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β-Secretase (BACE1) Purification by Refolding Method and Complex with Hispidin

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ABSTRACT. Alzheimer's disease (AD) is a devastating neurodegenerative disease that represents the most common form of dementia among the elderly population. The deposition of aggregated β-amyloid (Aβ) senile plaques in the human brain is a classic observation in the neuropathology of AD, yet an understanding of the mechanism of their formation remains elusive. Aβ is formed through endoproteolysis of the amyloid precursor protein (APP) by β-secretase (BACE1, β-site APP-cleaving enzyme) and γ-secretase. In this study, BACE1 protein was successfully over-expressed, purified, and refolded and utilized in a binding study with hispidin. We developed a simpler refolding method using a urea gradient and size-exclusion gel filtration to purify an active BACE1 protein variant, in larger quantities than that reported previously, and measured the binding affinity of hispidin to the BACE1 protein variant through isothermal titration calorimetry.

Key words: Alzheimer's disease, β-Secretase, Refolding, Hispidin, ITC

INTRODUCTION

Alzheimer's disease (AD) is a progressive and ultimately fatal condition that causes debilitating memory loss and extensive deterioration of cognitive and functional abilities.[1] With an increase in the aging population, neurodegenerative diseases are becoming more prevalent. Aging is a major risk factor for AD, and estimates of the impact of the increasing aged population worldwide reveal that 35.6 million people were living with dementia in 2010, with a projection of 65.7 million by 2030 and 115.4 million by 2050. AD is a highly debilitating disorder; AD patients older than 60 years live with an estimated 11.2% of years of disability.[2] In addition, it is now becoming a public health priority in developing countries.[3] AD is currently the most common form of dementia in the U.S., and consistently is one of the top 10 causes of death in the elderly.[4] The disease is characterized by memory loss and cognitive dysfunction. Examination of patient's brains reveals an abundance of two neuropathological features: senile plaques and neurofibrillary tangles. Plaques and tangles, and their components, β-amyloid (Aβ) and abnormally phosphorylated tau (P-tau), respectively, are thought to be central to disease pathogenesis. However, the mechanisms involved and thus the underlying cause of AD are unknown.[5]

AD is characterized by the formation of senile plaques in the brain. The major components of these senile plaques are 39–43 amino acid Aβ peptides, which are proteolytically derived from AD β-amyloid precursor protein (APP).[6] Aβ is the main component of the senile plaques found in the brain of patients with AD.[7] Aβ is composed of a portion of both the transmembrane domain and the extracellular domain of APP and is produced by sequential cleavage of APP by β-secretase at the amino terminus, followed by γ-secretase at the carboxyl terminus.[8] β-secretase has been characterized as an aspartic protease and is also referred to as β-site amyloid precursor protein cleaving enzyme 1 (BACE1), Asp2 (for novel aspartic protease 2), and memapsin 2 (for membrane aspartic protease/pepsin 2).[9] Cleavage of APP by β-secretase results in production of a soluble N-terminus (sAPPβ).[10–14] A third enzyme, α-secretase, then cleaves APP within the Aβ sequence to produce soluble sAPPα[15] and a 10-kDa membrane-bound carboxyl terminal fragment, C83.[16] Aβ accumulation is known to be toxic and can induce AD pathologies, such as accumulation of tau neurofibrillary tangles and neuronal cell death.[17] On the basis of this amyloid model of AD pathology, BACE1 and γ-secretase, which mediate the amyloidogenic processing of APP, are considered prime drug targets for the treatment of AD.[17,18] Therefore, *in vitro* expression and purification of BACE1 is a crucial step for the development of an AD drug. Although BACE1 protein variants have been purified previously,[19] a method for purifying large quantities of the protein remains to be established.

Phellinus linteus, a well-known fungus in the Hymenochaetaceae family, is increasingly being used to treat a wide variety of diseases such as oral ulcers, gastrointestinal disorders, inflammation, lymphatic disease, and various cancers. Interestingly, *P. linteus* commonly produces yellow, antioxidant pigments that are composed of hispidin derivatives and other polyphenols.[20] Hispidin itself has been shown to possess strong antioxidant, anticancer, protein kinase C (PKC) inhibitor, anti-dementia, and antiviral properties, making it a promising therapeutic agent for the treatment of dementia and AD.[21–23] As such, there is a need to investigate the effect of hispidin on AD related proteins, for which *in vitro* binding studies between hispidin and BACE1 are required.

