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Generic application of an aqueous two-phase process for protein recovery from animal blood

Marco Rito-Palomares [a,*], Christopher Dale [b], Andrew Lyddiatt [b]

[a] *Centro de Biotecnología, Instituto Tecnológico y de Estudios Superiores de Monterrey (I.T.E.S.M.), Campus Monterrey. Sucursal de Correos J, Monterrey, NL 64849, Mexico*

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

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Abstract

The recovery of proteins from animal blood using an established two-stage extraction process was selected as a practical model system to study the generic application of polyethylene glycol (PEG)-phosphate aqueous two-phase systems (ATPS). Processing of whole bovine blood in the ATPS two-stage process resulted in the partition of soluble protein (e.g. bovine serum albumin (BSA), haemoglobin, IgG; partition coefficient $K = 55$) into a PEG-rich top phase and cell debris into a phosphate-rich bottom phase. Subsequent back extraction of soluble protein into a second phosphate-rich bottom phase resulted in a maximum overall protein recovery of 62%. The increased protein concentration within the ATPS (from 1.2 to 7.0 mg/g) caused a decreased in the recovery to 44%. Recycling of PEG into the initial extraction stage did not significantly influence the partition behaviour of protein over the equivalent of 20 operational cycles, but protein recovery decreased from 44 to 37%. The extreme conditions (waste material characterised by the presence of solids and impurities) in which the implementation of this ATPS process was tested, confirms the potential for the generic application of ATPS for processing complex biological suspensions to achieve a simple primary recovery and partial purification of target protein solutes.

Keywords: Aqueous two-phase systems; Protein recovery; Bovine blood; Polymer recycling

1. Introduction

Aqueous two-phase systems (ATPS) comprising mixtures of poly-(ethylene glycol) (PEG) and potassium phosphate has been widely used for the recovery of macromolecules from fermentation broth and biological extracts. Previous work [1,2] has demonstrated aqueous two-phase partition as an alternative to conventional process for concomitant particle (debris) and solute (macromolecule) handling. However, adoption and commercial application of ATPS processes necessarily requires a practical approach to process design and economic re-use of phase components in subsequent operational cycles.

The practical implementation of ATPS process for the recovery of products will most simply involve the development of robust two-stage processes. In a typical two-stage ATPS process, the first extraction yields a bottom phase containing cell debris and contaminants and a top phase containing the product of interest (protein). In the second stage (back extraction), the protein is concentrated in the bottom phase and further processing of that phase by ultrafiltration yields a protein product concentrate [3]. In the context of process design, the limited understanding of the molecular behaviour of proteins in ATPS severely limits the predictive design of extraction process using this technique. However, a practical approach can be adopted which exploits the known effect of operating parameters such as PEG and phosphate concentration and volume ratio of the phases upon protein partition.

The generic application of two-stage ATPS to a variety of product solids requires systems that remain

* Corresponding author. Tel.: +52-8-3284132; fax: +52-8-3284136.

E-mail address: mrito@campus.mty.itesm.mx (M. Rito-Palomares)

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stable in performance in the face of varied feedstocks. In order to evaluate the generic application of this technology as a primary purification step, a two-stage ATPS process was previously established for the recovery of protein from yeast [4]. For this second study a different system was selected for investigation. It was decided that to be representative of the problems of bioprocessing, this new waste material would be characterised by a high solids concentration (cells or cell debris) and a heterogeneous mixture of proteins, other macromolecules and pigments. On that basis, raw bovine blood was chosen as a representative material typical of abattoir waste produced from the meat industry. Real abattoir waste is potentially very hazardous in terms of pathogen contamination and other aspects. Diluted bovine blood (a key component of such waste) was judged to be the closest and safest raw material suited to extensive laboratory studies characteristic of this work. Animal blood (and related material) is characterised by a wide range of potential protein products such as inmunoglobulin G (IgG), haemoglobin, bovine serum albumin (BSA) and clotting factors [5,6] which all have potential added-value in the purified state. In addition to the pigment content in the bovine blood, the presence of solids (i.e. blood cells) made this feedstock particularly appropriate for the implementation study as a test of the applicability of ATPS.

