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Purification of normal cellular prion protein from human platelets and the formation of a high molecular weight prion protein complex following platelet activation

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Abstract

A method for the extraction and purification of PrP^C, in its native monomeric form, from outdated human platelet concentrates is described. Both calcium ionophore platelet activation and lysis in Triton X-100 were evaluated as methods for the extraction of soluble platelet PrP^C in its monomeric form. Following platelet activation, the majority of released PrP^C was detected as a disulphide linked high molecular weight complex, which under reducing conditions could be separated into what appear to be stable non-disulphide linked PrP dimers or PrP covalently linked to another as yet unidentified protein. This phenomenon appears to be unique to activation since only monomeric PrP^C was detected following lysis of resting platelets. Subsequently, PrP^C was purified from the Triton X-100 lysate by sequential cation ion exchange and Cu^{2+} affinity chromatography. From 10 L of outdated platelet concentrate, we were able to recover 1.29 mg PrP^C at a purity of 92%.

Keywords: Human prion protein; Purification; Blood; Platelet; Calcium ionophore activation; Creutzfeldt-Jakob disease; Copper affinity; Conformation change; Detergent lysis; Dimer; Cation exchange

vCJD belongs to a group of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs or prion diseases) which are characterised by the post-translational modification of the normal cellular host derived prion protein (PrP^C) to an abnormal form (PrP^{Sc}) [1]. Whilst no differences in the covalent structure have been observed between PrP^C and PrP^{Sc} [2], the two isoforms have profoundly different biochemical and biophysical properties. PrP^C consists largely of α-helices, is soluble in mild detergents and is easily degraded by proteinase K. In contrast, PrP^{Sc} is rich in β-sheet structure, is insoluble in mild detergents, and is highly resistant to proteinase K digestion [3]. Following the discovery that PrP^{Sc} and infectivity co-purified [4], it is now widely assumed by some that PrP^{Sc} is the infectious agent and a requirement for the expression of host PrP^C in the dissemination of infection has been demonstrated [5]. Many questions remain about the mechanisms involved in TSE propagation, however research has been hampered by difficulties in purifying large amounts of native PrP^C from the host.

PrP^C is a glycosylphosphatidylinositol (GPI)-anchored membrane protein [6], which is expressed in many cell

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types and tissues [7] with the highest amounts located in the brain. Attempts to extract and purify native PrP^C from brain tissue have resulted in poor recoveries [8–10] and researchers have relied upon recombinant PrP^C expressed in a variety of cell culture systems [11]. The main disadvantage of these systems is that whilst large amounts of recombinant PrP^C can be expressed and purified, it is unlikely to be truly representative of the PrP^C expressed in the host. Furthermore, it has been suggested [12] that PrP^C structure may vary both with species and within species, with PrP genotype, with tissue or cell-specified PrP transcripts, post-translational modifications or topology. For example, differences in the size of the fully glycosylated and aglycosyl isoforms of PrP^C and differences in the proteolytic processing from human brain and non-neural tissue (tonsils and platelets) have been reported [13]. Such tissue-specific differences could have an impact upon the energetics of PrP^C conversion to PrP^{Sc} and might also affect the uptake and dissemination of infectivity.

Following the recent reports of transmission of vCJD infectivity by blood transfusion [14,15], the need to identify the markers of vCJD infectivity in blood has never been more important. Since the best available marker for prion infectivity is PrP^{Sc}, there is a need to develop reagents which can be used to detect this abnormal isoform in blood and blood components. A source of native PrP^C purified from human blood or a blood component would be a valuable tool in these studies, for example, for use as an immunogen to produce monoclonal antibodies.

In normal human blood, the majority (95%) of PrP^C is distributed between the plasma (68.5%) and the platelet (26.5%) compartments [16]. Flow-cytometry studies [17] revealed the presence of PrP^C on the surface of human platelets and that the platelet PrP^C accounted for at least 96% of the cellular-associated PrP^C in normal human blood. Furthermore, human platelets also contain a significant intracellular concentration of PrP^C stored within the platelet α-granules [18]. Upon platelet activation, this PrP^C is translocated from the α-granules to the plasma membrane [19] leading to eventual release of soluble PrP^C [20,21]. The fact that human platelets contain a significant intracellular store of PrP^C and that large amounts of outdated platelet concentrates (OPC) can be obtained make human platelets an attractive source from which to attempt to purify native PrP^C. It is the development of such a purification strategy for routine use that is described in this paper.

Materials and methods

Source of outdated platelets. Outdated (5-day old) leucodepleted pooled platelet concentrates were supplied by Edinburgh SNBTS Components. These platelet concentrates had passed their expiry date for clinical use, were due for disposal, and donor consent had been obtained for their use in research. Those platelets not used immediately were incubated on a shaker at 60 rpm at 22 °C for a maximum of a further 48 h. Any platelets not used in this period were discarded.