In this study, we developed a simplified method for the purification of large quantities of a BACE1 protein variant. Furthermore, we utilized an *in vitro* binding assay to evaluate the interaction between hispidin and recombinant BACE1 protein.

EXPERIMENTAL PROCEDURE

Materials

DNA encoding β-secretase (BACE1) and the pET28-MHL inducible-expression vector were obtained from Structure Genomics Consortium (Toronto, ONT, Canada). *E. coli* strains DH5α, BL21(DE3), and PfuUltra DNA polymerase were purchased from Stratagene (Santa Clara, CA, USA). GE Hitrap™ 5-mL Q-Sepharose HP and HiLoad™ 16/60 Superdex™ 200 columns were purchased from Amersham Biosciences (Pittsburgh, PA, USA). Kanamycin, isopropyl-β-D-1-thiogalactopyranoside (IPTG), urea, Benzonase and protease inhibitor were purchased from Sigma (St. Louis, MO, USA). A Ni-NTA column was purchased from Qiagen (Venlo, Netherlands). BseRI restriction enzyme was purchased from New England Biolabs (Ipswich, MA, USA).

Cloning of Human *BACE1* Gene

Residues 53–441 and 58–441 of the *BACE1* gene product were cloned by PCR amplification using PfuUltra Hotstart. The PCR primers utilized were designed to add 21bp overhangs that were homologous to the ends of BseRI linearized pET28-MHL vector. For site-directed mutagenesis reactions, *BACE1* was PCR-amplified as two fragments, using mutagenic primers that included the altered codons and added 21 bp of overhanging sequence homologous to the sequential fragment of the gene. PCR products were directionally assembled into the pET28-MHL vector in a single reaction using the In-Fusion cloning enzyme (Clontech, Mountain View, CA, USA). All genes were cloned into a modified pET28-MHL vector and the mutation was confirmed by sequencing.

Purification of Inclusion Body of Recombinant BACE1 Protein

E. coli BL21(DE3) cells were transformed with the pET28-MHL-BACE1$_{53–441}$ and pET28-MHL-BACE1$_{58–441}$ plasmids by heat shock treatment. Transformants were grown in 12-L cultures of Terrific broth (TB) medium, supplemented with 50 μg/ml kanamycin, at 37 °C to an approximate OD_{600} of 0.9. Expression of BACE$_{53–441}$ and BACE$_{58–441}$ was then induced by addition of IPTG to a final concentration of 1 mM, followed by incubation for 3 h at 37 °C. Cells were harvested by centrifugation and stored at −80 °C until used. Harvested cells were suspended in 150 mL lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl) containing Benzonase and PMSF/Benzamidine solution, and lysed by sonication (15 min: on-time 10 s, off-time 10 s). The cell lysate was centrifuged at 15,500 rpm for 30 min at 4 °C. The resulting pellet was re-suspended in 150 mL lysis buffer, homogenized, and centrifuged as before. The washed pellet was re-suspended in 150-mL lysis buffer containing 0.5% Triton X-100, homogenized, incubated on ice for 15 min, and then centrifuged at 15000 g * 10 min. This step was repeated using lysis buffer containing 0.5% Triton X-100 and again with lysis buffer lacking Triton X-100. After washing, the pellet was stored at −80 °C until use.

