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# Aqueous two-phase partition of complex protein feedstocks derived from brain tissue homogenates

Simon G. Walker*, Christopher J. Dale, Andrew Lyddiatt

*Biochemical Recovery Group, BBSRC Centre for Biochemical Engineering, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*

**Abstract**

This study describes the application of aqueous two-phase partition using polyethylene glycol (PEG)–potassium phosphate systems for the direct recovery of proteins, and aggregates thereof, from mammalian brain tissue homogenates. Investigation of established methodologies for the purification of prion proteins (PrP) from bovine brain affected with transmissible spongiform encephalopathy (BSE) has identified an alternative purification regime based on aqueous two-phase partition. This circumvents energy-intensive and rate-limiting unit operations of ultracentrifugation conventionally used for isolation of PrP. Selectivity of various PEG–phosphate systems varied inversely with polymer molecular mass. The maximum protein recovery from bovine brain extracts was obtained with systems containing PEG 300. Manipulation of the aqueous environment, to back-extract protein product from the PEG-rich top phase into the phosphate-rich lower phase, enabled integration of ATPS with conventional hydrophobic interaction chromatography (HIC) which selectively removes obdurate contaminating proteins (i.e. ferritin).

*Keywords:* Aqueous two-phase systems; Partitioning; Proteins

## 1. Introduction

A characteristic of all scrapie-like diseases (transmissible spongiform encephalopathies, TSE) is the presence in detergent extracts from brain tissue of fibril structures comprising aggregates of an abnormal isoform of a membrane glycoprotein (PrP). It is unclear whether these aggregates, termed scrapie-associated fibrils (SAF), form during detergent extraction of brain tissue [1] or are present as pathological structures in vivo [2]. However, they are of considerable importance, since SAF and TSE infectivity co-purify [3], and are widely employed for mechanistic and diagnostic investigations of the diseased state.

The conventional procedure for the isolation of SAF from TSE-affected tissue comprises a series of centrifugation stages for the clarification of tissue homogenate and differential ultracentrifugation of protein fractions [1,3,4]. Preliminary studies at the University of Birmingham and the Central Veterinary Laboratory suggested that the concentration of PrP in bovine brain tissue is low (less than 2 mg/kg) and necessitated the processing of large amounts of tissue to obtain sufficient yield of SAF. This exposed the chief limitations of ultracentrifugation methodologies where severe constraints upon product throughput

*Corresponding author.

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arise as a result of low rotor capacities coupled with extended time scales and high-energy requirements.

Aqueous two-phase systems (ATPS) are suited to a number of solid–liquid handling problems (e.g. virus-like particles [5], membrane synaptosomes [6,7] and inclusion body processing). The major benefits conferred are the combination of a low-intensity clarification stage with the selective fractionation of soluble proteins and molecular aggregates from cell debris in processes suited to increases in scale and throughput. Furthermore, the biocompatible environment of ATPS facilitates the preservation of biological activity of protein extracts [8–10]. This confirms that ATPS could provide an efficient alternative methodology for the large-scale isolation of TSE-infective fractions. The results presented herein constitute part of an ongoing study of the definition and exploitation of a generic role for ATPS in the efficient processing and recovery of proteins, molecular aggregates and particulates from tissue homogenates.

## 2. Experimental

### *2.1. Processing of brain stem tissue*

Histopathologically diagnosed BSE positive and negative bovine brain stem tissue was supplied by the Veterinary Investigation Service, in conjunction with the Central Veterinary Laboratory (courtesy of R. Jackman, Weybridge) and stored at −40°C. Brain stem was selected for the preparation of SAF since preliminary studies demonstrated that this tissue yielded the greatest concentration of PrP [11].

The SAF preparation procedure was adapted from published reports [1,3,4,12]. Homogenates of brain stem tissue (33% w/v) were prepared by extraction in 10 m*M* sodium phosphate, pH 7.4 containing 10% w/v N-lauryl sarcosine, 0.08 m*M* phenylmethyl sulphonyl fluoride (PMSF) and 0.08 m*M* N-ethylmaleimide (NEM) using a Polytron PT 1000 homogeniser. The homogenate was clarified by centrifugation (20 000 *g* at 10°C for 50 min), the pellet discarded and the supernatant subjected to ultracentrifugation (177 000 *g* at 10°C for 150 min). The supernatant was discarded and the pellet (P1) resuspended in 20 m*M* Tris-HCl, pH 7.4 and agitated at 37°C for 30 min. The resuspended pellet was incubated in potassium iodide buffer (137 m*M* potassium iodide containing 17.5 m*M* sodium thiosulphate and 1% w/v N-lauryl sarcosine) at 37°C for 60 min. Samples were then overlaid onto sucrose cushions (20% w/v sucrose) and centrifuged at 189 000 *g*, 10°C, for 90 min. The supernatant was discarded and the pellet (containing SAF) was retained at −40°C.

