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Evaluation of superior BACE1 cleavage sequences containing unnatural amino acids

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ABSTRACT

A recombinant form of BACE1 (β-site amyloid precursor protein cleaving enzyme-1) corresponding to positions 46–454 of the extracellular domain of the original membrane enzyme was prepared. The recombinant BACE1 (rBACE1) had the kinetic parameters K_m = 5.5 μM and k_{cat} = 1719 s^{-1}. Using several libraries of substrates containing unnatural amino acids, the specificity of rBACE1 was evaluated. LC/MS of digests derived from the libraries clarified that a dodecapeptide containing unnatural amino acids at P_2 to $P_{1'}$ was a superior cleavage sequence.

1. Introduction

The increased production of amyloid peptide (Aβ) followed by oligomerization and accumulation in the brain is a major factor underlying the pathogenesis of Alzheimer's disease (AD).[1] Aβ 39–43 residues) derives from a larger membrane protein (amyloid precursor protein, APP) which is cleaved by two proteases, β-secretase (β-site APP cleaving enzyme, BACE1) and γ-secretase.[2] BACE1 cleaves APP at the N-terminus of Aβ to yield a membrane-bound C99 fragment, which is then cleaved by γ-secretase to produce Aβ. Since BACE1-mediated cleavage of APP is the first and rate-limiting step in the processing of APP to yield Aβ, BACE1 is considered a major therapeutic target for treating AD.

BACE1 is a transmembrane aspartic protease and its catalytic domain is located in the extra-cellular domain.[3–6] Although numerous X-ray structural analyses of the catalytic domain with inhibitors have been conducted for the design of BACE1 inhibitors,[7,8] limited results on substrate specificity were obtained.[9–11] The specificity was evaluated mainly by substituting parts of the APP sequence with natural α-amino acids. More detailed information on substrate specificity especially at the site of cleavage by BACE1 would provide a useful starting point for the design of small and tightly binding inhibitors. We report here superior BACE1 cleavage sequences containing unnatural amino acids based on the combination of a conventional LC/MS assay procedure and a recombinant BACE1 (rBACE1).

2. Results and discussion

To evaluate specificity with an assay system using conventional HPLC, we first needed to obtain enough amount of highly active rBACE1 corresponding to positions 46–454 of an extracellular domain containing the pro sequence (Fig. 1). The corresponding plasmid was introduced into *Escherichia coli*, and the 49-kDa pro-BACE1 was obtained as a major product in inclusion bodies. The

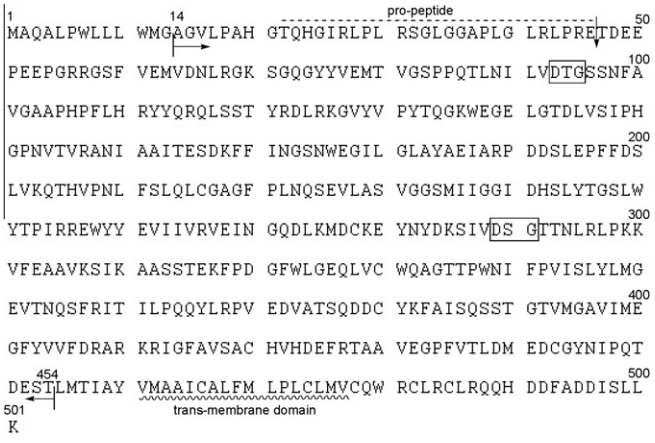

Figure 1. Amino acid sequence of BACE1: ↓ shows the site of cleavage by clostripain; boxed sequences show the sites of catalytic Asp residues of the aspartic protease BACE1; expressed sequence is 14–454.

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product in a solubilization buffer was refolded by dilution, and the refolded pro-BACE1 was then treated with clostripain[12] to yield rBACE1. The desired 45-kDa product was further purified by anion exchange chromatography to give a homogeneous rBACE1. The purified protease was finally concentrated by ultrafiltration, and an equal volume of glycerol was added. The rBACE1 solution was stored at −20 °C without any loss of catalytic activity for at least several years.