Refolding and Chromatographic Purification of Recombinant BACE1 Protein

The protein pellets obtained from the 12-L cultures were solubilized in 6.25 mL of solubilization buffer (50 mM Tris-HCl, pH 9.0, 8 M urea, and 10 mM β-mercaptoethanol) per liter of cell media. Refolding of the solubilized protein was accomplished using a dilution method (8 M-6 M-4 M-2 M-1 M-0.5 M) by sequential addition of the refolding buffer (20 mM Tris-HCl, pH 9.0, 0.5 mM oxidized glutathione, and 1.25 mM reduced glutathione) to a final volume of 1.2 L. The resulting protein solution was concentrated using Millipore Centripreps. The refolded protein was purified using a HiTrap 5-mL Q-Sepharose HP that was pre-equilibrated in buffer A (20 mM Tris-HCl, pH 7.5, 0.4 M urea). The majority of the aggregated BACE1 protein failed to bind to the column, but the bound protein was eluted with buffer B (20 mM Tris-HCl, pH 7.5, 0.4 M urea, and 0.5 M NaCl). The eluted protein was concentrated using a Millipore Centriprep and loaded onto a HiLoad™

16/60 Superdex^{TM} 200 gel filtration column pre-equilibrated in buffer A. For better resolution, the sample loading volume was kept to 5 mL or less. The peak protein-containing fractions were pooled and analyzed by SDS-PAGE followed by Coomassie blue staining.

Binding of Recombinant BACE1 Protein to Hispidin

Isothermal titration calorimetry (ITC) measurements were performed in duplicate at 25 °C using a VP-ITC microcalorimeter (MicroCal Inc., Commerce, CA, USA). Experiments were performed by injecting 10 μL of 1 mM hispidin into a sample cell containing 100 μM BACE1 protein that was previously dialyzed in de-gassed ITC buffer (20 mM HEPES, pH 7.4, 150 mM NaCl). A total of 25 injections were performed at an interval of 180 s and a reference power of 13 μcal/s. Hispidin was first dissolved to a concentration of 1 mM in 1% DMSO and then diluted in ITC buffer. Heat of dilution generated from the injected compound was subtracted from the experimental curves, and binding isotherms were plotted and analyzed using Origin software (MicroCal Inc., Commerce). The resulting data were then fit to a one-site binding model equation.

RESULTS AND DISCUSSION

Cloning of Constructs Encoding Recombinant *BACE1* Gene Fragments

Prior to cloning, the pET28-MHL vector was PCR amplified and run on a 1.5% DNA agarose gel. pET28-MHL is derived from the pET28a-LIC expression plasmid (Structure Genomics Consortium, Toronto, ONT, Canada). These vectors were designed to allow cloning of recombinant genes encoding N-terminal 6X His-tags, followed by a TEV cleavage site. Expression from pET28-MHL is driven by a T7 promoter and the vector encodes a kanamycin resistance cassette for selective purposes. In addition, pET28-MHL vector was linearized using BseRI restriction enzyme for insertion of *BACE1*. The pET28-MHL vector backbone also contains *SacB*, which encodes the secreted enzyme levansucrase. This enzyme catalyzes the hydrolysis of sucrose and synthesis of levans, which are high-molecular-weight fructose polymers. In the gram-negative bacteria *Escherichia coli*, *Erwinia chrysanthemi*, and *Legionella pneumophila*, expression of SacB in the presence of sucrose is lethal. BACE1 PCR products were directionally assembled into the vector in a single reaction using the In-Fusion cloning enzyme. We designed two BACE1 truncation constructs ($BACE1_{53–441}$ and $BACE1_{58–441}$) that are distinct from those used in a previous study ($BACE1_{14–454}$, $BACE1_{22–454}$, $BACE1_{41–454}$, $BACE1_{43–454}$, and $BACE1_{56–454}$),[16] and which we predict will express soluble protein fragments. *E. coli* DH5α cells were then transformed with pET28-MHL vectors containing the BACE1 constructs. Transformants were spread on LB agar plates containing 50 μg/mL kanamycin and 5% sucrose, and incubated at 37 °C. Transformants were then screened for the presence of the correct insert by colony PCR. As a result, all 10 selected colonies of the $BACE1_{53–441}$ construct were available, whereas only 5 colonies of $BACE1_{58–441}$ construct were available.

