ 40 to the conditioned medium. A $\beta$ 40 was significantly decreased by 50 and 80% in the conditioned medium at 24 and 48 h, respectively (data not shown). We next evaluated A $\beta$ -degrading activity in human serum because A $\beta$ 40 was also significantly decreased by 20% to 40% in the 3% human serum (Fig. 1b). We first purified human serum by DEAE chromatography, and measured the A $\beta$ -degrading activity by western blotting, autoradiography and ELISA (Fig. 1c-e). Proteins with A $\beta$ -degrading activity in human serum were eluted with a linear gradient containing 0 to 1 M NaCl. We selected ELISA to explore purified fractions of the A $\beta$ -degrading activity, termed blood amyloid- $\beta$ -degrading activity (BADA), in human serum as it is the simplest method.



**Fig. 1** A $\beta$ 40-degrading activity was observed in human serum

a A $\beta$ 40-degrading activity in HUVEC-conditioned medium. b A $\beta$ 40-degrading activity in human serum. c The remaining A $\beta$ 40 was measured by western blotting and d autoradiography in purified HS using ion exchange column chromatography. e The remaining A $\beta$ 40 was compared among measurement methods using ELISA.

### Purification of BADA fractions

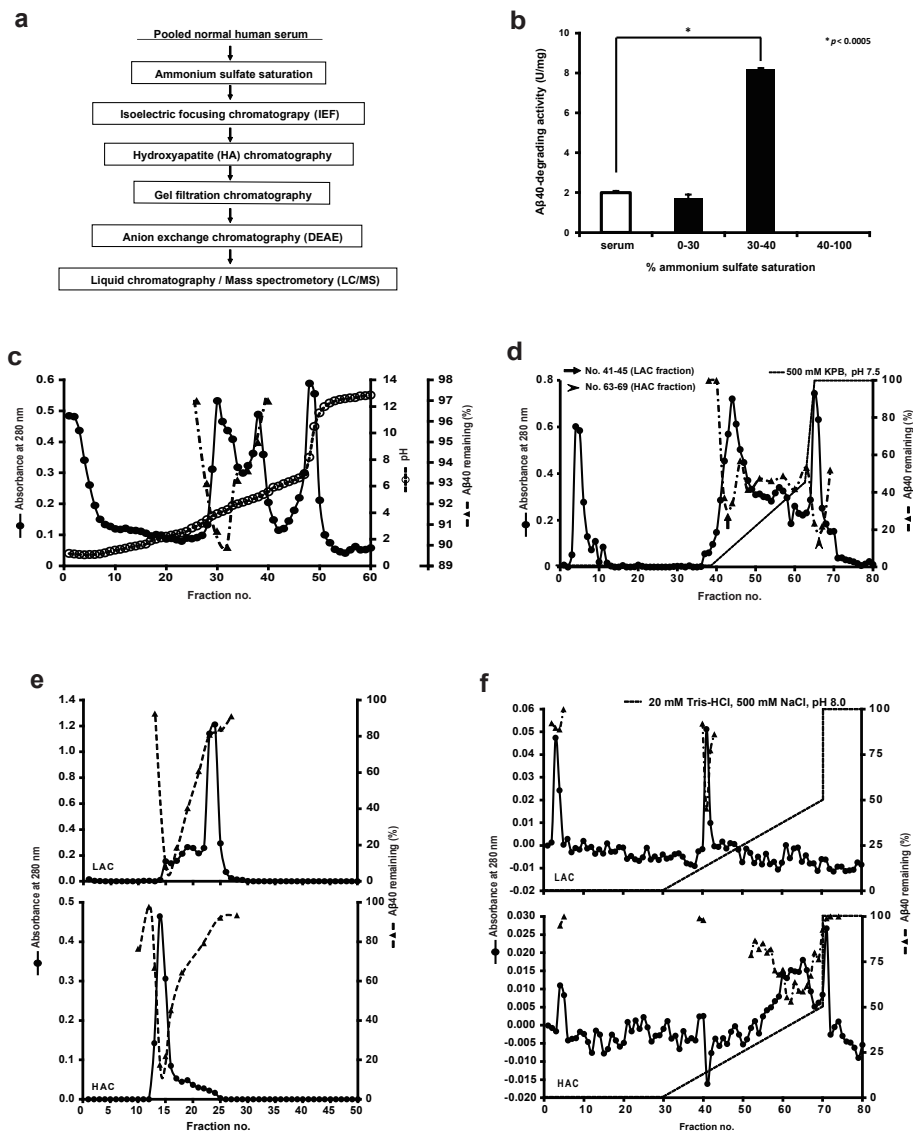
