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Expression, purification and characterization of a synthetic gene encoding human amyloid ß (Aß1-42) in *Escherichia coli*

Sarada Subramanian* and A N Divya Shree

Department of Neurochemistry, National Institute of Mental Health & Neurosciences, Bangalore 560 029, India

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of cognitive function. Existing evidence indicates that abnormal processing and extracellular deposition of the longer form of the amyloid peptide Aß(1-42), a proteolytic derivative of the amyloid precursor protein (APP), is a key step in the pathogenesis of AD. Active immunization with Aß(1-42) has been shown to decrease brain Aß deposition and improve cognitive performance in mouse models of AD. In the present study, we sought to express the synthetic gene encoding Aß in *Escherichia coli* to enable rapid production of the antigen and its purification. The synthetic gene has been constructed from six oligonucleotides by employing overlapping PCR strategy and expressed in *E. coli* using the T7 promoter system. The recombinant peptide has been purified to homogeneity by a single step Ni^{+2} affinity chromatography. Enzyme-linked immunosorbent assay (ELISA) using polyclonal anti-Aß(1-42) sera confirms that the corresponding linear B-cell epitopic sequences are available for immunorecognition in the recombinant peptide. This methodology enables rapid, continuous production and purification in bulk amounts of human Aß sequence by employing bacterial expression system

Keywords*:* Alzheimer's disease, Amyloid ß peptide, *Escherichia coli*, Overlapping PCR, Synthetic gene, ELISA, Expression, Purification and characterization

Alzheimer's disease (AD) is the most common cause of age-related cognitive decline, affecting >12 million people worldwide[1]. An invariant pathological hallmark of AD is the deposition of plaques consisting largely of the 40-42 amino acid amyloid ß (Aß) peptide. Aß peptide is generated by the consecutive cleavage of a family of ubiquitously expressed membrane-spanning proteins, the amyloid precursor proteins (APP)[2] by proteases ß- and γ-secretases. Another protease α-secretase cleaves APP within the Aß domain and therefore precludes Aß formation. Under normal conditions, Aß(1-40) peptide is the most abundant species in the brain, though, fibrillar Aß is mainly composed of the longer, more fibrillogenic Aß(1-42) peptide[2]. The physiological role of Aß(1-42) remains largely unknown. However, recently soluble Aß(1-42) has been found to be an endogenous regulator of neural activity[3].

*Corresponding author
Tel: 91 80 26995165; Fax: 91 80 26564830
E-mail: sarada@nimhans.kar.nic.in; saradabs@yahoo.com

Abbreviations used: Aß, amyloid ß peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; IPTG, isopropyl thiogalactoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride.

The aetiology of AD is thought to be the result of an imbalance between Aß(1-42) production and clearance[4,5]. The accumulated peptides undergo conformational change and polymerize into an aggregated and toxic form, rich in ß-structure[6]. Prevention and/or reversal of this aggregation process may serve as a treatment for AD. Aß deposition and its toxic effects could be prevented or reversed by decreasing its production, preventing its conversion to insoluble forms (inhibiting ß-sheet formation), by changing the dynamics of extracellular brain Aß either locally within the brain or by altering net flux of Aß between the central nervous system and plasma compartment[7]. The active immunization with pre-aggregated form of the Aß peptide decreases brain Aß deposition[8] and improves cognitive performance in transgenic mouse models of AD[9,10]. Certain peripherally administered anti-Aß antibodies have also shown similar effects[11].

Many important aspects of Aß biology remain unknown because the intrinsic biochemical and physical properties of Aß make it difficult to investigate its function and pathological effects. In particular, the tendency of Aß to form aggregates has led to suggestions that fibrils or soluble oligomers are the active components of Aß that ultimately cause synaptic loss and dementia associated with AD[12-14].

Although chemical methods for synthesizing Aß have improved[15], its production in bulk amounts still remains a challenge, primarily due to its aggregating nature. Here, we report molecular cloning and production of Aß(1-42) by expressing it in *E. coli.* The gene product has been characterized for its antigenic properties.

