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Protein recovery using gas–liquid dispersions

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Abstract

Two separation techniques, foam separation and colloidal gas aphrons (CGAs), both of which are based on gas–liquid dispersions, are compared as potential applications for protein recovery in downstream processing. The potential advantages of each method are described and the concentration and selectivity achieved with each method, for a range of proteins is discussed. The physical basis of foam separation is the preferential adsorption of surface active species at a gas–liquid interface, with surface inactive species remaining in bulk solution. When a solution containing surface active species is sparged with gas, a foam is produced at the surface: this foam can be collected, and upon collapse contains surface active species in a concentrated form. CGAs are microbubble dispersions (bubble diameters 10–100 μm) with high gas hold ups (>50%) and relatively high stability, which are formed by stirring a surfactant solution at speeds above a critical value (typically around 5000 rpm). It is expected that when proteins are brought into contact with aphrons, protein adsorbs to the surfactant through electrostatic and/or hydrophobic forces. The aphron phase can be separated easily from the bulk solution due to its buoyancy, thus allowing separation of protein in a concentrated form.

Keywords: Foam separation; Colloidal gas aphrons; Proteins

1. Introduction

With the production of novel proteins within the biotechnology industry, there is an on-going need for new cost-effective and simple separation techniques. Relatively novel bioseparation techniques such as reverse micelles and aqueous two-phase systems are finding application for protein separation. Protein separation using foam fractionation or colloidal gas aphrons (CGAs) may be attractive alternatives.

Foam fractionation shows potential for the recovery of proteins from dilute solutions, is inexpensive to operate and mechanically simple. Foam fractionation can be operated in batch or continuous mode. Foaming purifies proteins according to their behaviour at the gas–liquid interface, i.e. their surface activity. Protein surface activity is dependent on its physico–chemical characteristics, namely size, charge, hydrophobicity and environmental conditions such as ionic strength, pH and the presence of detergents, salts, sugars, and other additives [1]. There has been considerable research into foam separation of single protein solutions using the batch mode of operation [2–4], some workers have used the continuous system with single protein solutions [5–8]. Only limited work exists on the purification of binary mixtures of proteins [9,10].

Colloidal gas aphrons, named by Sebba [11,12], are microbubbles, 10–100 μm in diameter composed of a gas core, surrounded by a surfactant shell (Fig. 1) [12]. They are created by the intense stirring (5000–10000 rpm) of a surfactant solution. They

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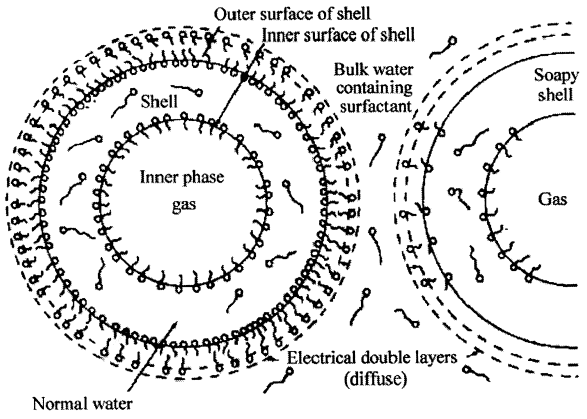

Fig. 1. Proposed structure of CGAs [12].

have a high surface area and are relatively stable; this may be due to the presence of multiple surfactant shells, as proposed by Sebba [12]. It is thought that CGAs can interact electrostatically and/or hydrophobically with proteins and because they are less dense than the bulk solution, the aphron–protein complex floats to the surface and is then easily separated from the bulk liquid, providing a potential protein separation system. Colloidal gas aphrons can be created using ionic or nonionic surfactants and it may be possible by altering the conditions of, e.g., the buffer (ionic strength and pH), to achieve conditions for selective separation of a desired protein from a mixture of proteins. CGAs have previously been used for other applications including the removal of sulphur [13], heavy metals [14] and dyes [15] from waste water, clarification of suspensions [16] and solvent sublimation [17]. A complete list of uses up to 1995 has been compiled by Jauregi et al. [18]. Currently CGAs have only been used for the separation of single proteins from solution [19], however the data available is very limited.

In this paper, results are presented for foam fractionation of BSA using both batch and continuous modes: behaviour in terms of enrichment and recovery in the two modes of operation is compared. The main variables considered are gas flow-rate and initial bulk concentration; these operating parameters were chosen as they highlight differences between operating modes and also significantly influence process performance [20,21]. Other operating parameters which also influence foam separation/fractionation include feed flow-rate (continuous mode), foam height and bubble size. For BSA these are considered in detail by Haryono [20] who found that conditions which lead to drier foams (i.e. low levels of initial protein concentration, gas flow-rate, feed-flow rate and high foam height) generally result in higher enrichments, but lower recoveries, and vice versa.

In addition, the ability of foam fractionation to partition binary mixtures [BSA–lysozyme (batch and continuous) and β-casein–BSA (batch mode only)] is discussed. These protein mixtures were chosen as these proteins are well characterised [22], readily available and adsorption behaviour at gas–liquid interfaces has been extensively studied for each protein [23–25]. There are currently few reported studies of protein recovery/separation from multi-component solutions using foams or CGAs, and use of binary model systems is the first step in understanding the potential role of this technique for more complex solutions, e.g., fermentation broths.

Results for foam fractionation of protein solutions are compared with protein recovery using CGAs. Whilst for some applications foam separation may be a valuable technique, the foaming process may not be applicable where proteins to be separated have similar surface properties or are possibly denatured by foaming. Protein denaturation caused by foam separation, has been reported for various enzymes [8,26], however, there is work where foam separation had no effect upon enzyme activity [27]. It may be possible to overcome these apparent limitations by using CGAs, where protein adsorption is likely to be driven by electrostatic protein–surfactant interactions, rather than adsorption driven by predominantly hydrophobic interactions in the foam separation process. Unfolding of the protein at the gas–liquid interface can lead to protein denaturation, especially with relatively flexible and very hydrophobic proteins. However, the protein–surfactant interactions at the aphron surface, may not involve protein unfolding and may therefore be less likely to denature the protein.

