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Production and Amyloid fibril formation of tandem repeats of recombinant Yeast Prion like protein fragment

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Abstract : Amyloid fibrils have long been known to be the well known α-helix to β-sheet transition characterizing the conversion of cellular to scrapie forms of the prion protein. A very short sequence of Yeast prion-like protein, GNNQQNY (SupN), is responsible for aggregation that induces diseases. KSI-fused tandem repeats of SupN vector are constructed and used to express SupN peptide in *Escherichia coli* (*E.Coli*). A method for a production, purification, and cleavage of tandem repeats of recombinant isotopically enriched SupN in *E. coli* is described. This method yields as much as 20 mg/L of isotope-enriched fusion proteins in minimal media. Synthetic SupN peptides and ^{13}C Gly labeled SupN peptides are studied by Congo Red staining, Birefringence and transmission electron microscopy to characterize amyloid fibril formation. To get a better understanding of aggregation-structure relationship of 7 residues of Yeast prion-like protein, the change of a conformational structure will be studied by ^{13}C solid-state nmr spectroscopy as powder of both amorphous and fibrillar forms.

Keywords : Amyloid, fibril, tandem repeats, Prion like protein

INTRODUCTION

A group of diseases are induced where proteins or fragments of proteins convert from their normally soluble forms to insoluble fibrils or plaques, which accumulate in a variety of organs including the liver, spleen, and brain. The final forms of these aggregates often have a well-defined fibrillar nature, and known as amyloid.[1-4] A partial list includes alzheimer's diseases, parkinson's

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diseases, the spongiform encephalopathies such as Creutzfeldt-Jakob disease, type II diabetes, fatal familial insomnia. The range of proteins involved in these diseases is such as lysozyme, transthyretin and the prions.

A convenient model for studying both amyloid formation and the prion-like transmission of protein conformation is the yeast protein Sup35. Sup35 protein has some properties typical of mammalian prions, such as aggregation and resistance to protease.[1-2] Since a short peptide (SupN) from N terminus is very critical for maintenance of the phenotype, we expressed and reveal the molecular level of aggregation of SupN.

EXPERIMENTAL

Gene construction -fused-SupN multimer inserted pET31b(+) vector were constructed as below. Oligonucleotides for peptide SupN-Met-SupN-Met were 5’-GGT AAT AAC CAA CAG AAT TAC ATG GGT AAT AAC CAA CAG AAT TAC ATG-3' (forward sequence) and 5'-GTA ATT CTG TTG GTT ATT ACC CAT GTA ATT CTG TTG GTT ATT ACC CAT-3' (reverse sequence). Annealed oligonucleotides were purified by agarose gel electrophoresis and extracted using Gel Extraction Kit (Bioneer, Korea). Purified oligonucleotides were ligated with AlwN I digested dephosphorylated pET-31b(+)vector (Novagen, USA). Ligated mixture was transformed to a non-expressed NovaBlue strain of *E.coli*. (Novagen, USA). The number (n) of SupN dimer units of the

resulting clones was ascertained by agarose gel electrophoresis. The appropriate vectors were then transformed into BLR(DE3)pLysS (Novagen, USA) for overproduction. All KSI-fused genes were sequenced to verify mutations and the number of multiple SupN units.

Expression and purification of the fusion proteins with tandem repeats of supN - All proteins with KSI fused (SupN)n His-tags were expressed in *Escherichia coli* BLR(DE3)pLysS cells. Production of the KSI-$(SupN)_{2,4,6}$-His-tags fusion protein was performed in M9 minimal medium. ^{13}C Glucose and $(^{15}NH_4)_2SO_4$ were used for preparation of uniformly ^{13}C and uniformly ^{15}N enriched peptide SupN separately. When the OD_{600} is at 0.5, transcription of the gene was induced by the addition of 1mM IPTG. The cells were harvested 4 to 5 hours after the induction when OD_{600} is decreased. Cells were suspened in the lysis buffer (20 mM Tris·HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9~8.0) and cells and DNA fragmented by sonication (Sonics, 500W). Cell lysate was pelleted for 20 min at 12,000 rpm at 4 ℃ and pellet of inclusion body was redissolved in the lysis buffer with 6 M Guanidine·HCl for overnight at room temperature. After centrifugation for 30 min at 12,000 rpm at 4 ℃, the supernatant was loaded onto Ni^{2+}-NTA-His·Bind Resins (Novagen, USA). Column had been charged with 50 mM $NiSO_4$ and equilibrated buffer containing 20 mM Tris.HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9~8.0 with 6 M Guanidine-HCl. The column was washed with 5 column volumns of 20 mM Tris.HCl, 500 mM NaCl, 16 mM imidazole, pH 7.9~8.0 with 6 M Guanidine-HCl. KSI-fused (SupN)n His-tags was eluted with 20 mM Tris.HCl, 500 mM NaCl, 500 mM Imidazole,