Materials and Methods

Animals

Albino rabbits used for production of polyclonal antibodies were from the Institute's Central Animal Research Facility. They were fed pelleted diet and water *ad libitum* and were exposed to 12 h light/12 h dark schedule under controlled conditions of humidity and ambient temperature.

Materials

Restriction enzymes, T4 DNA ligase, and 100 bp DNA ladder were purchased from MBI Fermentas (Lithuania). Deep Vent DNA polymerase was obtained from New England BioLabs (UK). Aß synthetic peptide was custom synthesized by Xcyton Diagnostics Pvt. Ltd. (Bangalore) and its purity (>95%) was established by reverse-phase HPLC. Plasmid pRSET B was purchased from Invitrogen (Carlsbad, CA, USA). Agarose, ampicillin, phenylmethanesulfonyl fluoride (PMSF), isopropyl thiogalactoside (IPTG), urea, ß-mercaptoethanol, imidazole, Ni CAM HC resin, 3,3',5,5'-tetramethyldiamino benzidine, goat anti-rabbit IgG conjugated to horseradish peroxidase, bovine serum albumin (fraction V), Freund's complete and incomplete adjuvants (FCA/FIA) were from Sigma Chemical Co. (St. Louis, USA). Enterokinase cleavage capture kit was purchased from Novagen (USA). Oligonucleotides, Genei Quick PCR purification columns and low molecular weight protein standards for SDS-PAGE were purchased from Bangalore Genei Pvt Ltd (Bangalore). High-binding microtitre plates were purchased from Greiner Labortechnik Ltd. (Germany). All other reagents used were of analytical grade and obtained locally.

Synthesis of Aß minigene by PCR amplification

PCR was performed in a 50 µl final volume containing PCR buffer (10 m*M* Tris-Cl, *p*H 8.8, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, and 0.1% Triton X-100) and dNTPs (200 µ*M*) in the presence of 1 U Deep Vent DNA polymerase for 20 cycles. Each cycle consisted of a 1 min denaturation at 96°C, followed by 1 min annealing (at 50°C for synthesis of "core template" and at 45°C for second and third rounds of PCR) and a 2 min extension at 72°C. The final PCR product was purified through Genei Quick PCR purification columns as per manufacturer's instructions.

Construction of recombinant plasmid

Bacterial cultures, plasmid purification and transformations were performed using standard protocols[16]. An aliquot of the purified synthetic DNA obtained by the PCR amplification was digested with *Bam*H1 and *Eco*R1. The resulting fragment was purified through LMP-agarose gels and cloned into pRSET B vector pre-digested with the same restriction endonucleases. The ligation mixture was used to transform competent DH5α cells and the transformants were selected by ampicillin resistance. Sequence of the recombinant plasmid DNA was obtained by automated DNA sequencing performed in the DNA sequencing facility at Indian Institute of Science, Bangalore. The recombinant plasmid was used to transform competent BL21 (DE3) pLysS cells, plated on to LB/ampicillin plates.

Expression and purification of recombinant Aß

One recombinant clone was inoculated into LB medium supplemented with 50 µg/ml ampicillin and grown at 37°C till OD_{600} reached 0.6. For induction of protein, IPTG was added to 1 m*M* final concentration and growth of the culture continued for an additional 2 h. Cells from 100 ml of induced culture were harvested by centrifugation for 10 min at 5520 *g* at 4°C, suspended in 5 ml of lysis buffer (50 m*M* Tris-Cl, *p*H 8.0 containing 300 m*M* NaCl, 5 m*M* ß-mercaptoethanol and 2 m*M* PMSF) on ice and disrupted by sonication (6 × 10 s cycles, on ice). The soluble fraction was separated from the insoluble fraction by centrifugation at 17,400 *g* for 20 min at 4°C and the latter was dissolved in lysis buffer containing 8 *M* urea. After centrifugation at 10,000 *g* for 30 min, the supernatant was loaded onto Ni^{+2}-CAM affinity matrix (1 × 5 cm) pre-equilibrated with lysis buffer containing 10 m*M* imidazole. Bound proteins were eluted with lysis buffer containing 400 m*M* imidazole. Fractions of 1 ml were collected and subjected to 15% SDS-PAGE analysis. For cleavage of the N-terminal fusion tag, Ni^{+2}-affinity purified recombinant protein was dialyzed against 20 m*M* Tris-Cl (*p*H 8.0) and subjected to digestion with enterokinase enzyme (1 Unit/50 µg of recombinant protein) for 20 h at room temperature as per the manufacturer's instructions.