There has been comparatively little work undertaken using aqueous two-phase systems for the extraction of blood proteins from human or animal origin (but see Refs. [5,6]). This may be attributed to the fact that many of the established methods for the preparation of blood products were developed in the period 1950–1970 and have been exhaustively refined using older technologies. Regulatory considerations have promoted a reluctance to embrace a totally new technology in this and other fields of biochemical engineering and biotechnology. For example, the traditional method of IgG isolation involves operations of precipitation, centrifugation, dialysis and microfiltration. Despite the fact that improved procedures have reduced the number of extraction steps [7], unit operations for the removal of cells and cell debris remain mandatory. In this context, the implementation of a two-stage ATPS process study might be expected to enhance the application of this technique as an alternative and combinatorial primary step for the production of blood products.

In the present study, a two-stage ATPS process was used to process bovine blood suspensions. The effect of varied operating conditions on ATPS performance was evaluated by monitoring the partition coefficient of bulk protein and protein blood products (IgG, haemoglobin and bovine serum albumin). Further advanced implementation of an ATPS process characterised by partial polymer recycling was examined using a previously developed spray column extraction system [4]. These results were then used to assess the overall operational stability of the process in the face of this new challenge with a complex feedstock.

2. Materials and methods

2.1. Identification of protein products in bovine blood

Samples of bovine blood suspension (Advanced Protein Products, UK) were used for protein quantitation and SDS-PAGE (12% T/2.65% C) analysis (as described by Laemmli [8]). After SDS-PAGE analysis, the Coomassie blue positive bands that exhibited the highest colour intensity were blotted, excised from the membrane and used for N-terminal amino acid sequencing in situ. The N-terminal sequencing was conducted by the commercial facilities of Alta Bioscience (School of Biochemistry, University of Birmingham) using a fully automated, pulse liquid protein sequencer (Applied Biosystems Incorporated).

2.2. The effect of protein concentration on ATPS process performance

The bovine blood was processed using the operating conditions previously established [4] for a two-stage ATPS process (i.e. first extraction: PEG 27% w/w, phosphate 14% w/w, pH 9.0 and back extraction: PEG 10% w/w, phosphate 14% w/w, pH 6.5). The variable studied in these experiments was the concentration of total protein present in the feedstock comprising bovine blood suspension. The first extraction was assembled using PEG molecular mass of 1000 daltons (PEG 1000, Sigma), phosphate (K_2HPO_4, BDH) and bovine blood feedstock characterised by an increasing protein concentration within the ATPS. The PEG and phosphate were dissolved directly in the blood feed by mixing and the phases were separated by centrifugation. Samples were taken from the phases for protein analysis [9] and subsequent estimation of the protein partition coefficient (K or $\ln K$; [4]). The top phases generated from the first extraction ATPS experiments were processed further under the back extraction conditions. The second two-phase systems were formed using fresh phosphate solution and the pH was adjusted to 6.5 using orthophosphoric acid. The protein recovery from each extraction stage (first and back extraction) was estimated as the amount of protein present in the phases (protein concentration $\times$ volume of the phase) expressed relative to the total protein loaded from bovine blood suspension or the top phase from the first extraction, respectively. Subsequently, the top phase product recovery from the first extraction, and that from the bottom phase products from the back extraction, were then used to calculate the overall process recovery.

2.3. Partition of bovine blood products in an ATPS process

The partition experiments of protein products from bovine blood in ATPS were performed at the system protein concentrations established from the previous studies described above. Samples were taken from each phase and the proteins precipitated using trichloroacetic acid (final concentration 20% by volume). The supernatant was discarded (containing PEG and phosphate) and the proteins were resolubilised in 2% SDS and 5% β-mercaptoethanol in 62.5 mM Tris–HCl, pH 6.8 and fractionated in SDS-PAGE slab gels of monomer concentration of 7.5% T/2.65% C [10]. Fractionated samples, were then transferred to polyvinylidene difluoride (PVDF) membranes using the wet blotting method [11] followed by immunoblotting analysis for the presence of bovine IgG.

2.4. Studies of polymer recycling

The impact of polymer recycling upon the partition of protein products from bovine blood in multiple cycle experiments, was assessed using a spray column system (Fig. 1 and Table 1). The design, assembly and operation of this extraction system have been previously discussed [4]. Such ATPS process was continuously operated for the equivalent of 20 operational cycles using PEG, phosphate and bovine blood suspension. Samples were taken from the phases of the first and back extraction stages throughout the experiment to determine the protein concentration and establish the protein partition coefficient. The bottom phase products from the back extraction after a selected number of equivalent cycles (i.e. 1, 4, 8, 12, 16 and 20) were fractionation on 12% T/2.65% C SDS-PAGE slab gels.

