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## A capillary electrophoresis method for evaluation of A $\beta$ proteolysis *in vitro*

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### Abstract

According to the amyloid hypothesis, A $\beta$  peptides are neurotoxic and underlie development and progression of Alzheimer's disease (AD). Multiple A $\beta$  clearance mechanisms, including destruction of the peptides by proteolytic enzymes, are hypothesized to regulate physiological A $\beta$  peptide levels. The insulin-degrading enzyme (IDE) is considered one of the predominant enzymes having A $\beta$  degrading activity. Despite its putative role in protecting against AD, relatively few methods exist for studying IDE activity *in vitro*. We report the application of capillary electrophoresis (CE) as a novel method for evaluating IDE-mediated A $\beta$  1–40 proteolysis. This method employs chemically unmodified substrates that are readily obtained from commercial sources. It involves minimal sample preparation, and requires no specialized equipment beyond a CE instrument equipped with a standard fused silica capillary. In the present analysis, we demonstrate that this CE-based method is amenable to kinetic analysis, and show that IDE-mediated A $\beta$  proteolysis is significantly and disproportionately inhibited in the presence of insulin, an alternative IDE substrate.

### Keywords

Alzheimer's disease; Amyloid beta; Insulin-degrading enzyme; Capillary electrophoresis

### 1. Introduction

Amyloid beta (A $\beta$ ) peptides are short, hydrophobic peptides that can aggregate to form neuronal plaques, which are symptomatic of Alzheimer's disease (AD) post-mortem. A $\beta$  peptides are derived from the amyloid precursor protein (APP), a type I integral membrane protein expressed within most tissues and particularly abundant within the brain (Glennner and Wong, 1984; Tanzi et al., 1987). Posttranslational processing of APP by the  $\beta$  and  $\gamma$ -secretases generates multiple A $\beta$  peptide species that are subsequently released to the extracellular matrix (Greenfield et al., 1999). Soluble A $\beta$  peptides are normally present within human plasma and cerebrospinal fluid, but under pathological conditions, these peptides accumulate within the brain, due to aggregation and fibrillogenesis (Seubert et al., 1992; Shoji et al., 1992; Hardy and Selkoe, 2002). Of the multiple A $\beta$  peptide species, A $\beta$  1–40 is the most physiologically abundant and is a principal component of neuronal plaque deposits (Sticht et al., 1995; Neve et al., 2000).

The broadly accepted mechanism for AD progression due to neuronal A $\beta$  accumulation has been termed the amyloid hypothesis (Hardy and Selkoe, 2002). According to this hypothesis, it is not the inherent toxicity of A $\beta$  *per se*, but an imbalance in the production and degradation

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of these amyloidogenic peptides that leads to their neuronal accumulation, plaque formation and resultant disease pathology. A number of biochemical strategies aimed at limiting accumulation of A $\beta$  have thus been pursued as potential AD therapies. Included among these are therapies that seek to increase the rate of A $\beta$  clearance via proteolytic degradation of the peptide. It has been postulated that enhancement of A $\beta$  proteolysis could decrease the amount of peptide available for neuronal accumulation, and hence might be able to forestall, halt, or even reverse the progression of AD (Leissring and Selkoe, 2006; Hardy and Selkoe, 2002).

One A $\beta$ -degrading enzyme that has received considerable biomedical attention is the insulin-degrading enzyme (IDE; EC 3.4.24.56). IDE is a zinc protease with broad substrate specificity. In addition to degrading A $\beta$  and insulin, IDE has been shown to degrade amylin,  $\beta$ -endorphin, and several other amyloidogenic peptides (Kurochkin, 2001). In animal models, partial and complete loss of IDE function have been correlated with increased levels of A $\beta$  and insulin, and increased incidence of AD and type II diabetes mellitus (DM2) (Farris et al., 2003, 2004; Miller et al., 2003). Similarly, levels of A $\beta$  and premature death rates are significantly reduced in a transgenic mouse AD model when IDE is overexpressed (Leissring et al., 2003a). The various substrates of IDE can compete with one another. Substrates with higher affinity for IDE may impair degradation of lesser affinity substrates physiologically. Consistent with this hypothesis, insulin has been shown to function as a potent inhibitor of IDE-mediated A $\beta$  proteolysis ( $K_m$  of 0.0657  $\mu$ M vs. 1.23  $\mu$ M, respectively) (Qiu et al., 1998; Farris et al., 2005). Furthermore, an association between the frequently hyperinsulinaemic DM2 condition and AD has been reported within human population studies (Haan, 2006; Janson et al., 2004).