The catalytic activity of rBACE1 was confirmed by the cleavage of an eicosa peptide amide (IKTEEISEVNL∗DAEFRHDSG-NH_2; ∗ shows the site of cleavage by BACE1) corresponding to the initial cleavage site of Swedish (SW) mutant APP (Fig. 2). The peptide amide (105 μM) completely disappeared after digestion with rBACE1 (100 nM) to give the expected fragments identified by MALDI-TOF MS. From measurements of the initial digestion rate and the plotting of [S]/v against [S], the kinetic parameters were calculated to be K_m = 5.5 μM and k_{cat} = 1719 s^{-1}. Compared to published results,[9–11] the catalytic activity of rBACE1 was considered high enough to achieve peptide-library digestion and evaluation with a conventional LC/MS procedure.

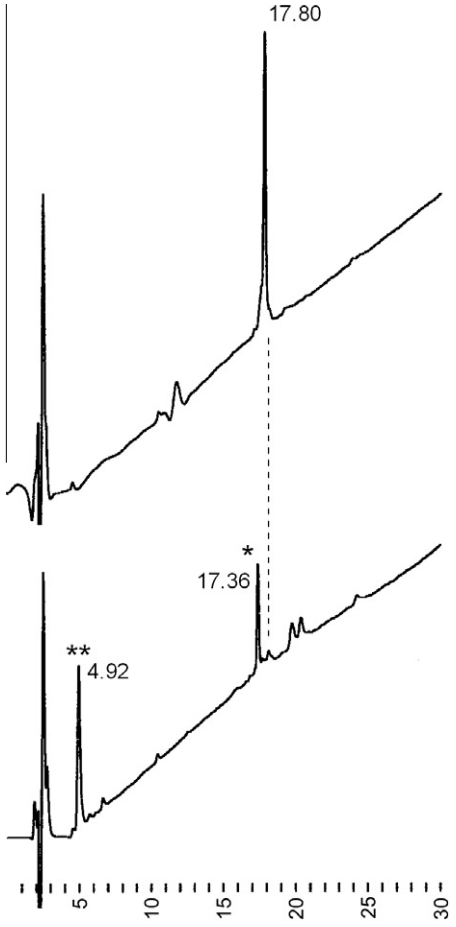

Figure 2. Cleavage of the eicosa peptide amide (IKTEEISEVNLDAEFRHDSG-NH_2): HPLC [System A, CH_3CN (10–40%)/30 min] t_R 17.80 min. The asterisks * and ** indicate a cleaved N-terminal fragment (IKTEEISEVNL, t_R 17.36 min) and a C-terminal fragment (DAEFRHDSG-NH_2, t_R 4.92 min), respectively.

In the present evaluation of the specificity of rBACE1, we focused on the P_4 to $P_{1'}$ sites of its substrate and introduced unnatural side-chain structures. Using a dodecapeptide amide, ISEVNL∗DAEFRH-NH_2, deduced from the SW mutant APP sequence as a template, libraries of peptides containing diversity at $P_{1'}$ to P_4 were designed (Fig. 3). Focusing mainly on the steric effects, side-chain structures containing aliphatic or aromatic substitutions were predominantly selected.

At position P_1, the corresponding library sequence is ISEVN$\mathbf{X_1}$∗DAEFRH-NH_2, where $\mathbf{X_1}$ is replaced by the unnatural amino acids listed in Figure 3B. For comparison, replacements with previously reported favorable amino acids (Leu (in SW APP), Phe and Tyr)[9–11] were also included. The peptide library was prepared by conventional Fmoc-based solid phase peptide synthesis (SPPS)[13] using the split & mix procedure.[14] The AcONa (pH 4.5, 40 mM) buffered solution of the P_1 library (300 μM) was then incubated with rBACE1 (120 nM) at 37 °C. The mixture was analyzed with an analytical HPLC system connected online with an ESI-MS detector. A typical HPLC profile of the reaction mixture is shown in Figure 4B. The cleavage rates for substrates containing Thi as well as Leu (in SW APP) and Phe at position P_1 were much higher than for other P_1 components. Consistent with the reductions in peaks, the corresponding fragments were clearly detected by LC/MS. For quantitative evaluation, three substrates containing P_1-Leu (SW), Phe, or Thi were independently synthesized, purified, and then mixed at an equal molar ratio. The mixture (300 μM) was treated with rBACE1 (60 nM), and the decrease in each substrate was monitored by HPLC. As summarized in Figure 4C, P_1-Thi was most rapidly cleaved by rBACE1 (ca. 70% cleavage after 3 h) compared to the SW and P_1-Phe substrates (ca. 50% after 3 h).