3. Results and discussion

3.1. Identification of protein products in bovine blood

The total bulk protein content in the suspensions of bovine blood can be simply estimated using the method of Bradford [9]. However, such complex biological system is characterised by the presence of protein products of different molecular weight together with cells and/or cell debris. Therefore, further characterisation of these

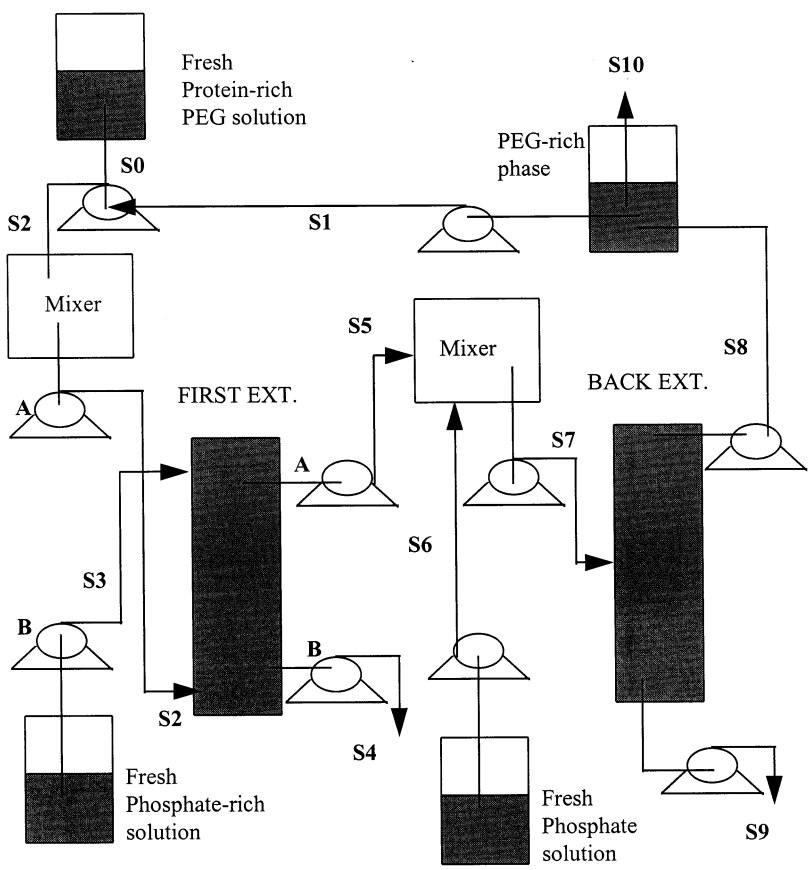

Fig. 1. Process scheme of the ATPS spray column system. The streams of the process were identified by S0–S10, and their flow rates and composition are detailed in Table 1. The dimensions of the spray columns are a total height of 600 mm, an inner diameter of 25 mm and a total volume of 250 ml.

Table 1
Flow rate and stream conditions of the ATPS spray column process

Stream	Flow rate (ml/min)	PEG (% w/w)	Phosphate (% w/w)	System pH
S0 (protein-rich PEG solution)	3.08	52.0	5.60	9.0
S1 (recycled PEG-rich phase)	3.08	32.0	2.20	6.5
S2 (mixture of S0 and S1)	6.12	42.0	3.90	9.0
S3 (fresh phosphate-rich solution)	3.70	1.00	33.0	9.0
S4 (bottom phosphate-rich phase)	3.70	1.00	33.0	9.0
S5 (top PEG-rich phase)	6.12	42.0	3.90	9.0
S6 (fresh phosphate solution)	19.0	0.0	18.0	7.0
S7 (mixture of S5 and S6)	25.0	10.0	14.0	6.5
S8 (top PEG-rich phase)	8.33	32.0	2.20	6.5
S9 (bottom phosphate-rich phase)	16.67	2.80	18.2	6.5
S10 (same as S8)	5.25	32.0	2.20	6.5

