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Cloning, Sequencing, and Expression of cDNA Encoding Bovine Prion Protein

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Abstract A normal prion protein (PrPc) is converted to a protease resistant isoform (PrPsc) by an apparent self-propagating activity in bovine spongiform encephalopathies (BSE), which is a neurodegenerative disease. The cDNA encoding bovine PrP open reading frame (ORF) in Korean cattle was cloned by polymerase chain reaction (PCR). The cloned cDNA had a length of 795 base pairs which coded for a protein of 264 amino acid residues with a calculated molecular mass of 28.6 kDa. Identities of 90, 90, 79 and 78% on nucleotide and 94, 94, 84, and 84% on amino acid sequence were shown to PrP genes from sheep, goat, human, and mouse, respectively. The cloned DNA was ligated into the pQE30 expression vector and transformed into *E. coli* M15. The PrP was expressed by induction with isopropyl-β-D-thiogalactoside (IPTG) and purified on the Ni-NTA affinity column. High specific activities of the recombinant PrP were observed in the fraction of pH 5.8 eluate and showed a molecular mass of ~29 kDa on SDS-PAGE and Western blot analysis.

Key words: Prion protein, cDNA, BSE, Korean cattle

Bovine spongiform encephalopathy (BSE) is thought to be the group of transmissible spongiform encephalopathy (TSE), which comprises a group of slow degenerative diseases of the central nervous system (CNS). It is characterized by neuronal vacuolation and accumulation of the abnormal isoform (PrPsc) of a host-encoded celluar membrane protein, reffered to as prion protein (PrPc), in CNS [7, 26].

The epidemic that arose is considered to be due to a change in the rendering process used to prepare cattle feed from ruminant offal [8]. Since an epidemic of BSE occured in Britain in 1986 and caused the deaths of almost 200,000 cattles it has now become a subject of general interest, because of the potential risk it poses to public health [1, 19, 25]. Even though there is no report concerning an emergency of BSE in Korea, its risk in cattle and pubic heath still remains.

PrPc is a normal cellular protein that is expressed in the neurons and glia of the brain and spinal cord, as well as in several peripheral tissues and leukocytes [3, 4, 6, 9, 12, 13]. The normal function of PrPc still remains to be revealed. However, its localization on the cell surface would be consistent with roles in cell adhesion and recognition, ligand uptake, or transmembrane signaling [8]. Because the PrPc may fail to perform its normal function when it is converted to the PrPsc isoform, the key to understanding the pathogenesis of this disease state is the conformational conversion of a normal isoform of PrPc (a cell surface glycoprotein) into a pathogenic isoform of PrPsc [16–18]. The two isoforms, PrPc and PrPsc, differ mainly in their secondary structures, sensitivity to proteinase K (PK), and solubility [5], although these two PrP isoforms (PrPsc and PrPc) are encoded by the same chromosomal locus and have the same amino acid sequence and molecular weight [2].

The different size of PrP fragments represents a suitable tool to determine the cellular and molecular effects of PrPsc *in vitro* and the position change in the tertiary structure of PrP, which has become an important subject in the pathogenesis [5, 18]. Thus, attempts are made nowadays to produce PrP fragments using recombinant cDNA techniques.

In the present study, as the first step in PrP research, we described cloning of cDNA encoding bovine prion protein from Korean cattle, comparative sequence analysis of the gene with PrP genes from other animals, and expression of a large fragment of the recombinant PrP in *E. coli* and its purification.

Total RNA was isolated from the fetal fibroblast of Korean cattle using Trizol Reagent (Gibco, CA, U.S.A.)

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Table 1. Synthetic oligonucleotide sequence used for PCR amplication of bovine PrP.