A protocol was developed for the high-yield purification of BADA. Using four chromatographic steps, highly pure fractions of BADA were obtained (Fig. 2a). We first purified by ammonium sulfate precipitation to eliminate contaminating proteins from the human serum. Saturated 30–40% ammonium sulfate separated the BADA (Fig. 2b). We were able to remove contaminating proteins while maintaining the A $\beta$ -degrading activity in human serum. Next, following dialyzed saturated 30–40% ammonium sulfate precipitation in PBS. The fractions of BADA were purified by column isoelectric focusing (IEF) in immobilized pH gradients. pH gradients ranging from 2.5 to 5.0 were formed using carrier ampholytes in sucrose solutions. The BADA was present at pH 4.3 among the three elution peaks (Fig. 2c). The separated proteins by IEF gained a positive charge in low pH environments. Subsequently, BADA at pH 4.3 was applied to hydroxyapatite (HA) chromatography after exchanging the potassium phosphate buffer using membrane ultrafiltration. The fraction of BADA was eluted by a potassium phosphate buffer gradient. The BADA was found to have two different affinities to the HA column in a low affinity cation fraction (LAC) and high affinity cation fraction (HAC) (Fig. 2d). The BADA was increased by 11.3- and 45.9-fold by HA chromatography relative to IEF in the LAC and HAC fractions, respectively (Table 1).