Despite significant biomedical interest in IDE-mediated A $\beta$  proteolysis and in A $\beta$  proteolysis in general, relatively few methods exist for studying A $\beta$  degradation *in vitro*. Assay development has been complicated by the considerable difficulties inherent to working with the amyloidogenic A $\beta$  peptide, described by at least one researcher as “the peptide from hell” (Zagorski et al., 1999). Existing methods for measuring A $\beta$  proteolysis commonly rely on chemically modified substrates that are difficult to obtain or synthesize. For example, one of the most frequently cited methods used to conduct kinetic analyses of A $\beta$  proteolysis relies on a fluorogenic, biotinylated A $\beta$  1–40 derivative (Leissring et al., 2003b) that is inconsistently available on the commercial market. Other methods used to study A $\beta$  proteolysis have relied upon radiolabeled substrates or indirect immunological detection methods (Qiu et al., 1998; Vekrellis et al., 2000). These methods can be hazardous, expensive, or require careful handling to prevent spontaneous aggregation of the peptide.

Capillary electrophoresis (CE) has been reported as a chromatographic tool for qualitative and quantitative studies of the A $\beta$  peptides. CE methods have been used to measure the amount of A $\beta$  1–40 present within biological fluids (Varesio et al., 2002), to evaluate the enantiomeric condition of A $\beta$  1–40 (Thorsen et al., 2001), and to better understand the aggregation process of both A $\beta$  1–40 and A $\beta$  1–42 (Sabella et al., 2004). CE methods have thus significantly contributed to the contemporary understanding of the biological and biochemical character of the A $\beta$  peptides.

We now report a CE method for evaluating IDE-mediated A $\beta$  1–40 proteolysis *in vitro*. This method has multiple advantages over alternative techniques that have historically been used for this purpose. It relies upon unmodified substrates that are readily obtained from multiple commercial sources, consumes small amounts of costly experimental reagents, involves minimal experimental preparation and handling, and requires no specialized equipment beyond a conventional CE instrument equipped with a fused silica capillary. We have used CE to confirm that IDE-mediated A $\beta$  proteolysis is a progressive enzymatic process that is subject to alternative substrate inhibition, specifically by insulin. Given the considerable interest in the

study of A $\beta$  proteolysis and the suitability of CE for studies of this process, we expect that the reported method will prove to be of significant biomedical utility.

## 2. Experimental

### 2.1. Plasmid construction

*Rattus norvegicus* IDE cDNA was provided by R.A. Roth (Department of Chemical and Systems Biology, Stanford University) (Bondy et al., 1994). The rat IDE ORF corresponding with amino acid residues M<sub>42</sub>-L<sub>1019</sub> of the annotated protein sequence was encoded within a pET-30b(+) expression vector to generate 6XHis-IDE<sub>M42-L1019</sub>. DNA sequencing analysis of the selected 6XHis-IDE<sub>M42-L1019</sub> clone revealed single base pair substitutions encoding Y248C and E768A mutations relative to the published rat IDE sequence. Structural analysis of the human IDE homologue reveals that these residues are positioned distal to the enzyme's catalytic pocket. Hence, they are not believed to have had a detrimental impact on enzyme function.

### 2.2. Enzyme purification

Plasmids encoding 6XHis-IDE<sub>M42-L1019</sub> were transformed into expression-competent BL21 DE3 *Escherichia coli*, and a transformed colony was cultured under kanamycin selection. Protein expression was induced by adding 1mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) to the media, and induction was allowed to proceed at 30 °C for approximately 3 h. Bacterial cells were harvested by centrifugation, resuspended within lysis buffer (50mM HEPES, 140mM NaCl, pH 7.4) containing 1  $\mu$ g/ml chymostatin, leupeptin, pepstatin, and aprotinin, and 1 mM phenylmethanesulphonylfluoride, and lysed using a chilled French pressure cell. The cellular lysate was cleared of insoluble material by high-speed centrifugation (100,000  $g \times 1$  h), and filtered using a Steriflip™ 0.22  $\mu$ m vacuum filter (Millipore Corp., Bedford, MA). Clarified lysate was subjected to immobilized nickel affinity chromatography using an AKTApurifier™ FPLC system and a HisTrap™ Fast Flow column (GE Health, Piscataway, NJ). Following purification, IDE was concentrated and excess imidazole displaced by filtration using an Amicon® Ultra 100 kDa MW-cutoff filter (Millipore Corp.) after repeated dilution with lysis buffer. The final sample was diluted to 1 mg/ml in lysis buffer containing 20% glycerol (v/v) and stored at -80 °C.