The use of colloidal gas aphrons, with ionic surfactants, for protein separation relies on electrostatic interactions (hydrophobic interactions are exploited for nonionic surfactants) between the surfac-

tant (in the aphrons) and the protein. Initial experiments have been carried out with the anionic surfactant AOT [sodium bis-(2-ethylhexyl) sulfosuccinate]. Further experiments have also been carried out using the cationic surfactant CTAB (cetyltrimethylammonium bromide), and the nonionic surfactant Triton X-100. AOT and CTAB were chosen as they are readily available in pure form, and have previously been used for the formation of colloidal gas aphrons [15,16,18,19,28,29]. Triton X-100 was chosen to investigate the role of hydrophobic interactions in these systems, and is known to show some interactions with proteins [28].

Proteins used in experiments with ionic surfactants were chosen on the basis of their isoelectric points (p*I*) (Table 1), availability and purity, additionally the proteins chosen have been well characterised. The pH of the protein and surfactant solutions were manipulated so that the protein would exhibit a net charge opposite to that of the surfactant, thus allowing electrostatic interactions between the protein and surfactant in the aphron phase. Proteins used in the experiments with nonionic surfactants were chosen on the basis of hydrophobicity rather than the p*I*. The interactions between protein and nonionic surfactant are likely to be hydrophobic, therefore it was thought that by using relatively hydrophobic proteins, the interactions between protein and surfactant could be maximised.

So far, there has been very little investigation into the use of colloidal gas aphrons for protein separation [19]. It is the aim of these investigations to determine which surfactant–protein systems give high enrichment and recovery of protein into the aphron phase and to examine the effect of a limited number of process variables (protein concentration, surfactant concentration, ionic strength and pH) on the enrichment and recovery of proteins into the aphron phase.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA, 44004 3J), cetyltrimethylammonium bromide (CTAB) and Triton X-100 were supplied by BDH (Poole, Dorset, UK), β-casein (C9605), lysozyme (L6876), thaumatin (T7658), α-chymotrypsinogen A (C4879) bicinchoninic acid (BCA) solution and copper (II) sulphate pentahydrate (4% w/v) were supplied by Sigma (Poole, Dorset, UK), AOT [sodium bis-(2-ethylhexyl) sulfosuccinate] was obtained from Fisons (Loughborough, Leics, UK). The laboratory mixer (SL2T model) fitted with four blade impeller ($D=30$ mm) surrounded by a high sheer screen and with digital speed readout was supplied by Silverson (Waterside, Bucks, UK). All other chemicals were of analytical grade and were supplied by Sigma.

2.2. Foams

2.2.1. Batch mode

Proteins were dissolved in the appropriate buffer at a pH corresponding to the p*I* of the protein to be purified. The apparatus used for batch foaming was similar to that used for continuous foaming (Fig. 2) except that the feed reservoir and pump were removed. Initially, 50 ml of protein solution was poured into the glass column prior to gas sparging. Prehumidified compressed air was continuously in-

Table 1
Physico–chemical properties of proteins used

	p*I*	Molecular mass (kDa)	Disulphide bridges	Native conformation	Average hydrophobicity[a]
BSA	4.6	67	17	Globular	1120
β-Casein	5.3	24	0	Random coil	1330
Lysozyme	10.1	14.5	4	Globular	970
Thaumatin	12.0	22.2	8	Globular	1050
α-Chymotrypsinogen	8.7	23.6	5	Globular	1040

[a] Hydrophobicity of amino acid residues defined by Bigelow [37].