Platelet activation. Outdated platelet concentrate (50 ml) was centrifuged at 2000*g* for 20 min at 20 °C and the supernatant discarded. The platelet pellet was resuspended in 1/5 the starting volume platelet wash buffer (10 ml of 50 mM Hepes, 150 mM NaCl, 5 mM glucose, and 2 mM EDTA, pH 7.4), centrifuged as previously described and the supernatant was discarded. The washed platelet pellet was resuspended in 1/5 the starting volume platelet activation buffer (10 ml of 50 mM Hepes, 150 mM CH_3COONa, 5 mM glucose, and 10 mM $CaCl_2$, pH 7.4) supplemented with 10 μl Protease Inhibitor Cocktail Set III (Calbiochem). Calcium ionophore A23187 (Sigma), prepared as a 2 mM stock solution in absolute ethanol, was then added to a final concentration of 2 μM. Following incubation at 37 °C for 2 min, the sample was centrifuged (2000*g*, 20 min, 4 °C) and the activated outdated platelet concentrate supernatant was (AOPCS) collected.

Platelet lysis. Platelets were pelleted from outdated platelet concentrate (50 ml) and washed as described for platelet activation. The washed platelet pellet was resuspended in 1/5 the starting volume ice-cold platelet lysis buffer (10 ml of 50 mM Hepes, 150 mM CH_3COONa, 5 mM glucose, 10 mM $CaCl_2$, and 1% (v/v) Triton X-100, pH 7.4) supplemented with 10 μl Protease Inhibitor Cocktail Set III (Calbiochem). Lysis was carried out on ice for 30 min, the sample was centrifuged (2000*g*, 20 min, 20 °C) and the supernatant (lysate) was collected. For large-scale extractions the volumes used were adjusted accordingly.

Estimation of total protein concentration. The total protein concentration was determined using the Bicinchoninic acid (BCA) protein assay kit (Pierce) following the manufacturer's instructions using bovine serum albumin standards.

Denaturation experiments. Aliquots of both the AOPCS and Triton X-100 lysate (100 μl lots) were diluted 1:1 with either distilled water or 6 M guanidine hydrochloride (GdnHCl) and incubated for 60 min at room temperature.

Slot-blotting. Samples (10 μl) were applied to Hybond-ECL nitrocellulose membrane (Amersham Biosciences) using a manifold filtration unit. The membrane was blocked for 60 min at room temperature in 5% (w/v) nonfat dried milk powder in 10 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.4 (TBST). The membrane was incubated in anti-PrP monoclonal antibody (mAb) 3F4 [22] (DAKO), diluted 1/1000 in TBTS for 60 min at room temperature, and washed three times with TBTS (5 min per wash). The membrane was incubated in anti-mouse IgG (whole molecule) peroxidase conjugate (DAKO), diluted 1/1000 in TBST for 60 min at room temperature, washed three times in TBTS, and finally incubated in TMB 1-Step Blotting Substrate (Pierce) at room temperature until staining was of a suitable intensity.

SDS–PAGE and Western blotting. Samples were diluted 1:1 with either 2× non-reducing sample buffer or 2× reducing sample buffer and boiled for 10 min. Aliquots (10 μl) were separated on an excel gel SDS gradient 8–18 precast gel (Amersham Biosciences) as per the manufacturer's instructions. The separated proteins were electrophoretically transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Following transfer, the membrane was blocked and probed with mAb 3F4 as described for the slot-blot assay. For quantitative Western blots to determine PrP^C concentration, aliquots (10 μl) of twofold serial dilutions of recombinant human prion protein (Calbiochem), in the range 10–0.625 μg/ml, were run alongside test samples on a Western blot as described above. The PrP^C concentration in the test samples was estimated based on their staining intensities relative to the standards.

SP-Sepharose XL cation exchange chromatography. Triton X-100 lysate (200 ml) prepared from 1 L of outdated platelet concentrate was adjusted to pH 6.0 with acetic acid and clarified by centrifugation (2000*g*, 20 min, 20 °C). The supernatant was loaded onto a SP-Sepharose XL (Amersham Biosciences) column (C10/20 column, 14 ml packed gel volume), pre-equilibrated in 150 mM CH_3COONa, 1% (v/v) Triton X-100, pH 6.0, at a flow-rate of 2 ml/min collecting the

flow-through. After all the sample was loaded, the column was washed sequentially with buffers containing 150, 250, 500 mM, and 1 M CH_3COONa in 1% (v/v) Triton X-100 and adjusted to pH 6.0, collecting 2 ml fractions. Protein elution was monitored using the BCA protein assay kit reagent, 25 μl of each fraction plus 200 μl BCA reagent, incubating at 37 °C for 30 min and measuring the absorbance at 570 nm. Fractions containing protein from each wash step were then pooled and the PrP^C distribution determined by Western blotting of the pooled samples.