Production of polyclonal antibodies

Synthetic Aß emulsified in FCA was injected into rabbits at a dose of 500 µg/animal for primary injection. For subsequent boosters at 3-weeks intervals, 250 µg of protein emulsified in FIA was administered subcutaneously. After each booster, the rabbits were bled, sera collected and stored in aliquots at –20°C until use. Animal Ethics Committee of this Institute approved this study.

Characterization of antiserum

The immune recognition patterns of rabbit anti-Aß serum towards the recombinant peptide and synthetic Aß was determined by direct ELISA[17]. Briefly, 2 µg of each peptide in PBS was coated in a high-binding ELISA plate. After blocking the unoccupied sites with 0.3% (w/v) BSA, these wells were probed with serially diluted antiserum. The antigen-antibody complex was visualized by employing peroxidase conjugated goat anti-rabbit IgG and 3,3′,5,5′-tetramethyldiamino benzidine as the chromogen. The colour developed was monitored at 450 nm.

Results and Discussion

In the present study, we preferred to synthesize Aß gene instead of subcloning the gene by conventional cloning strategies for the following advantages: i) the designed gene contained the desired restriction sites and with a specific codon usage; and ii) it was optimized for heterologous expression in prokaryotic system. The methodology employed in this study was fast, reliable and economical based on PCR. This procedure could be completed within 1 working day, since no ligations were required, and the PCR product could be used for the amplification of the synthetic DNA.

Synthesis and cloning of the gene for Aß

An *E. coli* optimized 135 bp nucleotide sequence encompassing the ORF of Aß was designed (Fig. 1). The approach employed in the synthesis of Aß minigene was similar to that described earlier[18]. A cDNA encoding Aß was deduced from its amino acid sequence. The sequence was then divided into four oligonucleotides (45 bases each) (Fig. 2) for a total of 135 bases. Two end primers of 30 nucleotides length were employed to introduce the recognition sites for *Bam*H1 and *Eco*R1 at 5′- and 3′-ends respectively. The overlaps between the oligonucleotide pairs were 15 bases with T_m values of 45-50°C.

In the first round of PCR, two primer oligonucleotides A and B (Fig. 2) were used at a concentration of 0.1 m*M* for the synthesis of a central template. A total of 1/20 of this mixture was used as a template for the subsequent PCR experiments. After each PCR run, 1/20 aliquot of the reaction mixture was withdrawn and added to fresh PCR buffer and dNTPs. New primers (i.e., C and D) in the second round of PCR were then added at 1 m*M* final concentration along with 1 U Deep Vent DNA polymerase, and the new elongation continued for additional 20 cycles. The same procedure was repeated until the final end primers i.e., E and F were used. The products of each PCR experiment and their purity were analyzed by 2% agarose gel electrophoresis. A stepwise elongation of the core template (75 nt) occurred after the second round (135 nt) and finally yielding a 155 nt product after third round of PCR (Fig. 3). The final PCR product was double-digested with *Bam*H1 and *Eco*R1 and cloned into pRSET B vector.

