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Generation of ovine recombinant prion protein (25-232): Characterisation via anti-PrP monoclonal antibodies and CD spectroscopy

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Abstract

In prion pathogenesis, the structural conversion of the cellular prion protein (PrP^{C}) to its abnormal isomer (PrP^{Sc}) is believed to be a major event. The susceptibility or resistance to natural sheep scrapie is associated with polymorphisms of host PrP gene (*PRNP*) at amino acid residues 136, to a lesser extent 154. The 112 residue in ovine PrP displays a natural polymorphism, Methionine to Threonine, which has not been thoroughly investigated. However the cell-free conversion assay showed that ARQ with Thr112 (T_{112}ARQ)[1)] presents lower convertibility to PrP^{Sc} than wild type ARQ (M_{112}ARQ) [1]. In this study we generated ovine recombinant PrPs of 112 allelic variants by metal chelate affinity chromatography and cation exchange chromatography. The final purity of the ovine PrP ARQ was more than 95%. These variants showed similar immunoreactivity against anti-PrP monoclonal antibodies in Western blot and ELISA. The refolded M_{112}ARQ and T_{112}ARQ presented the secondary structural content to similar extent via CD spectroscopy analysis. The inherited structural features of M_{112}ARQ and T_{112}ARQ under the different biophysical conditions are in the middle of investigation.

Key words: Ovine PrP, Polymorphism, Recombinant PrP

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Introduction

TSEs such as the Creutzfeldt Jakob disease in humans, scrapie in sheep and BSE in cattle are recognised to be caused by a novel infectious proteinaceous pathogen, the 'prion' [1-3]. These are chronic, neurodegenerative and typically fatal diseases of the central nervous system (CNS), which exist in both humans and animals and they can manifest as infectious, sporadic and/or inherited disorders [4].

During prion disease, PrP^{Sc}, an abnormal isomer of a normal host protein PrP^{C}, accumulates in the brain of infected individuals, which mainly explained the protein only hypothesis [3]. Ultimately the major event in the pathogenesis of prion diseases is the structural conversion of PrP^{C} to PrP^{Sc}. The normal form of PrP consists of predominantly a-helical (42%) with little β-sheet structure (3%) whilst PrP^{Sc} has increased β-sheet content (43%) and a similar α-helical content (30%) [5].

PrP, the prion protein is approximately 250 amino acids long glycoprotein (varies depending upon the species type) with an approximate molecular weight of 33 - 35 kD. The C-terminal region of the protein contains three α-helices and two short anti parallel β-sheets. The two longest α-helices are stabilized by a connecting single disulfide bridge. The N-terminal domain of PrP containing the octarepeat region has not structurally well defined [5].

Scrapie in sheep is affected by allelic variants of the host PrP gene (*Prnp*). The major polymorphisms associated with differences in susceptibility to natural scrapie occur at amino acid residues 136, 171, and to a lesser extent 154. Animals that express the allelic variant $V_{136}R_{154}Q_{171}$ (PrP^{VRQ}) or $A_{136}R_{154}Q_{171}$ (PrP^{ARQ}) show susceptibility to scrapie whilst those with $A_{136}R_{154}R_{171}$ (PrP^{ARR}) show resistance. These polymorphic residues are located within or close to that region of PrP that undergoes the major conformational change associated with conversion of PrP^{C} to PrP^{Sc} during prion disease [6]. Little is known about the association of the allelic variants $PrP^{T_{112}ARQ}$, $PrP^{AT_{137}RQ}$, PrP^{AHQ}, PrP^{ARH}, and $PrP^{ARQQ_{211}}$ regarding susceptibility to scrapie [1, 6, 7].

Studies by Bossers et al [6] found that scrapie susceptibility - linked polymorphisms modulate the *in vitro* conversion of sheep PrP^{C} to PrP^{Sc}. Ovine PrP^{VRQ} was efficiently converted into PrP^{Sc}, whereas the PrP^{ARR} variant was poorly converted. Interestingly, the codon 112 polymorphism, Met to Thr, decreases the convertibility of PrP^{C} to PrP^{Sc}. In the proposed 3D-structure of sheep PrP, this is located in the highly flexible N-terminal region [6]. The larger side chain of Met, the polarity of Thr, or the shorter hydrogen bond between amino acids at codon 110 and 112 may affect the stability of PrP^{C} and/or interaction with PrP^{Sc} due to their side chains affecting any potential interaction sites.