Cu^{2+} affinity chromatography. Platelet lysate, SP-Sepharose flow-through or the SP-Sepharose 500 mM CH_3COONa eluant was adjusted to pH 7.0, 20 mM imidazole and centrifuged (2000*g*, 20 min, 20 °C) to clarify. Each sample was then loaded onto a Cu^{2+} ion charged 1 ml Hi-Trap Chelating column (Amersham Biosciences), pre-equilibrated in 20 mM imidazole, 150 mM CH_3COONa, and 1% Triton X-100, pH 7.0, at a flow-rate of 1 ml/min collecting the column flow-through. The column was washed sequentially with 10 ml lots of buffers containing 20, 40, 100 mM imidazole and 50 mM EDTA, respectively, all prepared in 150 mM CH_3COONa, 1% (v/v) Triton X-100 and adjusted to pH 7.0, collecting 1 ml fractions. The protein elution profile was determined as previously described using the BCA protein assay reagent. Fractions containing protein from each wash step were then pooled and the PrP^C distribution determined by Western blotting of the pooled samples.

PrP^C size distribution. Aliquots of the Cu^{2+} affinity 100 mM imidazole eluant were loaded onto Amicon Centricon centrifugal filter devices (Millipore) with molecular weight cut-offs of 10, 50, and 300 kDa, respectively. The filter devices were spun at 2000*g*, 20 °C until the entire sample had passed through each filter. Aliquots of each filtrate were slot-blotted onto nitrocellulose membrane and probed with mAb 3F4 as previously described.

Large-scale purification of PrP^C. Outdated platelets (1 L lots) were lysed and the lysate loaded onto a SP-Sepharose XL column as previously described. The column was washed with 40 ml wash buffer (250 mM CH_3COONa, 1% (v/v) Triton X-100, pH 6.0). Bound PrP^C was eluted in elution buffer (500 mM CH_3COONa, 1% (v/v) Triton X-100, pH 6.0), discarding the first 10 ml to elute and collecting the next 25 ml. Each eluant (25 ml) collected was stored frozen at −40 °C until loaded onto a Cu^{2+} affinity column. The eluants from five such SP-Sepharose XL fractionations were pooled, adjusted to pH 7.0, 20 mM imidazole, centrifuged to clarify, and loaded onto a Cu^{2+} affinity column as previously described. The column was washed with 10 ml Cu^{2+} affinity wash buffer (40 mM imidazole, 150 mM CH_3COONa, and 1% (v/v) Triton X-100, pH 7.0). Bound PrP^C was then eluted in Cu^{2+} affinity elution buffer (100 mM imidazole, 150 mM CH_3COONa, and 1% Triton X-100, pH 7.0) collecting the first 5 ml to elute from the column. The Cu^{2+} affinity eluant was stored at 4 °C. Eluants from two such Cu^{2+} affinity fractionations were pooled and concentrated 10-fold through an Amicon Ultra 10,000 MWCO centrifugal filter device (Millipore). The concentrated sample was centrifuged at 14,000*g* for 10 min to clarify. The total protein concentration was determined by BCA protein assay, the PrP^C concentration determined by quantitative Western blotting and the purity assessed by silver-stained SDS–PAGE.

Results and discussion

Platelet PrP^C extraction by calcium ionophore platelet activation

PrP^C release from platelets following activation with various agonists has been reported [20,21] and therefore activation with calcium ionophore was investigated as a method of extracting PrP^C from platelets. Aliquots of the AOPCS obtained post-activation were slot-blotted onto nitrocellulose and probed with mAb 3F4. PrP^C could only be detected in the AOPCS following denaturation with 3 M GdnHCl (Fig. 1A). The fact that PrP^C was not detected in the AOPCS under native conditions suggested that the PrP^C had adopted a conformation similar to that of PrP^{Sc} [23] in that the mAb 3F4 epitope was no longer exposed. Immobilised recombinant PrP^C has been shown to undergo a spontaneous rearrangement to a conformation having features in common with PrP^{Sc} [24]. It was possible that the platelet PrP^C had undergone such a rearrangement upon binding to the