The DNA sequence of the recombinant plasmid revealed that the inserted sequence was correct and corresponded to the gene as designed, except for a single change from C to G in the third position in the codon for glycine (residue 34) that did not change the coded amino acid. This plasmid allowed the expression of the recombinant peptide with an N-terminal fusion peptide (40 amino acids) containing 6x His tag and an

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ATG GAT GCC GAG TTC AGA CAT GAT TCT GGC TAT GAA GTG CAT CAT CAG AAA   51
 M  D  A  E  F  R  H  D  S  G  Y  E  V  H  H  Q  K                  17

CTG GTG TTC TTC GCC GAA GAC GTG GGC TCT AAC AAA GGC GCC ATC ATC GGC  102
 L  V  F  F  A  E  D  V  G  S  N  K  G  A  I  I  G                  34

CTG ATG GTG GGC GGC GTG GTG ATC GCC TAA TAA                         135
 L  M  V  G  G  V  V  I  A stop stop                                 43
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Fig. 1—Complete DNA sequence of the synthetic gene encoding Aß and its primary amino acid sequence represented in single letter code

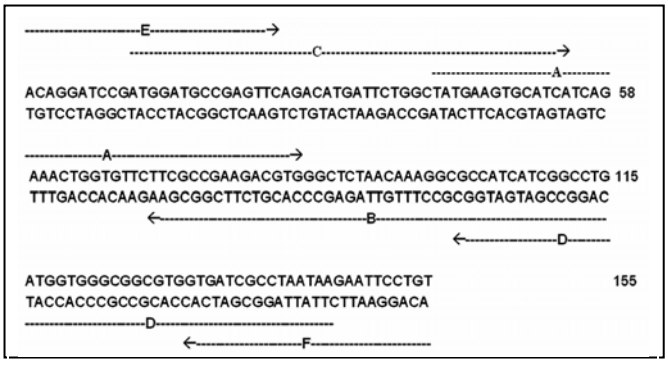

Fig. 2—Sequence of the synthetic gene encoding Aß [The underlined nucleotides indicate the sequences recognized by *Bam*H1 and *Eco*R1 at 5′- and 3′-ends respectively. Arrows indicate the sequence of oligonucleotides used in the synthesis of the gene]

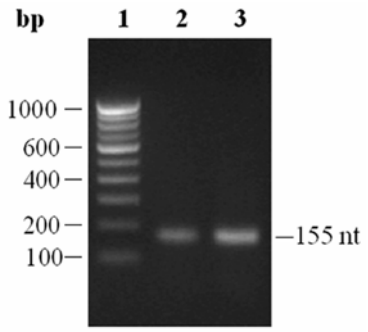

Fig. 3—Final product of consecutive PCR experiments for the synthesis of Aß cDNA [Lane 1, 100 bp DNA ladder was loaded as marker; lane 2, 5 µl of the final PCR product was loaded; and lane 3, column purified Aß DNA]

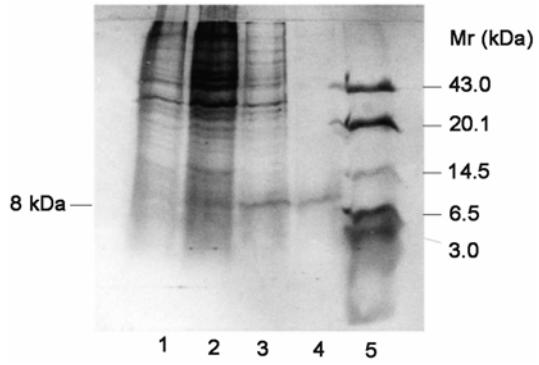

Fig. 5—SDS-PAGE (15%) analysis of Aß produced in *E. coli* [Lane 1 and 2, staining patterns obtained with cell lysates from 250 µl of non-induced and induced cultures; lane 3, proteins dissolved in 8 *M* urea; lane 4, metal-ion affinity chromatography purified Aß (5 µg); and lane 5, low molecular weight markers]

enterokinase cleavage site (Fig. 4). Expression of Aß was achieved by transformation and induction of BL 21 (DE3) cells by 1 m*M* IPTG. SDS-PAGE analysis of the induced and non-induced cells extracted in electrophoresis loading buffer showed that a protein species with an anticipated molecular mass of approximately 8 kDa was produced in the induced cells (Fig. 5).