P_4 to $P_{1'}$ libraries were then prepared and the cleavage by rBACE1 was analyzed by LC/MS (Figs. S1–S4). Several substrates cleaved more rapidly than the others were selected from each library and synthesized independently for quantitative evaluation. These selected substrates were P_2-Asn (in SW APP), -Asp, -Nva, -Thi, -Met; P_3-Val (in SW APP), -Nva, -Ile; P_4-Glu, -Cit, -Phg; and $P_{1'}$-Asp, -Phg, -Cit, -Met, -Abu, -Nva. The residues at P_3 to $P_{1'}$ were clearly preferred by rBACE1 over the SW substrate: P_3, Ile and Nva; P_2, Thi, Nva, and Met; $P_{1'}$, Nva, Abu, and Met (Fig. 5). The P_4-site Glu of the SW substrate was most preferred.

Finally, a dodecapeptide amide with the most preferred residues at P_4 to $P_{1'}$, Ile-Ser-Glu-Ile-Thi-Thi∗Nva-Ala-Glu-Phe-Arg-His-NH_2

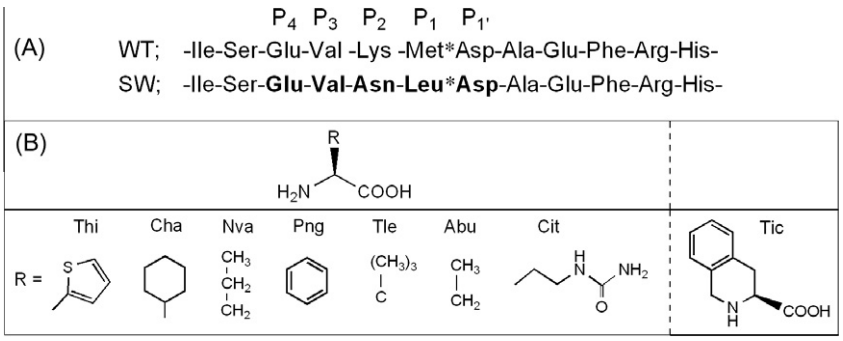

Figure 3. (A) β-Site cleavage sequences of APP (WT; Wild-type, SW; Swedish mutant). (B) Unnatural amino acids used for the construction of libraries.