In this paper, we report the purification of the recombinant ovine PrP^{C} protein of ARQ genotype with a disulfide bond and structural verification by CD spectroscopy. Moreover each allelic variant was characterized by monoclonal antibodies generated in our lab.

Materials and Methods

Genomic DNA extraction from ARQ homozygous sheep tissue

Approximately 2 ㎖ of ARQ homozygous sheep brain tissue was incubated in 0.4 ㎖ digestion buffer with 150 ㎎/㎖ PK overnight at 37℃. The sample was treated with 0.4 ㎖ water saturated phenol (pH 8.0) and 0.4 ㎖ chloroform / iso-amylalcohol (24 : 1). The aqueous layer was retained and chloroform / iso-amyl-alcohol extracted prior to precipitation of the genomic DNA in 2 volumes of 100% ethanol. Precipitated DNA was pelleted at 16,000 *g* for 1 minute, air dried for 5 minutes and resuspended in 100 ㎖ distilled water.

Generation of ovine PrP^{ARQ} DNA by PCR

The genomic DNA was used to amplify a desired region of PrP DNA (*PRNP*) by the polymerase chain reaction using the oligonucleotide primers as listed in Table 1a. The reactions were set up in total volume of 25 ㎕ as follows: 1 ㎕ of template DNA (2 ng/㎕), 2.5 ㎕ of *Pfu* 10 X buffer with 20 mM $MgSO_4$ (Promega), 2.5 ㎕ of dNTPs (2 mM), 1 ㎕ of forward primer (100 ng/㎕), 1 ㎕ of reverse primer (100 ng/㎕), 0.25 ㎕ of *Pfu* DNA polymerase (3 U/㎕) (Promega) and 16.75 ㎕ of water. The reaction mixtures were subjected into the PCR thermal cycler as follows: denatured for 2 minutes at 94℃, 30 cycles of 1 minute at 94℃, 30 sec at 55℃ and 2 minutes at 72℃ and final extension step for 7 minutes at 72℃. Reaction products were then purified using a Qiagen QIAquick PCR purification kit. Purified PCR products were analysed by agarose gel (1%) electrophoresis.

Purification of recombinant mature PrPs

Recombinant PrP were purified from BL21(DE3)pLysS bacteria transformed with the prokaryotic expression vector pET-23b (Novagen) that contained the open reading frame coding sequence of PrP ARQ (residues 25-232) in a method adapted from Hornemann et al [8]. To increase the possibility of cloning the site directed mutagenesis technique was used using a previously cloned vector containing the gene encoding ovine PrP^{VRQ} as substrate which finally generated mutant strands containing one nucleotide substitution (thymine to cytosine). The mutagenic primers were synthesized as listed in Table 1b. The reactions were set up using various concentrations of DNA template (5 ng, 10 ng, 20 ng and 50 ng) as manufacture's instructions.

Table 1. Oligonucleotide primers for cloning technique

a) Primers used to generate DNA encoding ovine PrPARQ (25-232)
Primer #1: 5′ CAAGCTTGGGCCATATGAGCAAGCGACCAAAACC 3′
Primer #2: 5′ GGGAATTCCTTATTACCCCTTTGGTAATAAG 3′
b) Primers used for site-directed mutagenesis (SDM)
Primer #3: 5′ CTGGGAAGTGCCATGAGCAGGCCTC 3′
Primer #4: 5′ GAGGCCTGCTCATGGCACTTCCCAG 3′

Enzyme linked immunosorbent assay (ELISA)

Ovine recombinant $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ were two fold diluted in PBS from the concentration of 2 ㎕/ml. ELISA plates were coated with diluted proteins and incubated overnight at 4℃. Excess recombinant PrP was removed, the plates were washed with PBS and wells blocked with 5% Marvel solution in PBS for 1 hour at 37℃. The plates were then washed three times with PBS containing 0.1% Tween 80. A panel of monoclonal antibodies (T325, 683, A516, 249 at 2 ㎍/㎖) [9, 10] were added and the plates were incubated at 37℃ for 1 hour. After washing the plates, biotinylated anti mouse IgG (Sigma) was added (diluted 1 : 1000 in PBS) and the plates were incubated at 37℃ for 45 minutes. After a further wash step, streptavidin alkaline phosphatase conjugate (Sigma) was added (diluted 1 : 1000 in PBS) and the plates were incubated at 37℃ for 45 minutes. The plates were washed as before and then washed with ELISA buffer (0.05 M glycine, 0.03 M NaOH, 0.25 mM $MgCl_2$, 0.25 mM $ZnCl_2$). Colour was developed with 0.5 ㎎/㎖ p-nitrophenyl phosphate in ELISA buffer and the absorbance read at 405 ㎚ on a MR5000 microELISA plate reader (Dynatech).