### 2.3. Peptides and handling

Recombinant human A $\beta$  1-40 was a gift from rPeptide, Inc. (Athens, GA). The lyophilized peptide was treated with hexafluoroisopropanol (HFIP) to ensure an initial monomeric state in accordance with reported methods (Dahlgren et al., 2002). After complete removal of HFIP under a nitrogen atmosphere, the peptide was resuspended to 4 mg/ml in dimethylsulfoxide (DMSO) and stored at -80 °C as aliquots that were thawed as needed. DMSO was chosen as a solvent because it permitted complete solubilization of the amyloidogenic peptide but did not affect the pH of the overall reaction mixture. Strongly acidic or basic solvents, such as those commonly cited for resuspension of A $\beta$  peptides (i.e., 1% NH<sub>4</sub>OH), were detrimental to proteolysis under the weak buffering conditions of our assay. Recombinant human insulin was obtained commercially (Sigma-Aldrich, Inc., St. Louis, MO). Lyophilized insulin was resuspended in DMSO to 4 mg/ml. As the peptide did not immediately dissolve within this solvent, the mixture was sonicated using a bath sonicator for approximately 5 min (Branson Ultrasonics Corporation, Danbury, CT) until it appeared clear. Aliquots were then frozen at -80 °C and thawed as needed.

## 2.4. Preparation of buffers for analytical chromatography

All buffers were prepared using 18  $\Omega$  nanopure deionized water. Buffers used for CE experiments were filtered through 0.22  $\mu\text{m}$  Steriflip™ or Steritop™ vacuum filters (Millipore Corp.) prior to using. Reagents for SDS–PAGE were prepared according to standard recipes (Sambrook et al., 1989). The background electrolyte for all CE experiments (background electrolyte) was 100 mM potassium phosphate, pH 7.6. The buffer for all proteolysis assays (proteolysis buffer) was 10 mM potassium phosphate, pH 7.6. This solution was prepared by diluting the background electrolyte with water, and additionally served as the injection buffer during CE experiments.

## 2.5. SDS–PAGE

Slab gel electrophoresis was conducted under denaturing conditions using a 10% polyacrylamide gel. Proteins and peptides were detected by staining with Coomassie® Brilliant Blue R-250. Two-fold serial dilutions of IDE were prepared in SDS–PAGE sample buffer, and heated to 100 °C for 3 min prior to analysis. SeeBlue® Plus 2 Pre-Stained Standard (Invitrogen Corp., San Diego, CA) was used as a size marker.

## 2.6. Enzymatic proteolysis

Proteolytic assays were conducted in proteolysis buffer at 37 °C. IDE concentration was maintained at 11 nM, and initial A $\beta$  1–40 concentration was 12  $\mu\text{M}$ . Final reaction volume was 40  $\mu\text{l}$ . Prior to mixing, thawed stocks of IDE and A $\beta$  1–40 were independently diluted into proteolysis buffer to prepare 2 $\times$  pre-mixtures. For insulin competition experiments, insulin was included in the 2 $\times$  A $\beta$  1–40 pre-mixture to twice the final reaction concentration indicated (0–8.6  $\mu\text{M}$ ). 2 $\times$  pre-mixtures were warmed at 37 °C for 15 min, and proteolysis was initiated by combining enzyme and substrate(s) within a pre-warmed PCR tube that was placed in a PCR thermocycler at a constant 37 °C. Reactions were terminated by flash freezing samples in liquid nitrogen, and subsequently heating them to 80 °C for 3 min to inactivate IDE. Samples were stored at –80 °C prior to evaluation by CE or HPLC. For quantitative experiments, chromatographic peak areas were related to peptide concentration by means of a standard curve.

## 2.7. Standardization

For CE experiments, a standard curve was derived using 2-fold dilutions of a 24  $\mu\text{M}$  A $\beta$  1–40 stock solution. Dilutions were prepared in proteolysis buffer. A 12 nl portion of each sample was analyzed by CE. The chromatographic profile of the substrate solvent, DMSO, was highly reproducible, and did not overlap with the profiles of either the A $\beta$  1–40 or insulin peptides. Thus, DMSO peak area was used as an internal standard, providing a loading control for normalization of volume input. For HPLC experiments, a standard curve was generated by preparing 2-fold serial dilutions of a 40  $\mu\text{l}$  stock containing 5  $\mu\text{g}$  A $\beta$  1–40, corresponding with a maximal peptide concentration of approximately 30  $\mu\text{M}$ . Each sample was loaded onto the HPLC column in its entirety. In both instances, chromatographic peak areas observed were graphed vs. the amount of peptide input, and standard curves generated using the 4th-order polynomial regression function of Microsoft Excel™ (see Supplementary Figs. S1 and S2).

## 2.8. CE instrumentation and chromatographic conditions

All CE experiments were conducted using an Agilent G1600AX CE instrument (Agilent Technologies Inc., Santa Clara, CA) equipped with an uncoated fused silica extended light path capillary (55 cm eff. length, 50  $\mu\text{m}$  ID). Prior to initial use, the capillary was subjected to a series of regenerative washes for maximization of electroosmotic flow. The following rinses were sequentially applied at ~1 bar: HPLC grade methanol  $\times$  8 min, 1 N hydrochloric acid  $\times$  8 min, deionized water  $\times$  8 min, 0.1 N sodium hydroxide  $\times$  8 min, deionized water  $\times$  2 min, and background electrolyte  $\times$  8 min. Between runs, capillary conditioning consisted of