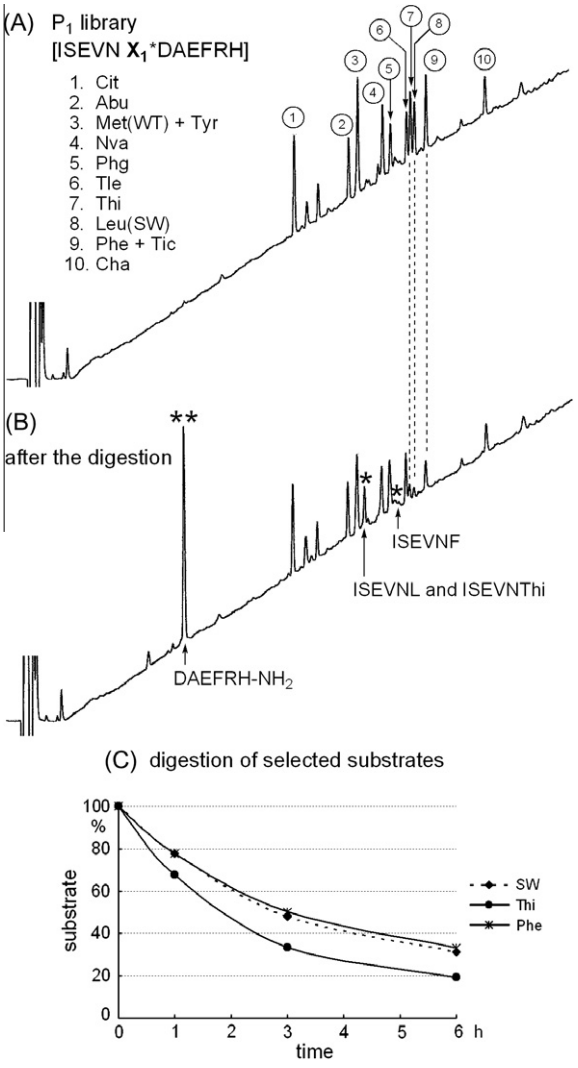

Figure 4. (A) HPLC profiles of the P_1 library before (A) and after (B) digestion (37 °C, 3 h) with rBACE1 (120 nM) [HPLC; Cosmosil 5C18 AR column (4.6 × 150 mm), linear gradient of CH_3CN containing 0.1% TFA (5–35% for 45 min) in 0.1% aqueous TFA at a flow rate of 1.0 mL min^{-1}, detected at 220 nm]. The asterisks * and ** indicate a cleaved N-terminal fragment (ISEVNL and ISEVNThi, t_R 25.75 min; ISEVNF, t_R 27.64 min) and a C-terminal fragment (DAEFRH-NH_2, t_R 13.00 min), respectively. (C) Digestion of selected substrates of the P_1 library by rBACE1.

1, was synthesized and its cleavage by rBACE1 was evaluated. For comparison, other sequences containing a preferred residue at P_3 to $P_{1'}$ were prepared and evaluated (Fig. 6). As expected, all the substrates (300 μM) were cleaved more quickly than the SW substrate. The cleavage rate for **1** was highest, with ca. 90% of the substrate cleaved within 3 h even using a decreased amount of rBACE (10 nM), whereas only ca 10% of the SW substrate was cleaved under the same conditions. The results also suggested the $P_{1'}$ substitution to have a significant effect on the recognition of the substrate by rBACE1.

3. Conclusion

Using small libraries containing substitutions at specific sites, several improved substrates for rBACE1 were obtained. Substitutions by unnatural amino acids at P_2 to $P_{1'}$ had remarkable effects on how well the substrate was recognized. rBACE1 was useful for on-line MS analyses of library digests using conventional HPLC. Among the sequences examined, Ile-Ser-Glu-Ile-Thi-Thi-Nva-Ala-Glu-Phe-Arg-His-NH_2 was the best substrate. The results should facilitate the design and evaluation of substrate-based BACE1 inhibitors.

4. Experimental

Reagents were purchased commercially and used without further purification. Fmoc amino acid derivatives were purchased from Watanabe Chemical Inc. (Hiroshima, Japan). MALDI TOF-MS spectra were obtained on an Autoflex-II (Bruker Daltonics), and ESI-MS spectra, on a HCT plus spectrometer (Bruker Daltonics). Analytical HPLC was performed using a C18 reversed phase column in two different systems (system A: Cosmosil 5C18-AR-II column (4.6 × 150 mm) with a binary solvent system: linear gradient of CH_3CN containing 0.1% trifluoroacetic acid (TFA) in 0.1% aqueous TFA at a flow rate of 1.0 mL/min, detected at UV 220 nm; system B: ZORBAX SB-C18 column (0.5 × 150 mm) with a binary solvent system: linear gradient of CH_3CN containing 0.1% TFA in 0.1% aqueous TFA at a flow rate of 20.0 μL/min, detected at UV 220 nm).

4.1. Construction of plasmid and expression

A cDNA fragment encoding residues 14–454 including the Pre-(residues 14–21) and Pro(residues 22–45) regions[6] of BACE was synthesized (GenScript, USA) with a restriction enzyme sequence at both N- and C-terminal sites. The cDNA was then subcloned into the pET-11a expression vector (Novagen). The expression vector containing the BACE construct was introduced into *E. coli* strain *BL21(DE3)* (Novagen) by heat shock. The cells were grown at 37 °C for approximately 2.5 h in LB medium containing 100 μg/mL of ampicillin to an optical density of 0.5. Protein expression was induced by the addition of isopropyl-D(−)-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. The expression was allowed to continue at 37 °C for 4 h. Cells were harvested by centrifugation at 6500 rpm for 15 min at 4 °C, washed with Phosphate-buffered Saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3), centrifuged again under the same conditions, and frozen at −80 °C.