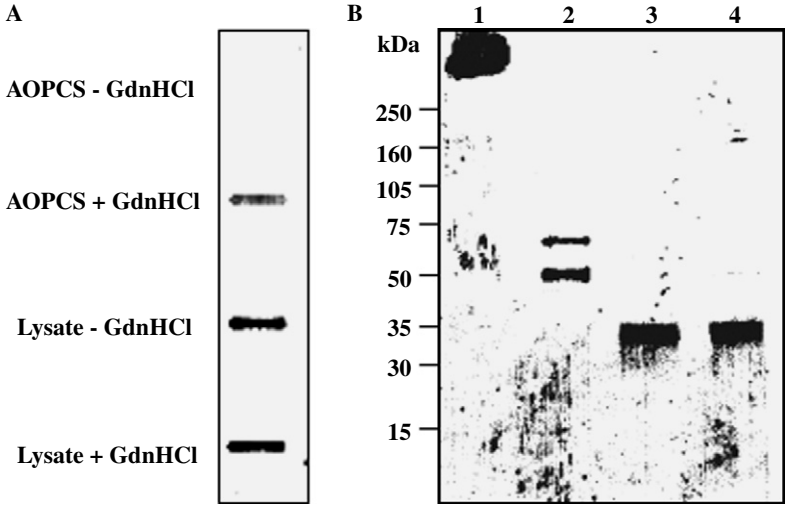

Fig. 1. Detection of soluble PrP^C in the AOPCS and Triton X-100 lysate. (A) Slot-blot probed with mAb 3F4 to detect PrP^C in the supernatants obtained after platelet activation (AOPCS) and lysis (lysate) under both native and denaturing conditions. Samples were denatured by incubating in 3 M GdnHCl for 60 min at room temperature. (B) Western blot probed with mAb 3F4, of AOPCS and Triton X-100 lystate, after separation on an excel gel SDS gradient 8–18% precast gel under both non-reducing and reducing conditions. Lane 1, AOPCS under non-reducing conditions; lane 2, AOPCS under reducing condition; lane 3, lysate under non-reducing conditions; and lane 4, lysate under reducing conditions.

nitrocellulose. However, Western blot analysis (Fig. 1B) suggested a different explanation. Under non-reducing conditions, the majority of PrP^C in the AOPCS migrated as a high molecular weight (>250 kDa) complex (Fig. 1B, lane 1) which upon reduction migrated as two bands with molecular weights between 50 and 75 kDa (Fig. 1B, lane 2). Trace amounts of monomeric PrP^C, migrating between 30 and 35 kDa, were also detected under both reducing and non-reducing conditions, although the bands were barely visible on the Western blot. An identical Western blot probed with mAb 3F4, pre-incubated with an excess of a synthetic peptide spanning the 3F4 epitope within PrP amino acids 106–120, failed to detect the bands seen in Fig. 1B (results not shown) confirming that the binding observed was specific for PrP^C. Based on these observations, it appears probable that upon platelet activation PrP^C forms a high molecular weight disulphide linked complex. This complex may be composed of either highly stable covalently linked PrP^C dimers or PrP^C linked to another as yet unidentified protein. The fact that these bands were not dissociated into monomeric PrP^C after boiling in SDS–PAGE sample buffer under reducing conditions suggest a covalent linkage other than a disulphide bond might be involved. The formation of PrP^C dimers in vivo has been well documented. Meyer et al. [25] demonstrated that native bovine PrP^C, but not recombinant PrP^C, formed a non-covalently linked dimer. The difference observed between the native PrP^C and the recombinant PrP^C was attributed to the glycosylation of the native PrP^C. It was suggested that the presence of the carbohydrate induced a specific PrP^C conformation that exposed the amino acid residues responsible for the dimerisation. A further report [26] describes the formation of stable PrP^C dimers in murine neuroblastoma cells expressing hamster PrP^C. These dimers had properties associated with both PrP^C (proteinase K sensitivity) and PrP^{Sc} (readily formed aggregates). The dimers were also resistant to dissociation under harsh conditions, including boiling in SDS–PAGE sample buffer, suggesting a covalent linkage other than a disulphide bond. It has been speculated [25] that the covalent linkage observed was enzymatically induced by the neuroblastoma cells and that such a process could explain the results obtained upon platelet activation. Formation of a number of disulphide linked and transglutaminase-catalysed protein assemblies have been described following platelet activation [27–29], and it is possible that the PrP^C is involved either directly or indirectly in these events. The mechanism by which platelet activation induces the formation of the disulphide linked high molecular weight PrP^C complex and what appear to be non-disulphide covalently linked PrP^C dimers merits further investigation. However, for the purpose of this study, activation was not a suitable method to release PrP^C in its monomeric native conformation from platelets for subsequent purification. Therefore, alternative approaches were investigated.

Platelet PrP^C extraction by platelet lysis in Triton X-100

Of a number of detergents evaluated for their ability to solubilise PrP^C from hamster brain [9], β-octylglucopyranoside and Zwittergent 3–12 resulted in the highest recovery of soluble PrP^C and have been used to purify PrP^C from hamster brain [9,10,30]. However, the use of these detergents is expensive and we therefore chose to use Triton X-100, a much cheaper alternative. Triton X-100 had been reported to solubilise 60% of the available PrP^C from hamster brain [9] and had been used in the purification of a number of platelet proteins [31,32]. PrP^C in the platelet lysate was detected on a slot-blot under both native and denaturing conditions (Fig. 1A), suggesting that the native conformation had been retained. Upon Western blotting, PrP^C was detected mainly as a monomer, running between 30 and 35 kDa, under both non-reducing (Fig. 1B, lane 3) and reducing conditions (Fig. 1B, lane 4) although very faint bands corresponding in size to potential PrP^C dimers were also detected. From these results it appears that PrP^C in resting platelets is in a monomeric state and that the changes in size distribution observed following platelet activation must be a direct result of the activation process. For the purpose of PrP^C purification, Triton X-100 lysis appeared to be a suitable method for the extraction of PrP^C in its native monomeric state from quiescent human platelets.