Before proceeding with purification of the BACE1 protein fragments, we sequenced *BACE1* (data not shown) and discovered the presence of a single nucleotide base change at position. This mutation (TCC to TTC) would affect the protein sequence by altering the serine residue at position to a phenylalanine. Phenylalanine, tryptophan, and tyrosine contain an aromatic ring, which can repel neighboring amino acids and affect protein stability and folding. Therefore, to restore this region to its native sequence, site-directed mutagenesis was performed using the following primers: Forward primer 5′-AGGCAGCTGTCCAGCACATAC-3′, and reverse primer: 5′-GTATGTGCTGGACAGCTGCCT-3′. We then confirmed the presence of the correct base through DNA sequencing (data not shown).

Solubility Test and Refolding of Recombinant BACE1 Protein

E. coli BL21(DE3) cells containing the pET28-MHL-BACE1 constructs were utilized for small-scale production (~100 mL) of BACE1 protein fragments. Upon induction, SDS-PAGE analysis followed by Coomassie blue staining of whole cell lysates was performed to verify that both recombinant $BACE1_{53–441}$ and $BACE1_{58–441}$ were stably expressed (*Fig*. 1A). Both proteins were expressed at high levels and $BACE1_{53–441}$ appeared to be expressed at a slightly higher level than $BACE1_{58–441}$. For large-scale production of $BACE1_{53–441}$ and $BACE1_{58–441}$ proteins, *E. coli* BL21(DE3) cells containing the pET28-MHL constructs were grown overnight in 50 mL of LB medium, supplemented with kanamycin, at 37 °C in an orbital shaker at 220 rpm and then subcultured in six bottles containing 2 L of fresh TB medium supplemented with 50 μg/mL kanamycin. Cultures were grown at 37 °C to an OD_{600} of ~0.9 (4–5 h) and induced by the addition of IPTG to a final concentration of 0.5 mM. Next, the culture was incubated for 48 h at 4 °C (induction condition 1), or IPTG was added to a concentration of 1 mM followed by incubation for 18 h at 15 °C (induction condition 2). Cells were harvested and lysed by sonication. To purify the His-tagged BACE1 vari-

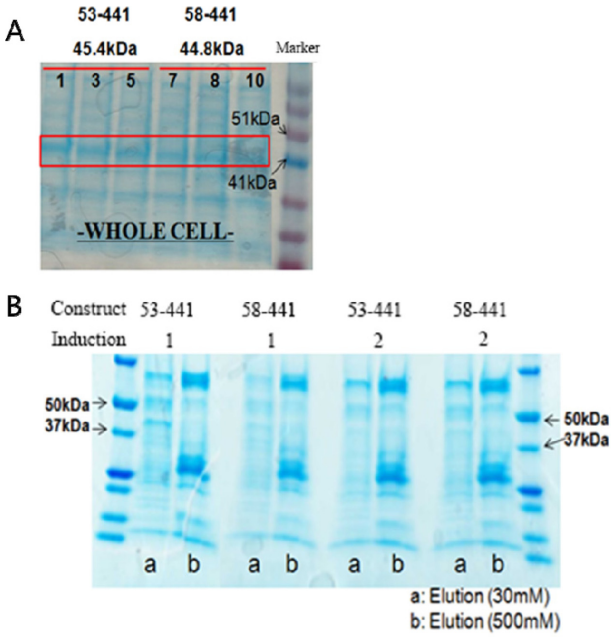

***Figure* 1.** Solubility of recombinant BACE1 protein (A) Small scale induction of BACE1 protein fragments. Whole cell lysates were analyzed by SDS-PAGE followed by Coomassie blue staining. The estimated molecular weights of $BACE1_{53–441}$ and $BACE1_{58–441}$ are 45.4 kDa and 44.8 kDa, respectively. BACE1 protein fragments are indicated with a red box. Numbers denote individual replicates. (B) Solubility test of BACE1 protein in large-scale preparations. For induction condition 1, cultures were induced with 0.5 mM IPTG for 48 h at 4 °C, whereas for condition 2, cultures were induced with 1 mM IPTG for 18 h at 15 °C. $BACE1_{53–441}$ and $BACE1_{58–441}$ were purified using Ni-NTA resin and eluted using the following buffer compositions: 10 mM HEPES, pH 7.5, 500 mM NaCl, 30 mM (a) or 500 mM (b) imidazole, 10% glycerol, and 1 mM TCEP.