sequential rinses with 0.1 N sodium hydroxide and background electrolyte, with each rinse lasting 8 min. Prior to analysis, samples were thawed, sonicated for 10 min, and centrifuged for 1 min at  $16,000 \times g$ . The supernatant from each sample was withdrawn and transferred to the sample-loading carousel of the CE instrument. Sample loading onto the capillary was achieved by a  $20 \text{ mbar} \times 20 \text{ s}$  pressure-controlled injection. Positive polarity electrophoretic separation proceeded for 40 min and was regulated to a maximum current of  $50 \mu\text{A}$ . A 1 min current-regulated ramping period directly followed sample injection. The capillary cassette was thermostated to  $25 \text{ }^\circ\text{C}$ . During separation, analyte absorbance was measured at  $200 \text{ nm}$  wavelength using a diode array detector. Peak area measurements were determined by parabolic interpolation using HP 3D-CE ChemStation software. Subsequent data analysis was performed using Microsoft Excel<sup>TM</sup>, PRISM<sup>TM</sup>, and Plot<sup>TM</sup> graphical analysis software.

### 2.9. HPLC instrumentation and chromatographic conditions

HPLC experiments (see Supplementary data) were performed based on previously established methods (Neuhaus et al., 1999). Reversed-phase separation of A $\beta$  1–40 was achieved using a Vydac low-TFA silica-based C18 column (model 218MS54 C18, Grace Davison Discovery Sciences, Deerfield, IL), which was heated to  $60 \text{ }^\circ\text{C}$  under acidic conditions in the presence of organic solvent. Separation was driven by a Rainin Dynamax solvent delivery system (model SD-200; Rainin Instrument LLC, Oakland, CA). Data analysis was achieved using Varian Star<sup>TM</sup> 6.4.1 graphical analysis software (Varian Inc., Walnut Creek, CA). Prior to sample injection, samples were diluted 1:1 in 0.075% trifluoroacetic acid and 5% MeCN. During separation, analyte absorbance was monitored at  $215 \text{ nM}$  using a UVIS-201 detection system (PerSeptive Biosystems Inc., United Kingdom). A $\beta$  1–40 standard curve and proteolysis experiments were otherwise conducted in an analogous manner to that previously described for CE experiments, with the notable exception that samples were wholly consumed during HPLC analysis.

## 3. Results and discussion

*R. norvegicus* IDE was isolated primarily as a single species whose apparent molecular weight was consistent with expectations (Fig. 1). The N-terminally truncated form of rat IDE used for this study is similar to that described in other studies of the functional enzyme (Shen et al., 2006; Song et al., 2005). The primary IDE species comprises greater than 80% of sample input, as judged using NIH ImageJ densitometry software. This species retains the polyhistidine tag used for purification, as evidenced by Western blotting (Alper and Schmidt, unpublished observation).

Chromatographic profiles for individual components of the IDE proteolytic reaction mixture and IDE-mediated proteolysis products were determined by CE (Fig. 2). Profile A depicts proteolysis buffer, which was used for all CE experiments and typically contained 2.5% DMSO. A peak attributable to DMSO passes the detection window after 20 min electrophoretic migration time, immediately following a chromatographic trough generated by bulk flow of the sample injection plug. Profile B depicts A $\beta$  1–40 as it appears in proteolysis buffer. The characteristic trough and DMSO peak are apparent and are followed by a single new peak, presumably A $\beta$ , which passes the detection window after 31 min migration time. Importantly, the area of this peak remained essentially constant between sample injections, inclusive of samples injected up to 8 h apart, suggesting that peptide aggregation did not appreciably occur during sample storage and processing. Profile C depicts IDE. Besides the trough and DMSO peak, a single peak is observed that passes the detection window at 36 min migration time. DMSO is not intrinsic to the IDE storage buffer but was included in the sample for consistency with other profiles. Profile D depicts the components present in a reaction mixture after co-incubation of IDE and A $\beta$  1–40 for 60 min at  $37 \text{ }^\circ\text{C}$ . The characteristic trough, DMSO, and



IDE peaks are present. However, the A $\beta$  1–40 peak expected at 31 min migration time was nearly eliminated (see arrowhead) and has been replaced by multiple novel peaks that are presumably reflective of the peptide degradation products. While the identity of these species was not determined, IDE is known to cleave A $\beta$  1–40 at multiple sites, which have been previously evaluated (McDermott and Gibson, 1997; Mukherjee et al., 2000). For comparison, chromatographic profiles for components and products of the proteolytic reaction were also determined by HPLC (Fig. S3).