SDS PAGE of protein and Western Blotting

Protein samples were resuspended in Laemmli buffer, boiled for 5 minutes and separated on 15% acrylamide SDS. For Western blots, proteins were transferred to nitrocellulose membranes (BioRad) pre-soaked in transfer buffer (3.03 g Tris Base, 14.42 g glycine, 200 ㎖ methanol, water up to 1 L) using semi dry blotting at 20 V 125 mA for 45 minutes. Membranes were blocked with 5% (*w/v*) non fat milk in 1 X TBS T for 1 hour. Primary antibodies used were monoclonals 683, 249, 968, T325 [9, 10] at a concentration of 2 ㎍/㎖. An anti-mouse IgG biotin conjugate (1 : 3000) was added, incubated for 45 minutes and followed by extravidin-horseradish peroxidase (1 : 2000) (Sigma). PrP bands were finally detected by ECL.

CD spectra

Samples of recombinant PrP at concentration of 25 ㎛ were used for CD spectroscopic analysis. CD spectra were recorded in a 0.1 ㎝ length quartz cuvette at either 20℃ under constant nitrogen flushing using a Jasco J720 spectrophotometer. At least 10 spectra were accumulated and the appropriate blanks were subtracted. Values are expressed as molar ellipticity (θ). The program CDNN [11] was used to estimate secondary structure contents based on the advanced data base of reference spectra.

Results

Generation of PrP^{ARQ} DNA from brain material

Genomic DNA prepared from an ARQ/ARQ sheep brain was used as the template in this reaction. The PCR product of DNA encoding mature (amino acid residues 25-232) PrP^{ARQ} is shown with all together full length and truncated form in Fig 1a and were found to be of a size corresponding to their predicted length.

The PCR primers used to generate mature PrP^{ARQ} DNA were designed to contain recognition sites for restriction enzymes *EcoRI* and *NdeI*. This would enable directional ligation into the vector pET-23b via cohesive ends. The plasmid map for pET-23b is shown in Fig 1b. Both pET-23b and the PCR product containing mature PrP^{ARQ} DNA were cleaved with *EcoRI* and *NdeI*, subsequently ligated, transformed into DH5α cells and transformants were selected on LB Amp plates. Resultant bacterial colonies were screened for inserts within vector DNA by mini-prep and restriction enzyme digest analysis of plasmid DNA.

Restriction digest fragments were predicted with knowledge of the DNA sequence of the pET-23b vector and of PrP^{ARQ} DNA from published sequences (data not shown).

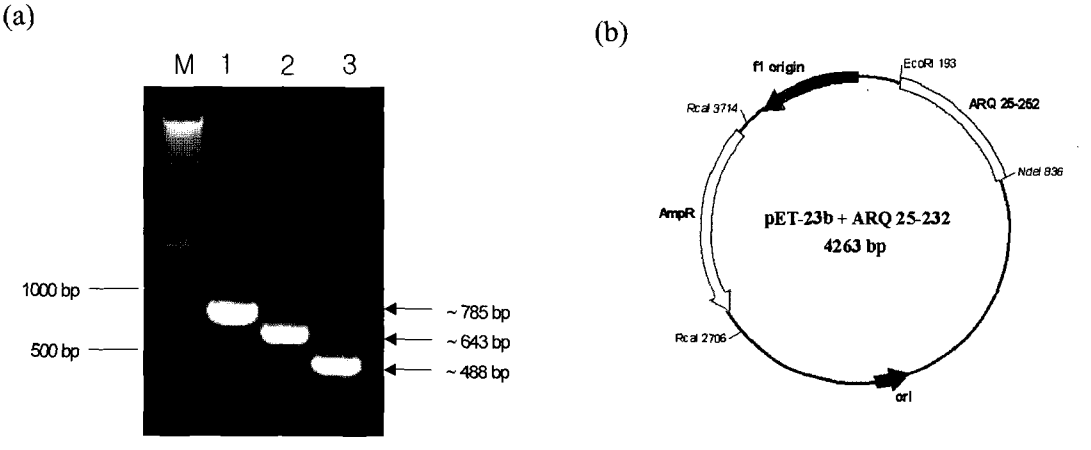

Fig 1. (a) Agarose gel electrophoresis of PCR products encoding full-length (lane 1), mature (lane 2) and truncated PrP ARQ proteins (lane 3). *(b)* Plasmid map of pET-23b with insert mature ARQ (in fact a.a 25-232) ligated into the EcoR I and Nde I restriction sites. Rca I restriction sites exist at 2661 and 3669 bp.

