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Purification of recombinantly expressed and cytotoxic human amyloid-beta peptide 1–42

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Abstract

The amyloid cascade hypothesis assigns the amyloid-beta peptide (Aβ) a central role in the pathogenesis of Alzheimer's disease (AD). Although there are strong efforts to biophysically characterize formation of Aβ aggregates and fibrils, as well as their prevention, progress is still severly hampered by the availability of tens of milligrams of recombinant Aβ(1–42). Here, we describe a reliable and easy procedure to recombinantly express and purify Aβ(1–42), which is fully cytotoxic and able to form fibrils without any further refolding steps. The yield of the procedure is 5–8 mg of tag-less peptide per liter culture volume.

Keywords: Purification; Amyloid-beta peptide; Cytotoxicity; Aggregation; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which is characterized by memory loss, confusion and a variety of cognitive disabilities [1]. The most reliable diagnosis of a person having AD is the *post mortem* identification of amyloid plaques and neurofibrillary tangles in the respective brain. The major component of the amyloid plaques is Aβ peptide consisting of 39–43 amino acid residues, predominantly Aβ(1–42). Aβ is produced from the amyloid precursor protein (APP) by two distinct proteolytic activities, called β- and γ-secretases [2–4]. These formerly proposed proteolytic activities have been assigned to certain proteins, called BACE [5,6] and presenilin-1 [7], the latter possibly in combination with other factors like nicastrin [8].

As originally suggested by the amyloid cascade hypothesis [9], it appears likely that Aβ peptides and their aggregates initiate cellular events leading to the pathologic effects of AD. This hypothesis is strictly supported by the identification of numerous mutations linked to early-onset familial AD, increasing either the level of Aβ production (especially Aβ(1–42)) or the propensity of mutated Aβ to form aggregates [10,11].

Substances that are able to inhibit Aβ aggregation and reduce its toxic effects are still highly desirable. A variety of such substances were described, e.g. congo red, haloperidol, nicotine, hexadecyl-*N*-methylpiperidiniumbromid, laminin and rifampicin. Small peptides that exhibit such effects are also described [12,1].

For diagnosis and potential therapy, compounds that directly address or specifically bind to Aβ peptide, are highly desired. We earlier described a mirror image phage display with the D-amino acid enantiomer of Aβ(1–42) as a target, we identified an Aβ binding D-peptide (Dpep) and characterized its Aβ plaque binding properties [13].

In order to study the interactions of Aβ(1–42) with D-peptides by biophysical methods, large amounts of Aβ(1–42) are needed. Especially, for investigations by nuclear magnetic resonance (NMR) spectroscopy, stable isotope labelled Aβ(1–42) is required. Aβ(1–42) is characterized by its extreme tendency to aggregate into fibers or to precipitate. Thus, large-scale production of the recombinant amyloidogenic peptide has proven particularly difficult. We constructed an expression system for production and developed a reliable procedure for purification of recombinant Aβ(1–42).

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2. Experimental

2.1. Cloning

2.1.1. Oligonucleotides

All oligonucleotides were obtained (HPLC-grade) from BioTeZ (Berlin, Germany).

Abeta_1 GGAGGAGGTACCCATATGGACGCTGAATTTC-GCCACGACTCCGGCTATGAGGTACACCACCA-GAAACTGGTTTTTTTTGCTGAGGACGTTGGC (93 mer)

Abeta_2 TCCAACAAAGGTGCTATCATCGGTCTGATGG-TTGGCGGCGTTGTTATCGCTTAAGCTGAGCG-AGCTCAGGAGG (73 mer)

Abeta_3 AAAAAAAACCAGTTTCTGGTGGTGTACCTCA-TAGCCGGAGTCGTGGCGAAATTCAGCGTCCA-TATGGGTACCTCCTCC (78 mer)

Abeta_4 CCTCCTGAGCTCGCTCAGCTTAAGCGATAAC-AACGCCGCCAACCATCAGACCGATGATAGCA-CCTTTGTTGGAGCCAACGTCCTCAGC (88 mer)