Platelet PrP^C purification by SP-Sepharose cation exchange chromatography

Cation exchange has been used in a number of published methods describing the purification of both native [9,10,29] and recombinant PrP^C [33]. All of these published methods have used sodium chloride as the source of the Na^+ counter ions required for the cation exchange. However, a recent report [34] has shown that common salts such as sodium chloride, even at low concentrations, could destabilise recombinant human PrP resulting in its irreversible conversion to the oligomeric β-sheet rich structure. Aperti and Surewicz [35] reported that other salts including sodium fluoride, sodium sulphate, and sodium acetate at low concentrations also decreased the stability of recombinant human PrP. However, unlike sodium chloride, the effect of these salts was biphasic and as the salt concentration increased above 50 mM the destabilising effect was gradually reversed. We therefore decided to include sodium acetate instead of sodium chloride as the source of Na^+ counter ions in the platelet PrP^C cation exchange fractionation and sodium chloride was also eliminated from all buffers used during the extraction and purification.

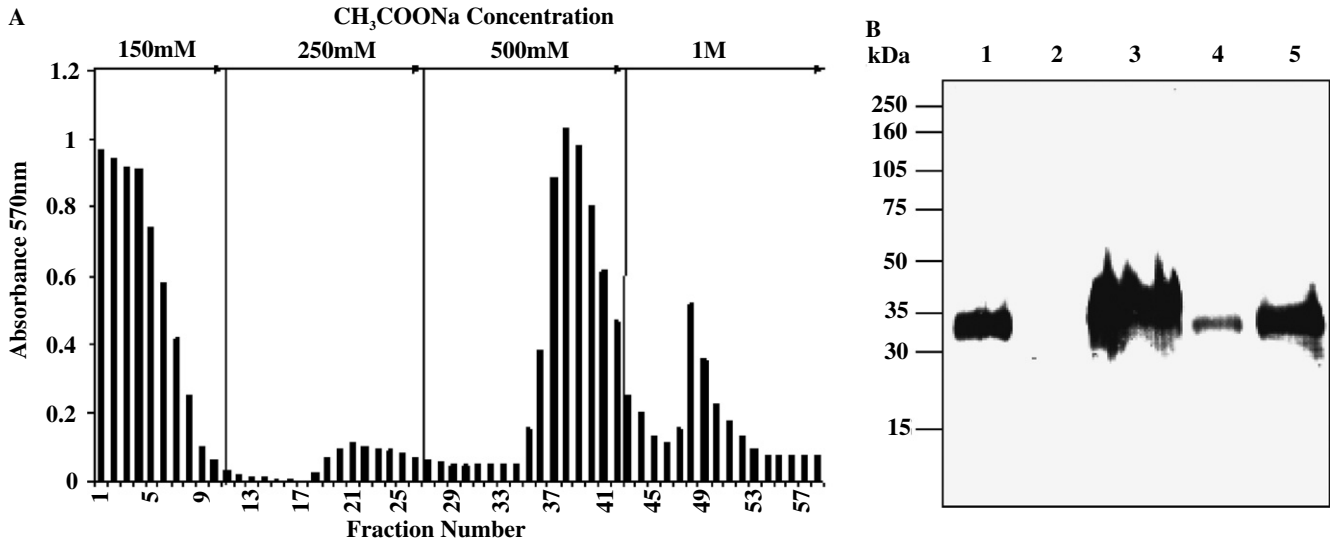

Fig. 2. Purification of PrP^C by SP-Sepharose cation exchange chromatography. (A) Protein elution profile from the SP-Sepharose cation exchange column after loading platelet lysate from 1 L of outdated platelet concentrate as determined by BCA protein assay. Based on the elution profile fractions were pooled as follows: fractions 1–12 (150 mM CH_3COONa eluant) which was added to the column flow-through, fractions 18–29 (250 mM CH_3COONa eluant), fractions 35–45 (500 mM CH_3COONa eluant), and fractions 46–53 (1 M CH_3COONa eluant). (B) Western blot probed with mAb 3F4, of pooled fractions obtained from the SP-Sepharose cation exchange column, following separation on an excel gel SDS gradient 8–18 precast gel under non-reducing conditions. Lane 1, SP-Sepharose XL column flow-through; lane 2, SP-Sepharose 250 mM CH_3COONa eluant, lane 3, SP-Sepharose 500 mM CH_3COONa eluant; lane 4, SP-Sepharose 1 M CH_3COONa eluant; and lane 5, platelet lysate.

Lysate (200 ml) from 1 L of outdated platelet concentrate was fractionated on a SP-Sepharose XL column. Based on the protein elution profile (Fig. 2A), fractions containing protein from each elution step were pooled. Western blot analysis (Fig. 2B) showed that some PrP^C failed to bind to the column eluting in the flow-through. The PrP^C detected in the column flow-through was not due to overloading of the column. When the column flow-through was loaded onto a fresh SP-Sepharose XL column, the PrP^C present in this fraction also failed to bind to the column (results not shown). The majority of the bound PrP^C was eluted from the column in 500 mM CH_3COONa and trace amounts were eluted in 1 M CH_3COONa (Fig. 2B).