Cloning, expression and purification of ovine recombinant PrPs; M_{112}ARQ and T_{112}ARQ

The 112 allelic variants of ovine recombinant $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ were cloned. The clone containing *PRNP* encoding allelic variant $PrP^{M_{112}ARQ}$ (M_{112}ARQ) was generated via site directed mutagenesis using previously cloned pET-23b-PrP^{VRQ}. The other allelic variant $PrP^{M_{112}ARQ}$ was made by traditional cloning method using genomic DNA as described in Meterials and methods. Verification of these constructs (pET-23b-$PrP^{M_{112}ARQ}$ and pET-23b-$PrP^{T_{112}ARQ}$) was performed by restriction endonuclease digestion analysis (Fig 2) and DNA sequencing (Fig 3). The clones were transformed into protein expression host bacteria, BL21(DE3)pLysS and prion proteins were overexpressed and accumulated as cytoplasmic inclusion bodies. Ovine full length recombinant PrPs (25-232) were purified by metal affinity and ion exchange chromatography using a method adapted from Hornemann et al. [8]

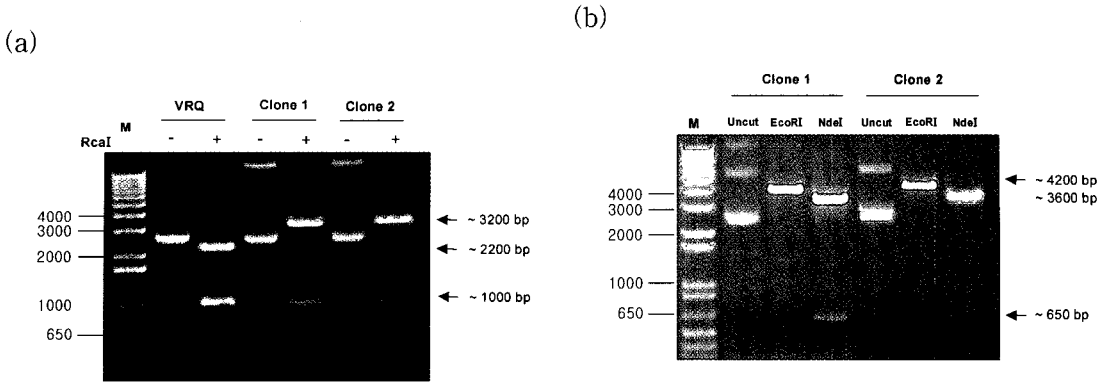

Fig 2. Agarose gel electrophoresis of pET-23b-PrPARQ generated by two different methods, conventional cloning (Clone 1) and site-directed mutagenesis (SDM) (Clone 2). *(a)* Digest with *RcaI* of pET-23b-PrPARQ clone 1 and 2 in comparison with pET-23b-PrPVRQ. *(b)* Verification of pET-23b-PrPARQ by restriction digest with *EcoRI* and *NdeI* as shown by the presence of a DNA fragment of ~650 bp. Markers in bp are shown on the left hand side.

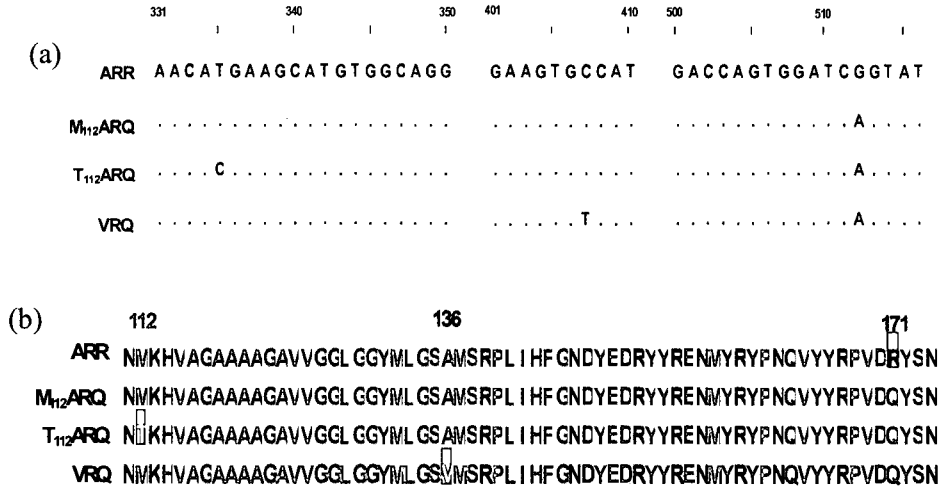

Fig 3. DNA sequence alignment of $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ *(a)*, $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ translation *(b)* There is one single nucleotide difference, thymine (T) or cytosine (C) between methionine or threonine at codon 112, which is considered to be a natural polymorphism