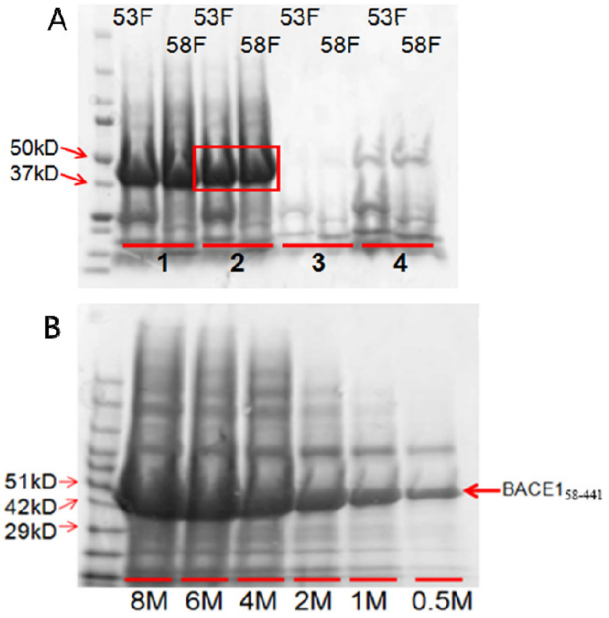

***Figure* 2.** Refolding of recombinant BACE1 protein (A) SDS-PAGE and Coomassie blue staining was used to assess purification of BACE1 protein by Ni-NTA affinity chromatography. *E. coli* cells were disrupted by sonication and protein was solubilized in solubilization buffer. Lane 1: Proteins solubilized in 8 M urea, 2: Column flow-through, 3: Washing fraction, 4: Eluted fractions; $BACE1_{53–441}$ (53F), and $BACE1_{58–441}$ (58F) constructs. Solubilization buffer: 50 mM Tris-HCl, pH 9.0, 8 M urea, 10 mM β-mercaptoethanol. Washing buffer: 20 mM Tris-HCl, pH 7.5, 8 M urea, 30 mM imidazole. Elution buffer: 20 mM Tris-HCl, pH 7.5, 8 M urea, 500 mM imidazole. (B) Refolding of $BACE1_{58–441}$ protein in refolding buffer using a urea gradient. Red bars denote the distinct urea concentrations of the refolding buffer at each step. The predicted molecular weight of $BACE1_{58–441}$ is ~44.8 kDa. Refolding buffer: 20 mM Tris-HCl, pH 9.0, 0.5 mM oxidized glutathione, 1.25 mM reduced glutathione.

ants, the soluble portion of each lysate was applied to a Ni-NTA column. Bound proteins were then eluted with either 30 mM or 500 mM imidazole. Analysis of the eluted fractions by SDS-PAGE followed by Coomassie blue staining failed to detect expression of $BACE1_{53–441}$ or $BACE1_{58–441}$ under any of the conditions tested (*Fig.* 1B). Thus, we could not acquire soluble forms of BACE1 protein with shorter constructs. We therefore opted to alter the purification strategy to include a refolding step to increase the solubility of the BACE1 variants.

We hypothesized that all BACE1 protein variants may be aggregated in inclusion bodies. To address this possibility, *E. coli* pellets obtained on lysis were thoroughly washed as described in Experimental Procedure. $BACE1_{53–441}$ and $BACE1_{58–441}$ proteins in the inclusion bodies were denatured using a solubilization buffer (50 mM Tris-HCl, pH 9.0, 8 M urea, 10 mM β-mercaptoethanol). The solubilized protein was then applied to Ni-NTA affinity chromatography columns and processed. However, $BACE1_{53–441}$ and $BACE1_{58–441}$ proteins (red box) were again not detected in any of the fractions (*Fig.* 2A). We speculated that the lack of binding of solubilized proteins to the Ni-NTA resin could be due to inaccessibility of the His-tag. Therefore, we decided to utilize a combination of ion-exchange and gel filtration chromatography columns to purify solubilized BACE1 proteins. In addition, we focused on the purification of $BACE1_{58–441}$ fraction because both $BACE1_{53–441}$ and $BACE1_{58–441}$ constructs appeared to behave similarly.