2.1.2. Construction of pET15b_Aβ(1–42)

To generate a synthetic gene coding for Aβ(1–42), oligonucleotides Abeta_1, Abeta_2, Abeta_3 and Abeta_4 were annealed. The annealing was performed by the following procedure: 1000 pmol of the four oligonucleotides were mixed with buffer (10 mM Tris–HCl; 10 mM $MgCl_2$; 0.1 mg/ml BSA, pH 7.5). The annealing reaction conditions were 95 °C for 5 min, 90 °C for 5 min, 85 °C for 5 min, 80 °C for 5 min, 75 °C for 5 min, 70 °C for 90 min, 65 °C for 5 min, 60 °C for 10 min, 55 °C for 5 min, 50 °C for 5 min, 45 °C for 5 min, 40 °C for 5 min. The annealing product was separated by electrophoresis in 3% (w/v) agarose gel and extracted (Qiagen Gel Extraktion Kit, Qiagen, Hilden, Germany). The resulting gene coding for Aβ(1–42) flanked by NdeI and Bpu1102I restriction sites was restricted by NdeI and Bpu1102I. After restriction, the DNA was separated by electrophoresis in 3% (w/v) agarose gel and extracted (Qiagen Gel Extraktion Kit). This purified DNA was ligated with NdeI/Bpu1102I restricted pET15b. The ligation product was electroporated into *E. coli* DH5a. The correctness of the gene coding for Aβ(1–42) was verified by DNA sequencing. DNA sequence analysis was performed by Seqlab (Göttingen, Germany). Finally, *E. coli* BL21(DE3) Star cells (Invitrogen, Leek, Netherlands) were transformed with plasmid pET15b_Aβ(1–42) coding for polyhistidine-tagged Aβ(1–42) with following amino acid sequence: GSSHHHHHHSSGLVPRGSHMDAEFRHDS-GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA.

2.2. Expression

A 1:50 inoculum of an overnight *E. coli* BL21(DE3) Star pET15b_Aβ culture in 1 l LB broth (10 g tryptone; 5 g yeast extract; and 5 g NaCl/l) containing 200 μg/ml ampicillin was allowed to grow aerobically at 37 °C to an OD_{600} of about 0.6. Expression of Aβ(1–42) was then induced with 0.5 mM IPTG. After further growth overnight at 37 °C, cells were harvested by centrifugation (5000 × *g*, 30 min) and sonicated in 5–10 ml urea buffer (8 M urea in 100 mM NaH_2PO_4 and 10 mM Tris–HCl, pH 8.0). The extract was clarified by centrifugation (20,000 × *g*, 45 min).

2.3. Immobilized metal affinity chromatography/cleavage

The cell-free extract was applied to a column filled with 2.5 ml Ni-NTA material (Qiagen), equilibrated with urea buffer (8 M urea in 100 mM NaH_2PO_4 and 10 mM Tris–HCl, pH 8.0). After washing with 2 column volumes of urea buffer, non-specifically bound protein was removed by washing with 2 column volumes of each 10, 20 and 50 mM imidazole in urea buffer. The flow rate was 1 ml/min. The column was washed with 5 column volumes PBS buffer (140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4). Obviously, removal of urea did not result in precipitation of the Aβ peptide bound to the resin. We did not observe any decrease in flow rate of the column. Approximately, 200 units of thrombin (Sigma–Aldrich, Deisenhofen, Germany), dissolved in PBS buffer were added and the column was shaken very carefully 1 h at 37 °C, following incubation at room temperature overnight. After completion of cleavage, the column was washed again with 5 column volumes of PBS buffer and 3 column volumes of urea buffer.

Aβ(1–42) was eluted by application of 2 column volumes of urea buffer pH 2.0 (8 M urea in 100 mM NaH_2PO_4 and 10 mM Tris–HCl, pH 2.0). The eluate was dialysed twice for at least 5 h in water containing 0.1% (v/v) trifluoroacetic acid (TFA) using a Spectra/Por membrane (molecular mass cut-off 1000 M_r; Spectrum Laboratories, Compton, USA). The sample was then frozen at −80 °C and lyophilized.