Purification of $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ proteins

Small scale screening expression of ovine recombinant PrP^{ARQ} proteins : The *E coli* expression host BL21(DE3)pLysS was transformed with either mature ovine pET-23b-$PrP^{M_{112}ARQ}$ or pET-23b-$PrP^{T_{112}ARQ}$

and PrP protein expressed by IPTG induction. Initially, small scale protein expression was conducted in order to verify the presence of PrP protein. Lysates from transformed and induced bacteria were analysed by SDS PAGE and the results are shown in Fig 4. A protein band of ≈23Kd, the predicted size of recombinant ovine PrP^{ARQ}, was seen in those bacterial lysates from appropriately transformed and induced bacteria. Western blot of these lysates showed that the band of interest at ≈23Kd reacted with an anti PrP monoclonal antibody strongly suggesting that it was indeed ovine PrP^{ARQ} (data not shown).

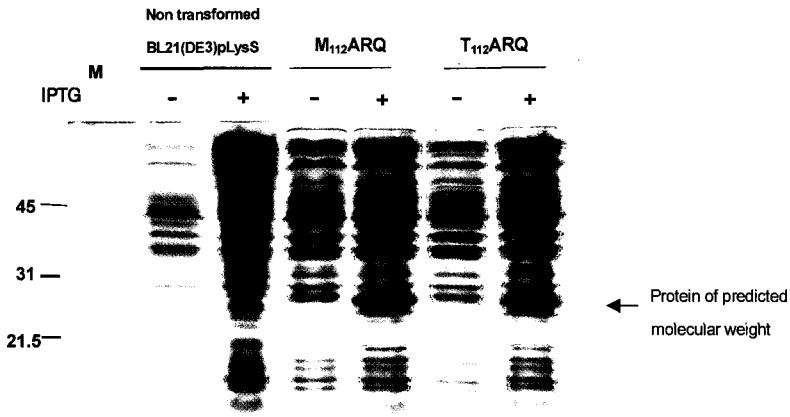

Fig 4. Small scale of IPTG induction; expression of mature $PrP^{M_{112}ARQ}$ (M_{112}ARQ) and $PrP^{T_{112}ARQ}$ (T_{112}ARQ) proteins from transformed BL21(DE3)pLysS cells detected by SDS PAGE followed by Coomassie stain, before (-) and after (+) induction by IPTG (1mM). ~ 27 kD is the predicted molecular weight of the proteins.

Large scale purification of recombinant $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ proteins : Large scale purification of recombinant $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ was achieved through the use of a standardised protocol used in the laboratory for the purification of recombinant PrP. This involved inclusion body preparation of appropriately transformed BL21 (DE3)pLysS cells followed by protein purification using immobilized metal affinity chromatography (IMAC) and ion exchange chromatography (IEC). A nickel ion charged sepharose column was used for IMAC. The purity of material within individual eluted fractions was further monitored by SDS PAGE as shown in Fig 5. It can be seen that protein eluted from the IMAC column by low pH buffer, which by experience is known to be recombinant PrP, was of reasonably high purity and of the predicted molecular weight for this protein as shown in Figure 5, lane e. Mature recombinant PrP^{ARQ} was further purified by selective elution from an IEC column (Fig 5, lane f). The final purity of the protein was estimated to be more than 95%.

Purified mature PrP^{ARQ} was oxidized by the addition of copper [8] and subsequently dialysed against 50 mM sodium acetate buffer (pH 5.5 with glacial acetic acid) to

remove the denaturant (8 M urea) and imidazole in the resolubilisation buffer used during the purification steps. Consequently, two allelic forms of refolded ovine recombinant PrP^{ARQ} protein were successfully purified. These mature recombinant PrP^{ARQ} proteins were studied to characterise the reactivity of anti PrP monoclonal antibodies for comparison with existing mature PrP^{VRQ} and PrP^{ARR} protein.