### *2.2. Aqueous two-phase systems*

The phase-forming behaviour of PEG–phosphate systems was investigated using a cloud point method [13] to define the binodal curve and identify suitable systems for study. The feedstock for ATPS studies comprised bovine brain stem homogenate as described above and elsewhere [12,14]. ATPS were formed directly in brain homogenate through the addition of solid polyethylene glycol (PEG, nominal $M_r$ 300, 600, 1450, 3350, and 8000; Sigma, St. Louis, MO, USA) and dipotassium hydrogen orthophosphate ($K_2HPO_4$; Sigma) to yield system compositions of 27% PEG–14% phosphate (w/w) at pH 9.0. Mixtures of this composition had similar tie-line lengths (TLL 49–51% w/w [15]) and volume ratios (0.95–1.2). After vigorous mixing, phase definition was enhanced by a brief low-speed centrifugation step (2000 *g*, 5 min at 10°C in a Beckman JA-17 rotor).

### *2.3. Hydrophobic interaction chromatography (HIC)*

Material recovered from ATPS was applied in phosphate-rich bottom phases containing 1.0 *M* ammonium sulphate to a pre-packed 1 ml Phenyl Sepharose HiTrap column (Pharmacia Biotechnology, Uppsala, Sweden). The column was loaded and eluted at 0.2 ml/min with a stepwise linear gradient (1.0 *M* ammonium sulphate to deionised water in a total volume of 20 ml). The 280 nm absorbance of the eluate was monitored using a VWM 2141 UV detector (Pharmacia Biotechnology).

### *2.4. Protein assay*

The protein content of isolated-phase samples was estimated using a bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA) and the results expressed

relative to a calibration plot derived from the assay of standard concentrations of BSA.

### *2.5. SDS-PAGE analysis of TCA precipitated fractions*

Proteins from each phase were precipitated with 20% v/v TCA and analysed by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) [16]. Samples were resuspended in denaturing buffer containing 2% SDS and 5% $\beta$-mercaptoethanol in 62.5 m*M* Tris-HCl, pH 6.8 and heated at 100°C for 2 min prior to electrophoresis in 12% T–2.65% C polyacrylamide gels arranged in a vertical apparatus (Bio-Rad Laboratories). Proteins were transferred from gels onto PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA) using a wet electro-blotting method described by Towbin et al. [17]. Protein bands were visualised on blots with Coomassie Brilliant Blue R-250 and estimates of the relative molecular mass of the protein subunits in SAF preparations were derived from the electrophoretic mobility of protein standards (BSA $M_r$ 67 kDa, ovalbumin $M_r$ 44 kDa, carbonic anhydrase $M_r$ 31 kDa, soybean trypsin inhibitor $M_r$ 21 kDa, lysozyme $M_r$ 14 kDa).

Quantitation of Coomassie Blue stained protein bands in SDS-PAGE gels was achieved by scanning laser densitometry using an Ultrascan XL (Pharmacia LKB, Uppsala, Sweden). Results were expressed relative to a BSA standard curve (0–2.5 $\mu$g total loading). Immunochemical detection of PrP on PVDF membranes exploited polyclonal antisera (SP40) raised against a synthetic peptide corresponding to amino acid residues 219–233 of the human sequence of PrP, supplied by Central Veterinary Laboratories, Weybridge, courtesy of R. Jackman.

### *2.6. Amino acid analysis and sequencing of bovine brain proteins*

Discrete protein components were excised from Coomassie Blue stained PVDF membranes and were hydrolysed in 6 *M* HCl at 110°C for 24 h. The amino acid digest was lyophilised, resuspended in 0.2 *M* sodium citrate and aliquots fractionated on an Aminex A8 ion-exchange column under elution conditions comprising a series of sodium citrate buffers of increasing pH and ionic strength. Amino acids in the eluate were detected by post-column reaction with ninhydrin and absorption at 440 nm and 570 nm. Discrete zones of Coomassie Blue stained PVDF blots were also subjected to N-terminal amino acid sequencing in an automated Applied Bioscience gas-phase instrument.