2.4. Reversed phase HPLC (RP-HPLC)

Lyophilized Aβ(1–42) was dissolved in 0.5 ml buffer A (water containing 5% (v/v) acetonitrile and 0.05% (v/v) TFA). RP-HPLC was performed employing a heated (60 °C) Sephasil peptide C18 column (12 μm ST4.6/250, Macherey-Nagel, Düren, Germany) using a Merck Hitachi system composed of L-5025 column thermostat, L-4500A diode array detector, D-600 interface module and L-6220 intelligent pump (Merck Hitachi, Darmstadt, Germany). The peptide was eluted using following gradient at a flow rate of 1 ml/min and a running time of 45 min; 0 min: 100% buffer A, 0% buffer B (water containing 90% (v/v) acetonitrile, 0.05% (v/v) TFA); 8 min: 75% buffer A, 25% buffer B; 29 min: 71% buffer A, 29% buffer B. Absorption was detected at 280 nm. Fractions containing Aβ(1–42) were dialyzed and lyophilized to be stored at −20 °C.

2.5. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tricine-sodium dodecyl sulfate-polyacryamide gel electrophoresis (Tricine-SDS-PAGE) was performed on 12% slab gels (70 mm × 82 mm × 7.5 mm) [14]. Gels were stained with Coomassie brilliant blue [15].

2.6. Western blotting

After tricine-SDS-PAGE Aβ(1–42) containing fractions were blotted onto a Sequi-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, USA). Western blotting was carried out on a semi-dry blotter (Hoefer SemiPhor, Amersham Pharmacia Biotech, Uppsala, Sweden) using 25 mM Tris; 192 mM glycine; 15% (v/v) methanol as transfer buffer. After blotting, the membrane was blocked in PBS buffer (140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4) containing 0.1% (v/v) Tween 20 and 10% (v/v) fetal calf serum. Subsequently, the membrane was incubated for 1 h with mouse anti-amyloid-beta 1–16 monoclonal antibody (Chemicon, Hofheim, Germany) diluted 1:500 in blocking solution. The detection was carried out with alkaline phosphatase conjugated secondary antibody (sheep anti-mouse IgG, Sigma, Deisenhofen, Germany) and a substrate composed of 10 ml alkaline phosphatase buffer (100 mM NaCl; 5 mM $MgCl_2$; 100 mM Tris–HCl, pH 9.5), 66 μl nitro-blue tetrazolium solution (5% (w/v) in 70% (v/v) dimethylformamide) and 33 μl of 5-bromo-4-chloro-3-indyl-phosphate solution (5% (w/v) in dimethylformamide).

2.7. Preparation of Aβ-samples for cell toxicity assays

Lyophilized Aβ(1–42) was dissolved in 2 mM NaOH-buffer. The pH of the sample was adjusted to 7.4. The sample was incubated for 10 min in an ultrasonic bath and diluted with PBS buffer (140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4) to different concentrations. The samples were incubated in a thermomixer at 37 °C for 24 h.

2.8. Cell toxicity assay

For the investigation of the cellular toxicity of Aβ(1–42), the 3-(4,5-dimethyl-thiazol-2-yl)2-5-diphenyl-tetrazolium bromide (MTT) assay was accomplished in this way: 20 μl of each of the Aβ-solutions were added to a collagen-IV-coated microtiter plate (BD Biosciences, Franklin Lakes, USA) well containing 80 μl DMEM ("Dulbecco's modified Eagle medium" with acetyl-alanine–glutamine; 10% fetal bovine serum; 5% horse serum and 10 mg/ml gentamycin) and 2×10^4 PC12 cells grown for 24 h at 37 °C in a 7.5% (v/v) CO_2 atmosphere.