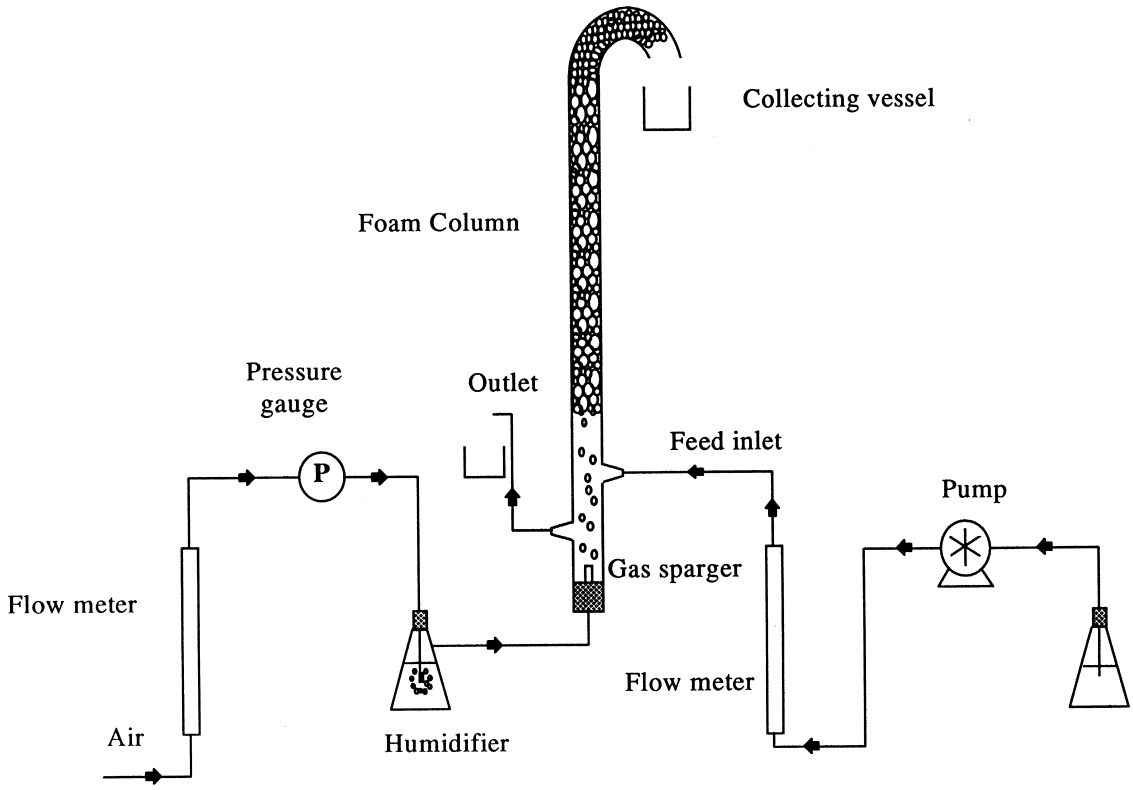

Fig. 2. Apparatus for continuous foam fractionation.

jected through a sintered glass sparger (pore size 16–40 μm) at the base of the column, and all foam was collected at the column exit until no more was produced. The residual liquid remaining after foam separation (retentate) was collected for analysis. Collected foam was allowed to collapse, unaided, at 4°C before analysis.

2.2.2. Continuous mode

For continuous foaming of a protein, the apparatus shown in Fig. 2 was used. The protein solution was pumped into the foaming apparatus at a constant foam flow-rate via a digitally controlled peristaltic pump. Once the required liquid level in the column was reached, air was passed through a sparger at a constant flow-rate. After the system reached a steady state (1 h), the foam produced was collected for analysis. Retentate samples were taken by collecting liquid overflowing from the outlet.

2.2.3. Analysis of foams

In the batch system, protein concentration was determined via absorbance measurements at 280 nm, using the published absorbance coefficients. For binary protein mixtures (continuous mode), a fast protein liquid chromatographic system (FPLC) fitted with a Resource Q anion-exchange column was employed. For binary protein mixtures (batch mode), protein concentration was determined using size-exclusion chromatography (Gilson column GF 250, Dupont). Both of these analysis techniques measured protein absorbance at 280 nm.

2.3. Colloidal gas aphrons

Colloidal gas aphrons were created by mixing 400 ml of surfactant solution at 8000 rpm, (chosen after extensive characterisation of CGAs created using AOT [19]), in a fully baffled beaker using a Silver-

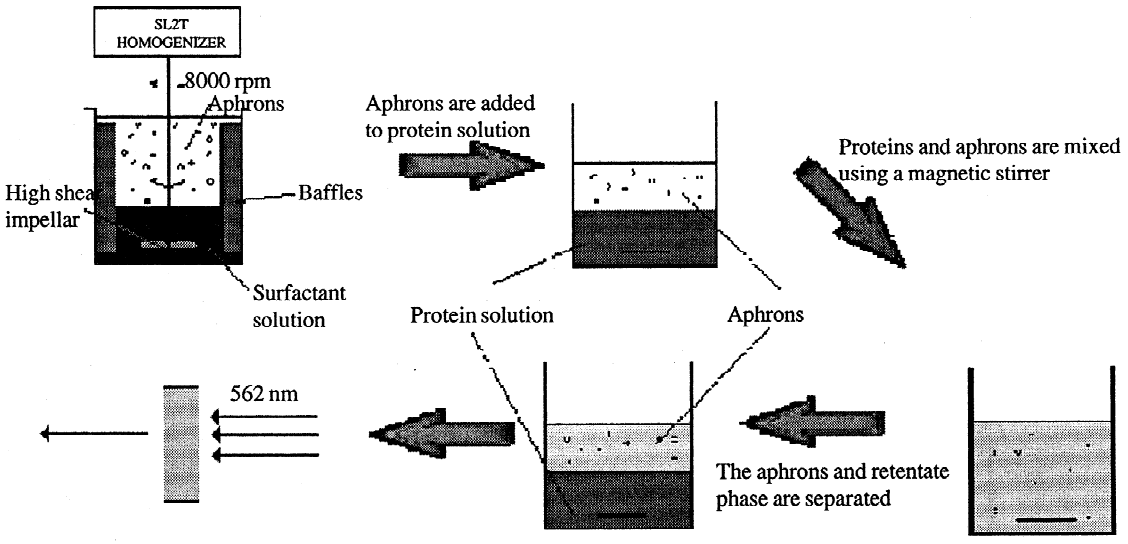

Fig. 3. Scheme for protein separation using colloidal gas aphrons.

son SL2T homogenizer, fitted with a high shear impeller. Protein separation was achieved (Fig. 3) by adding 10 ml of aphrons to a beaker containing 5 ml of protein solution [30]. This was then mixed for a set amount of time using a magnetic stirrer. The aphron phase separates from the mixture (being less dense than the bulk solution), and as soon as stirring is stopped the two phases are readily discernible. The concentration of protein in each phase was determined by use of the BCA assay [31] using the Sigma protocol (Procedure number TPRO-562).

2.3.1. Process variables

Enrichment ratio (Er), (foam or aphrons)

$$= \frac{\text{Concentration of protein in the } \textit{foam} \text{ or } \textit{aphron} \text{ phase}}{\text{Concentration of protein in the } \textit{initial} \text{ bulk solution}} \quad (1)$$

Separation ratio (Sr), (aphrons)

$$= \frac{\text{Concentration of protein in the } \textit{aphron} \text{ phase}}{\text{Concentration of protein in the } \textit{retained} \text{ liquid phase}} \quad (2)$$

Protein recovery [R_p(%)], (foams or aphrons)

$$= \frac{\text{Mass of protein in the foam or aphron phase}}{\text{Total initial mass of protein}}. \quad (3)$$

For foam separation of binary protein mixtures (i.e. protein A and protein B):

Concentration ratio (Cr), (protein A:protein B)

$$= \frac{\text{Concentration of protein A in } \textit{foam}}{\text{Concentration of protein B in } \textit{foam}}. \quad (4)$$

Concentration ratios are also calculated for retentate and bulk phases.

