The Expression of Recombinant Sheep Prion Protein (RecShPrPC) and its Detection Using Western Blot and Immuno-PCR

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The objective of this study was to develop a protocol for the production of the non-pathogenic recombinant sheep prion protein (RecShPrPC) using a bacterial expression system and confirm its production using Western Blot and Immuno-PCR. Prion protein is the causative agent of many neuro-degenerative diseases including Bovine Spongiform Encephalopathy (BSE) in cattle and scrapie in sheep. The first case of BSE in the United States occurred in December of 2003, and, as a result, beef sales plummeted significantly, which threatened to put thousands of farmers out of business. The study of the structure and the biochemistry of the prion protein should solve some of the unanswered questions about this infectious protein which, in turn, would help in the discovery of better detection methods and ante mortem testing techniques of BSE in animals. However, researchers have faced significant difficulties with the extraction of the purified prion protein directly from tissues. This project developed a protocol to produce normal prions as a His-tagged fusion protein (His-RecShPrPC) in E. coli cells and solublize them from the inclusion bodies using 6 N Guanidinium Chloride. The solublized protein was purified using Nickel based Affinity Chromatography and achieved a final yield of 28 mg of His-RecShPrPC per liter of bacterial broth. Western Blot using a specific anti-prion antibody targeted against the RecShPrPC identified up to 1 μg of purified protein. Immuno-PCR detected up to 200 fg of purified RecShPrPC. In vitro production of recombinant sheep prions is important to furthering research that studies the biochemistry and structure of prion proteins.

Key Words: Prion, PrP, Sheep, Ovine, Expression

Introduction

Scientists around the globe have shown intense interest in a group of transmissible neuro-degenerative diseases among which are spongiform diseases of cattle, scrapie of sheep, and Creutzfeld-Jacob disease of humans. The agents responsible for transmitting these diseases are called prions, which are classified as infectious proteins (Prusiner, 1994). In order to understand the biochemistry and molecular mechanism of these proteins, it is important to study the structural properties of PrPC and PrPSc. There have been many attempts made in the past to extract and purify PrPC from tissues and from expression bacterial systems; however, very low expression and yield has hindered the process (Caughey et al., 1988, Scott et al., 1988). To overcome these issues, we have expressed in bacteria, the full-length mature sheep PrP (25-234) and fused at its C-terminus a polyhistidine extension (His-PrP). The His-PrPC was recovered from inclusion bodies with guanidinium chloride, purified by Ni-NTA affinity chromatography and confirmed using Western Blotting and Immuno-PCR.

Materials and Method

Genomic DNA was extracted from a gram of sheep brain tissue obtained from the Food and Agriculture Products Research Center, Oklahoma State University (OSU), Stillwater. The primers for ovine prion gene were generated with the help of a computer from the following sites: http://www.ncbi.nlm.nih.gov and http://frodo.wi.mit.edu/cgi-

bin/primer3/primer3_www.cgi. The complete coding sequence of the prion gene was copied from the NCBI site to the Sequence Input Box on the Primer3 site. The various parameters, like the length of the primers, self-complementarity of the sequence, GC content, Tm difference and other parameters were optimized while designing the primers. Obtained primers were analyzed in IDT's Oligo Analyzer 3.0 at http://207.32.43.70/biotools/oligocalc/oligocalc.asp for various parameters to ensure maximum efficiency.

The ovine prion gene was amplified by the polymerase chain reaction using the following oligonucleotide primers: forward 5'-TGCTGCAGACTTTAAGTGATT-3' and reverse 5'-CCCCAACCTGGCAAAG-3'. The PCR conditions used were the following: (i) initial denaturation at 95 º C for 1.5 min, (ii) denaturation at 94 º C for 30 min, (iii) annealing at 58 º C for 30 s and (iv) extension at 72º C for 30 s. The PCR product which was 893 bp was analyzed by 1.2% agarose gel electrophoresis for confirmation of the quality of PCR. The gel was analyzed by an imaging system (GDS 8000 system, UVP BioImaging Systems, USA).

The PCR product was cloned into the TOPO vector by Chemical Transformation using Invitrogen's Zero blunt PCR cloning kit (Carlsbad, CA) as described in manufacturer's instructions. Cloning was done by heat-shocking the cells for 30 s at 42 º C in a water bath without shaking, after which the tubes were immediately transferred to ice. 250 µL of S.O.C. medium was added to the tube at room temperature and was shaken horizontally (200 rpm) at 37 º C for 1h. Clones that had taken the PCR product were picked using blue-white screening and cultured again. The confirmation of the inserted PCR product was carried out by running the plasmid extracted from positive clones on a 1.2% agarose gel. The integrity of the inserted PCR product was performed using dideoxy chain termination sequencing reaction at the Core Facility in Noble Research Center located at Oklahoma State University.