Determination of cellular MTT reduction was carried out after a 24 h incubation period of the cells together with the Aβ(1–42)–DMEM mixtures described above at 37 °C in a 7.5% (v/v) CO_2 atmosphere. Then, 10 μl sterile filtered solution of 5 mg/ml MTT in PBS buffer was added and incubated for another 3 h. Medium was removed and 100 μl cell lysis buffer (99.4 ml DMSO; 0.6 ml 100% acetic acid; 10 g SDS) were added and incubated for 30 min, while gently shaking. MTT reduction was determined by measuring the difference between absorbencies at 570 and 630 nm. A cell viability value of 100% was defined that corresponds to MTT reduction of control cells not treated with Aβ(1–42). A cell viability value of 0% was defined by treatment of the PC12 cells with 0.2% Triton-X. The percentage of MTT reduction for each measurement was calculated as the fraction of the value relative to the 100 and 0% values.

2.9. Thioflavin assay

Five microliters of each Aβ(1–42) sample was added to 195 μl 5 μM thioflavinT (ThT, Sigma, Deisenhofen, Germany) in 50 mM glycine–NaOH, pH 8.5. Fluorescence was monitored with a microplate reader at excitation and emission wavelengths of 440 and 490 nm, respectively (Polarstar Optima, BMG, Offenburg, Germany). Fluorescence of the ThT solution without addition of Aβ(1–42) was subtracted from each value to correct for the fluorescence background.

3. Results and discussion

The neural deposits characteristic for Alzheimer's disease (AD) consist predominantly of a 42 amino acid peptide, the amyloid-beta peptide Aβ(1–42). Aβ(1–42) polymerization has been identified as a major feature of AD pathogenesis [16].

Recombinant production of Aβ(1–42) is required for biophysical studies elucidating Aβ(1–42) interaction with itself and other ligands. NMR is known to be very useful for structural characterization of proteins or peptides or interactions of proteins/ligands [17], but often requires stable isotope-labeled proteins.

Recombinant Aβ(1–42) purification methods described so far are complicated [18], end with Aβ(1–42) fusion proteins [19] or only parts of Aβ(1–42) are expressed and purified [20].

The hereby-described expression and purification procedure allows preparation of recombinant Aβ(1–42) from an efficient *E. coli* expression system. We used synthetic oligonucleotides for the construction of the gene coding for Aβ(1–42) peptide. The described purification system yielded 5–8 mg peptide per liter culture volume.

Denaturing conditions in the first purification step were necessary to avoid fibril formation, as Aβ(1–42) has an exceptional tendency to aggregate and is therefore very difficult to purify. Step-wise washing with increasing imidazole concentrations was an efficient method to obtain pure Aβ(1–42) peptide on the Ni-NTA column, leading to the removal of virtually all non-specifically bound protein (data not shown). Addition of thrombin, however, leads to new impurities (Fig. 1) caused by reaction side products.

Cleavage by thrombin was performed directly on the Ni-NTA column. The Ni-NTA material was rinsed with PBS buffer, leading to conditions enabling thrombin activity. Conditions for cleavage by thrombin had to be adjusted very carefully, as variations of the described conditions lead to partial cleavage or to unspecific cleavage of the Aβ(1–42) peptide. After cleavage, Aβ(1–42) was eluted from the column material using acidic urea buffer. This seemed to be beneficial, as low pH values inhibit Aβ(1–42) aggregation [21].

Urea was removed by dialysis against water containing 0.1% (v/v) TFA to decrease the aggregation tendency of Aβ(1–42), which is characteristically decreased at low pH values [21]. Aggregation is known to be maximal at pH values close to the isoelectric point of 5.5 [22,23].

