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Quantification of Amyloid Precursor Protein Isoforms Using Quantification Concatamer Internal Standard

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Abstract

It is likely that expression and/or post-translational generation of various protein isoforms can be indicative of initial pathological changes or pathology development. However, selective quantification of individual protein isoforms remains a challenge, because they simultaneously possess common and unique amino acid sequences. Quantification concatamer (QconCAT) internal standards were originally designed for a large-scale proteome quantification and are artificial proteins that are concatamers of tryptic peptides for several proteins. We developed a QconCAT for quantification of various isoforms of amyloid precursor protein (APP). APP-QconCAT includes tryptic peptides that are common for all isoforms of APP concatenated with those tryptic peptides that are unique for specific APP isoforms. Isotope-labeled APP-QconCAT was expressed, purified, characterized, and further used for quantification of total APP, APP695, and amyloid- β (A β) in the human frontal cortex from control and severe Alzheimer's disease donors. Potential biological implications of our quantitative measurements are discussed. It is also expected that using APP-QconCAT(s) will advance our understanding of biological mechanism by which various APP isoforms involved in the pathogenesis of Alzheimer's disease.

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ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interest.

The pathogenesis of Alzheimer's disease (AD) is marked by deposition of insoluble amyloid- β (A β) resulting from proteolytic processing of amyloid- β protein precursor (APP).¹ Three major isoforms of APP are derived from alternative splicing of exons 7 and/or 8. Two of them, APP770 and APP751, contain a Kunitz-type serine protease inhibitor domain (APP-KPI), whereas the third isoform, APP695, lacks this domain. Studies on variation in APP isoform expression have found either no change in total APP transcript levels or modest reductions in AD-affected areas of the brain. Nevertheless, relative increase in APP-KPI to the total APP has been reported.²⁻⁵ Since APP-KPI may be more amyloidogenic than other APP isoforms, precise quantification of changes in APP isoform expression may be indicative of AD pathogenesis. Traditional approaches to access expression levels of APP include quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), but measurement of specific isoforms remains a challenge.

In addition to measuring full-length APP isoforms, a tremendous need exists for accurate quantification of the 40–42 amino acid long A β . Converging evidence suggests that A β deposition in the brain is the initial pathological feature of AD and that various events occur downstream of A β deposition in the process of cognitive decline. For example, there is strong evidence that apolipoprotein E (apoE) isoforms differentially modulate A β accumulation;^{6,7} several studies have reported an increase in senile plaques in patients with apoE ϵ 4 allele compared with those without the allele.^{8,9} Consequently, quantitative study on apoE-isoform-dependent A β accumulation remains relevant in understanding how apoE genotype influences AD risk. Overall, further investigation of A β involvement in AD would be aided by the development of techniques to precisely quantify level of A β in brain tissues. Currently, there are a number of immunoassays that are routinely employed for measuring A β ; however, these assays give inconsistent values for the level of A β .¹⁰ Better accuracy of quantification has been demonstrated by using liquid chromatography-tandem mass spectrometry (LC-MS/MS).^{11,12}

Multiple reaction monitoring (MRM) mass spectrometry in combination with isotope-labeled quantification concatamer (QconCAT) standards has been proven to be an effective method for quantification of specific proteins in biological samples.¹³⁻¹⁵ In the present study, we have expressed, purified, and characterized isotope-labeled QconCAT (APP-QconCAT) containing tryptic peptides, which are unique for total APP and two APP isoforms, APP695 and A β . We further used APP-QconCAT as an internal standard in MRM assay to quantify the total APP, APP695, and A β in the frontal cortex from control and severe AD donors.

EXPERIMENTAL SECTION

Materials

The Expressway cell-free *E.coli* expression system was from Invitrogen (Carlsbad, CA). The L-[¹³C₆, ¹⁵N₂]lysine and L-[¹³C₆, ¹⁵N₄]arginine (>95% purity) were from Spectral Stable Isotopes (Columbia, MD). The DC protein assay kit was from Bio-Rad Laboratories (Hercules, CA). Sequencing grade modified trypsin was obtained from Promega Corp. (Madison, WI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Expression, Purification, and Characterization of Isotope-Labeled APP-QconCAT