Time-course experiments indicate that IDE-mediated A $\beta$  1–40 proteolysis is a progressive enzymatic process (Fig. 3A). Peaks representative of intact A $\beta$  1–40 peptides were gradually converted into multiple proteolytic species over the course of a 120 min proteolysis experiment. Importantly, chromatographic profiles of the A $\beta$  1–40 degradation products were not observed to overlap with those of the intact peptide species. This allowed for accurate quantification of the amount of A $\beta$  degradation, as inferred from the amount of A $\beta$  remaining (Fig. 3B). A maximal reaction velocity for IDE-mediated A $\beta$  1–40 proteolysis of  $0.16 \mu\text{mol min}^{-1} \text{mg}^{-1}$  was observed under conditions of our assay. This value can be compared with IDE-mediated cleavage of the synthetic fluorogenic Abz-GGFLRKGVQ-EDDnp peptide, which exhibits a  $V_{\text{max}}$  of  $\sim 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , and  $\beta$ -endorphin, which exhibits a  $V_{\text{max}}$  of  $2.6 \mu\text{mol min}^{-1} \text{mg}^{-1}$  (Song et al., 2001). The A $\beta$  degradation progress curve was best fit by a 4-parameter Boltzman sigmoidal response curve.

IDE is capable of degrading multiple substrates, with high affinity substrates being capable of slowing the degradation of low affinity substrates (Qiu et al., 1998). We thus examined the utility of our CE method for evaluating such competition by including insulin in varying amounts in our standard proteolytic reaction (Fig. 4). A $\beta$  1–40 proteolysis was significantly reduced in the presence of insulin, with near to complete inhibition observed under the highest insulin concentration evaluated. Importantly, intact insulin and A $\beta$  1–40 exhibited distinct chromatographic profiles, and peaks representative of the cleavage products generated from proteolysis of either peptide were not found to overlap to a meaningful extent with the intact A $\beta$  1–40 species (Alper and Schmidt, unpublished observation). Increasing DMSO concentrations that accompanied input of increasing amounts of insulin to the proteolysis mixture also did not significantly affect the rate of substrate proteolysis. IDE is reported to have significantly higher affinity for insulin than for A $\beta$  1–40 (Farris et al., 2005), yet, under the conditions employed within this analysis, we observed that insulin proteolysis occurred at a significantly slower rate than A $\beta$  1–40 proteolysis (maximal velocities of  $0.024$  and  $0.16 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , respectively). Thus, inclusion of even small amounts of insulin in the IDE proteolysis mixture had a disproportionate effect upon the extent of A $\beta$  1–40 proteolysis (Fig. 4). Observations such as ours support the hypothesis that alternative substrates can inhibit IDE-mediated A $\beta$  proteolysis, which may provide a mechanistic basis for the risk association linkage between DM2 and AD.

#### 4. Conclusions

We have developed a CE-based method for evaluation of IDE-mediated A $\beta$  1–40 proteolysis *in vitro*. This method is amenable to kinetic analysis and is suitable for demonstrating the effects of alternative substrate competition on A $\beta$  proteolysis. In particular, we have demonstrated that insulin strongly and disproportionately inhibits IDE-mediated A $\beta$  1–40 degradation. This observation provides evidence for IDE function being central to the risk association linking DM2 and AD, and potentially other amyloidopathies.

Our CE-based method offers significant advantages over methods that have previously been used to study A $\beta$  proteolysis. The method requires little specialized equipment, and relies solely upon unmodified and commercially available substrates that are consumed in limited amounts.

This may be contrasted with HPLC-based methods that consume greater amounts of substrate, yet yield lower resolution, while requiring the use of specialized columns, organic solvents, and potentially hazardous reaction components (McDermott and Gibson, 1997; Mukherjee et al., 2000; Neuhaus et al., 1999; also see Supplementary Figs. S1–S3). For example, we estimate that 4–11  $\mu\text{g}$  of A $\beta$  1–40 were consumed upon sample injection during individual runs of reported HPLC-based methods, whereas our CE-based method requires injection of less than 1 ng A $\beta$  1–40 per run for adequate sample detection. Comparative limitations of our CE-based method include increased time of data collection, lack of direct sample collection, and the potential for peak overlap, particularly among uncharged sample components.