Primer	Oligonucleotide Sequence	Length (bases)
Forward	5'-GCATGCATGGTGAAAAGCCACATAGGCAGTTGG-3' *Sph*I	33
Reverse	5'-AAGCTTCTATCCTACTATGAGAAAAATGAGGAA-3' *Hin*dIII	33

and chloroform, and aqueous total RNA was precipitated by isoprophenol and centrifugation at 12,000 ×g for 10 min. The RNA pellets were washed once with 75% ethanol and dissolved in diethylpyrocarbonate (DEPC)-treated water. The RNA was treated with 2 units of RNase-free DNase at 37°C for 30 min to remove residual DNA and was purified again with Trizol Reagent.

Single-stranded cDNA was synthesized using Superscript Preamplification System for First Strand cDNA synthesis Kit (Gibco, CA, U.S.A.). Five μg of purified total RNA was incubated with 50 units of Superscript II reverse transcriptase at 42°C for 50 min in the presence of 10 × RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 10 mM dNTP, and 0.5 μg/μl oligo (DT). Samples were treated with 2 units of RNase H at 37°C for 20 min and the synthesized single-stranded cDNA was used as a template in the polymerase chain reaction (PCR).

Primers were designed to obtain *Sph*I and *Hin*dIII enzyme sites by modification of the flanks representing nucleotides of the GenBank nucleotide entry (S55629) for bovine PrP (Table 1). The PCR solution was composed of 2 μl of 10 × buffer, 0.4 μl of dNTPs (2.5 mM), 0.5 μl each of 5'CS/3'CS (10 pmol/μl), 0.2 μl of *Taq* DNA polymerase (5 U/μl, Promega, Wisconsin, U.S.A.), 15.4 μl of distilled water, and 1 μl of template DNA (50 ng/μl). The PCR amplication consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min and 30 sec, and then a final extension at 72°C for 15 min. PCR products were analyzed through electrophoresis on 1.0% agarose gel. The PCR product identical with bovine PrP in size (795 bp) was extracted by QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands), and the purified amplicon was ligated with TA cloning vector (Invitrogen, California, U.S.A.) and transformed into One Shot Cell (Invitrogen, California, U.S.A.) for cloning. The cloned DNA fragment was sequenced using an automated DNA sequences (ABI PRISM 377 × L, perkin Elmer, U.S.A.) and compared with the GenBank database using the software DNassist.

PCR product was digested by *Sph*I and *Hin*dIII to obtain an insert DNA fragment with a staggered ends. This fragment was ligated with pQE30 prokaryote expression vector (Qiagen, Venlo, Netherlands), digested by the same enzymes, and transformed into *E. coli* M15 strain. Twenty ml of overnight culture of the transformed M15 were inoculated to 1 l of LB medium containing both 100 μg/ml of ampicillin and 25 μg/ml of kanamycin, and then grown at 37°C with vigorous shaking until an OD_{600} of 0.6 was reached. The expression of the mass cultured M15 was induced by 1 mM final concentration of isopropyl-β-D-thiogalactoside (IPTG), and they were incubated for additional 4 h. The M15 was harvested, lysed by lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 8.0), and sonicated for 10 min. The lysate was applied onto Ni-NTA agarose column (Qiagen, Venlo, Netherlands) and eluted with elution buffers having a series of pH from 6.3 to 4.5. To confirm the expression of recombinant PrP, each eluat was loaded, resolved on 15% polyacrylamide gel (SDS-PAGE), and then transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4°C with mouse anti-PrP monoclonal antibody (F89/160.1.5) [15]. Bound mAbs were detected with an alkaline phosphate (AP) conjugated goat anti-mouse IgG (Bio-Rad, CA, U.S.A.), and the blots were developed using AP-Substrate Kit (Bio-Rad, CA, U.S.A.).

Prion is a proteinaceous infectious particle that is thought to be the causative agent of transmissible spongiform encephalopathies (TSEs), comprising a group of fatal neurodegenerative disorders, and these disorders can arise sporadically or can have an infectious or genetic etiology [10, 18, 24]. PrP encoded by bovine PrP cDNA comprised 264 amino acids and has a predicted unglycosylated molecular weight of 28,614 Da [20].