For each system all experiments were carried out in duplicate with the average results reported.

3. Results and discussion

3.1. Foam separation: single proteins

Fig. 4 shows protein recovery [R_p(%)] and enrichment ratio (Er) of batch and continuously foamed BSA, as a function of gas flow-rate. For the batch mode, the enrichment ratio decreases from 3.5 to 1.6 as the gas flow-rate is increased from 60–240 ml/min but protein recovery increases from 65 to 82%. Similar trends for enrichment and recovery are obtained with the continuous mode, where enrich-

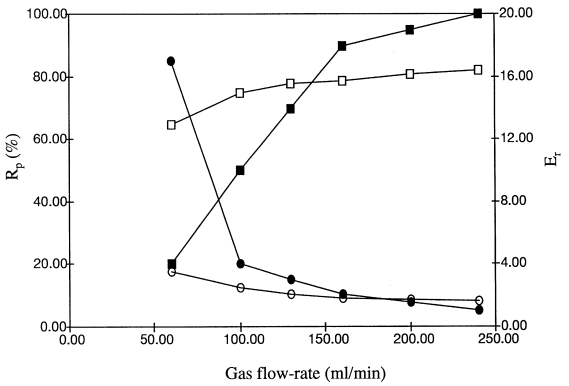

Fig. 4. Protein recovery (□/■) and enrichment ratio (○/●) as a function of gas flow-rate for batch (open) and continuous (closed) foam separated BSA; foam height = 0.8 m; pH = 4.6; feed flow-rate = 5 mg/ml (continuous); BSA concentration = 0.25 mg/ml.

ment decreases from 17 to 1 and recovery increases from 20 to 100% within the gas flow-rate range studied. At gas flow-rates below 150 ml/min, BSA enrichment in the continuous mode is greater than in the corresponding batch mode value. However, BSA recovery in the batch mode is greater than the corresponding continuous mode value below 150 ml/min. These trends for batch and continuous modes agree with previous work [2–5,20].

At high gas flow-rates, the liquid hold-up in the foam increases, causing (i) low enrichments, as protein adsorbed into the foam phase is effectively ''diluted'' and (ii) high protein recoveries, as protein is removed from the bulk, into the foam interstitial liquid, where the protein concentration is equal to that in the bulk. At low gas flow-rates, the foam has lower liquid hold-up due to (i) greater drainage time, as foams take longer to rise up the column and (ii) less bulk liquid enters the interstitial liquid.

Fig. 4 indicates that significant differences between batch and continuous operating modes are observed at low gas flow-rates. In the batch system, foaming continues until the protein concentration in the residual liquid falls below that required for stable foam formation (BSA~0.001 mg/ml), upon which, no further foam is produced. In batch mode, the total foam volume produced during the whole process divided by the total foaming time, is greater than the foam volume produced per unit time in the continuous system. Hence, in the batch mode, especially at low gas flow-rates, the presence of more liquid in the foam decreases enrichment and increases protein recovery. It may be possible by operating the batch mode over shorter time periods to improve enrichment, for a lower recovery, thus illustrating the importance of how the batch mode is operated. At higher gas flow-rates, volumes of foam produced in continuous mode increase, resulting in lower enrichment and higher recovery and results that are in closer agreement with those for batch experiments.

Fig. 5 shows protein recovery and enrichment of batch and continuous foam separated BSA, as a function of the initial bulk concentration. For the batch mode, enrichment decreases from 10 to 2.5 as the initial bulk concentration is increased from 0.08–0.50 mg/ml and protein recovery increases from 60 to 90%. Similar trends for enrichment and recovery are obtained with the continuous mode; enrichment decreases from 18 to 2 and recovery increases from 45 to 53% within the initial bulk concentration range studied. These trends are due to increased foam stability and hence greater foam volume produced at higher bulk concentrations. The effect of increasing foam volume on enrichment and recovery has been discussed above.

The results above show that foam separation can be operated to achieve relatively high enrichments

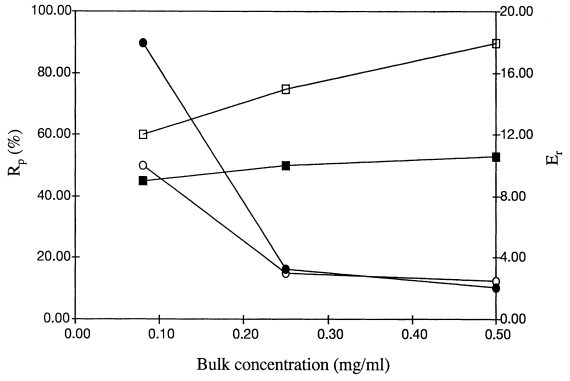

Fig. 5. Protein recovery (□/■) and enrichment ratio (○/●) as a function of bulk concentration for batch (open) and continuous (closed) foam separated BSA. Gas flow-rate = 100 ml/min; foam height (batch) = 1.30 m; foam height (continuous) = 1.20 m; feed flow-rate = 10 ml/min; pH = 4.6.

and protein recoveries. The effect of foaming on protein conformation may also be important. There is evidence that foaming can cause protein denaturation [8,24], however there is also evidence to the contrary [27,32]. Some indirect evidence for conformational change to BSA has been found in the experiments described above; when residual BSA in the retentate (at an initial concentration well above that required for minimum foam production) was resparged with air; stable foam formation was not achieved, suggesting that protein denaturation may have occurred during the preceding foam experiment [20], compromising the ''foamability'' of BSA. Previous work conducted by the authors, has examined the effect of foaming on β-casein [33]. Qualitative fluorescence studies by the authors, with foamed β-casein indicated that buried tryptophans in the protein had become exposed to the aqueous environment; indicating that denaturation to some of the protein molecules had occurred, i.e. a primary layer of unfolded β-casein molecules situated at gas–liquid interface (due to protein reorientating to expose hydrophobic regions into the gas phase and polar regions into the aqueous phase) and native β-casein layers situated in adsorbed multilayers [22]. These results indicate that the effect of foaming on protein structure cannot be neglected, the importance of any such effects depending on the end use of the protein product.