Purification and characterization of recombinant Aß

Using a Ni^{+2}-charged resin, it was possible to purify Aß to homogeneity from the 8 *M* urea dissolved insoluble fraction (Fig. 5) in a single step. Yields were about 10 mg of fusion protein per litre of bacterial culture. The N-terminal fusion tag of the recombinant protein was subjected to enterokinase digestion. It was observed that the fusion protein underwent cleavage (Fig. 6). The apparent molecular mass of processed Aß was approximately 4 kDa according to SDS-PAGE (Fig. 6). Cleaved Aß was separated from the digestion mixture by using Ni^{+2}-affinity column. Final yields of

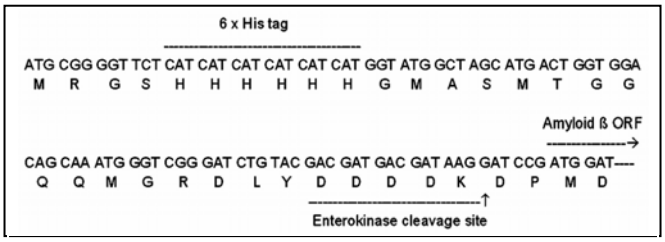

Fig. 4—N-terminal amino acid sequence of recombinant Aß aligned with cDNA sequence expressed by the plasmid pRSET B

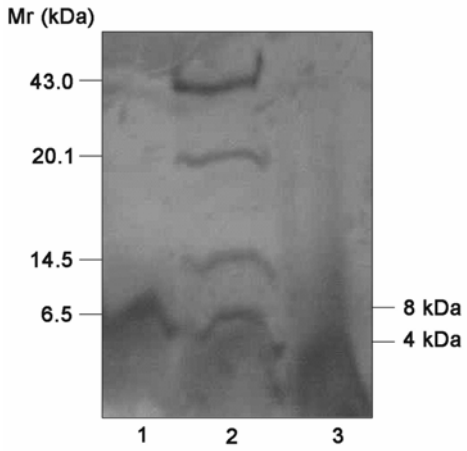

Fig. 6—15% SDS-PAGE analysis of the cleavage of Aß fusion protein with enterokinase [Lanes 1 and 3, the reaction mixture before and after digestion with enterokinase; and lane 2, low molecular weight markers]

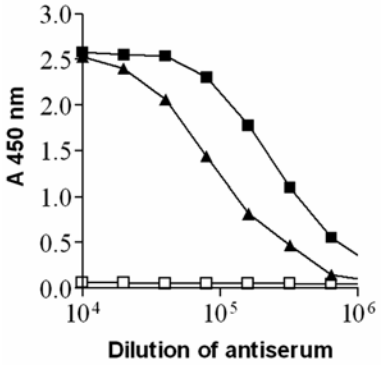

Fig. 7—Reactivity of rabbit polyclonal anti-Aß serum as determined by ELISA [Antibody dilution curves obtained with 2 µg/well of recombinant Aß (▲) or chemically synthesized Aß (■), and values obtained with pre-immune serum (□)]

the recombinant Aß peptide were approximately 4 mg/L of bacterial culture.

To examine whether recombinant Aß harboured the epitopes dictated by the chemically synthesized Aß, an ELISA was carried out. Analysis included polyclonal antiserum raised against chemically synthesized Aß peptide in the rabbits. It was evident from the closely parallel binding curves (Fig. 7) that the B-cell

epitopes in the recombinant Aß were shared with the chemically synthesized Aß.

In conclusion, we report a simple procedure for production of bulk amounts of Aß by employing bacterial expression system, as a first step towards the development of Alzheimer's vaccine. In addition, recombinant Aß is likely to be a valuable tool for investigating the physiological role of Aß *in vitro* and *in vivo.*

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