Proteolytic processes other than IDE-mediated A $\beta$  1–40 and insulin degradation can likely also be evaluated by CE with minimal modification of the current methodology. For example, investigators seeking to study the proteolytic activity of neprilysin, another A $\beta$ -degrading enzyme, or those who study the degradation of amylin and other short amyloidogenic peptides may find the reported method to be of considerable interest. Indeed, the reported method requires only a satisfactory source of proteolytic activity, adequate analyte mobility and detectability, and sufficiently limited peak overlap among components of the sample mixture to permit tractable analysis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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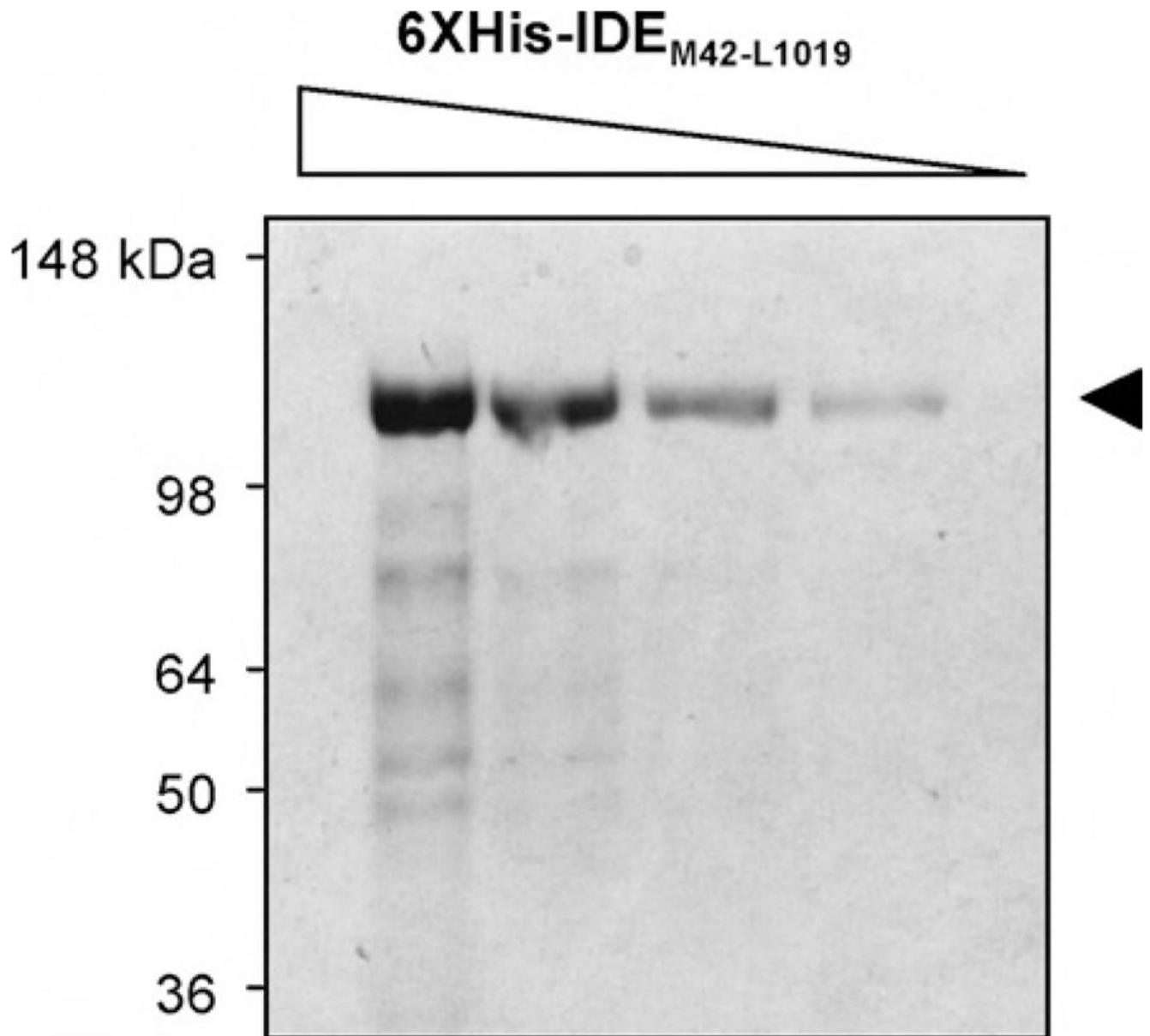