pH 7.9~8.0 with 6 M Guanidine-HCl. Eluted fusion peptides were dialyzed overnight against H_2O in 10 kDa MWCO dialysis bag. White precipitate from dialysis was lyophilized and dissolved into 70% formic acid. Prestabilized CNBr at room temperature was added to the sample solution at 100-fold molar excess over all methionyl residues in the fusion protein. The flask was wrapped in aluminum foil and incubated for at least 3 hrs at room temperature. Reaction products were 10 fold diluted with H_2O and lyophilized under vacuum. Lyophilized mixture was dissolved into 20 mM KH_2PO_4 and 100 mM NaCl, pH 7.4 and centrifuged at 5000 g for 1 hr at 4 ℃. Soluble peptide SupN in the Supernatant was purified by RP-HPLC on a Waters Delta-Pak C18 column (7.8x300 mm ID). Elution was carried out at room temperature with a linear gradient from 0% to 100% of buffer B over 60 mins at a flow rate of 3 ml/min with the absorbance monitored at 220 nm and 280 nm using a DIONEX P580. Buffer A was 5% acetonitrile and 0.1% TFA in H_2O and buffer B was 95% acetonitrile and 0.1% TFA in water. The resultant SupN peptide fractions were collected and dried under vacuum in a lyophilizer (Labconco, USA). Peptide concentrations were determined by measuring absorbance at 276 nm. Identity of peptide was confirmed by ESI or MALDI mass spectrometry.

Fibrillization of GNNQQNY(M) peptide from KSI-fused (SupN)$_n$-His6 peptide -Lyophilized SupN peptide was dissolved in 20 mM KH_2PO_4 and 100 mM NaCl, pH 7.4 to a final concentration of 5-10 mg/ml. GNNQQNY peptides were fibrillized by incubation of the peptide solution for 5-30 days at 24 ℃. Fibrillized solutions were lyophilized for NMR measurements. Synthesized peptides which

were performed by Anygen, Inc. (Daejeon, Korea) were also fibrillized. The correct identity of the synthetic peptide was confirmed by ion spray mass spectrometry, and the purity of the peptides was confirmed by reverse phase high pressure liquid chromatography. Various conditions were used to obtain fiber. The best results were obtained as below. The peptides were dissolved in DMSO, and then diluted with buffer (0.02 M NaCl/0.01 M Tris, pH 7.2) to a final concentration of approximately 2 mM. And peptide were incubated at room temperature for 7~13 days.

Congo Red staining and Birefringence – Birefringence was determined using the same solution of peptides that were used for fibrillization experiments. A 10 $\mu\ell$ aliquot of the suspension of peptide aged for 10 days was allowed to dry on a glass microscope slide. Staining was performed by addition of a solution of 80% ethanol saturated with Congo Red and NaCl. Birefringence was determined with an Axioskop-40 (Carl Zeiss Inc.) stereoscope equipped with a polarizing stage.

Transmission Electron Microscope(TEM) –A drop (~10 $\mu\ell$) of fibrillized peptide solution is applied to a carbon-coated film on a 200-mesh copper grid. After 1 min, excess fluid was removed, and the grid was then negatively stained with 2% uranyl acetate in water and dried for 30 min. Samples were viewed on a Jeol JEM 1010 transmission electron microscope at an accelerating voltage of 80 kV program.

RESULTS AND DISCUSSION

Gene construction The overall strategy for construction and production of the SupN proteins is shown in Figure 1.[5-6] Complementary oligonucleotides encoding SupN peptide-Met-SupN peptide-Met were synthesized, annealed, and unidirectionally self-ligated using three-base-ATG-3’, 3’-TAC-. These cohesive ends form tandem repeated SupN units spaced by single methionine codons, ATG.