## 3. Results and discussion

The centrifugation methodology for the isolation of abnormal PrP in bovine brain affected with TSE restricts the process throughput. The initial clarification of crude brain homogenate in a preparative ultracentrifuge (see Section 2) is limited to batch sizes of 3.2 l homogenate or 160 g tissue. Limitations were further compounded by a ten-fold reduction in batch volume due to the constraints of rotor capacity available to the high-speed ultracentrifugation stages employed for the selective purification and isolation of SAF. Circumvention of either of these stages would increase productivity. However, the complexity of the protein composition of ultracentrifugation derived pellets and the starting titre of PrP require that alternative strategies possess comparative or higher degrees of selectivity.

### *3.1. Screening of ATPS for the primary processing of brain homogenate*

The extraction of soluble protein from bovine brain homogenate by aqueous two-phase partition was investigated in trials using a series of PEG–phosphate systems comprising PEG of nominal molecular mass 8000, 3350, 1450, 600 and 300. The highest recovery of brain protein in PEG-rich top phases was obtained with PEG 300 and the protein composition and distribution compared favourably in SDS-PAGE analyses with fractions obtained from conventional first high-speed ultracentrifugation stages (P1, see Section 2 and see Fig. 1). The phosphate-rich bottom phase was shown, by BCA assay, to contain only low concentrations of protein (<0.5 mg/ml) which was not readily visualised in Coomassie Blue stained SDS-PAGE analyses. In all systems studied, material comprising protein and cellular debris precipitated at the interphase and this effect was particularly evident with ATPS containing

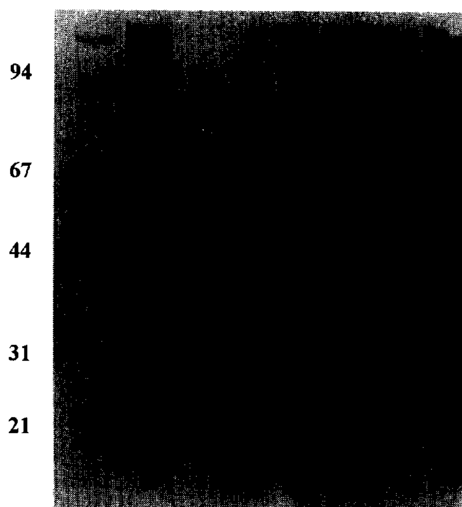


Fig. 1. Effect of changing the molecular mass of PEG on the partition characteristics of crude brain homogenate in two-phase systems. Lane 2 contains centrifugation-prepared material (P1) as a control. All ATPS had composition 27–14% (w/w). Aliquots of 1 ml of isolated phase was TCA-precipitated and disrupted (see Section 2). All lanes were loaded with 25 $\mu$l of sample. The gel was Coomassie Blue stained and Bio-Rad pre-stained molecular mass standards correspond to BSA 67 kDa, ovalbumin 44 kDa, carbonic anhydrase 31 kDa, trypsin inhibitor 21 kDa and lysozyme 14 kDa.

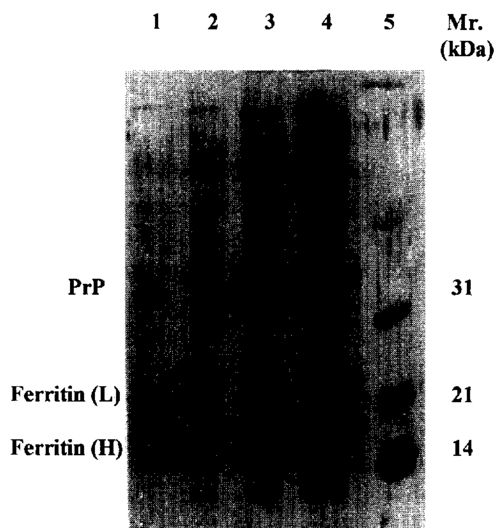


Fig. 2. An immunochemical comparison of PrP isolation and purification from bovine brain homogenate achieved by aqueous two-phase or ultracentrifugation approaches. Lanes 1 and 2 were loaded with centrifuge controls (10 $\mu$l and 25 $\mu$l), lanes 3 and 4 contain equivalent loads of isolated top phase (i.e. 10 $\mu$l and 25 $\mu$l). Samples were prepared as described in Section 2. Lane 5 contains molecular mass standards as described in Fig. 1.

high molecular mass polymer (PEG>1450). However, given the low $g$-forces applied to maximise phase resolution (e.g. 2000 $g$), this approach represents an efficient and simple solution to the problems of solid–liquid separation at this stage.