Refolding of the $BACE1_{58–441}$ protein fragment was performed by serial dilution as described in Experimental Procedures. Briefly, the protein was first suspended in refolding buffer containing 8 M urea. The urea concentration was then reduced to a final concentration of 0.5 M by serial addition of refolding buffer. Each dilution step included an incubation for 3 h at 4 °C and the total refolding time

was 18 h. A protein sample was harvested at each dilution step and analyzed by SDS-PAGE and Coomassie blue staining (*Fig*. 2B). The dilution steps resulted in a final volume of 1.2 L refolding buffer solution containing approximately 240 mg of $BACE1_{58–441}$ protein (~20 mg protein from 1L culture). Compared to a previously published refolding strategy,[16] which utilized a combination of urea and pH gradients for refolding of BACE1 fragments, our method is superior in that it requires only the use of a urea gradient and results in a higher yield of BACE1 than was obtained in the previous study.[16] Lastly, we verified that our method resulted in refolding of $BACE1_{53–441}$ in a manner similar to that in refolding of $BACE1_{58–441}$ (data not shown).

Purification of Recombinant $BACE1_{58–441}$ Protein

The refolded $BACE1_{58–441}$ protein was first concentrated using a HiTrap 5-mL Q-Sepharose HP column and eluted in buffer containing 20 mM Tris-HCl, pH 7.5, 0.4 M urea, and 0.5 M NaCl (*Fig*. 3A). After SDS-PAGE analysis of the eluted fractions, we found that the majority of $BACE1_{58–441}$ failed to bind to the column. We speculated that this lack of binding could again be due to aggregation of $BACE1_{58–441}$. We therefore decided to separate the monomers of $BACE1_{58–441}$ from any higher molecular-weight aggregates using size exclusion chromatography. For this experiment, the fractions eluted from the HiTrap column (6 fractions * 2 mL in 0.25 M NaCl), were concentrated using a Millipore Centriprep and loaded on a HiLoad Superdex 200(16/60) gel filtration column, pre-equilibrated with 20 mM Tris-HCl, pH 7.5, 0.4 M urea. In the eight 2 mL fractions collected (*Fig*. 3B), we obtained approximately 96 mg of $BACE1_{58–441}$ protein. The chromatograph from the gel filtration column indicated a greater purity of $BACE1_{58–441}$ than was obtained by ion-exchange chromatography (*Fig*. 3B).

The eight fractions (2 mL each) containing the purified $BACE1_{58–441}$ were collected, concentrated, and assessed for purity by SDS-PAGE (*Fig*. 3C). This purification strategy yielded a 4-mL solution containing approximately 48 mg of $BACE1_{58–441}$ protein (~4 mg protein from 1-L culture, ~48 mg protein in total). In a previous study, Sardana *et al.* purified BACE1 fragments using gel filtration followed by ion exchange.[16] In contrast, we opted to perform ion exchange followed by gel filtration chromatography and found that this approach may provide a greater yield of BACE1 protein. The purified BACE1 protein was found to quantitatively bind to a Ni-NTA column for further purification and crystallization studies of human BACE1 protein (data not shown). Therefore, we developed a method