products is desirable in order to evaluate their partition in ATPS. The most detailed step in identifying and characterising a protein is the determination of its amino acid sequence. The primary structure identifies a protein unambiguously and ultimately determines all its chemical and biological properties. Since every protein has a unique amino acid sequence, the identification of the most common species can be achieved by using the computer databases of known sequences [12]. Fig. 2 illustrates the fractionation of protein products present in the bovine blood suspension in SDS-PAGE 12% T/2.65% C. It is clear that at least four protein sub-units (due to detergent dissociation) of native oligomeric products are well defined (as the Coomassie blue stained bands) and can be identified within the molecular weight range of 70 000–15 000 Daltons. The sample A may be identified as BSA on the basis of a molecular weight comparable with that of the standard protein. This conclusion was confirmed by the protein sequencing analysis (Table 2). Similar analysis identified subunits of haemoglobin (α and β). The appearance of several Coomasie stained bands for haemoglobin (Fig. 2 and Table 2) may be attributed to various processing factors. These include: (i) an artifact of sample preparation from the SDS-PAGE analysis where oxidation of products under sample boiling promotes covalent links between protein sub-units; (ii) insufficient of SDS in the face of the high protein concentration in the starting material (approximately 6.0 mg/ml, prior to sample preparation for SDS-PAGE analysis); or (iii) a storage effect of the material where again covalent links between the haemoglobin subunits are established. It is important to emphasise that SDS-PAGE was exploited here as a technique to qualitatively identify the partition of the protein blood products in ATPS. It is anticipated that in ATPS these protein products will behave as native macromolecules (aggregates of monomers) and not as the protein subunits seen in SDS-PAGE.

Haemoglobin has an quaternary structure (of native molecular weight approximately 64 000 Daltons) of a dimer of αβ units. The α and β chains are not identical but have similar functional properties [12]. On the basis of the molecular weight of the Coomassie stained band identified as sample B (approximately 45 000 Daltons

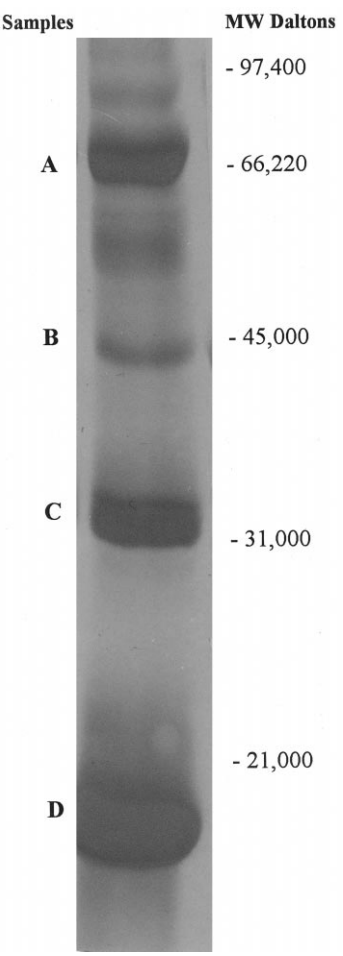

Fig. 2. SDS-PAGE analysis of protein products in bovine blood. Samples from bovine blood suspension were fractionated in a 12% T, 2.65% C SDS-PAGE.

Table 2
Characterisation of SDS bands from protein products in bovine blood

Sample	N-terminal amino acid sequence	Identification
A	D T H K S	BSA
B	M L T A E V L X A A	β globin α globin
C	M L T A E E K A V L S A A D K G	β globin α globin
D	M L T A E E K A V L S A A D K G	β globin α globin

relative to the mobility of the standard proteins, Fig. 2), and the data from the sequencing analysis, it can be suggested that the haemoglobin protein of this particular electrophoretic sample was in a trimeric form (i.e. 2αβ or α2β). Furthermore, the proportion of the α and β haemoglobin chains in the sequence analyses indicates that the subunit stoichiometry is likely to be at least one to two, respectively (i.e. α:2β). Such a conclusion could be supported by the reduced signals obtained from the α globin chain in the sequencing analysis when compared with that from the β chain (data not shown). Following similar analysis (as for sample B) for the Coomassie stained band identified as C (Fig. 2 and Table 2), it is proposed that the haemoglobin identified from this sample was in a dimeric form (with a molecular weight of approximately 33 000 Daltons), with an equal proportion of α and β globin chains. The sample identified as D with a molecular weight of approximately 19 000 Daltons (relative to the mobility of the standard protein in Fig. 2) is likely to be in a monomeric form. According to the sequencing analysis (Table 2), this is a mixture containing similar proportions of both globin chains (i.e. α and β) in a free-state denatured by SDS. It is interesting to note that the detection of inmunoglobulin G (IgG) was not possible by this technique. This may be due to the low concentration of IgG in the sample (less than 0.5 mg of bulk protein/ml, prior to sample preparation for SDS-PAGE analysis) with a 0.08-ml loading which could not produce a positive response by Coomassie blue staining. This recommended a different more sensitive technique such a Western blotting. However, the Coomassie positive band between A and B in Fig. 2 does possess a molecular mass (approximately 50 000 Daltons) similar to that for the heavy chain (H) of IgG. Sequencing analysis of this stained band proved impossible because of the presence of a blocked N-terminal amino acid (data not shown). It is interesting to note that a diffuse band of Coomassie blue stain above band D in Fig. 2 has a mobility (25 000 Daltons) appropriate to the light (L) chain of bovine inmunoglobulin (IgG). These observations require further study.