The principal protein impurities extracted with detectable PrP immunoreactivity in the top-phase fractions ($M_r$ 22–23 and $M_r$ 24–26 kDa) were characterised by amino acid sequence analysis and selective immunorecognition. They were identified as the H and L sub-units of the iron storage protein ferritin. It is interesting to note that ferritin displays similar characteristics of aggregation and centrifugal sedimentation to the PrP molecules associated with SAF [14]. Earlier work reported by Dale et al. [14] also identified other contaminants which co-purified with PrP, as tubulin, creatine kinase and glyceraldehyde-3-phosphate dehydrogenase. Identification of the ferritin, PrP and other protein bands on SDS-PAGE gels and immunoblots of isolated top-phase samples (Fig. 2) demonstrated a comparable degree of molecular selectivity in ATPS to that seen in centrifugation procedures. Coupled with the success of the solid–liquid separation, this confirms ATPS as a direct route for the primary isolation and partial purification of both soluble and particulate proteins from crude brain homogenates. Similar molecular characterisation of contaminant proteins was used in subsequent stages as a measure of selectivity for the partitioning of crude homogenates in ATPS.

### 3.2. Recovery of soluble protein by back-extraction and integration of process streams into conventional protein purification regimes

Proteins extracted into the PEG-300-rich top phase were stripped into a phosphate-rich bottom phase by addition of potassium phosphate to yield a new ATPS of composition 14% PEG–24% phosphate (w/w) pH 6.5 supplemented with 0.5 $M$ NaCl. No conditions employed for back-extraction from a

PEG-300-rich top phase exhibited partition coefficients strongly favouring partition of total protein to the bottom phase. Repeated cycles of back-extraction increased product yield, but also increased process volumes and processing times (greater than two-fold). An alternative approach to product recovery from PEG 300 is the exclusion of protein from the top phase by addition of PEG 8000 to raise the average molecular mass of PEG to about 4000. This increased the total protein recovery figure from 18 to 27% (i.e. greater than that for centrifugation, 19%).

The protein stripped from the top phase of the initial ATPS extraction of brain homogenate was directly applied in bottom phase containing 1.0 *M* ammonium sulphate to a Phenyl Sepharose HIC column (Fig. 3). The elution profile was dominated by a single peak of UV absorbance which was shown by SDS-PAGE analysis to comprise a complex array of protein components ($M_r$ range 10–100 kDa). Characterisation of the principal protein constituents, visualised by Coomassie Blue staining confirmed the presence of H and L sub-units of ferritin (Fig. 4).

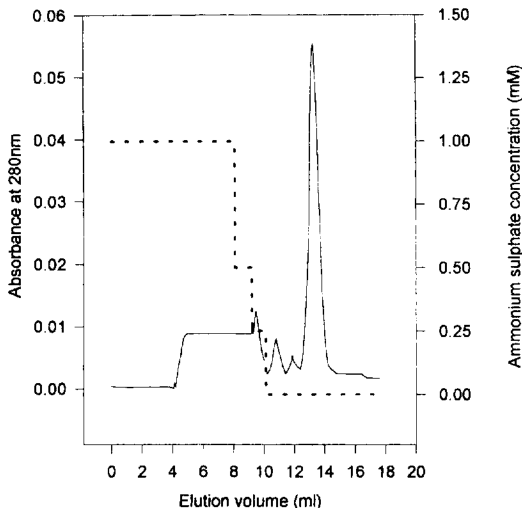


Fig. 3. Elution profile from a Phenyl Sepharose HIC column of a sample of back-extracted lower phase. Product stripped to the phosphate-rich phase of a PEG 300–phosphate (14/24%, w/w) ATPS was applied in 1 *M* ammonium sulphate to a 1-ml pre-packed Phenyl Sepharose (low sub) HiTrap column. Loading and elution was at a flow-rate of 0.2 ml/min. Elution was achieved by a stepwise gradient of ammonium sulphate (1 *M* to 0 *M*) (dashed line). The eluate was continuously monitored for UV absorbance at 280 nm (solid line) and subsequently TCA-treated before electrophoretic analysis of precipitates (see Fig. 4).