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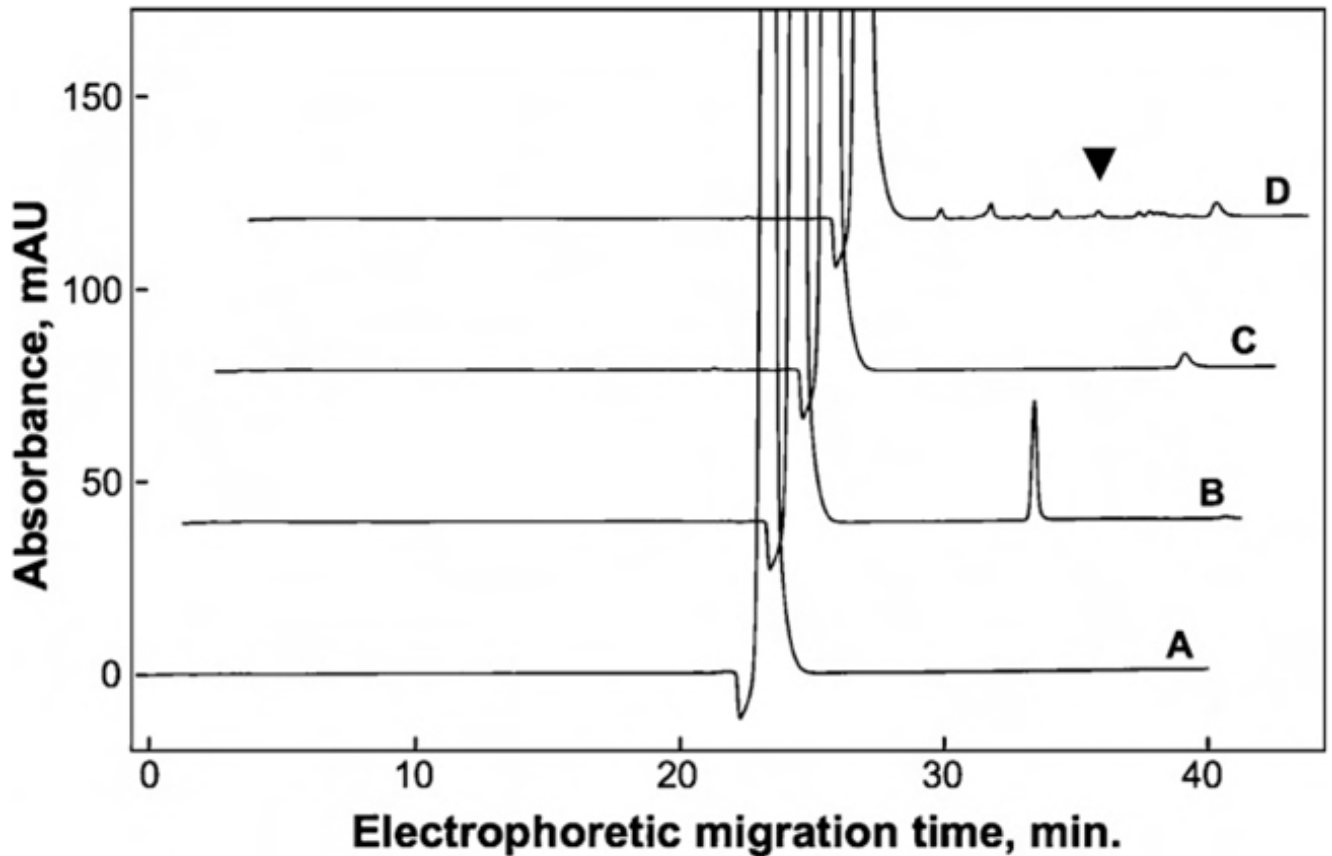
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## Appendix A. Supplementary data

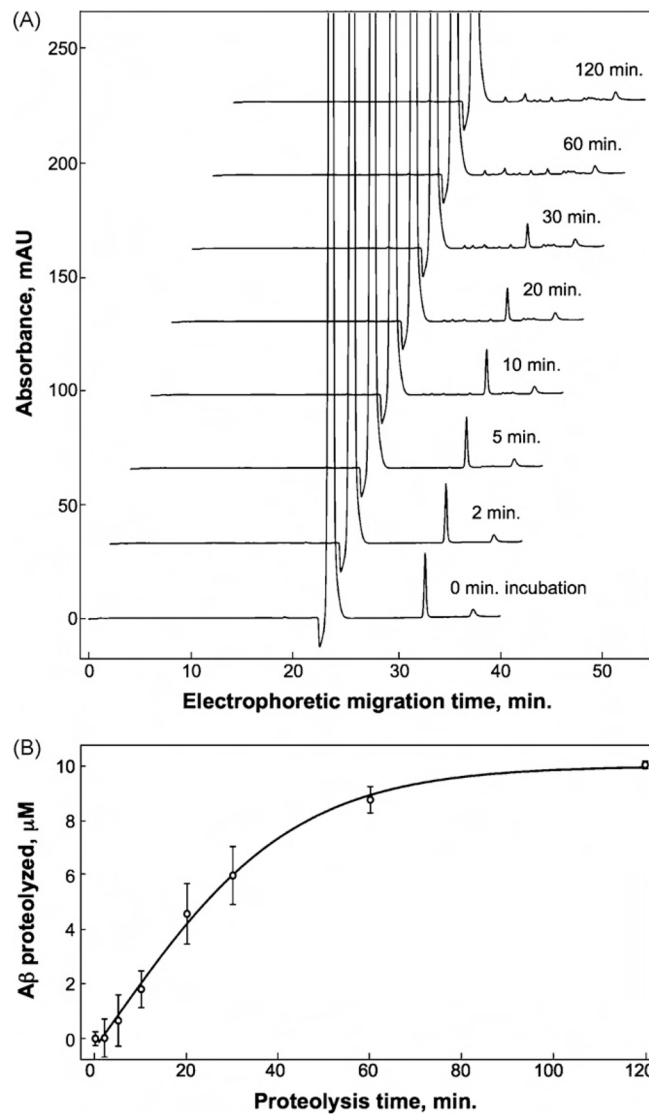
Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.jneumeth.2008.11.010.