Based on the SP-Sepharose XL fractionation, the PrP^C in the platelet lysate could be separated into two distinct types (Type I and Type II). Type I bound strongly to the column eluting in 500 mM CH_3COONa, whereas Type II failed to bind under the loading conditions used (150 mM CH_3COONa, pH 6.0). A similar observation had previously been reported when using cation exchange to purify PrP^C from hamster brain [30]. In this case, the PrP^C could be separated into two distinct species, which had different mobilities on SDS–PAGE gels, with the lower molecular weight species eluting from the cation exchange column at a lower molarity of NaCl compared to the higher molecular weight species. These differences were attributed to either N-terminal cleavage or molecules with different post-translational modifications. In addition, human platelets contain at least two PrP^C isoforms, full-length PrP^C and an N-terminal truncated $PrP_{27–30}$ form [20]. This observation could explain the results obtained upon the SP-Sepharose XL fractionation of platelet PrP^C, with the full-length PrP^C binding to the column and the $PrP_{27–30}$ form failing to bind under the loading conditions used. If this is the case then we might expect to see a size difference between the PrP^C detected in the SP-Sepharose flow-through and in the 500 mM CH_3COONa eluant. While no discernible size differences were observed on the Western blot shown in Fig. 2B, such size differences might only be evident following deglycosylation.

Platelet PrP^C purification by Cu^{2+} affinity chromatography

The binding of PrP^C to Cu^{2+} loaded resins has been documented [36] and previously used in the purification of PrP^C from hamster brain [9,10]. It was therefore chosen for evaluation in the purification of the platelet PrP^C. Attempts to load either the platelet lysate or SP-Sepharose flow-through directly onto the Cu^{2+} affinity column failed when the columns became clogged. Evidently, there was something in both of these samples which interacted with the Cu^{2+} ions thus blocking the column and therefore Cu^{2+} affinity was not suitable as an initial PrP^C purification step. However, no such problem was observed when the SP-Sepharose 500 mM CH_3COONa eluant was loaded onto the copper column. Based on the protein elution profile (Fig. 3A), fractions containing protein from each