Both LAC- and HAC-BADA fractions were purified separately by gel filtration and size-exclusion chromatography. The BADA fractions were eluted by PBS, and the molecular weight was measured using a calibration curve. Both BADA fractions had a relative molecular mass of greater than 400 kDa (Fig. 2e). LAC-BADA was completely separated to an albumin peak (Fig. 2e, LAC). Although the BADA was increased more by gel filtration than HA chromatography, several gel bands were detected by SDS-PAGE (data not shown). The protein peaks were also not consistent with the BADA peak. We then additionally purified the BADA proteins by ion exchange chromatography.

The fractions of BADA were eluted using the weak anion exchanger DEAE (diethylaminoethyl). The fractions of LAC- and HAC-BADA were eluted with low- or high-salt buffer, respectively (Fig. 2f). The different salt concentrations were consistent with HA chromatography (Fig. 2d). The BADA of acidic proteins may have different surface charges. The LAC-BADA was finally

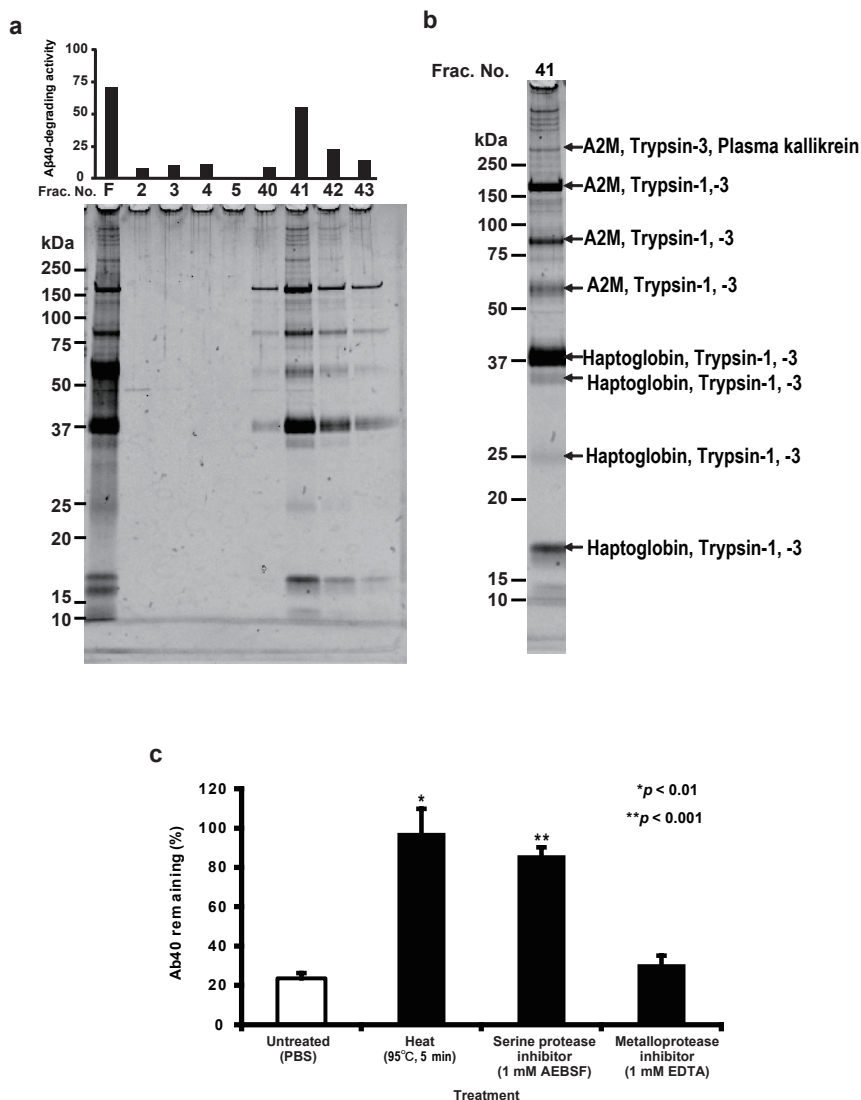
**Table 1 Partial purification of the BADA fraction from human serum**

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Pooled human (10 ml)	1335	66647	2	100	1
Ammonium sulfate saturation (30–40%)	1224	13556	9	92	5
Isoelectric focusing (pI 4.3)	170	1177	14	13	7
Low-affinity cation (LAC)					
Hydroxyapatite	456	2.90	157	34	79
Gel filtration	852	0.28	3048	64	1522
DEAE (fraction no.41)	330	0.03	10776	25	5380
High-affinity cation (HAC)					
Hydroxyapatite	422	0.66	643	32	321
Gel filtration	552	0.66	840	41	419
DEAE (fraction no.61)	72	0.01	9360	5	4673

Identification of Blood Amyloid- $\beta$  Degrading Activity

**Fig. 2 Purification of BADA fractions**

a Schematic representation of the purification protocol. b Fractionation of BADA with ammonium sulfate saturation from human serum. c Isoelectric focusing of BADA. d BADA from hydroxyapatite chromatography. BADA was separated into low- and high-affinity cation fractions (LAC and HAC). e BADA from gel-filtration chromatography. Each LAC and HAC fraction of BADA (upper, lower) was exchanged to PBS at pH 7.5, and then eluted into high molecular weight (400 kDa) fractions by gel filtration with PBS at pH 7.5. The molecular weight of the protein was measured using gel filtration markers. f LAC- and HAC-BADA were purified using a linear gradient of 250 mM sodium chloride in 20 mM Tris-HCl buffer (pH 7.5) by diethylaminoethyl (DEAE) anion exchange chromatography. LAC-BADA was eluted by DEAE chromatography with low levels of sodium chloride (upper). HAC-BADA was eluted by DEAE chromatography with high levels of sodium chloride (lower).