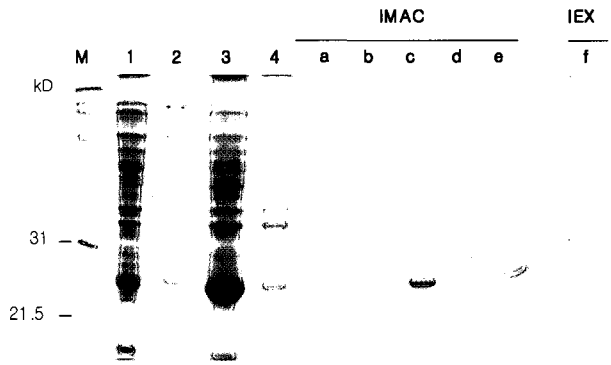

Fig 5. The representative of the purification of PrP^{ARQ} protein; SDS PAGE gel is showing different stages of the protein purification procedures through immobilized metal affinity chromatography (IMAC) and ion exchange chromatography (IEX). Lanes are: M: protein markers; track 1: crude bacterial pellet; track 2: supernatant after lysing cells; track 3: resolubilised inclusion bodies; track 4: the first flow through after loading onto IMAC column. track a: first wash; track b: 1st peak of non-specific protein by resolubilisation buffer; track c: 2nd peak of non-specific protein; track d: 2nd wash; track e: eluted protein through IMAC column; track f: eluted protein through IEX colum

Dominant α-helical structure of refolded ovine recombinant PrPs

Far UV CD spectral analysis was conducted to determine secondary structure of the two naturally existing PrP variants, namely M_{112}ARQ and T_{112}ARQ. The spectral intensity was converted into molar ellipticity (θ) in Jasco J810 spectrophotometer. Three different batches of each recombinant protein were used to verify their intrinsic conformations and to analyse each secondary structural contents.

The CD spectral patterns of each proteins represent two shoulders of minima at 208 and 222 nm of UV wavelength, indicating the predominantly **a**-helical structure expected for ovine refolded PrP [8, 12]. The three different batches of each allelic variant of PrP displayed almost identical shape of spectral patterns as shown in Fig 6. Table 2 shows the details of secondary structural contents deduced by CDNN software revealing that less than 1% structural difference between M_{112}ARQ and T_{112}ARQ.

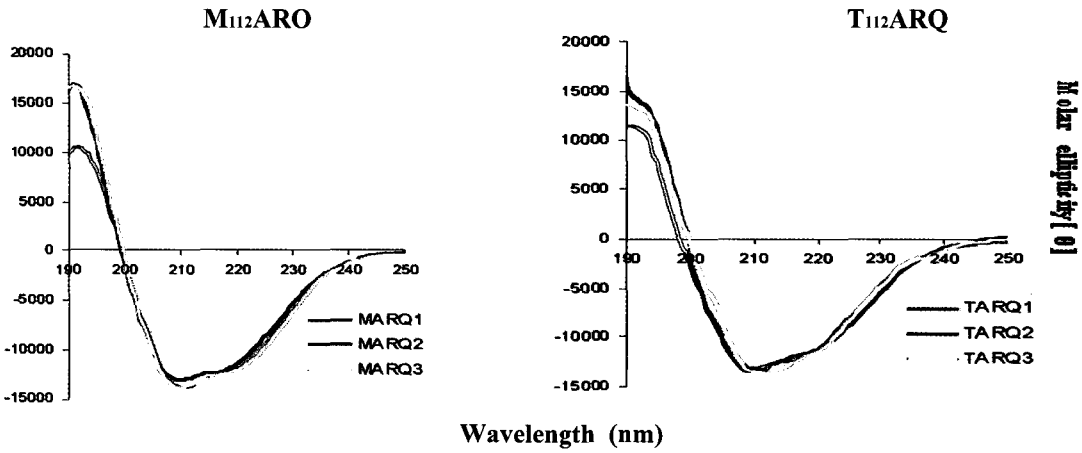

Fig 6. CD spectral analysis of refolded a-helical recombinant PrP; Three different batches of $PrP^{M112ARQ}$ (M112ARQ) and $PrP^{T112ARQ}$ (T112ARQ) were used to verify the secondary structure. The each batches were represented as different colours; Batch 1 (red line), Batch 2 (blue line), Batch 3 (yellow line).