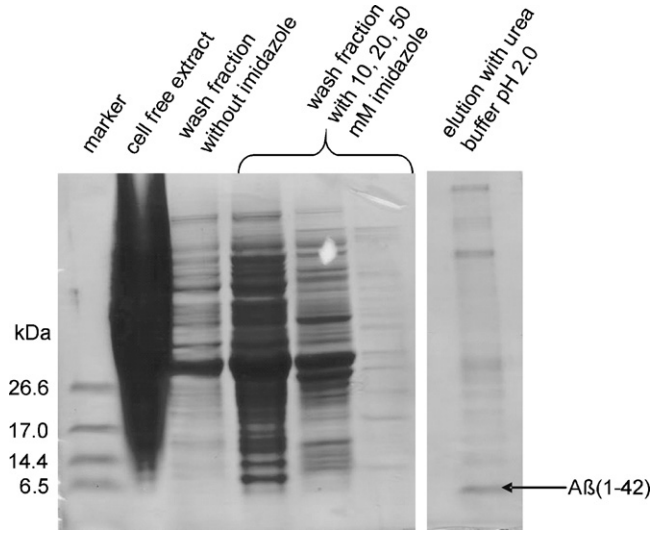

Fig. 1. Result of immobilized metal affinity chromatography of polyhistidine-tagged Aβ(1–42) on Ni-NTA as analyzed by tricine-SDS-PAGE. Aliquots (10 μl) of each fraction were applied to 12% polyacrylamide gels [14].

Thrombin, His-tag and proteolytic side products were separated from Aβ(1–42) by RP-HPLC carried out at 60 °C to overcome the problem of Aβ(1–42) aggregation.

The finally recombinant Aβ(1–42) is more than 95% pure as judged by tricine-SDS-PAGE (Fig. 2).

Identity of the purified protein was confirmed by Western blotting. Bands resulting from recombinant Aβ(1–42) from the RP-HPLC step were compared to those resulting from Aβ(1–42) purified by Ni-NTA without cleavage (Fig. 3).

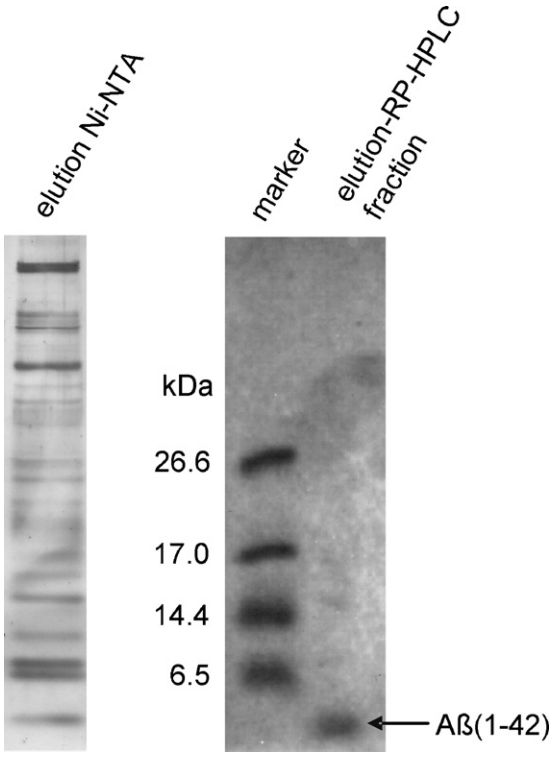

Fig. 2. Result of RP-HPLC of Aβ(1–42) as analyzed by tricine-SDS-PAGE. Aliquots (10 μl) of each fraction were applied to 12% polyacrylamide gels [14].

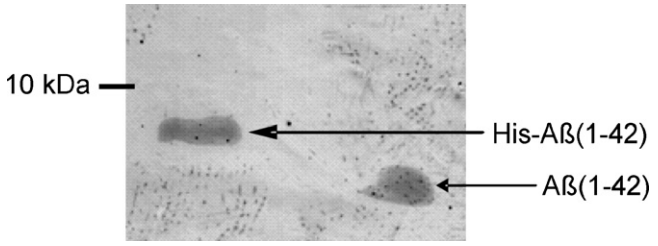

Fig. 3. Western blot of polyhistidine-tagged Aβ(1–42) after immobilized metal affinity chromatography without cleavage step and of Aβ(1–42) after RP-HPLC.

To investigate the fibril forming properties of recombinant Aβ(1–42), fibril formation was quantitatively determined by thioflavinT (ThT) assays [24]. Different Aβ(1–42) concentrations were diluted 1:40 with thioflavinT solution for determination of fibril formation (Fig. 4).

Recombinant Aβ(1–42) showed the typical properties described for natural [25] and synthetically prepared Aβ as fibril formation increased with higher Aβ concentrations.