A synthetic gene encoding 160 amino acids composed of the sequence NVQNGKWSDPSGTKTCIDTKGRKQCKTHPHFVIPYRCLVGE-FREKWYKEVHSGQARWLMLVEEVVRVPTTAASTPDAVDKYLETPGKADKKAVIQHFQEKVESLEQEAANERQQLVETDAERHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA was synthesized by Integrated DNA Technologies (Coralville, Iowa). The underlined segments represent the signature proteotypic peptides of APP (Q-peptides). The design of APP-QconCAT also includes six-amino acid long extensions from their natural sequences on both sides of the Q-peptides. The synthetic gene was cloned into the pEXP5CT/TOPO expression vector in-frame to the C-terminal His₆-tag. Isotope-labeled APP-QconCAT was expressed using an in vitro translation kit (Invitrogen) according to the manufacture's protocol. L-[¹³C₆, ¹⁵N₂]lysine and L-[¹³C₆, ¹⁵N₄]arginine were added to the amino acid mixture to replace unlabeled lysine and arginine. After in vitro translation, the labeled APP-QconCAT was purified by nickel–nitrilotriacetic acid resin (Qiagen, Valencia, CA) in batch mode. Finally, the purified APP-QconCAT was loaded onto a SpinTrap G-25 spin column (GE Healthcare, Waukesha, WI) to exchange buffer into 25 mM NH₄HCO₃ with 1% (m/V) sodium dodecyl sulfate (SDS). The protein concentration of APP-QconCAT was measured in the presence of 1% (m/V) SDS using the DC protein assay kit and bovine serum albumin as a standard. The final APP-QconCAT was aliquoted and kept frozen at –80 °C.

Human Tissues

Frozen samples of frontal cortex were received from the Washington University School of Medicine Alzheimer's Disease Research Center. All brain specimens were collected from deidentified donors following informed consent of the respective families. Demographic information on the donors is summarized in the Supporting Information (Table S1). The clinical dementia rating (CDR) listed in Supporting Information Table S1 is a numeric scale used to quantify the severity of symptoms of dementia. The composite score of CDR ranging from 0 through 3 with CDR equals 0 for no symptoms and CDR equals 3 for severe symptoms (<http://www.biostat.wustl.edu/~adrc/cdrpgm/index.html>).

Processing of Samples

The minced brain tissue was placed in 25 mM NH₄HCO₃/1% (m/V) SDS and homogenized by sonication at 30 W using five 10 s continuous cycles (Sonicator 3000, Misonix Inc., Farmingdale, NY). The homogenate was centrifuged at 2000g for 5 min to remove tissue debris. The supernatant was used to measure total protein concentration in the presence of 1% SDS using the DC protein assay kit and bovine serum albumin as a standard. The supernatant was then aliquoted to 0.2 mg of total tissue protein per tube and kept frozen at –80 °C. During the following experiments, samples of 0.2 mg of total tissue protein were supplemented with 20 mM DTT and various amounts of labeled APP-QconCAT, ranging from 0.5 to 10 pmol per sample. The mixture was incubated at room temperature for 60 min to allow reduction of cysteines and was then treated with 50 mM iodoacetamide for another 60 min to alkylate the reduced cysteines. Alkylated samples were precipitated with chloroform/methanol.¹⁶ The protein pellets obtained were sonicated in 100 μL of 25 mM

NH_4HCO_3 and treated with trypsin for 15 h at 37 °C. The substrate/trypsin ratio was 50:1 (m/m). After trypsinolysis, the samples were dried using a vacuum centrifuge (Vacufuge, Eppendorf AG, Hamburg, Germany).

LC–MS/MS Analysis

Dried peptides were reconstituted in 10 μL of 3% acetonitrile/97% water (v/v) containing 0.1% formic acid, and 2 μL was used for a single LC–MS/MS run. Instrumental analyses were performed on a hybrid triple-quadrupole/linear ion trap mass spectrometer (4000 QTRAP, ABI/MDS-Sciex) coupled to an Eksigent nanoLC-2D system (Dublin, CA). Separation of peptides was performed with an Eksigent cHiPLC-nanoflex system equipped with a nano cHiPLC column, 15 cm $\times$ 75 μm , packed with ReproSil-Pur C18-AQ, 3 μm (Dr. Maisch, Germany). Peptides were eluted over a 29 min gradient from 15% to 35% acetonitrile, containing 0.1% formic acid at a flow rate of 300 nL/min. The column effluent was continuously directed into the nanospray source of the mass spectrometer. All acquisition methods used the following parameters: an ion spray voltage of 2200 V, curtain gas of 105 kPa (15 psi), source gas of 140 kPa (20 psi), interface heating temperature of 170 °C, declustering potential of 76 V for +2 precursor ions and 65 V for +3 precursor ions, collision energy of 30 V for +2 precursor ions and 22 V for +3 precursor ions, and collision cell exit potential of 16 V for +2 precursor ions and 13 V for +3 precursor ions. The dwell time for all transitions was 40 ms.