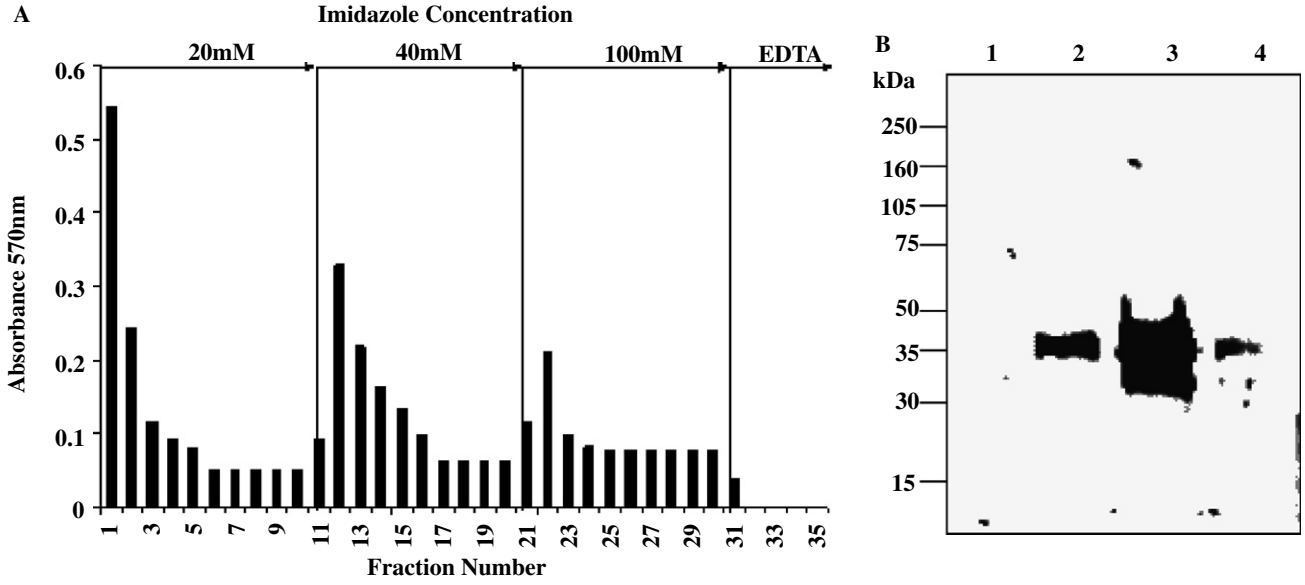

Fig. 3. Purification of PrP^C from the SP-Sepharose 500 mM CH_3COONa eluant by Cu^{2+} affinity chromatography. (A) Protein elution profile from the Cu^{2+} affinity column, after loading the SP-Sepharose 500 mM CH_3COONa eluant, as determined by BCA protein assay. Based on the elution profile fractions were pooled as follows: fractions 1–5 (20 mM imidazole eluant) and added to the column flow-through, fractions 11–16 (40 mM imidazole eluant), fractions 21–24 (100 mM imidazole eluant), and fractions 31–33 (50 mM EDTA eluant). (B) Western blot probed with mAb 3F4, of pooled fractions obtained from the Cu^{2+} affinity column, following separation on an excel gel SDS gradient 8–18 precast gel under non-reducing conditions. Lane 1, Cu^{2+} affinity column flow-through; lane 2, Cu^{2+} affinity 40 mM imidazole eluant; lane 3, Cu^{2+} affinity 100 mM imidazole eluant; and lane 4, Cu^{2+} affinity 50 mM EDTA eluant.

elution step were pooled. Western blot analysis (Fig. 3B) showed that all the available PrP^C had bound to the column and that while some PrP^C eluted in 40 mM imidazole, the majority eluted in 100 mM imidazole with only trace amounts eluting in 50 mM EDTA.

Estimation of PrP^C yield, recovery, purity, and size distribution

The total protein concentrations in the lysate, SP-Sepharose XL 500 mM CH_3COONa eluant, and Cu^{2+}

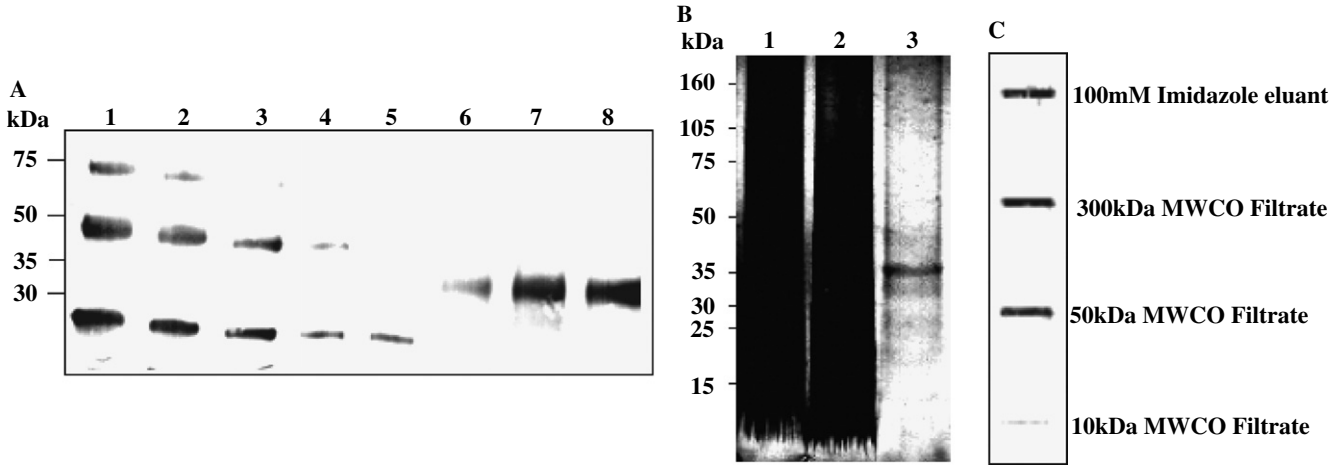

Fig. 4. Amount, purity, and size distribution of PrP^C purified from 1 L of outdated platelet concentrate. (A) Quantitative Western blot to determine the concentration of PrP^C in the platelet lysate, SP-Sepharose 500 mM CH_3COONa eluant and Cu^{2+} affinity 100 mM imidazole eluant. Lanes 1–5, twofold serial dilutions (10 μl per lane) of recombinant human PrP in the range 10–0.625 μg/ml prepared in 1× non-reducing SDS–PAGE sample buffer; lane 6, platelet lysate diluted 1:1 in 2× non-reducing SDS–PAGE sample buffer (20 μl); lane 7, SP-Sepharose XL 500 mM CH_3COONa eluant diluted 1:1 in 2× non-reducing SDS–PAGE sample buffer (10 μl); and lane 8, Cu^{2+} affinity 100 mM imidazole eluant diluted 1:10 in 1× SDS–PAGE sample buffer (10 μl). (B) Silver-stained SDS–PAGE confirming the purity of the PrP^C in the Cu^{2+} affinity 100 mM imidazole eluant. Lane 1, crude platelet lysate; lane 2, SP-Sepharose XL 500 mM CH_3COONa eluant; and lane 3, Cu^{2+} affinity 100 mM imidazole eluant. (C) Slot-blot to determine the size distribution of PrP^C in the Cu^{2+} affinity 100 mM imidazole eluant. Aliquots of the sample were passed through Amicon Centricon centrifugal filter devices (Millipore) with molecular weight cut-offs of 10, 50, and 300 kDa, respectively, 10 μl aliquots of each filtrate were slot-blotted onto nitrocellulose and probed with mAb 3F4.