**Fig. 3 Characteristics of LAC-BADA by LC/MS, heat and protease inhibitors**

a LAC-BADA was separated by 12.5% SDS-PAGE and stained by Oriole staining. Lane 1: before DEAE chromatography; Lane 2–5 (Fraction no.2–5): Flow-through, Lane 6–9: eluted using a linear gradient of 250 mM sodium chloride in 20 mM Tris-HCl (pH 7.5). Bar graph showing Aβ40-degrading activity of each sample. The molecular weight marker positions are shown to the left in kDa. b Protein identification of SDS-PAGE bands correlated with Aβ40-degrading activity (panel a. SDS-PAGE gel lane fraction no.41) using LC/MS. c Effects of heat and protease inhibitors on LAC-BADA.

purified from normal human serum at 5400-fold, and it exhibited an increased specific activity of approximately 10,000 U/mg protein (Table 1). The fraction of LAC-BADA was eluted as a single peak, which matched the activity peak, but several bands were observed on SDS-PAGE (Fig. 3a). Thus, we identified the LAC-BADA protein by LC-MS because HAC was eluted as a broad peak that was not consistent with the activity peak



*Identification of BADA by LC-MS and Suppression of BADA by Serine Protease Inhibitor*

To identify LAC-BADA among the several bands detected by SDS-PAGE, we identified proteins by LC/MS from several bands and found that the fraction correlated with A $\beta$ -degrading activity (Fig. 3a. frac. no.41). The numerous bands were identified as alpha-2 macroglobulin. The others were characterized as serine proteases such as trypsin and plasma kallikrein (Fig. 3b). To examine whether LAC-BADA is suppressed by serine protease inhibitors, we added 1 mM AEBSF with A $\beta$ 40 to the LAC-BADA fraction and measured residual A $\beta$ 40 by ELISA. The LAC-BADA was suppressed by AEBSF but not EDTA, a metalloproteinase inhibitor (Fig. 3c). These results suggest that the LAC-BADA includes a serine protease.

## DISCUSSION

The hallmark of AD is A $\beta$  accumulation with aging in the brain. A $\beta$  production and clearance are important to balance A $\beta$  deposition. There have been many studies on A $\beta$  deposition, including APP overexpression and excessive quantities of A $\beta$  exerting toxic effects other than A $\beta$  clearance. Although the roles of neprilysin (NEP) and insulin-degrading enzyme (IDE), which are major A $\beta$ -degrading enzymes in brain parenchyma, were demonstrated in transgenic AD model mice, data supporting an association between A $\beta$ -degrading enzymes and AD are limited.<sup>13</sup> We reported A $\beta$ -degrading activity in human serum (BADA). BADA was characterized by several chromatographic columns as large acidic proteins. The acidic protein having BADA was separated by different charges on the protein surface. Alpha-2 macroglobulin (a2M) containing some serine proteases was identified by LC-MS analysis in the LAC-BADA fraction. Previously reported major A $\beta$ -degrading enzymes in the brain (NEP and insulin-degrading enzyme) and blood (plasmin) were not found in LAC-BADA.

A2M is a well-known protease inhibitor present in the blood that forms a tetramer including a protease (monomer: 180 kDa).<sup>14</sup> However, a2M itself is not a protease, and several studies have reported that the a2M-protease complex plays a key role in A $\beta$  clearance. A2M, including trypsin, has degradation activity *in vitro*.<sup>15</sup> Furthermore, a2M binds A $\beta$  with high affinity and the a2M-protease complex inhibits amyloid formation.<sup>16,17</sup> The a2M-protease complex can degrade A $\beta$  deposited around cerebral vessel walls because a2M has a binding site on LRP1 (low density lipoprotein receptor-related protein 1), which transports A $\beta$  from the brain to the blood across the blood-brain barrier.<sup>18</sup> Although a2M-protease is assumed to play an essential role in A $\beta$  clearance, the relationship between AD and a2M-protease activity in human serum remains unknown. In the future, elucidation of its activity will be important for early diagnosis and new treatment strategies for AD.