4.2. Purification of the recombinant BACE1

Frozen cells (5 g) obtained from a 1-L culture were suspended in 50 mL of sonication buffer (50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5, and 50 μL of protease inhibitors (Sigma)), and lysed by sonication (10 times). The lysate was collected and centrifuged at 15,000 rpm for 1 h. An inclusion-body was obtained as precipitant, dissolved in 125 mL of solubilization buffer (100 mM Tris–HCl, 1 mM EDTA, 1 mM Glycine, 100 mM β-mercaptoethanol, and 8 M Urea, pH 10.0), and centrifuged at 15,000 rpm for 1 h. The supernatant was concentrated and then refolded at 4 °C by rapid dilution (20-fold) with refolding buffer (20 mM Tris–HCl, 0.5 mM oxidized glutathione, and 1.25 mM reduced glutathione, pH 10.0). After 48 h, the solution was adjusted to pH 9.0 and gently incubated at 4 °C for 24 h. The procedure was repeated to adjust the pH to 8.0. The solution was concentrated and the product was loaded onto a Sephacryl S-300 size exclusion column (GE Healthcare) pre-equilibrated in 20 mM Tris–HCl, 0.4 M Urea, pH 8.0. Eluted fractions containing refolded BACE were pooled, concentrated, and treated with clostripain (Sigma).[12] The product solution was loaded onto a Resource-Q column (packed with SOURCE 15Q, GE Healthcare) pre-equilibrated in 20 mM Tris–HCl, 0.4 M Urea, pH 8.0. rBACE1 was eluted with a linear gradient from 0 to 500 mM NaCl in 20 mM Tris–HCl, 0.4 M Urea, pH 8.0 at a flow rate of 0.2 mL/min. The desired fractions were pooled and concentrated to a volume of 3 mL. This