In conclusion, conditions can be chosen for substantial BSA enrichment or recovery into the foam phase in both batch and continuous operating modes. Due to the nature of the batch process, enrichments at low gas flow-rates and bulk concentrations are greater for the continuous mode. In addition, the possibility of some protein denaturation during foam separation cannot be discounted.

3.2. Foams: protein mixtures

3.2.1. BSA–lysozyme

Fig. 6, illustrates batch foam fractionation of BSA–lysozyme mixtures; foam and retentate concentration ratios are plotted as a function of bulk concentration ratio {Cr(all phases)=[BSA]/[lysozyme]}. The solid line indicates ''no separation''. Maximum separation of the two proteins would be indicated by high foam concentration ratios and low

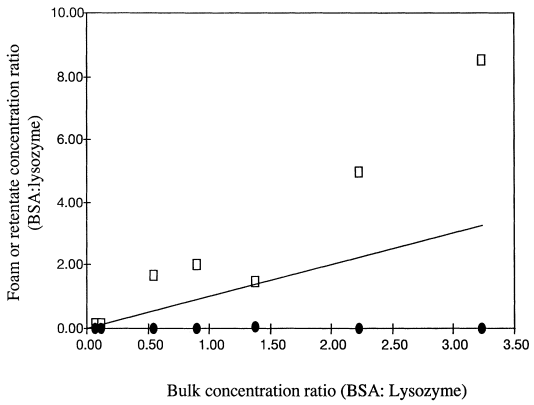

Fig. 6. Foam (open) and retentate (closed) concentration ratios (BSA–lysozyme) as a function of bulk concentration ratio (BSA–lysozyme) in batch mode; gas flow-rate=100 ml/min; foam height=0.33 m; pH=4.6.

retentate concentration ratios relative to the bulk concentration ratio. Fig. 6 shows clearly that for all bulk concentration ratios, foam concentration ratios are greater than the ''no separation'' line, and the maximum foam concentration ratio of 8.5 occurs at an initial bulk concentration ratio of 3.25. Also, all retentate concentration ratios are no greater than 0.05.

At all bulk concentration ratios, foam concentration ratios are greater than the ''no separation'' line and retentate concentration ratios are consistently lower, indicating BSA partitions into the foam, leaving lysozyme in the retentate [9]. In addition, retentate concentration ratio values indicate that most of the BSA has entered the foam, with corresponding high recoveries and enrichment ratios. During batch foam separation of BSA only, bulk BSA concentration eventually decreases below the required level for stable foam formation, thus foam separation ceases with this fraction of BSA unrecovered. In the binary protein mixture, the presence of lysozyme within the foam interstitial liquid increases foam stability. This stability increase, due to the presence of lysozyme, enables foaming to continue beyond the point where BSA concentration limits stable foam formation, hence greater BSA recovery is observed in the mixed system than when BSA is foamed on its own.

Fig. 7 shows the continuous foam fractionation of two series of BSA–lysozyme mixtures. The data is

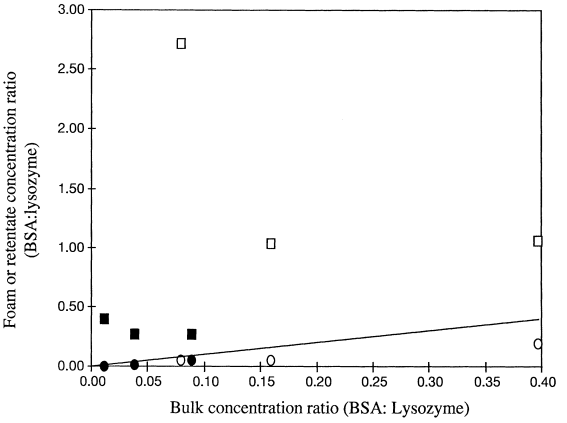

Fig. 7. Foam (□/■) and retentate (○/●) concentration ratios (BSA–lysozyme) as a function of bulk concentration ratio (BSA–lysozyme) in continuous mode. Closed symbols are mixture series at constant 2 mg/ml initial lysozyme concentrtation. Open symbols are mixture series at constant 0.5 mg/ml; gas flow-rate = 60 ml/min; foam height = 0.34 m; feed flow-rate = 2.5 ml/min; pH = 4.6.