Quantitative Analysis and Validation

The initial list of MRM transitions was selected as previously described¹⁷ and was experimentally screened for the three most intensive transitions per peptide. These transitions were further used for quantification. The relative signal ratios of the three transitions monitored in the 25 mM NH_4HCO_3 for labeled APP-QconCAT were similar to those observed by spiking labeled APP-QconCAT into the frontal lobe homogenate. This indicated no obvious interference from tissue for the quantification based on selected transitions. The identities of the measured peptides were confirmed based on the equivalence of retention time of the three MRM peaks from a given peptide and the ratio among the three MRM peaks. The mean and standard deviation of the protein concentration were calculated by treating the three transitions for each of the different target peptides and the three experimental replicates all as independent measurements.

RESULTS AND DISCUSSION

Characterization of APP-QconCAT Standard

Amino acid sequence of expressed APP-QconCAT includes eight Q-peptides from APP for selective measuring of a total APP (four peptides), $A\beta$ (two peptides), APP695 (one peptide), and APP305 (one peptide). Preliminary measurements on frontal cortex showed that three out of these eight peptides were never detected. APP isoforms and those five peptides that were detected are shown schematically in Figure 1. The APP770 isoform is the canonical full-length sequence of APP. Alternative splicing that removes exon 8 (346–364) and generates APP751 isoform does not produce a unique tryptic peptide suitable for MRM quantification. Therefore, APP770 and APP751 are indistinguishable in the assay

used. Alternative splicing that removes exons 7 and 8 (290–364)) and generates APP695 isoform produces a unique VPTTAASTPDAVDK tryptic peptide (Q2) for a selective measuring of APP695. Truncation (306–770)) and a different from canonical C-terminal sequence of the APP305 isoform produce a unique EVHSGQAR tryptic peptide (Q3) for a selective measuring of APP305. THPHFVIPYR (Q1) and VESLEQEAAANER (Q4) tryptic peptides were used for measuring total APP. LVFFAEDVGSNK tryptic peptide (Q5), which is present in both total APP and A β was used for measuring A β by subtracting values obtained for the total APP (Q1, Q4) from those measured based on Q5.

The purity of used APP-QconCAT was considered to be 95% based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 2A), and no correction for protein concentration of APP-QconCAT was made during data analysis. Optimal MRM transitions for Q-peptides were experimentally selected from analysis of APP-QconCAT tryptic digestion (Table S2, Supporting Information). These transitions were then used to determine the level of stable isotope incorporation into the APP-QconCAT. Figure 2B shows representative extracted ion chromatograms for Q2 and Q4 peptides. Two transitions per peptide are shown. It is evident that both a light and heavy version of peptides is present, but the heavy version of peptides is dominant. The isotope incorporation was calculated as the percentile of the area of the labeled peak to the sum of the unlabeled and labeled peaks. Calculation based on all five Q-peptides resulted in a total of $98.1\% \pm 0.4\%$ isotope incorporation in the APP-QconCAT. This value was accepted as a complete labeling, and no correction for labeling efficiency was applied during data analysis.

To further characterize the APP-QconCAT, linearity of the optional transitions was verified by spiking temporal lobe samples with 0.5–10.0 pmol of the labeled APP-QconCAT. All standard curves showed a low scatter and linearity over the concentration range tested. Figure 2C shows representative standard curves for two transitions of Q1. Standard curves were also used to calculate a limit of quantification (LOQ), which was defined as the lowest calibration point of the curve that could be measured with a coefficient of variance less than 20%.¹⁸ The LOQ for total APP, APP695, and A β was 1.0 pmol/mg of tissue protein.

Quantification of APP Isoforms

We were not able to detect the peptide specific to APP305 isoform. Presumably, this short isoform does not exist in tested frontal cortex samples or the level of APP305 is lower than our LOQ. Quantifications of the total APP (APP770 + APP751 + APP695), specific APP695, and A β are summarized in Figures 3 and 4.

Parts A and B of Figure 3 show data for total APP and APP695 in the control group (CDR 0) and severe AD group (CDR 3), respectively. Although there is a donor-to-donor variability in absolute levels, it seems that the mean value of total APP remains similar for both donor groups: 3.7 ± 0.6 pmol/mg of tissue protein for the control group and 3.3 ± 0.4 pmol/mg of tissue protein for the severe AD group. At the same time, the mean value for APP695 seems to decline from 3.0 ± 0.7 pmol/mg of tissue protein in the control group to 1.9 ± 0.4 pmol/mg of tissue protein in the severe AD group (significantly different with $p < 0.05$, $n = 6$). In other words, APP695 represents 80% of total APP in our control group (leaving 20% for APP770/APP751) and only 60% of total APP in our severe AD group

(leaving 40% for APP770/APP751). On the whole, our data point to elevated level of APP700/APP751 (KPI-APP) in severe AD.