3.2. The effect of protein concentration on ATPS process performance

Since the levels of protein concentration (more than 6.0 mg/ml) in bovine blood feedstock may affect the performance of the two-stage ATPS process, it was decided to examine the impact of high concentrations of protein products and solids upon the partition properties of the systems. Although the protein partition coefficient remained relative constant in all the systems studied under first extraction conditions ($K = 55$), the volume ratio was affected by increasing the loading of cells and/or cell debris upon the systems. The presence of cell debris in ATPS inevitably affects the phase in which they are present as reflected in a changing volume ratio. In the present study, the volume ratio varied from 1.7 for the most dilute system (1.2 mg/g) to 1.0 for the more concentrated (7 mg/g) ATPS. The changes in the volume ratio necessarily affect the practical recovery of bulk protein from the phases (protein concentration × volume of the phase). It is important to emphasise that, any change in the volume of the phase affects the recovery even when the protein concentration in that phase remains constant (i.e. as here when the protein partition coefficient remains constant). The changes in the volume ratio observed in the ATPS studied meant that the practical protein recovery for the top phase preference products from the first extraction stage decreased from 89% for the systems containing 1.2 mg protein/g of system mass to 75% in the system loaded with 7 mg/g. The top phases generated from the experiments discussed above were subsequently used to study the impact of system protein concentration upon the performance of the back extraction. Since the insoluble products (cells, debris and protein precipitate) were removed in the first extraction (as discarded interface and bottom phase) their previous influence upon the volume ratio was not encountered for this second stage of the process. Thus, the volume ratio of the systems studied remained constant (at 0.56 ± 0.02) as did the practical recovery of the bottom phase products. On the basis of the overall composite recovery of the top phase preference products from the first extraction and that of the bottom phase preference products from the back extraction, the practical recoveries from the two-stage processes were estimated (Table 3). It is clear that the increase in system protein concentration promoted a decrease in the overall practical recovery. Such a decreasing trend was mainly attributed to the impact of increasing solids concentration (cells) upon the recovery of the top phase preference products in the first extraction. The differences between the practical recoveries obtained from the different ATPS studied (43–62%) did not immediately justify the adoption of the system with the greater recovery (i.e. from the most dilute feed). It was decided,

Table 3
The impact of protein concentration upon overall recoveries of bulk protein from bovine blood in a two-stage process

Initial system protein concentration (mg/g ATPS)	Overall recovery (%)
1.2	62
1.4	62
1.7	52
2.3	43
3.5	43
7.0	44

in order to assess the generic implementation of the two-stage ATPS under extreme conditions of solids tolerance, the most possible concentrated system would be used. In the case of ATPS with 7 mg/g bulk protein concentration (the most concentrated studied here) problems of sampling the bottom phase were encountered. Consequently, the system with a 3.5-mg/g bulk protein concentration was selected for extensive experimental study.

3.3. Partition of bovine blood products in an ATPS process

The SDS-PAGE analysis of the partition of protein products from bovine blood in the two-stage ATPS process is represented in Fig. 3. The low concentration of bulk protein in the lower phase of the first extraction (0.1 mg/ml) produced after sample preparation for the SDS-PAGE analysis, a maximum of 2.5 μg of bulk protein to be loaded in each gel track. This was below the practical limit of reliable positive detection by Coomassie blue staining (approximately 0.5 mg/ml bulk protein in the sample, equivalent to 12.5 μg loaded in the tracks, [10]). Consequently, as it was expected in preliminary experiments, the SDS-PAGE fractionation of the protein present in the bottom phase was characterised by the absence of Coomasie blue bands (data not shown). Furthermore, by comparing tracks 2 and 3 in Fig. 3 (corresponding to the starting material and top phase from first extraction, respectively) it is clear that the majority of the Coomasie blue bands present in track 2, are also represented in track 3. This last