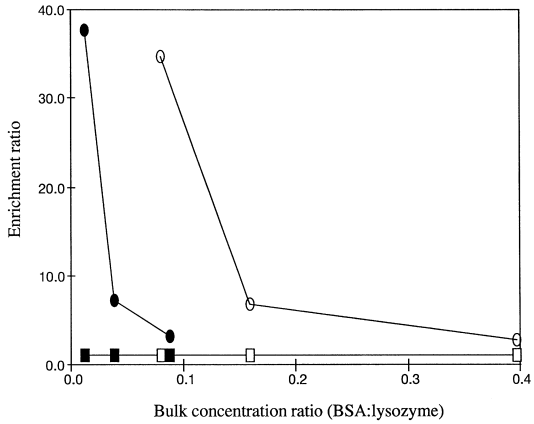

Fig. 8. Enrichment ratio as a function of bulk concentration ratio (BSA–lysozyme) at constant 0.5 mg/ml initial lysozyme concentrtation (□ lysozyme; ○ BSA); and at constant 2.0 mg/ml initial lysozyme concentration (■ lysozyne; ● BSA); gas flow-rate = 60 ml/min; foam height = 0.34 m; feed flow-rate = 2.5 ml/min; pH = 4.l6.

grouped into two series (see figure legend). Within each series, the concentration of lysozyme is constant. For each mixture, foam concentration ratios are always greater than the ''no separation'' line, again suggesting BSA partitions into the foam to a greater extent than lysozyme. In the series with lysozyme concentration = 0.5 mg/ml (open symbols), the maximum foam concentration ratio obtained is 2.72 at an initial bulk concentration ratio of 0.08, and foam concentration ratios decrease as bulk concentration ratios increase, due to the reduction in the concentrating effect of the process as more bulk liquid enters the foam. In addition, all retentate concentration ratios are below the ''no separation'' line and increase from 0.01 to 0.2 as bulk concentration ratio increases, due to an increase in BSA concentration in the retentate, probably due to gas–liquid interfacial area limiting the efficiency of protein removal to the foam. This highlights a potential limitation of the foaming process in continuous mode. The same trends are observed for mixtures in the series with lysozyme concentration = 2 mg/ml (closed symbols), i.e. BSA partitions into the foam at the expense of lysozyme, however, foam and retentate concentration ratios are lower due to a higher lysozyme concentration present in the initial bulk solution.

Fig. 8 shows enrichment ratio as a function of initial bulk concentration ratio for both mixture series. For mixtures where bulk lysozyme concentration is 2 mg/ml, BSA enrichment (Er 37.6) and therefore partitioning (Er_{BSA}/Er_{LYSO}) is maximum at the lowest initial bulk concentration ratio and decreases as initial bulk concentration ratio increases, as foam stability improves and greater liquid hold-up reduces the concentrating effect of the process. For mixtures where bulk lysozyme concentration is 0.5 mg/ml, the same trends are observed, however BSA enrichments are lower than with mixtures containing 2 mg/ml lysozyme; Poole et al. [34] observed that at a concentration of 1 mg/ml, lysozyme can enhance stability of BSA foams (BSA concentration: 5 mg/ml; pH 8.0), however, at higher lysozyme bulk concentrations, foam stability decreased. Foams with lower stability produce higher enrichment ratios. Lysozyme does not enrich at any initial bulk concentration ratio; a result of this protein's low surface activity compared to that of BSA.

Similar work has been conducted by the authors with β-casein–BSA mixtures [33]. It was found that as these proteins have very similar surface activities, i.e. both BSA and β-casein enriched into the foam phase, therefore limiting the degree of partitioning (i.e. $Er_{\beta\text{-CASEIN}}/Er_{BSA}$ were reduced). The lack of selectivity displayed within β-casein–BSA mixtures

highlights a potential limitation of foam fractionation, i.e. differences in physico–chemical properties of β-casein and BSA (Table 1) are not sufficient to selectively partition these proteins. Problems involving selectivity may be overcome by (i) modifying influential physico–chemical factors to increase the surface activity of the target protein; or (ii) using colloidal gas aphrons, where the nature of the interaction between protein and surfactant at the gas–liquid interface may be more specific.

3.3. Colloidal gas aphrons

Colloidal gas aphrons may be used as an alternative to foam separation of proteins. Unlike foams, the surface area of colloidal gas aphrons is greater due to their high surface area to volume ratio so that compared to foam systems, higher protein recovery should be obtained. By changing the type of surfactant used for the creation of the aphrons, and, e.g., the pH of the media, it may be possible to selectivity separate specific proteins from aqueous solution.

3.4. Characterisation of CGAs

The stability and gas hold-up of colloidal gas aphrons are likely to play an important role in the degree of enrichment of proteins into the aphron phase. The stability of the aphrons is defined as the time required for the volume of liquid to reach half the initial value after mixing has been stopped. To achieve high enrichment and recovery of proteins, it is desirable to have a stable aphron system to enable the maximum amount of interaction to take place before the two phases are separated. However, if aphron stability is too high the prolonged contact of protein and aphron may lead to protein denaturation.

Studies with AOT have shown that concentration of surfactant, concentration of salt, pH, temperature and time have an effect on stability and gas hold-up parameters [18]. Increasing surfactant concentration is shown to increase the stability of the CGA dispersion. This is likely to be due to an increase in repulsive forces between the surfactant molecules either in the surfactant shell or the bulk solution, and may also be due to the formation of multiple layers of surfactant around the aphrons [12].

The stability of the colloidal gas aphron dispersion is seen to decrease with increasing salt concentration. This is due to the salt ions suppressing the repulsive forces between the individual surfactant molecules, this will allow the aphrons to move closer together, aiding coalescence, thus reducing stability. The pH shows no significant effect on the stability of the aphrons, except at high surfactant concentrations. For the system studied, maximum stability of AOT aphrons was achieved at pH 4 and 61 m*M* surfactant concentration [18].

Gas hold-up (ε) is a measure of the amount of air in the aphron system, and is given by:

$$\varepsilon = \frac{V_f - V_i}{V_f} \tag{5}$$

where V_i is the initial volume of surfactant solution and V_f the volume after creation of aphrons.