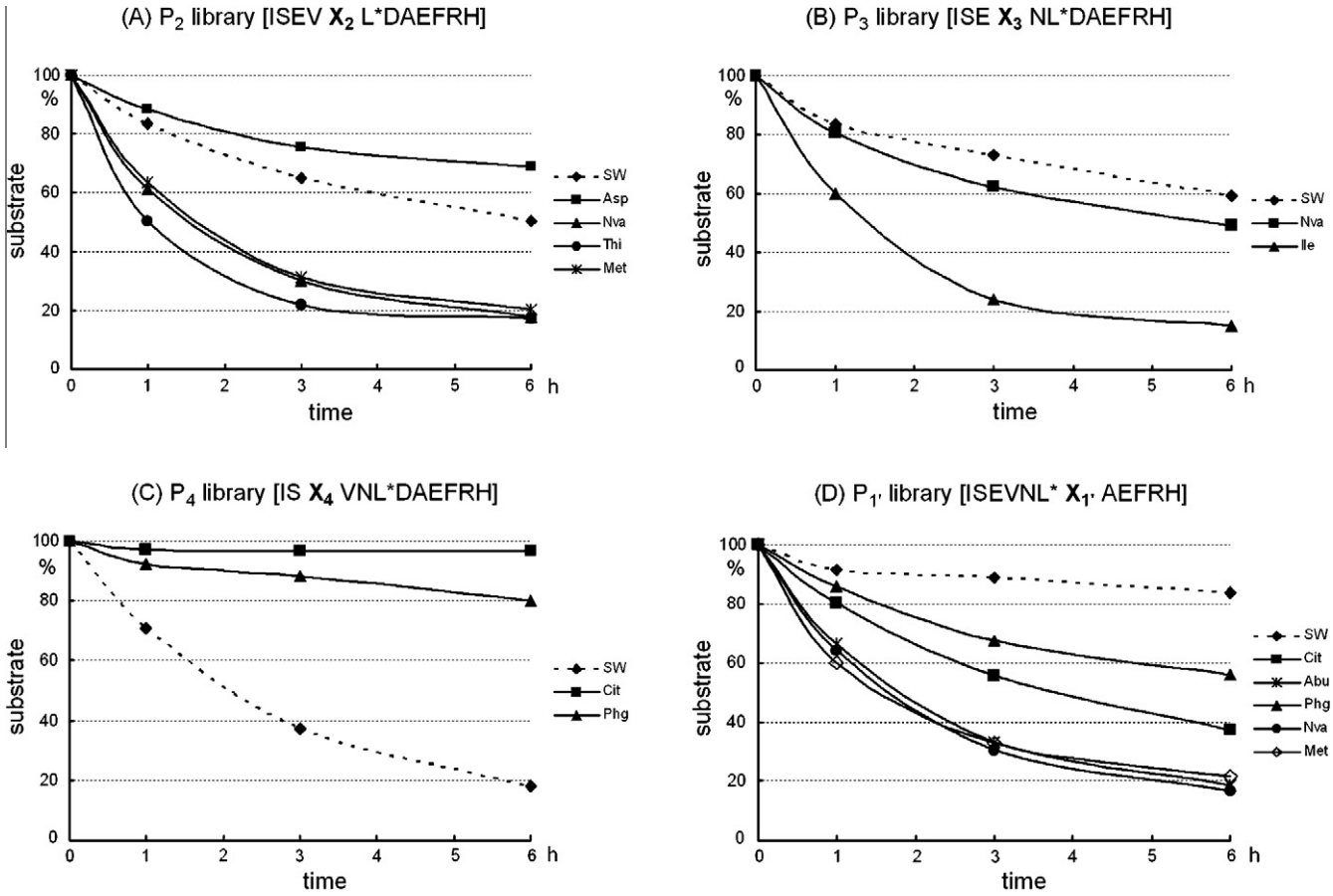

Figure 5. Digestion of selected substrates of the P_2-, P_3-, P_4-, and $P_{1'}$-libraries by rBACE1.

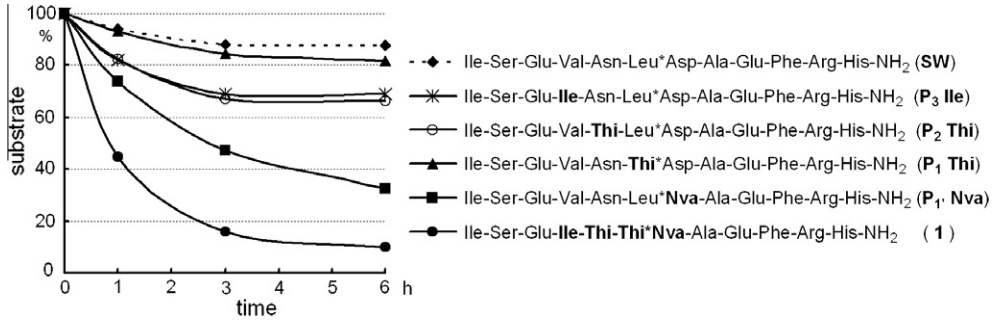

Figure 6. Digestion of a superior substrate, Ile-Ser-Glu-Ile-Thi-Thi-Nva-Ala-Glu-Phe-Arg-His-NH_2 (**1**) (300 μM), and related substrates by rBACE1 (10 nM).

solution was dialyzed for buffer exchange to 20 mM Tris–HCl, 150 mM NaCl, 2 mM DTT, pH 8.0.

4.3. Enzymatic activity

The substrate eicosapeptide amide (IKTEEISEVNLDAEFRHDSG-NH_2) was synthesized using Fmoc-based SPPS[13] starting with Rink amide resin (4-(2′,4′-dimethoxyphenyl-Fmoc aminomethyl)phenoxy resin)[15] according to the procedure described in Section 4.4: HPLC [System A, CH_3CN (10–40%)/30 min] t_R 17.80 min, MALDI TOF-MS, *m/z* 2288.478 for $[M+H]^+$ (calcd 2288.117 for $C_{97}H_{155}N_{28}O_{36}$), Amino acid analysis after 6 M HCl (110 °C, 20 h) hydrolysis; Asp 3.05, Thr 0.89, Ser 1.81, Glu 4.17, Gly 1.00, Ala 0.89, Val 1.03, Ile 1.82, Leu 1.03, Phe 1.05, Lys 0.90, His 1.01, Arg 1.00.