In the present study, the PrP ORF of Korean cattle amplified by PCR using cDNA of fetal fibroblast of healthy cattle was cloned into TA cloning vector. The plasmid vector was treated with *Sph*I and *Hin*dIII, and the insert DNA was determined to have 795 bp through electrophoresis on 1.0% agarose (Fig. 1). To avoid

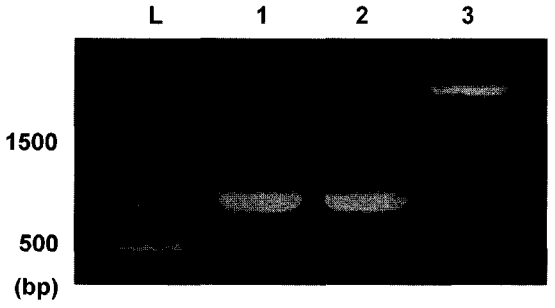

Fig. 1. Agarose gel (1.0%) electrophoresis pattern of the insert DNA and pQE30 expression vector digested with *Sph*I and *Hin*dIII.
Lane L, 100 bp DNA ladder (Promega); lanes 1 and 2, insert DNA; lane 3, pQE30 expression vector.

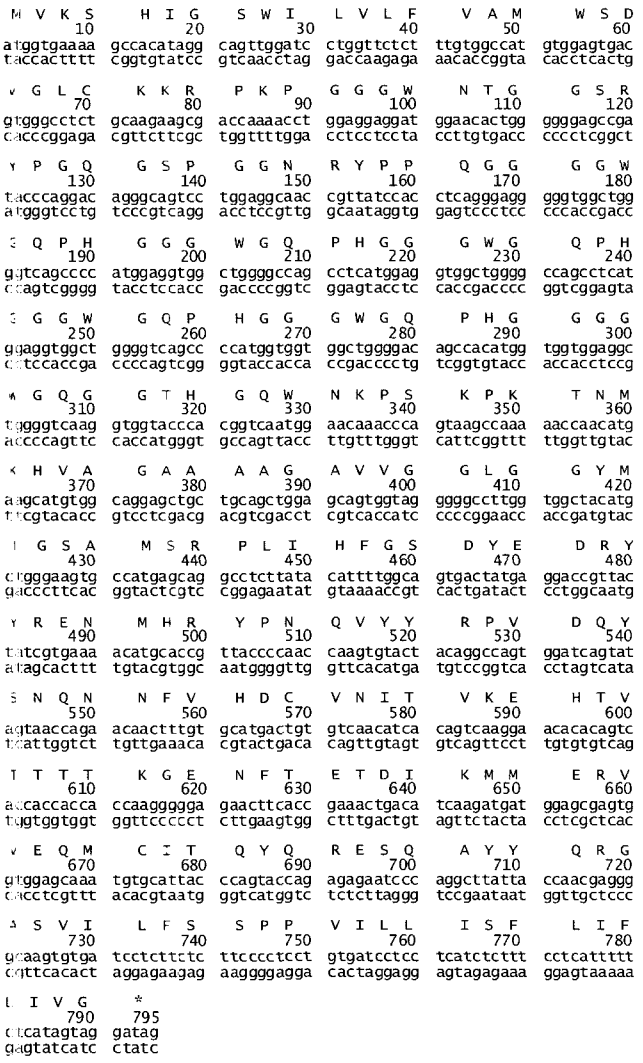

Fig. 2. Nucleotide and predicted amino acid sequence of bovine PrP.
The deduced amino acid sequence is shown by the single-letter amino acid codes over the nucleotide sequence. Stop codon is indicated by asterisk.

potential artifacts due to PCR, five positive clones from different trials were isolated, sequenced, and compared with each other. The confirmed plasmid vector with insert DNA was sequenced and reported to GenBank database

Table 2. Comparison of nucleotide and amino acid sequences of the clones with PrP genes originated from different animals.