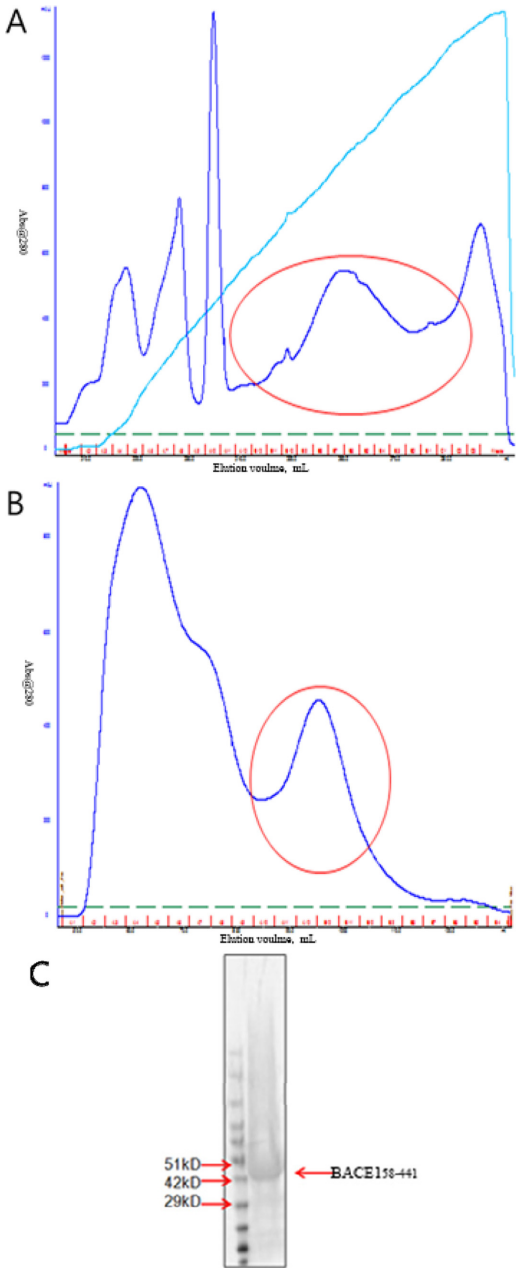

***Figure* 3.** Purification of $BACE1_{58–441}$ by ion exchange and gel filtration chromatography (A) Ion-exchange chromatography of solubilized and refolded $BACE1_{58–441}$. The refolded protein was loaded onto a 5-mL Q-Sepharose HP column, pre-equilibrated in 20 mM Tris-HCl, pH 7.5, 0.4 M urea, and the bound protein was eluted with 3-4 column volume of a 20 mM Tris-HCl, pH 7.5, 0.4 M urea, and 0.5 M NaCl buffer. Fractions within the red circle were collected and pooled. The X-axis represents elution volume (mL) and the Y-axis represents absorbance at 280 nm. (B) Size-exclusion chromatography. Protein harvested by ion-exchange chromatography was loaded onto a HiLoad Superdex 200(16/60) column, pre-equilibrated in 20 mM Tris-HCl, pH 7.5, 0.4 M urea. Again, fractions within the red circle were collected and pooled. The X-axis represents elution volume (mL) and the Y-axis represents absorbance at 280 nm. (C) SDS-PAGE of the purified $BACE1_{58–441}$ protein.

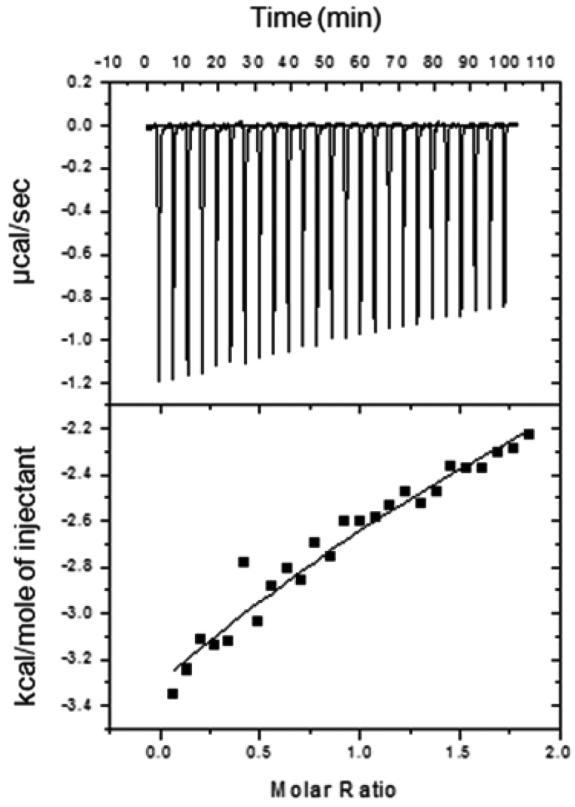

***Figure* 4.** Binding of recombinant $BACE1_{58–441}$ protein to hispidin. Representative ITC titration experiment for the inclusion complex between hispidin and the $BACE1_{58–441}$ protein. A 100 μM solution of $BACE1_{58–441}$ protein was titrated with 10-μL injections of hispidin. Each peak in the top panel illustrates the binding of hispidin to $BACE1_{58–441}$ protein. The bottom panel depicts the binding curve of hispidin to $BACE1_{58–441}$ as calculated using Origin software.

to achieve greater purification of the BACE1 protein in an active form (~48 mg from this study; ~12 or ~36 mg from previous study[16]). This concentrated protein was then used in *in vitro* binding studies.