The proteolytic activity of rBACE was detected by a cleavage assay with the eicosapeptide amide using analytical HPLC. The substrate (105 μM) in 40 mM AcONa buffer pH 4.5 was incubated with rBACE1 (100 nM) at 37 °C and an aliquot was injected into the analytical HPLC system [System A, CH_3CN (10–50%)/30 min]. After 1 h of incubation, the substrate's peak had decreased by approximately 20%. After 6 h, the fragments derived from the digestion [Fig. 2: detected by analytical HPLC, System A, CH_3CN (10–40%)/30 min] were identified by MALDI-TOF MS; IKTEEISEVNL, t_R 17.36 min, *m/z* 1274.361 for $[M+H]^+$ (calcd 1274.685 for $C_{55}H_{96}N_{13}O_{21}$), DAEFRHDSG-NH_2, t_R 4.92 min, *m/z* 1032.241 for $[M+H]^+$ (calcd 1032.450 for $C_{42}H_{62}N_{15}O_{16}$).

Kinetic parameters, K_m and k_{cat}, were determined by initial rate measurements of the hydrolysis of the substrate at 37 °C. The reaction was initiated by adding rBACE1 (33 nM) to various solutions

containing different final concentrations of the substrate (0–105 μM). After 15 min of incubation, aliquots were analyzed by HPLC. The initial digestion rates were calculated from the decrease in the peak area of the substrate. K_m was calculated from the plot of [S]/v versus [S], where [S] is the concentration of the substrate and v is the initial reaction rate. Reactions were repeated three times and the results were averaged.

4.4. Peptide library

The peptide chain was elongated on Rink amide resin (0.65 mmol/g resin) using a combination of Fmoc-deprotection and coupling. The deprotection was performed through treatment with piperidine/DMF (20% v/v) for 20 min at room temperature, followed by washing with DMF (×10). Fmoc-amino acid (5 equiv) was condensed in DMF in the presence of DIPCDI (5 equiv) and HOBt (5 equiv) for 2 h at room temperature. The library was prepared by the split and mix procedure by dividing the resin into equal portions at a specific position and by mixing after the coupling. After elongation of the corresponding peptide chain, the protected peptide-resin was treated with TFA/thioanisole (20:1, v/v) for 2 h at room temperature. The mixture was filtered, and the solution was concentrated in vacuo. Dry diethyl ether was added to the residue at 0 °C and the mixture was centrifuged. The precipitate was suspended in H_2O, and lyophilized to give a crude product. Each component of the library was identified by HPLC with ESI-MS. The data are depicted in Tables S1–S7 (see Supplementary data). Each library was used for the rBACE1 assay without further purification.

Selected library components were similarly synthesized as above, independently. The Fmoc-deprotection was carried out with DBU/DMF (2% v/v, 5 min), and Fmoc-amino acid (2 equiv) was coupled by a 1-h reaction using TBTU/HOBt[16] (2 equiv each in DMF) as coupling reagents. Each deprotected product was purified by preparative reverse-phase HPLC, and the purified compound was lyophilized to afford the target peptide as a white amorphous powder: Ile-Ser-Glu-Ile-Thi-Thi-Nva-Ala-Glu-Phe-Arg-His-NH_2 **1;** HPLC [System A, CH_3CN (5–35%)/45 min] t_R 39.47 min, ESI-MS, m/z 1505.7024 for $[M+H]^+$ (calcd 1505.7034 for $C_{68}H_{101}N_{18}O_{17}S_2$). Analytical data for other selected library components were identical to those in Tables S1–S7.

4.5. Digestion of libraries with rBACE1

A mixture of the P_x library (300 μM) and rBACE1 (120 nM for P_1 and P_4, 60 nM for P_2 and P_3, and 30 nM for P_4) in AcONa-buffered solution (pH 4.5, 40 mM, 100 μL) was incubated at 37 °C. At a specific time point (0, 1, 3 and 6 h), an aliquot (25 μL) was collected and the reaction was quenched by the addition of an equal volume of GuHCl solution (7 M, 25 μL). The mixture was analyzed by LC/ESI-MS.

A quantitative evaluation of selected sequences was performed as above using 300 μM of substrate and rBACE1 (60 nM for P_1- and P_4-substrates, 30 nM for P_2- and P_3-substrates, and 10 nM for 1 and $P_{1'}$-substrate).

Acknowledgments

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Supplementary data

Supplementary data (LC/MS data and HPLC profiles of P_x libraries and LC/MS data of the substrates) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.056.

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