Construct of pET31b(+) with SupN tandem inserts was confirmed by a 1.5% agarose gel electrophoresis after double digested with Xba1 and XhoI of purified plasmid DNA in Figure 2(a).

Figure 2(a, lane 3, 4, and 5) shows corresponding plasmid DNA inserts of SupN tandem inserts of n=1, 2, 3. Each of the different KSI-(SupNM-SupNM)n-His6 (n=1, 2, and 3) were expressed in the protease deficient strain BLR(DE3)pLysS successfully. The production levels are shown in Figure 2(b).

SupN G N N Q Q N Y M G N N Q Q N Y M
(+) Oligo: 5'- GGT-AAT- AAC-CAA-CAG-AAT-TAC-ATG-GGT-AAT-AAC-CAA-CAG-AAT-TAC-ATG-3'
(-) Oligo: 3'- TAC-CCA-TTA-TTG-GTT-GTC-TTA-ATG-TAC-CCA-TTA-TTG-GTT-GTC-TTA-ATG -5'

Anneal
Ligate
Gel purify

M M M
5'-(SupN -ATG SupN -ATG) -3'
3'-TAC -TAC)n -5'

+

T7 lac → KSI -CAG-ATG-3' 5'-CTG-CTC-GAG-(CAC)- TGA T7 Term
-GTC-5' 3'-TAC-GAC-GAG-CTC- (GTG)6 ACT

pET31b(+)/AlwN1

Ligate

M M M
T7 lac KSI (SupN SupN)n LLE His6 T7 Term →

pET31bKSI(+)/(SupN)n(His)6

Expression
Ni-Affinity Chromatography
CNBr Cleavage

KSI SupN x 2n , (n=0, 1, 2, 3) His6

RPHPLC (C18)
MS

SupN x 2n , n=0, 1, 2, 3

Figure 1. Schematic representation for construction, production, purification of Ketosteroid isomerase(KSI)-multiple supN peptide-His6 fused proteins. Oligonucleotides encoding both strands of the 7-13 amino acids of Sup35 were annealed, unidirectionally self-ligated to form an array of tandem repeats of SupN separated by ATG codons. These multimers(n=1, 2, and 3) were then lagated into the AlwNI site of pET31b(+) to make KSI-(supN-M-supN-M)n-His6 fusion genes where n=0, 1, 2, 3. Expression levels were tested by SDS-PAGE, the highest yielding fusion protein(n=3) was purified by Ni-affinity chromatography and cleaved by CNBr into supN, KSI, and His6. SupN was isolated by C18 reverse phase HPLC and checked by ESI MS.

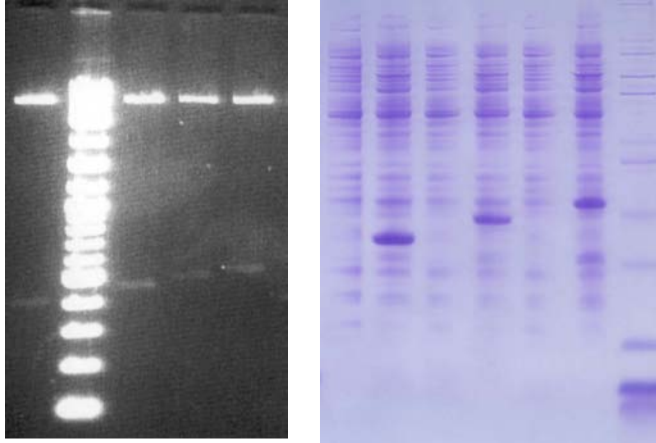

Figure 2. 1.5% agarose gel electrophoresis after double digested with Xba1 and XhoI of purified plasmid DNA and production of KSI fused SupN with tandem repeats in E. coli by 12% SDS-PAGE. (a) lane1, empty vector control (423 bp); lane 2, DNA ladder 100, 200, 300bp, … ; lane 3, 4, and 5 shows corresponding plasmid DNA inserts of SupN tandem inserts of n=1(48 bp), 2(96 bp), 3(144 bp). (b) Lane 2, 4, and 6 are insoluble protein fractions from IPTG induced BLR(DE3)pLysS where n=1, 2, and 3. Lane 1, 3, and 5 are protein fractions before induction. Molecular weight of KSI is about 14 kDa. Molecular weight of SupN dimmer is about 2 kDa.