It is desirable to have a high gas hold-up as the surface area (A) of the system will generally be higher than for a system with a lower gas hold-up, assuming the bubble size (d) is constant. A is given by the following equation [35]:

$$A = 6\frac{\varepsilon}{d}. \tag{6}$$

In addition, for high gas hold-ups, the volume of the collapsed aphron phase collected is likely to be less due to the higher content of air, thus enrichments will be higher. In the study outlined above [18], the main factors governing gas hold-up were shown to be surfactant concentration, salt concentration and time of stirring. Gas hold-up was seen to increase with surfactant concentration, and tends towards a maximum at 25 m*M*. A decrease in gas hold-up was observed with the addition of salt, and an increase was observed as the time of stirring was increased [18].

3.5. Protein recovery using CGAs

Initial studies carried out with a lysozyme/AOT system have indicated that ionic strength, pH, initial protein concentration and initial surfactant concentration are important factors for the optimisation of protein recovery in this system [19].

3.6. Effect of ionic strength

For an AOT/lysozyme system, similar recoveries were obtained at the different ionic strengths studied, (0.03, 0.10, 0.29 *M*), but enrichment ratios were doubled when working at the highest ionic strength. This was a consequence of the recovery of a lower volume of aphrons at this high ionic strength, which in turn was caused by a decrease in stability of the aphrons [18].

3.7. Effect of pH

pH also plays a role in optimising the recovery for this system. In a system comprising 0.56 m*M* AOT and 0.1 mg/ml α-chymotrypsinogen A, the highest enrichments were obtained at low pH values (pH 3 and 4) (Table 2), where the protein exhibits a large net positive charge, and so interacts more strongly with the surfactant. For an AOT lysozyme system, high enrichments and recoveries were observed at all pH values studied (pH values 4, 6 and 8) [19]. This range is broader than that observed for α-chymotrypsinogen A and is probably a consequence of the higher p*I* of lysozyme, i.e. the protein will exhibit a net positive charge over a broader pH range.

3.8. Effect of initial protein concentration

In an AOT/α-chymotrypsinogen A CGA/protein system, at constant pH, ionic strength and initial surfactant concentration, it can be seen that the enrichment and recovery of α-chymotrypsinogen A into the aphron phase is dependent on the initial concentration of the protein in the protein/aphron mixture (Table 3). At high initial protein concentration (0.5 mg/ml) there is no significant enrichment of the protein into the aphron phase (Er 1.07, Rp 12.7%). As the initial protein concentration decreases, both enrichment and recovery of the protein into the aphron phase increase, with a maximum enrichment of the protein being observed at an initial protein concentration of 0.1 mg/ml for the concentration range studied. The recovery of protein at 0.1 mg/ml is less than that observed for 0.2 and 0.4 mg/ml, but this is a consequence of a higher volume of aphrons being recovered at these higher concentrations.

Table 2
Effect of pH on recovery and enrichment of α-chymotrypsinogen A with AOT

pH	Vr (ml)	Er	Sr	R_p(%)
2	0.7	2.6	4.7	14.7
3	1.1	2.6	150.0	38.7[a]
4	0.8	4.8	11.4	40.8
5	0.8	3.2	7.1	22.5
6	0.9	1.1	1.2	6.0

Initial protein concentration, 0.1 mg/ml; 5 ml protein solution was mixed with 10 ml of aphrons for 5 min before separation of the phases.
Er: Enrichment ratio; Sr: Separation; R_p(%): Percentage recovery; Vr: Volume of recovered aphron phase.
[a] High degree of precipitation seen at this pH.

Table 3
Enrichment and recoveries at different concentrations of α-chymotrypsinogen A with 0.56 m*M* AOT at pH 4

[Protein] (mg/ml)	Ms_0/Mp_0	Vr (ml)	Er	Sr	R_p(%)
0.5	26.5	1.2	1.1	1.5	12.7
0.4	33.4	2	2.2	7.0	51.0
0.2	66.3	2.2	2.7	9.6	59.8
0.1	133	0.8	4.8	11.4	40.8

Five ml of protein solution was mixed with 10 ml of aphrons for 5 min before separation of the two phases.
Er: Enrichment ratio; Sr: Separation; R_p(%): Percentage recovery; Vr: Volume of recovered aphron phase. Ms_0/Mp_0: Molecular ratio of surfactant to protein.

3.9. Effect of initial surfactant concentration

In an AOT, α-chymotrypsinogen A CGA/protein system, at constant pH, ionic strength and initial protein concentration, protein enrichment and recovery are seen to vary with initial surfactant concentration. At high initial surfactant concentration, (above 2 m*M*), little enrichment of the protein into the aphron phase is observed (Table 4). Maximum protein enrichment is observed at 0.25–0.56 m*M* AOT, with lower protein enrichments being observed at lower or higher surfactant concentrations. Enrichments and recoveries at surfactant concentrations greater than 2 m*M* are very low.

It may be that at concentrations above 2 m*M* there is no enrichment of the protein because it may

Table 4
Enrichment and recoveries of α-chymotrypsinogen A at different AOT concentrations (pH 4)

[Surfactant] (m*M*)	Ms_0/Mp_0	Vr (ml)	Er	Sr	R_p(%)
0.10	11	0.7	2.8	3.3	16.7
0.20	22	1.2	3.2	3.9	34.0
0.25	28	1.0	4.5	8.5	46.2
0.56	63	0.9	5.1	11.4	43.5
1.00	112	2.1	3.5	8.5	85.0
2.00	224	1.1	1.2	1.1	13.2
2.50	280	1.1	1.3	1.0	14.4
5.00	560	1.3	1.4	1.0	19.2
10.00	1120	1.0	1.3	1.0	13.8

Initial protein concentration 0.1 mg/ml; 5 ml protein solution was mixed with 10 ml of aphrons for 10 min before separation of the two phases.
Er: Enrichment ratio; Sr: Separation; R_p(%): Percentage recovery; Vr: Volume of recovered aphron phase. Ms_o/Mp_o: Molecular ratio of surfactant to protein.

interact with surfactant micelles in the system rather than the aphrons (the critical micelle concentration of AOT is 2.4 m*M* in aqueous solution at 29°C [19]). At lower surfactant concentrations, there may not be enough surfactant present to stabilize the aphrons, or to allow sufficient interactions with the protein.