Further primers were developed (as described elsewhere in the article) to amplify the coding sequence of the full-length mature protein after the removal of the signal peptides from both N and C terminals. PCR was carried out using both with and without stop codons. PCR was carried out using the following primers: Forward primer: 5'-CAC CAA GAA GCG ACC AAA ACC TGG-3' and Reverse without stop codon; 5'- CAC ACT TGC CCC CCT TTG GTA-3'. The PCR was done using a high fidelity taq polymerase Platinum® pfx DNA polymerase and the PCR conditions were: (i) Initial denaturation at 95 º C for 2 min, (ii) denaturation at 95 º C for 30 s, (iii) annealing at 58 ºC for 30 s, (iv) extension at 72 º C for 45 s and (v) a final extension at 72 º C for 3 min. The PCR was run for 30 cycles. The amplified coding sequence of 633 bases were ligated to Shot®TOP10 Competent Cells from Invitrogen Corporation, Carlsbad, CA and analyzed for positive clones. The plasmids were extracted from the positive clones and were transformed into BL21 star *E. coli* cells which were used for expression of the protein. The expression and the Ni-column affinity chromatography was carried out according to the instructions on the manual supplied with the Champion™ pET Directional TOPO® Expression kit. Western Blotting and Immuno-PCR was performed on the purified protein using an anti-prion antibody to detect if the right protein has been expressed and purified.

Results and Discussion

The agarose gel electrophoresis of the amplified PCR product corresponding to the nucleotide sequence containing the sheep prion gene ORF (open reading frame) of approximately 893 bases showed a single band that confirmed the correct size of the expressed protein (Fig1).

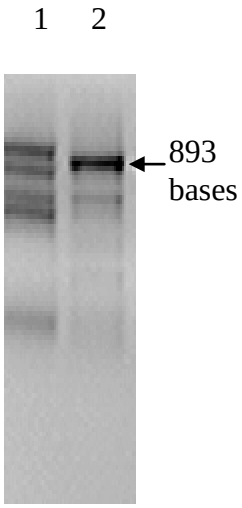

Fig 1: 1kb standard marker (lane 1) and 893 bases PCR product amplified off the genomic DNA (lane 2).

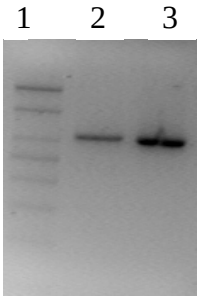

Fig 2: 1kb standard marker (lane 1), PCR products amplified off the ORF of PrP with stop codon (lane 2) and without stop codon (lane 3).

Nucleotide sequencing of the vector and the insert from all the positive clones verified its identity with the ovine prion protein (GenBank accession DQ345757). The analysis of the obtained sequence confirmed that the insert was in the right orientation and in the frame with the vector sequence. In Fig 2, PCR amplification of nucleotide sequence encoding the 210 amino acids (Lys 25 to Ser 234) is demonstrated where lane 2 and lane 3 are with and without the stop codon respectively. The elution fraction obtained after purification of proteins induced from His-PrP transformed bacterial clones contained the detectable protein with a mobility of 39-kDa (expected size 39.5-kDa) as shown by the Coomassie blue staining of SDS-PAGE (Fig 3).

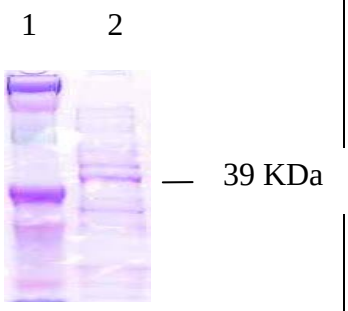

Fig 3. SDS-PAGE analysis of the purified RecShPrPC, Molecular (lane 1) and 1µg RecShPrPC (lar 2)

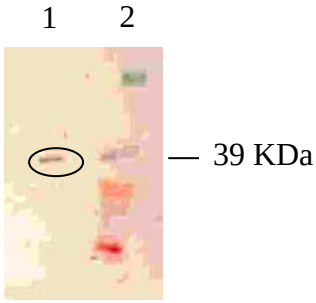

Fig. 4. Western Blot analysis of purified RecShPrP (1 µg) Mol mass Markers (lane 2)

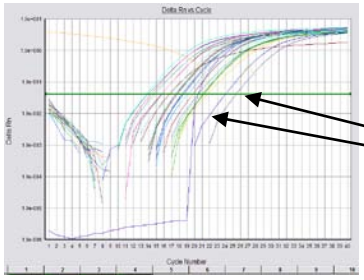

Fig 5. Change in fluorescence (y-axis) vs cycle number (x-axis). Note that each curve represents a sample and a smaller cycle number indicates the presence of higher concentration of the sample

The 39-kDa protein was detected and identified by Western Blot using a monoclonal antibody (mouse IgG F89 / 160.1.5) against the prion protein (Fig 4). Immuno-PCR is a highly sensitive antigen detection technique, which uses the enormous amplification property of PCR and highly specific binding feature of an antibody towards its antigen. Immuno-PCR results (Fig 5) indicated that it could detect up to 200 femto gram of purified prion protein which suggests it could be a useful detection tool for prion proteins. In conclusion, a protocol was developed for the in vitro production of Histidine tagged fusion sheep prion proteins. Our future work includes the transfection of a mammalian cell with the prion gene open reading frame and expressing the protein for structural studies.

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