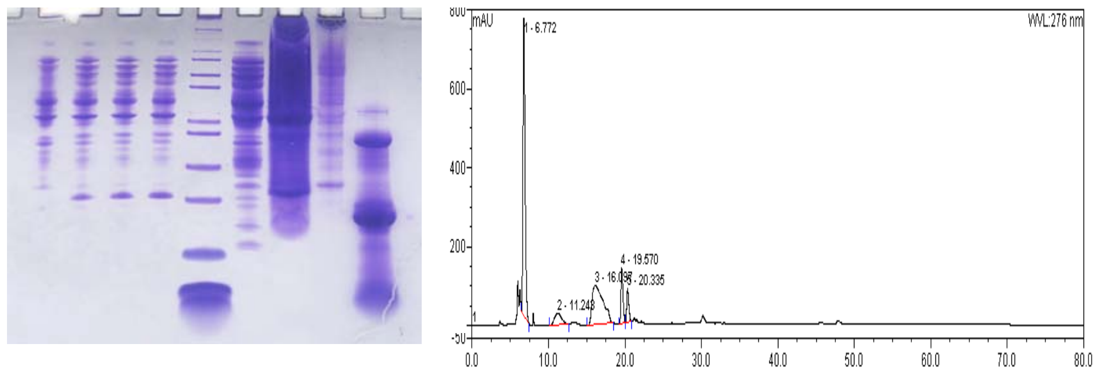

Figure 3. Coomassie blue stained (a) 12% tris-tricine PAGE showing samples from expression and Ni-NTA column purification of the uniformly ^{13}C labeled KSI fusion protein. Lane 1, cells before IPTG induction; Lane 2, cells after 1 hr IPTG induction; Lane 3, cells after 2 hrs IPTG induction;

Lane 4, cells after 3 hrs IPTG induction; Lane 5, Marker; Lane 6, soluble fraction after lysis; Lane 7, insoluble fraction after lysis; Lane 8, KSI-fused protein purified from a Ni–NTA affinity chromatography; Lane 9, reaction mixtures after CNBr cleavage. (b) Reversed phase HPLC chromatogram. Peptides were eluted with a linear gradient of ACN in water containing 0.1 % TFA on the C4 column. Eluent A: 95 % ddH_2O with 0.1% TFA and 5% ACN, Eluent B: 5 % ddH2O with 0.1% TFA and 95 % ACN.

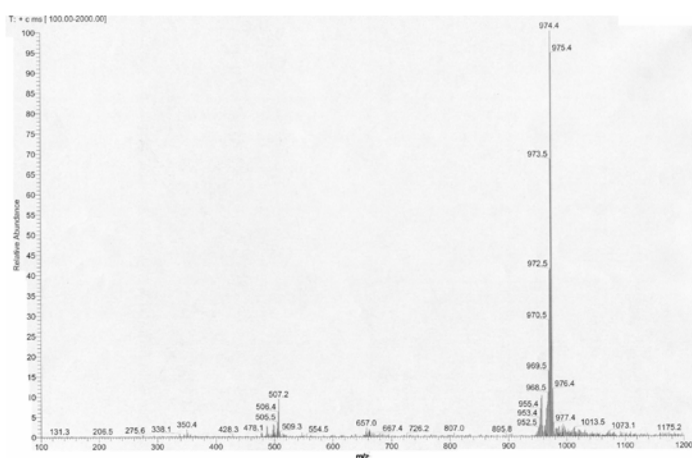

Figure 3.Electrospray Mass spectra of SupN peptide. Uniformly ^{13}C labeled GNNQQNYM, SupN peptide was expressed in *E. coli*. Major free acid form of 975 Da and minor homoserine lactone form of 957 Da are shown.

Fibrillization of GNNQQNY(M) peptide from KSI-fused (SupN)$_n$-His6 peptide –

Identification of Fibril-forming – The fibrillization potential of peptide fragments was examined by Congo Red Staining, Birefringence, and TEM(Transmission Electron Microscope).[7-11] Two fibrillar structures of synthetic and expressed peptides were obtained (Figure 4 a and b) by TEM. Green-gold birefringence after staining with Congo Red is one of the best characteristics of amyloid fibrils. The

studied peptides have showed some degree of birefringence. The green birefringence that was observed with the synthetic and expressed peptides was clear and strong. Fibrillar structures are similar to other amyloid fibrils which reported paper by other workers.[7-9] The expressed peptides were shown better fibrillar structures than that of synthetic peptides.