	Bovine *Bos taurus*	
	Identities of nucleotide (%)	Identities of amino acid (%)
Sheep *Ovis aries*	90	94
Goat *Capra hircus*	90	94
Human *Homo sapiens*	79	84
Mouse *Mus musculus*	78	84

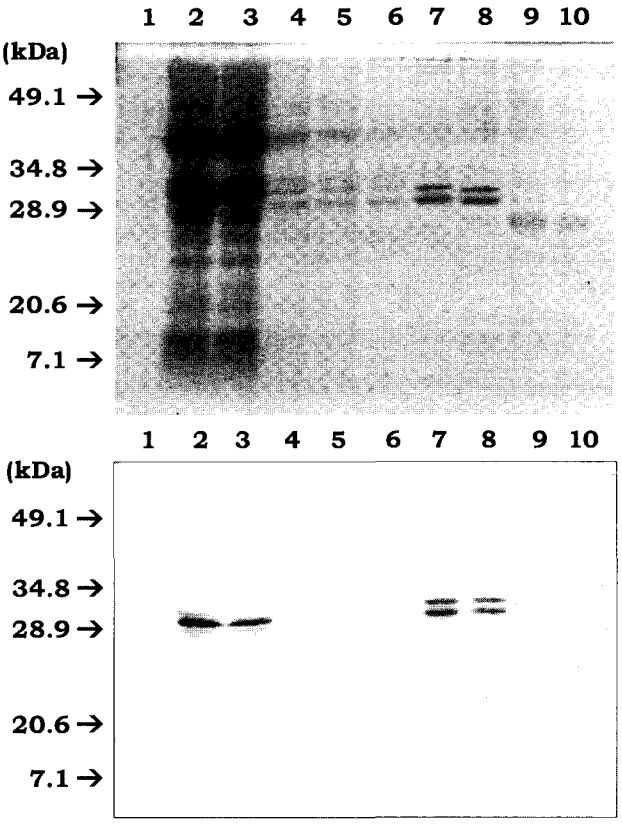

Fig. 3. SDS-PAGE (top) and Western blot (bottom) analysis.
Fractions were obtained in each purification step of Ni-NTA column chromatography. Lane 1, LB broth used for culturing M15; lane 2, lysate; lane 3, non-bound fraction; lane 4, 4th washing; lanes 5 and 6, pH 6.3 eluates; lanes 7 and 8, pH 5.8 eluates; lanes 9 and 10, pH 4.5 eluates.

with Accession No. AF517842 (Fig. 2). Comparison of the nucleotide sequences of the present bovine PrP ORF with that reported by Prusiner [20] (GenBank Accession No. S55629) revealed that both were identical. The nucleotide sequence of bovine PrP ORF in the cDNA was approximately 90, 90, 79 and 78% homologous and the predicted amino acid sequence of the cDNA was approximately 94, 94, 84, and 84% homologous to those from sheep, goat, human, and mouse [11, 14, 21, 22], respectively (Table 2).

To express the bovine PrP in *E. coli* M15, the cloned vecter plasmid was digested with *Sph*I and *Hin*dIII to obtain an insert DNA fragment with staggered ends and ligated into pQE30 prokaryote expression vector system. Although positive transformants were screened by antibiotics, both ampicillin and kanamycin in growth media, a false positive could not be avoided. Therefore, additional analysis with restriction enzymes and sequencing should be performed to confirm the presence of insert DNA. The recombinant PrP was expressed in *E. coli* M15 by IPTG induction and purified by applying it to an affinity chromatography. The expressed bovine PrP was found by

Western blot using mouse anti-PrP monoclonal antibody to be identical with that previously reported [15], and it was purified by immobilized-metal affinity chromatography column, Ni-NTA resin, with pH 5.8 elution buffer (Fig. 3). However, the protein concentration was found to be extremely low. Significant difficulties have been reported in the expression of recombinant PrP, probably due probably to their insolubility, aggregativity, sensitivity towards proteolytic digestion and instability, as well as environmental conditions [11, 12, 17]. In some cases, this was caused in mammalian cells by misfolding and accumulating into inclusion bodies [5, 23].

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