Table 2. Secondary structural contents deduced by CDNN program

(a) M112ARQ

	185-260 nm	190-260 nm	195-260 nm	200-260 nm	205-260 nm	210-260 nm
Helix	32.70%	32.70%	32.87%	36.70%	40.77%	39.37%
Antiparallel	11.50%	11.97%	10.63%	7.87%	6.50%	6.83%
Parallel	7.80%	7.77%	8.10%	7.57%	7.17%	7.47%
Beta-Turn	17.73%	17.63%	17.17%	16.50%	15.63%	15.87%
Random Coil	27.40%	27.27%	28.27%	28.17%	28.27%	29.33%
Total Sum	97.13%	97.33%	97.03%	96.80%	98.33%	98.87%

(b) T112ARQ

	185-260 nm	190-260 nm	195-260 nm	200-260 nm	205-260 nm	210-260 nm
Helix	32.10%	31.53%	31.70%	35.33%	39.50%	38.33%
Antiparallel	12.30%	13.40%	11.40%	8.23%	6.73%	7.07%
Parallel	7.90%	7.93%	8.37%	7.87%	7.47%	7.67%
Beta-Turn	17.90%	17.93%	17.40%	16.77%	15.83%	16.03%
Random Coil	27.57%	27.77%	28.83%	28.93%	29.00%	29.93%
Total Sum	97.77%	98.57%	97.70%	97.13%	98.53%	99.03%

Western blot reactivity of anti-PrP monoclonal antibodies with ovine recombinant PrP

The initial reactivity of the monoclonal antibodies T325, 683, 249, A516 and 968 with the 112 allelic variants of ovine recombinant PrP was first investigated by Western blot. The 112 allelic variants of ovine PrP, namely M_{112}ARQ and T_{112}ARQ displayed a similar secondary structure by CD spectroscopy. The two alleles were completely denatured under reducing conditions and a panel of antibody was used to detect subtle differences. The concentration of two proteins was measured by BCA assay and an equal amount of them were loaded. A SDS PAGE gel seen in Figure 7 illustrates equal loading of the two ovine recombinant PrP proteins used in the subsequent Western blots. As seen in Western blots of Fig 7, monoclonals T325, 683, 249 and 968 showed almost identical immunoreactivity in that they reacted with allelic variants of ovine PrP. Interestingly, monoclonals A516 did not react with both alleles in Western blot, which the protein itself totally denatured by reducing reagent and heat.

Monoclonal antibody A516 showed lack of reactivity with ovine PrPs [10] in the previous studies. Consistently, monoclonal antibody A516 represented no reactivity with 112 allelic variants of ovine PrP (Fig 7). This was not due to the absence of protein in these tracks as equal loading was shown in SDS PAGE. Monoclonal A516 was characterised as conformation dependent antibody in the previous study and the data shown in this study are showing consistency.

As a result a single amino acid change of Met to Thr at amino acid residue 112 of ovine PrP showed no alteration of immunoreactivity on epitopes of a panel of antibodies used.

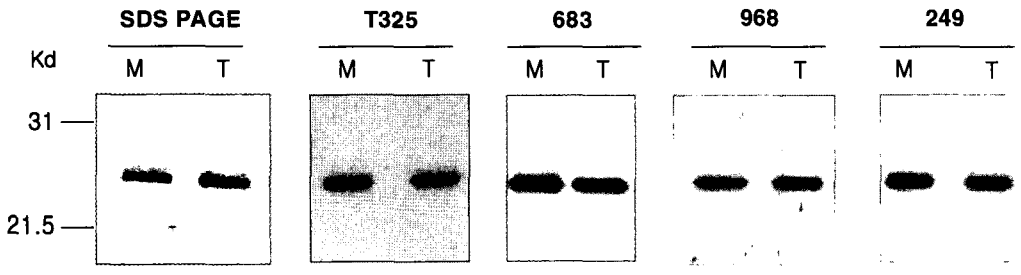

Fig 7. Western blot reactivity of monoclonal antibodies with ovine recombinant PrP; M_{112}ARQ (M) and T_{112}ARQ (T). SDS denaturing gel showed the equal loading of both proteins (~ 200 ng/track) and Western blots were conducted using similar quantities of each recombinant protein. The anti-PrP monoclonal antibodies, T325, 683, 968 and 249 were used either at 2 mg/ml concentration. Molecular weight markers in Kd are shown on the left hand side.

ELISA reactivity of anti-PrP monoclonal antibodies with ovine recombinant PrP

The protein used in ELISA was usually considered as a less denatured form compared with one used in Western blot. Therefore proteins investigated in this manner may retain some of their native structure.