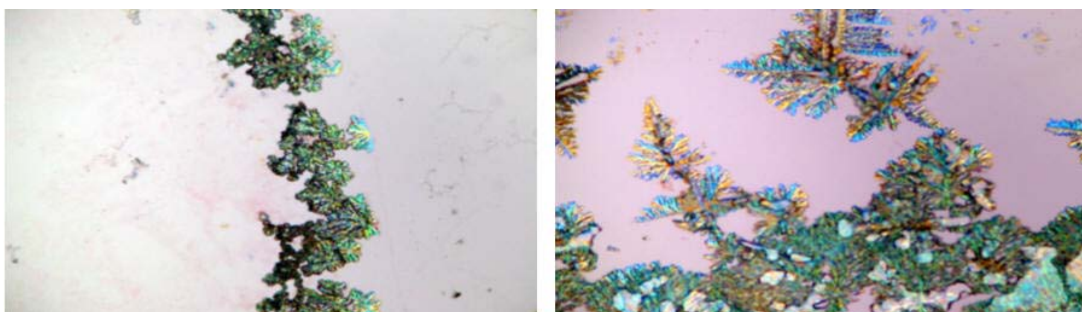

Figure 4. Green-gold birefringence of fibrillized synthetic SupN (a) and uniformly ^{13}C labeled expressed SupN (b) was measured with Polarizing Microscope: asioskop 40 (x100, Zeiss)

Transmission Electron Microscopy (TEM) TEM images were observed using the TEM visualization to further determine the properties of the fibrillar structure in figure 5. Expressed SupN were well observed more than synthetic SupN at fibrillar state. And fibrillization of peptide was confirmed by TEM. Therefore the sample solutions were used because it was studied conformation of fibrillar state SupN.

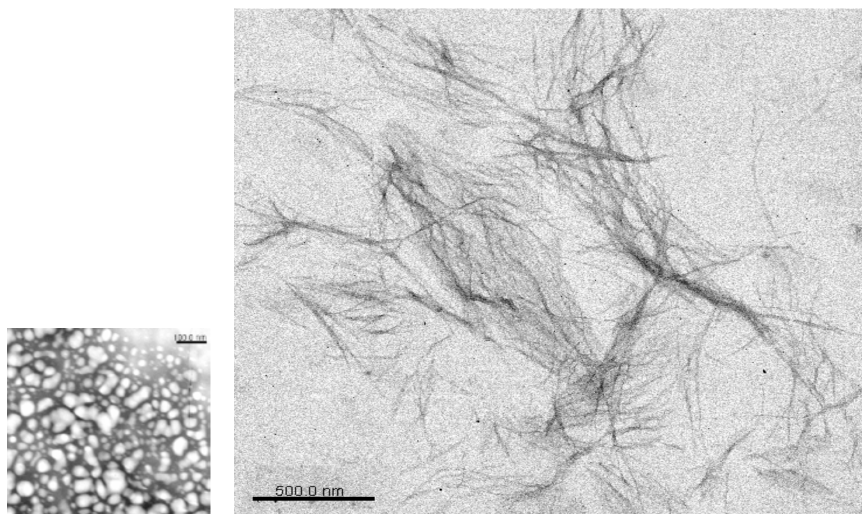

Figure 5. TEM images of amorphous aggregates (a) and insoluble fibrillar states (b) in an aged solution of SupN peptide. Scale bar represents 100nm.

CONCLUSION

In this paper, we demonstrate the optimization of expression, purification, and fibrillization of SupN peptides. Milligram quantities of SupN peptides were obtained by using preparative reversed-phase HPLC. Purified SupN peptides formed as a fibrillar state under incubating condition. Synthetic SupN peptides of unlabeled and expressed SupN peptides of ^{13}C Gly labeled were studied with Congo Red staining and Birefringence and Transmission Electron Microscopy that are used to characterize amyloid fibril formation. To get a better understanding of aggregation-structure relationship of 7 residues of Yeast prion-like protein, the change of a conformational structure will soon be studied by ^{13}C solid-state nmr spectroscopy as powder of both amorphous and fibrillar forms.

Acknowledgments

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