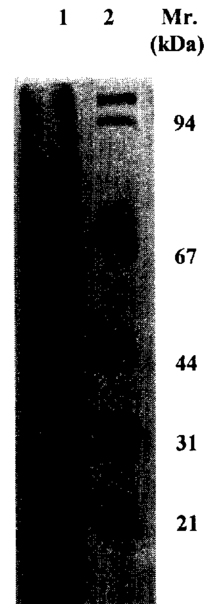


Fig. 4. SDS-PAGE analysis of a back-extracted bottom-phase sample eluted from a Phenyl Sepharose HIC column. Lane 1 contains eluate from the Phenyl Sepharose HIC column (1.2 mg/ml total protein). A 1-ml fraction was TCA-precipitated (20% v/v) then disrupted in SDS disruption mix (see Section 2). Lane 2 contains molecular mass marker standards as described in Fig. 1.

However, PrP was not similarly detected in back-extracted, bottom-phase material.

Ferritin has been shown to co-purify with PrP in conventional centrifugation-based methodologies and shares many molecular characteristics with PrP. This severely disadvantages conventional options for PrP purification from low-titre tissues (less than 2 mg/kg) [14]. Both ferritin sub-units critically cross-react with the best polyclonal antisera available for the detection of PrP and share the molecular mass range of PrP homologues in SDS-PAGE. Furthermore, both ferritin and PrP aggregates exhibit significant resistance to digestion by proteolytic enzymes [14] widely used for eliminating protein impurities from PrP preparations [1].

The selective removal of ferritin in back-extracted fractions (see above) prompted study of the partition behaviour of commercial ferritin in ATPS. Partition coefficients (*K*) were determined in selected PEG–phosphate systems at pH 9, whose initial composition (tie-line length and volume ratio) was identical to that in systems used for brain tissue. The be-

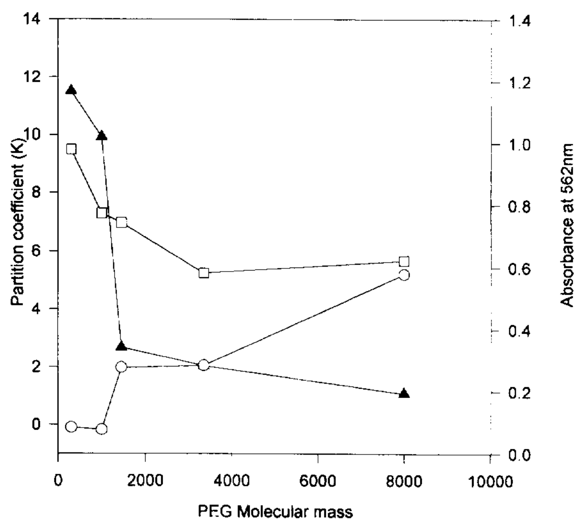


Fig. 5. Partition behaviour of commercial ferritin in model primary extraction systems. Solute concentrations were analysed spectrophotometrically using the BCA assay. Concentration of ferritin in the top phase ($C_t$) is represented by (□) and in the bottom phase ($C_b$) by (○). The symbol (▲) denotes the partition coefficient ($K$), where $K = C_t/C_b$. A stock ferritin solution of 1 mg/ml was used in 5 g ATPS (27% PEG–14% phosphate).

haviour of ferritin was consistent with the total protein recovery in the process systems (Fig. 5). The values of partition coefficients for ferritin in systems containing PEG in the molecular mass range 300–8000 varied between 12 and 2 (i.e. all favouring the top phase). The decreasing yield of protein with increasing PEG nominal molecular mass may be explained in part by hydrophobic exclusion phenomena [15]. The sequestration of ferritin by the application of the back-extraction condition may thus provide a valuable means of isolating the target PrP protein from the principal co-purifying contaminants.

## 4. Conclusions

A role for aqueous two-phase partition has been identified in the processing of crude bovine brain homogenate. This bypasses energy-intensive and rate-limiting unit operations of ultracentrifugation conventionally used for clarification of cell debris and differential fractionation of PrP-containing protein fractions. Back-extraction of protein from isolated top-phase samples provides an effective means for removal of the major co-purifying contaminants (H and L ferritin) from PrP. The successful application of this technique to the processing of bovine brain homogenate suggests that ATPS may provide a generic solution to difficult solid–liquid separations in suspension processing.

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