Hispidin Interaction with Recombinant $BACE1_{58–441}$ Protein

To determine whether hispidin interacts with the $BACE1_{58–441}$ protein, we titrated 1 mM hispidin against the 100 μM $BACE1_{58–441}$ protein. Each injection resulted in an exothermic reaction, and the temperature of the solution gradually decreased (*Fig*. 4). This observation suggests that hispidin does indeed bind the $BACE1_{58–441}$ protein fragment. Using origin software, we then found that the binding constant (K_d) of hispidin to the recombinant BACE1 protein is approximately 10 ± 2 μM. The binding constant is relatively weaker than we expected. However, this value is consistent with previously measured binding constants of hispidin bound to several kinases (~several μM), which further supports the conclusion that hispidin can bind to BACE1 *in vitro*.

In future studies it will be necessary to build on these results and identify compounds that bind BACEl with a higher affinity. Towards this aim, obtaining a crystal structure of hispidin bound to recombinant BACE1 protein could provide valuable information for designing BACE1 inhibitors. We intend to develop much stronger binding compounds based on this initial investigation of hispidin binding to $BACE1_{58–441}$ by performing crystallization studies of the recombinant BACE1 protein.

CONCLUSION

AD is the currently most common age-related disease.[24] The risk of AD increases dramatically in individuals above the age of 70. Current estimates predict that the incidence of this disease could increase 3-fold over the next 50 years. Aβ is generated following the sequential cleavage of its precursor, APP. The amyloidogenic processing of APP involves two sequential cleavages by the β- and γ-secretases at the N-and C-termini of Aβ, respectively.[25] Several lines of evidence support the central role of Aβ in the pathology of AD. Aggregates of Aβ are neurotoxic and initiate a series of events, including the hyperphosphorylation of tau, which result in neuronal cell dysfunction and death.[26] Moreover, soluble Aβ oligomers have been found to alter memory function in different murine models of AD.[27,28] The length of the Aβ peptide typically ranges from 39 to 42 residues. The two major isoforms of Aβ in the brain are Aβ40 and Aβ42, consisting of 40 and 42 residues, respectively.[18,29]

BACE1 is crucial for the formation of Aβ oligomers and insoluble plaques in the brains of patients with AD.[30] Despite the importance of BACE1 in the production of Aβ and the progression of AD, there is little experimental information on purification of BACE1 protein. Therefore, the goal of this study was to generate and optimize a simplified protocol to increase production of purified BACE1 protein. Unsurprisingly, upon over-expression in *E. coli*, the $BACE1_{58–441}$ variant formed insoluble inclusion bodies. Although bacteria still represent a convenient production system, most recombinant polypeptides produced in prokaryotic hosts undergo misfolding or incomplete folding processes that usually result in their accumulation as insoluble aggregates, called inclusion bodies.[31]

A general procedure for solubilization, refolding, and purification of human BACE1, expressed in bacteria, has

been described in detail. BACE1 protein purification from inclusion bodies as described in previous studies,[19,32] however, was vague in details and irreproducible in our hands. For our method, inclusion body proteins were solubilized in 8 M urea (*Fig*. 1) and refolded using a urea gradient (*Fig*. 2). We then successfully purified $BACE1_{58–441}$ by employing a combination of ion exchange chromatography followed by size exclusion chromatography (*Fig*. 3). This study demonstrates that our protocol provides a simpler method for producing greater amounts of BACE1 protein than was previously available. In addition, we confirmed by ITC analysis that hispidin binds the $BACE1_{58–441}$ protein fragment, *in vitro* (*Fig*. 4), suggesting that hispidin may be an effective inhibitor of BACE1 for treatment of AD.

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