The influence of the recombinant Aβ(1–42) on the viability of cells was assayed by the MTT reduction assay [24,26]. The reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is an indicator of Aβ amyloid-mediated cell death when compared with untreated cells [24]. The viability of PC12 cells was assayed by MTT reduction capability as a function of recombinant Aβ(1–42) peptide concentration in the cell culture media. Between 10 and 1000 μM of recombinant Aβ(1–42) the viability of the PC12 cells dropped to values between 65 and 45% (Fig. 5). Such values are frequently reported for synthetic Aβ at micromolar concentrations [27].

The described expression and purification system of human Aβ(1–42) allows the preparation of recombinant peptide at quantities allowing biophysical studies. The system is also suited to produce isotope labelled Aβ(1–42) for structural studies by

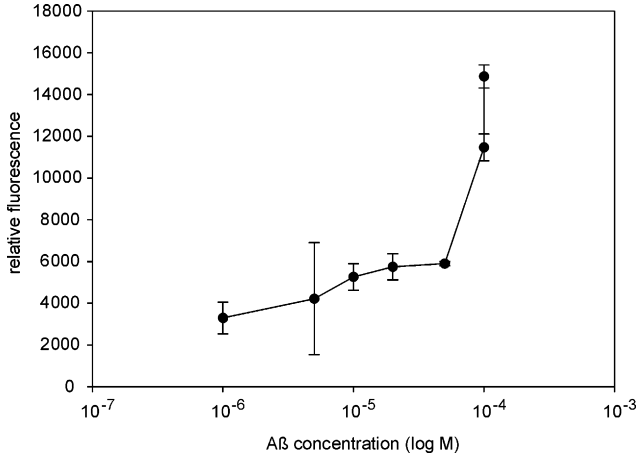

Fig. 4. Thioflavin T (ThT) measurement of recombinant Aβ(1–42) aggregation depending on Aβ(1–42) concentration. Binding of ThT to Aβ(1–42) fibrils was determined by ThT fluorescence at Ex 440/Em 490 and shown as relative fluorescence units. All values are given as means from six measurements with the respective standard deviation. All measurements were carried out on freshly prepared Aβ(1–42) incubated for 24 h at 37 °C.

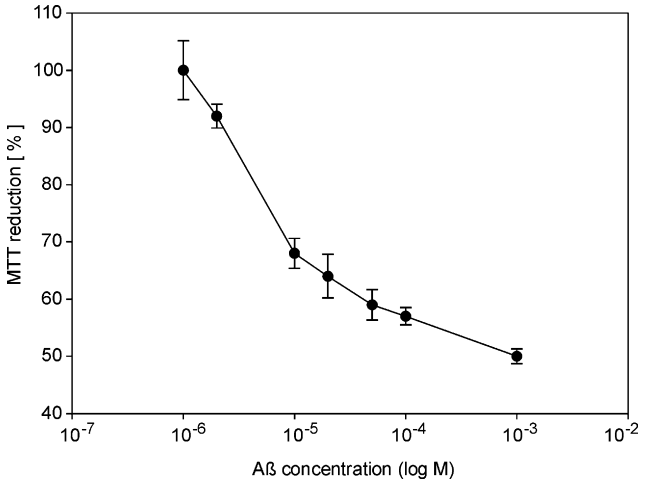

Fig. 5. Dependence of cell viability from recombinant Aβ(1–42) concentration. Cell viability for Aβ-treated cells was assayed using the MTT assay with PC12-cells. Percentages of cell viability were derived as follows: A value of 100% was obtained from cells not treated with recombinant Aβ(1–42); a value of 0% was obtained by treatment of the PC12 cells with 0.2% Triton-X. All values were determined from six independent measurements. The data points indicate the arithmetic means of the respective measurements with error bars according to the standard deviations.

NMR spectroscopy. The finally obtained Aβ(1–42) was cytotoxic and showed high propensity for fibril formation as shown in MTT and ThioflavinT assays. No refolding steps after purification were necessary.

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