From these results there is clearly an optimum initial concentration of surfactant and protein that gives good protein enrichment and recovery. For the above study, the highest enrichment and recovery of protein was obtained at an initial surfactant to protein molecular ratio of 30–110 molecules of surfactant per protein molecule. For an AOT/lysozyme system the authors found an initial surfactant to protein molecular ratio of around 12–30 molecules surfactant per protein molecule to give the highest enrichment and recovery [30].

These results indicate that the molecular mass of the protein, or the number of charged amino acids on the protein per unit mass may influence the amount of surfactant required for separation into the aphron phase, i.e. for a larger protein, or a protein with high charge density, more surfactant will be required to bind a unit mass of the protein.

3.10. Other surfactant and protein systems

Under the optimum conditions determined for α-chymotrypsinogen/AOT, a ribonuclease-A/AOT CGA/protein system also shows high recovery of protein. Enrichment is somewhat less than that obtained for α-chymotrypsinogen A, but this is probably due to the larger volume of the recovered aphron phase (Table 5). High enrichments and recoveries have also been observed for an AOT/lysozyme system [19].

Results with other surfactant systems show lower enrichment and recoveries than those obtained with AOT. In preliminary investigations using the cationic surfactant CTAB, experiments using CTAB–BSA and CTAB–α-chymotrypsinogen A show relatively low recoveries (R_p 24 and 26.5%, respectively) and enrichments (Er 1.9 and 2.3, respectively) of the protein into the aphron phase (Table 5) under the conditions studied. It should be noted that further

Table 5
Results for other protein/surfactant systems

Protein	Cp (mg/ml)	Surfactant	Cs (m*M*)	Vr (ml)	pH	Er	R_p(%)
Ribonuclease A	0.1	AOT	0.56	2.4	4	3.0	70.0
α-Chymotrypsinogen A	0.1	CTAB	0.1	1.2	10	2.3	26.5
BSA	0.1	CTAB	0.1	1.3	10	1.9	24.0
α-Chymotrypsinogen A	0.1	Triton X-100	0.2	1.1	8.9	1.9	25.0
β-Casein	0.2	Triton X-100	0.2	1.3	5.4	1.3	10.0
β-Casein	0.1	Triton X-100	0.2	1.4	5.4	1.5	34.0
Thaumatin	0.2	Triton X-100	0.2	2.2	10	1.9	65.0
Thaumatin	0.01	Triton X-100	0.2	2.3	10	3.9	74.0

Initial protein concentration (Cp), initial surfactant concentration (Cs) and pH as shown. Five ml protein was mixed with 10 ml of aphrons before separation of the two phases.
Er: Enrichment ratio, R_p(%): Percentage recovery, Vr: Volume of recovered aphron phase.

optimisation is needed before conclusions can be drawn for this system, i.e. the effect of variations of initial protein and surfactant concentrations has not yet been evaluated.

CGAs created from the nonionic surfactant Triton X-100 give very low enrichments (max. 1.9) and recoveries (max. 25%) when contacted with the majority of proteins studied (Table 5). It does appear that enrichment of the protein into the aphron phase increases as the hydrophobicity of the protein increases, with high enrichment (3.9) and recovery (74%) of thaumatin being obtainable at low initial protein concentration (Table 5). Again the use of nonionic surfactants needs further and more detailed investigation before definite conclusions can be drawn.

In experiments with AOT and lysozyme, some precipitation of the protein was observed in the aphron phase after separation, the extent of this precipitation depending on experimental conditions. The precipitate was easily solubilised by the addition of urea. It is not known if this precipitation causes irreversible denaturation of the protein but initial results with lysozyme show no significant loss of activity ($\pm 10\%$) after addition and subsequent removal of urea, indicating no damage to the protein during this process, however this is likely to depend on the protein being studied and the specific interactions between protein and surfactant. Some precipitation of the protein was observed with both α-chymotrypsinogen A and ribonuclease A (with CGA's created from AOT), but only under conditions where protein recovery was high (Rp values of $>90\%$ after resolubilization of the protein). This precipitate was easily solubilised by the addition of a small quantity of 4 *M* sodium hydroxide.

Another potential problem with the use of CGAs is the presence of surfactant which must be removed from the purified protein. For the AOT/lysozyme system, the protein has been successfully separated from the surfactant by the use of size-exclusion chromatography [36].

4. Conclusions

Foam separation in batch and continuous operating modes using BSA only and BSA–lysozyme mixtures has been successfully performed. By choosing appropriate process conditions, high enrichment ratios or recoveries can be obtained in both operating modes. Partitioning of proteins is clearly dependent on the relative surface activities of the proteins involved, as shown by the partitioning achievable in BSA–lysozyme mixtures. In addition, there is some evidence of conformational change to BSA as a result of foaming, which needs to be considered when using this process.

In cases where the relative surface activities between proteins are similar, protein recovery using CGAs may be a viable alternative to foam fractionation. In order to ascertain the potential of CGAs, preliminary work is presented employing various surfactant/protein systems. For CGA systems using anionic surfactants it is possible to obtain high enrichment and recovery of protein into the aphron phase at optimum conditions, although some precipitation of the protein can occur. Colloidal gas aphrons created from nonionic and cationic surfactants show some enrichment and recovery of the proteins studied, but this is less than that observed with AOT, corresponding to possibly reduced interaction of the tested proteins with nonionic and cationic surfactants compared to anionic ones. Research into CGAs is at a very early stage and results are promising enough to warrant further investigation, particularly in determining cationic and nonionic surfactant systems which will allow protein adsorption and recovery.

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