Table 1
Yield, purity, and recovery of PrP^C from 1 L of outdated platelet concentrate

Purification step	Total protein[c] (mg)	Total PrP^C[d] (μg)	Recovery of PrP^C (%)	Purity (%)
Platelet lysate	720	250	100	0.03
SP-Sepharose XL[a]	10.5	125	50	1.19
Cu^{2+} affinity[b]	0.114	100	40	87.72

[a] SP-Sepharose XL, SP-Sepharose 500 mM CH_3COONa eluant.
[b] Cu^{2+} affinity, Cu^{2+} affinity 100 mM imidazole eluant.
[c] Estimated by BCA protein assay against BSA standards prepared in the relevant buffers.
[d] Estimated by quantitative Western blotting against recombinant human PrP standards.

affinity 100 mM imidazole eluant were determined by BCA protein assay. The total PrP^C concentration in each of the samples was estimated from a quantitative Western blot (Fig. 4A). These results are summarised in Table 1. The PrP^C concentration in the Cu^{2+} affinity 100 mM imidazole eluant was estimated at 25 μg/ml (100 μg total PrP^C) at a purity of 88% and a recovery of 40%. The purity was confirmed on a silver-stained SDS–PAGE gel (Fig. 4B).

Aliquots of the Cu^{2+} affinity 100 mM imidazole eluant were passed through Amicon Centricon centrifugal filter devices (Millipore) with molecular weight cut-offs of 10, 50, and 300 kDa, respectively. The resulting filtrates were slot-blotted onto nitrocellulose and probed with mAb 3F4. From the result obtained (Fig. 4C) it was evident that the majority of PrP^C passed through both the 300 and 50 kDa MWCO filters but was retained by the 10 kDa MWCO filter, suggesting that the majority of the PrP^C in the sample was monomeric.

Large-scale PrP^C purification

The purification process was scaled up to purify PrP^C from 10 L of outdated platelet concentrate. However, outdated platelet concentrates were not always available and attempts to store either frozen platelet pellets prior to lysis or frozen lysates resulted in a significant loss of PrP^C compared to fresh lysates (results not shown). No significant loss of PrP^C was observed when the SP-Sepharose 500 mM CH_3COONa eluant was stored frozen. Therefore, lysis and SP-Sepharose fractionation were carried out on individual 1 L lots of platelet concentrates, the 500 mM CH_3COONa eluants stored frozen and pooled prior to loading onto the Cu^{2+} affinity column. The volumes loaded onto the Cu^{2+} affinity column were restricted to avoid overloading the column. A final 10-fold concentration step using an Amicon Ultra 10,000 MWCO centrifugal filter device was included into the purification method. Some protein precipitated out of solution during the concentration step and this was removed by centrifuging at 14,000*g* for 10 min. The majority of the PrP^C was retained in the supernatant (Fig. 5, lane 1) with only trace amounts detected in the pellet (Fig. 5, lane 2). From 10 L of outdated platelet concentrate, we were able to purify 1.25 mg PrP^C at a purity of 92% in a 1 ml final volume, which was confirmed by silver-stained SDS–PAGE (Fig. 5, lane 3).

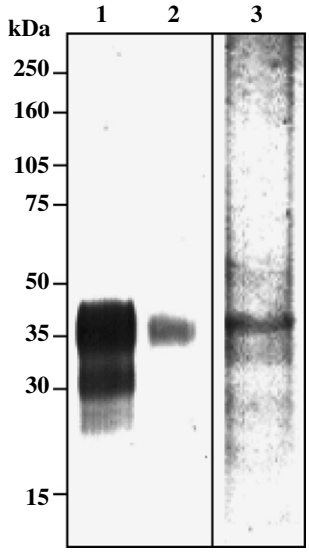

Fig. 5. Western blot and silver-stained SDS–PAGE of PrP^C purified from 10 L of outdated platelet concentrate post 10-fold concentration. Western blot probed with mAb 3F4 of supernatant (lane 1) and resuspended pellet (lane 2) obtained post 10-fold concentration and subsequent centrifugation to remove precipitated protein. Lane 3, silver-stained SDS–PAGE gel of the final purified PrP^C obtained from 10 L of outdated platelet concentrate.

Summary of results

We have developed a strategy for the extraction and purification of PrP^C from human platelets. This involves platelet lysis in Triton X-100 followed by sequential cation exchange and Cu^{2+} affinity chromatography. As far as we are aware this is the first reported method for the purification of endogenous human PrP^C and from a source other than brain tissue.

Furthermore, upon calcium ionophore platelet activation, PrP^C was shown to form a high molecular weight disulphide linked complex consisting of what ap-

pear to be non-disulphide covalently linked PrP^C dimers or PrP^C covalently linked to an other as yet unidentified protein. This phenomenon appears to be unique to platelet activation, since these complexes were not detected in resting platelets, and merits further investigation.

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