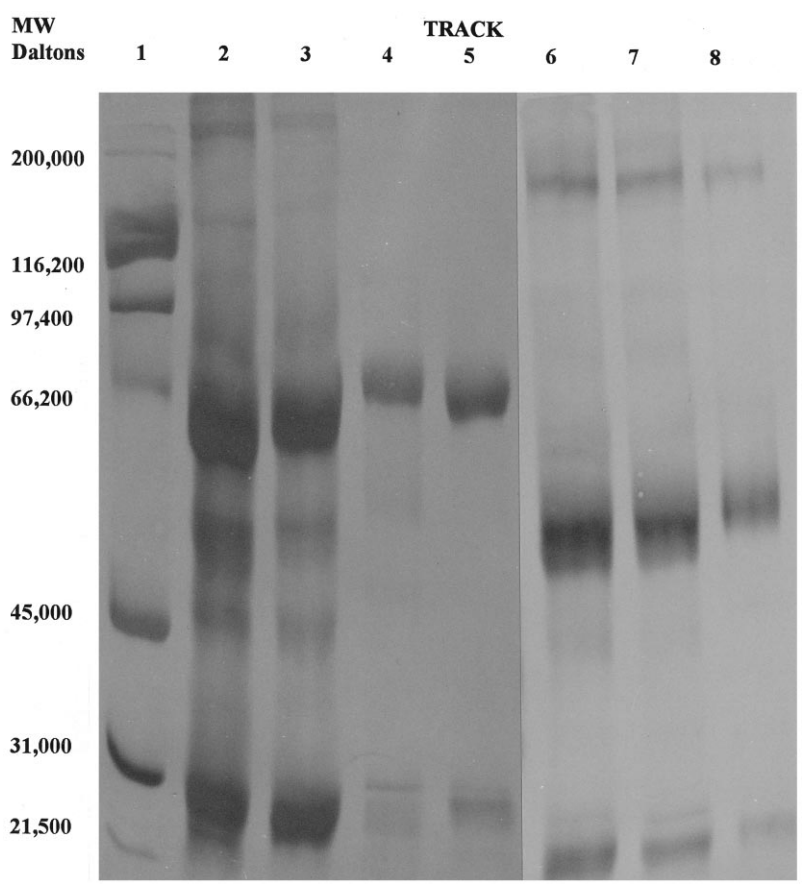

Fig. 3. SDS-PAGE and immunoblotting analysis of the partition of bovine blood protein products in a two-stage ATPS. Identical samples volume from top and bottom phases from the two-stage ATPS process were prepared and used for SDS-PAGE (7.5% T, 2.65% C) and immunoblotting analysis. (Track 1, standards; track 2, bovine blood suspension; track 3, top phase first extraction; track 4, top phase back extraction; track 5, top phase back extraction; tracks 6–8, inmunoblotting of tracks 3–5, respectively).

observation reinforced the conclusion that in the first extraction a solid–liquid separation (removal of cell debris in the bottom phase) occurs simultaneously with a partition of the majority of protein into the top phase. Any differences in protein representation and/or concentration between samples analysed in tracks 2 and 3 may be attributed to co-precipitation of protein with the cells and cell debris at the interface of the ATPS.

BSA exhibited a change in phase preference from top to bottom between the first and back extractions, respectively (see tracks 3 and 5 in Fig. 3). This observation agreed with results of the partition studies of pure preparations of BSA under the same operation conditions [13]. Such partition behaviour can be exploited for the recovery and purification of BSA from bovine whole blood feedstock by the implementation of the two-stage ATPS process (compare tracks 2 and 5 in Fig. 3). The partition of the red colour pigments (that has been associated with the presence of the heme group of haemoglobin, [13]) might imply that haemoglobin exhibits a behaviour similar to that of the BSA under back extraction conditions (changing phase). However, the intensity of the Coomassie blue bands associated with the heme proteins (Fig. 2) was considerably less (relative to BSA) into the back extraction, suggesting a possible precipitation of these products at the interface.