The Q5 peptide is present in both A β and in the total APP. Therefore, the measured concentration based on Q5 (Figure 4) results from contribution of A β and total APP. After subtraction of the signal coming from total APP, we observed a donor-to-donor variable level of A β in the control group ranging from approximately 1 pmol/mg of tissue protein to 27 pmol/mg of tissue protein (Figure 4A). In the severe AD group (Figure 4B), the level of A β is much higher, reaching 570 pmol/mg of tissue protein in donor 7.

For both Figures 3 and 4, the apoE genotype is shown for each donor. We used a Student's *t* test to compare groups with different apoE genotypes. For total APP and APP695 (Figure 3B), the *p*-values varied from 0.1 to 0.8 for different compared groups. We concluded that there is no correlation between apoE genotype and levels of total APP or APP695 (Figure 3) in our sample set. However, *p*-values for $\epsilon 3/\epsilon 3$ versus $\epsilon 3/\epsilon 4$ and $\epsilon 3/\epsilon 4$ versus $\epsilon 4/\epsilon 4$ were 0.005 and 0.006, respectively. This indicates a correlation between apoE genotype and A β accumulation in the severe AD group (Figure 4B). Donors with $\epsilon 3/\epsilon 3$ genotype possess a lower level of A β than those with $\epsilon 2/\epsilon 4$ or $\epsilon 3/\epsilon 4$, whereas donors with $\epsilon 4/\epsilon 4$ possess the highest level of A β (Figure 4B). At the same time, our limited data support the notice that A β accumulation is not always necessary for the development of AD. For example, donor 11 in the severe AD group has a basic level of A β .

In summary, QconCATs were initially proposed as a robust MRM approach for large-scale proteome quantification.¹⁹ In the present work, we aimed to demonstrate practical application of QconCAT technology to targeted quantification of various isoforms of a protein of medical significance. The obtained data illustrate the feasibility of applying QconCAT standards to quantify proteome isoforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

APP isoforms. Schematic presentation of selected Q-peptides for quantification of APP isoforms. Q-peptides are shown in rose. The Kunitz-type serine protease inhibitor domain (KPI) is shown in green. The unique C-terminal sequence of APP305 is shown in red. Aβ is shown in yellow. Sequence truncations are marked with Δ. Linear sizes of proteins and peptides are not in exact scale.

Figure 2.

Characterization of APP-QconCAT. (A) A 15% SDS-PAGE of His₆-tagged APP-QconCAT purified on nickel-nitrilotriacetic acid resin. The molecular mass standards are shown on the left. (B) Stable isotope incorporation into the APP-QconCAT. MRM spectra for two representative transitions per Q2 and Q4 peptides are shown. The pair transitions for the light (unlabeled) and heavy (labeled) form of each Q-peptide are color-coordinated. The inset for the light forms shows zoomed peaks of unlabeled Q-peptides. The isotope incorporation was calculated as the percentile of the area of the labeled peak to the sum of the unlabeled and labeled peaks. The final value was calculated based on combined data for all five Q-peptides and is presented as mean $\pm$ SD. (C) Standard curves. The area ratio of heavy to light peaks for a selected transition is plotted vs the supplemented APP-QconCAT amount for each Q-peptide. Two representative standard curves are shown. Transitions: Q1-t1 is 633.8/794.5 and 638.8/804.5; Q1-t2 is 633.8/931.5 and 638.8/941.5.

Figure 3.

Quantification of total APP and APP695. Measurements were performed on the control (A) and severe AD (B) frontal cortex. Total APP is represented by the average of both Q₁ and Q₄ peptides while APP695 was quantified based on Q₂ peptide. Control donor IDs are 1, 5, 12, 13, 14, and 18, and severe AD donor IDs are 2, 3, 8, 10, 11, and 19 (Supporting Information, Table S1). ApoE genotype is shown for each donor. The concentration was calculated for three experimental replicates by monitoring three transitions per individual peptide and is presented as mean $\pm$ SD.

Figure 4.

Quantification of A β . Measurements were performed on the control (A) and severe AD (B) frontal cortex based on Q₅ peptide. Control donor IDs are 1, 5, 12, 13, 14, and 18, and severe AD donor IDs are 2, 3, 6, 7, 8, 10, 11, 16, 17, and 19 (Supporting Information, Table S1). ApoE genotype is shown for each donor. The concentration was calculated for three experimental replicates by monitoring three transitions per individual peptide and is presented as mean $\pm$ SD.