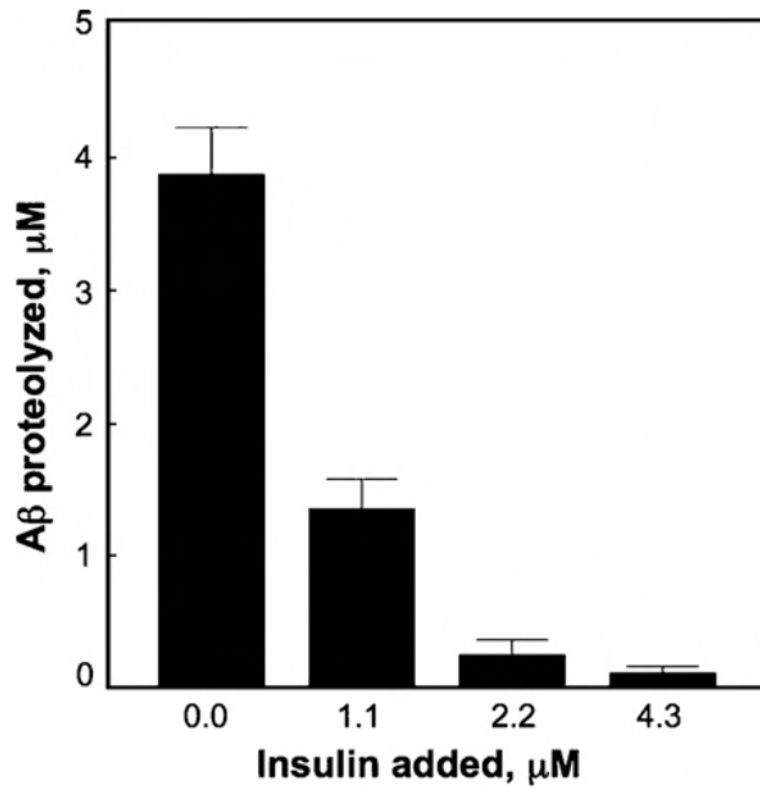
**Fig. 1.** Purification of recombinant IDE. Rat insulin-degrading enzyme (IDE) was heterologously expressed in *Escherichia coli* as a polyhistidine-tagged protein (6XHis-IDE<sub>M42-L1019</sub>) and purified by nickel affinity chromatography. Two-fold serial dilutions of the purified enzyme were analyzed by SDS-PAGE and stained using Coomassie® Brilliant Blue R-250. The leftmost sample of the dilution series contains 10 µg of total protein (lane 1). The isolated IDE species (arrowhead) migrates in a manner consistent with its predicted molecular mass of 114 kDa.



**Fig. 2.** IDE-mediated A $\beta$  1–40 proteolysis can be evaluated using CE. The components of the A $\beta$  1–40 degradation reaction were evaluated alone and in combination by capillary electrophoresis (CE). Shown are the chromatographic profiles of proteolysis buffer (A) alone, or containing (B) 12  $\mu$ M A $\beta$  1–40, (C) 11 nM IDE, and (D) a mixture of 12  $\mu$ M A $\beta$  1–40 and 11 nM IDE. The relative position of intact A $\beta$ , had it not been degraded, is marked (arrowhead). All samples contained 2.5% DMSO, which is responsible for the peak observed immediately after migration of a trough caused by bulk flow of the injection plug, and were incubated at 37 °C for 60 min prior to analysis. Chromatograms depict sample absorbance at 200 nm. Electrophoretic migration time and sample absorbance axes have been offset between chromatograms to facilitate data presentation.



**Fig. 3.** CE permits kinetic analysis of IDE-mediated A $\beta$  1–40 proteolysis. Reaction mixtures initially containing 12  $\mu$ M A $\beta$  1–40 and 11 nM IDE were incubated at 37  $^{\circ}$ C over a 120 min time course. Aliquots were removed at specific times indicated and enzyme activity was quenched. Chromatogram traces (A) were obtained for each sample as described in Fig. 2. A standard curve that correlates A $\beta$  concentration with peak area was used to derive the amount of A $\beta$  degraded at each time point, which is depicted graphically (B). The graphical representation of data derives from the averaged values of three experimental replicates, each having single points for each time point condition. Error bars represent standard deviation from the mean.



**Fig. 4.** Insulin inhibits IDE-mediated A $\beta$  1–40 proteolysis. Reaction mixtures initially containing 12  $\mu\text{M}$  A $\beta$  1–40 and the indicated amounts of insulin were incubated with 11 nM IDE at 37 °C for 20 min. The quantity of A $\beta$  1–40 remaining was determined as described in Fig. 3, which was used in turn to determine the amount degraded. The graphical representation of data derives from the averaged values of four experimental replicates. Error bars represent standard deviation from the mean values indicated.