In the case of the partition behaviour of IgG present in the bovine whole blood feedstock, it is clear that at least three stained bands (i.e. IgG positive) were identified by the immunoblotting analysis in samples taken from the top phase of the first extraction, and both top and bottom phase of the back extraction respectively (see tracks 6, 7 and 8 in Fig. 3). On the basis of comparison of the molecular weight with that of the standard proteins, stained bands of approximately 50 000 and 21 000 Daltons can be attributed to the heavy and light chain of IgG. The third band may be explained by the presence of undissociated IgG or a non-specific response of the antiserum with another high molecular weight protein. In the two-stage ATPS process, IgG initially partitioned into the top phase in the first extraction (track 6 in Fig. 3) and subsequently to both phases (top and bottom) in the back extraction (tracks 7 and 8 in Fig. 3). Relevant work conducted by Huddleston and Lyddiatt [14], suggested the top phase preference in PEG-phosphate ATPS for serum based mammalian cell culture enriched with monoclonal antibody anti-human IgG. The partition behaviour of the bovine blood protein products discussed here confirms the practicality of the two-stage ATPS process (as implemented here) for the primary extraction of protein products from a range of similar sources (fermentation broth, cell homogenates and biological extracts).

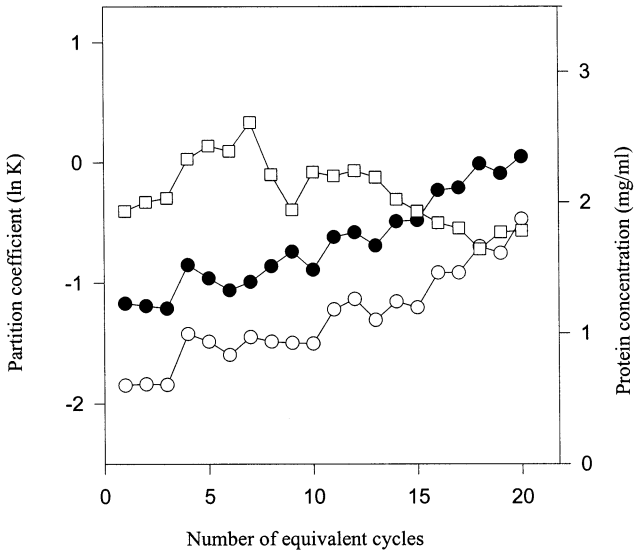

Fig. 4. The impact of polymer recycling upon the partition coefficient of protein from bovine blood in the back extraction. The protein partition coefficients (●) and the concentrations of bulk protein in the top and bottom phase (○, □) from the back extraction in spray columns experiments are represented relative to the number of equivalent operational cycles.

3.4. Studies of polymer recycling

Polymer recycling caused no significant impact upon the protein partition coefficient under first extraction conditions, and values remained relatively constant throughout the experiment. However, partition coefficient of protein products from bovine blood, under back extraction conditions exhibited a particular behaviour (Fig. 4). It is clear that both the protein partition coefficient and the protein concentration in the top phase exhibited similar and parallel increases in value. This phenomena can be attributed to the accumulation of top phase products. This product accumulation inevitably affects the initial composition of the systems. As a result at the start of each cycle, the position of the ATPS within the phase diagram relative to the binodal is changed and cause the partition coefficient to rise. The protein concentration in the bottom phase increased (a situation favourable for the recovery of bottom phase products) during the initial part of the experiment (up to seven cycles) under the influence of the increase of protein loading from the first extraction. Subsequently the protein concentration of the bottom phase decreased, which served to enhance the increasing trend of the protein partition coefficient. Such a decrease of the protein concentration in the lower phase, can be explained by changes in the free volume of the bottom phase [15] caused by phase saturation in the face of the accumulation of sodium hydroxide and orthophosphoric acid used to control system pH. The protein precipitation associated with the decrease of the protein concentration of the top

phase first extraction and that of the bottom phase back extraction severely affected the practical recovery of the soluble bovine blood protein products in the two-stage ATPS process. In the present study, the overall protein recovery decreased from 44 to 37% from the first to the twentieth equivalent cycle, respectively. However, SDS-PAGE analysis of bottom phase samples from back extraction (representing the final product of the two-stage ATPS process) shown similar bands patterns in samples from 1, 4, 8, 12, 16 and 20 equivalent cycles, respectively (Fig. 5). The changes in the intensity of the bands may likely be attributed to variation inherent in sample loading or handling procedures, together with some track spreading (particularly in track 6 in Fig. 5), but not to changes in partition conditions. These results confirm the findings obtained using the bulk protein concentration as reported above and indicate that the main contribution arises from purified BSA.