Ovine $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ were attached to microplates with various concentrations prior to addition of monoclonal antibodies. The data described in Fig 8 show that a panel of antibodies including N- and C-terminal specific monoclonals displayed identical reactivity against both allelic variants. Monoclonal A516 showed the similar pattern of reactivity to the 112 allelic variants and it indicates that conformational epitopes of these two variants to monoclonal A516 are commonly shared. N-terminal specific monoclonal antibody T325 also showed identical reactivity to both variants, indicating the substitution of methionine at the residue 112 did not alter their N-terminal conformation. Taken together ELISA data implied that the amino acid substitution at this site largely do not vary its C-terminal conformation. Moreover capture detector ELISA was also used to detect the possible conformational differences between these two proteins and the different pairs of capture and detector antibodies were used (data not shown). Most pairs of antibodies showed similar pattern of reactivity to both allelic variants.

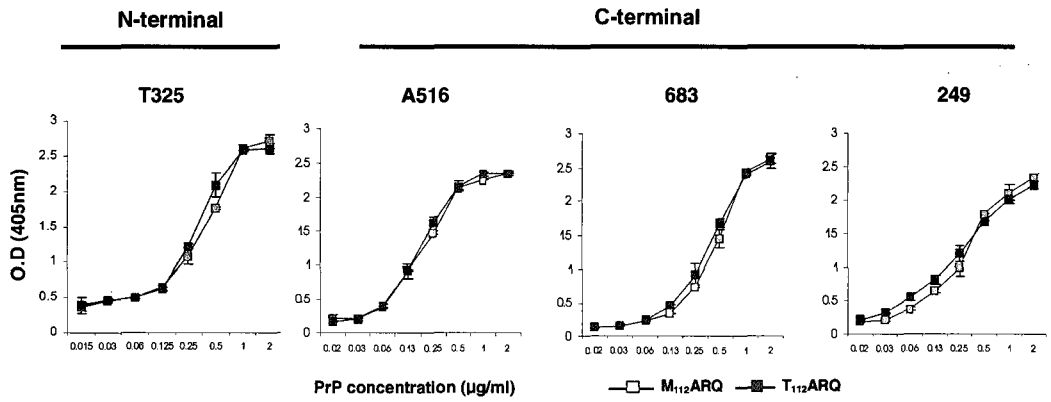

Fig 8. ELISA reactivity of monoclonal antibodies with $M_{112}ARQ$ and $T_{112}ARQ$; both N- and C-terminal specific monoclonal antibodies reacted with these variants to similar extent.

Discussion

Mature ovine PrP^{ARQ} protein was generated by recombinant DNA technology using the conventional PCR and site directed mutagenesis (SDM). DNA sequencing of these clones confirmed that mature ovine PrP^{ARQ} (A136, R154 and Q171) was generated and in the correct open reading frame for protein expression in a prokaryotic expression host. However,

and somewhat fortuitously, two allelic forms of PrP^{ARQ} (denoted as $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$) were obtained, that differed by a single amino acid alteration (Met to Thr) at codon 112. This polymorphism is known to exist naturally in ARQ homozygous sheep [1, 13, 14]. Studies *in vitro* have shown that the polymorphism at codon 112 has a reduced susceptibility to conversion from PrP^{C} to PrP^{Sc} [1]. As the existing ovine recombinant proteins PrP^{VRQ} and PrP^{ARR} to be used here both contained Met at codon position 112 only $PrP^{M_{112}ARQ}$ was used in the studies presented in this thesis.

Mature ovine PrP^{ARQ} was purified to a high level via metal affinity chromatography using the histidine residues present in the octapeptide repeat region of this protein. The final purify of PrP was more than 95%.

In the previous study the murine mature PrP (23-231) were generated with the high purity via metal affinity column and ion exchange column. Here we successfully generated the constructs expressing ovine recombinant prion protein, which are fortuitously dimorphic at codon 112 site. Biophysical and structural studies of prion proteins have been hampered by the lack of an efficiency of PrP expression and purification system. Therefore these allelic variants of ovine PrP can be structurally investigated as for their biophysical normality based on the method of purifying ovine PrPs.

Cellular form of ovine $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ were refolded into predominant a ⍺helical structure as shown by CD spectra and it was consistent with other prion proteins in many species [12, 15, 16] A comparison of $PrP^{M_{112}ARQ}$ and $PrP^{T_{112}ARQ}$ with respect to biophysical differences and reactivity with anti PrP monoclonal antibodies were carried out. However they were found to be very much identical in structure and immunoreactivity. The biological relevance *in vivo* on the susceptibility or resistance to scrapie should be further investigated in the future study.

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