## ACKNOWLEDGMENTS

The authors thank all of the staff members at the Department of the Mechanism of Aging and Laboratory of Radiation Safety, Research Institute, and the National Center for Geriatrics and Gerontology for their valuable support.

## CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

## REFERENCES

- 1 Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev.* 2001;81(2):741–766.
- 2 Tanzi RE. The genetics of Alzheimer disease. *Cold Spring Harb Perspect Med.* 2012;2(10):a006296.
- 3 Harman D. Alzheimer's disease pathogenesis: role of aging. *Ann N Y Acad Sci.* 2006;1067:454–460.
- 4 Sasaguri H, Nilsson P, Hashimoto S, et al. APP mouse models for Alzheimer's disease preclinical studies. *EMBO J.* 2017;36(17):2473–2487.
- 5 Kovari E, Herrmann FR, Hof PR, Bouras C. The relationship between cerebral amyloid angiopathy and cortical microinfarcts in brain ageing and Alzheimer's disease. *Neuropathol Appl Neurobiol.* 2013;39(5):498–509.
- 6 Ma JF, Wang HM, Li QY, et al. Starvation triggers Abeta42 generation from human umbilical vascular endothelial cells. *FEBS Lett.* 2010;584(14):3101–3106.
- 7 LeBlanc AC, Papadopoulos M, Bélair C, et al. Processing of amyloid precursor protein in human primary neuron and astrocyte cultures. *J Neurochem.* 2002;68(3):1183–1190.
- 8 Natté R, de Boer WI, Maat-Schieman MLC, et al. Amyloid  $\beta$  precursor protein-mRNA is expressed throughout cerebral vessel walls. *Brain Res.* 1999;828(1–2):179–183.
- 9 Qiu WQ, Borth W, Ye Z, Haass C, Teplow DB, Selkoe DJ. Degradation of amyloid -protein by a serine protease-alpha2-macroglobulin complex. *J Biol Chem.* 1996;271(14):8443–8451.
- 10 Du Y, Ni B, Glinn M, et al.  $\alpha$ 2-Macroglobulin as a  $\beta$ -amyloid peptide-binding plasma protein. *J Neurochem.* 2002;69(1):299–305.
- 11 Du Y, Bales KR, Dodel RC, et al.  $\alpha$ 2-Macroglobulin attenuates  $\beta$ -amyloid peptide 1–40 fibril formation and associated neurotoxicity of cultured fetal rat cortical neurons. *J Neurochem.* 2002;70(3):1182–1188.
- 12 Blacker D, Wilcox MA, Laird NM, et al. Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat Genet.* 1998;19(4):357–360.
- 13 Nalivaeva NN, Beckett C, Belyaev ND, Turner AJ. Are amyloid-degrading enzymes viable therapeutic targets in Alzheimer's disease? *J Neurochem.* 2012;120 Suppl 1:167–185.
- 14 Rehman AA, Ahsan H, Khan FH. alpha-2-Macroglobulin: a physiological guardian. *J Cell Physiol.* 2013;228(8):1665–1675.
- 15 Lauer D, Reichenbach A, Birkenmeier G. Alpha 2-macroglobulin-mediated degradation of amyloid beta 1–42: a mechanism to enhance amyloid beta catabolism. *Exp Neurol.* 2001;167(2):385–392.
- 16 Wyatt AR, Constantinescu P, Ecroyd H, et al. Protease-activated alpha-2-macroglobulin can inhibit amyloid formation via two distinct mechanisms. *FEBS Lett.* 2013;587(5):398–403.
- 17 Hughes SR, Khorkova O, Goyal S, et al. alpha(2)-macroglobulin associates with beta-amyloid peptide and prevents fibril formation. *P Natl Acad Sci USA.* 1998;95(6):3275–3280.
- 18 Deane R, Bell R, Sagare A, Zlokovic B. Clearance of amyloid- $\beta$ ; peptide across the blood-brain barrier: implication for therapies in Alzheimers disease. *CNS Neurol Disord Drug Targets.* 2009;8(1):16–30.