4. Conclusions

BSA and IgG exhibited a change in phase preference from top to bottom phase within the two-stage ATPS process conditions operated. In the case of the haemoglobin, this product shown initially top phase preference, but it is likely that some material was lost to the system interfaces as an intractable protein precipitate. The presence of cells and cell debris in the systems severely affected the practical protein recovery from the phases (decreased from 62 to 44%), but concentrated bovine blood suspensions were preferred for the chosen studies on the basis of the extreme solids and impurities tolerance appropriate to the generic implementation of the ATPS process. In the implementation of two-stage ATPS with partial polymer recycling using the spray column extraction system, operational stability was clearly demonstrated. The constant behaviour exhibited by the protein partition coefficient from first extraction minimised the influence of instabilities seen with the back extraction. However, during the multi-cycle continuous operation of the ATPS process with spray columns, the practical recovery of bulk protein decreased from 44 to 36% due to protein precipitation at ATPS interface. Overall it was shown the potential of the two-stage ATPS process for generic implementation for the processing of complex biological systems in the

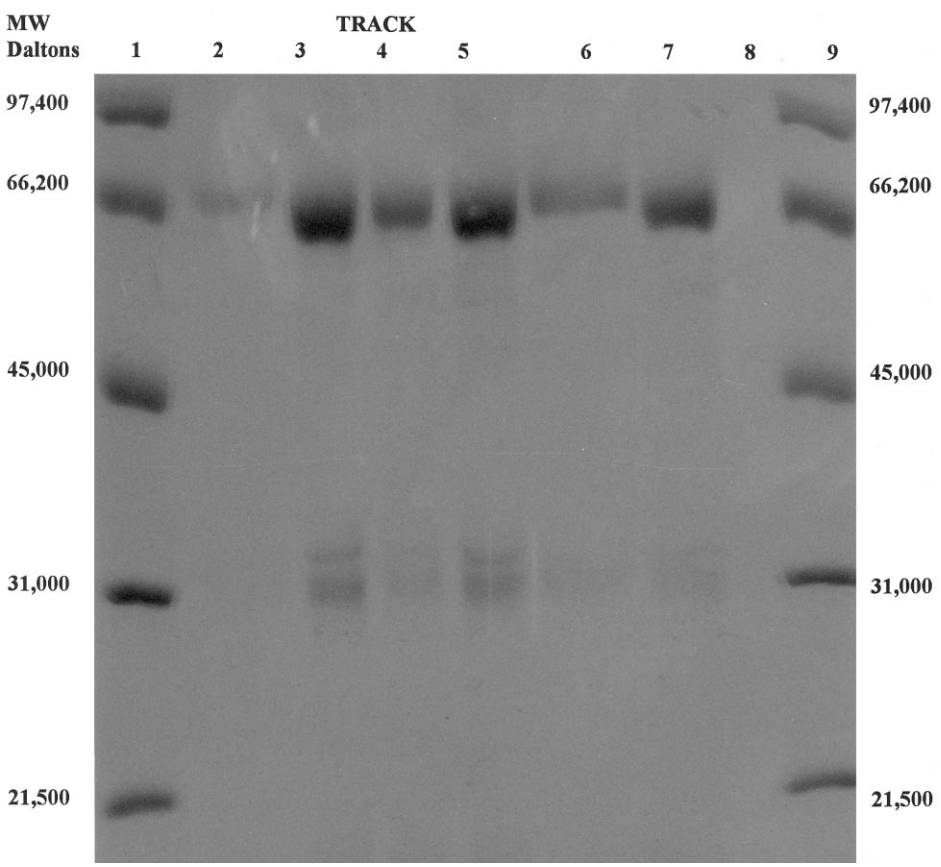

Fig. 5. SDS-PAGE analysis of the impact of polymer recycling upon products from bovine blood processed in a two-stage ATPS. Identical sample volumes from bottom phase of the back extraction from the two-stage ATPS process were fractionated in a 12% T, 2.65% C SDS-PAGE. (Tracks 1 and 9, standards; track 8, blank; and tracks 2–7, process products after one, four, eight, 12, 16 and 20 equivalent cycles).

primary step of the purification process. This ATPS process show benefits of tolerance, solid-liquid clarification of biological suspensions and reduction of chemical consumption during long term operation with partial polymer recycling.

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