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An insight into the mechanism of protein separation by colloidal gas aphrons (CGA) generated from ionic surfactants☆

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Abstract

Colloidal gas aphrons (CGA), which are surfactant stabilised microbubbles, have been previously applied for the recovery of proteins from model mixtures and a few studies have demonstrated the potential of these dispersions for the selective recovery of proteins from complex mixtures. However there is a lack of understanding of the mechanism of separation and forces governing the selectivity of the separation. In this paper a mechanistic study is carried out to determine the main factors and forces influencing the selectivity of separation of whey proteins with CGA generated from ionic surfactants. Two different separation strategies were followed: (i) separation of lactoferrin and lactoperoxidase by anionic CGA generated from a solution of sodium bis-(2-ethyl hexyl) sulfosuccinate (AOT); (ii) separation of β-lactoglobulin by cationic CGA generated from a solution of cetyltrimethylammonium bromide (CTAB). Separation results indicate that electrostatic interactions are the main forces determining the selectivity however these could not completely explain the selectivities obtained following both strategies. Protein–surfactant interactions were studied by measuring the zeta potential changes on individual proteins upon addition of surfactant and at varying pH. Interestingly strongest electrostatic interactions were measured at those pH and surfactant to protein mass ratios which were optimum for protein separation. Effect of surfactant on protein conformation was determined by measuring the change in fluorescence intensity upon addition of surfactant at varying pH. Differences in the fluorescence patterns were detected among proteins which were correlated to differences in their conformational features which could in turn explain their different separation behaviour. The effect of conformation on selectivity was further proven by experiments in which conformational changes were induced by pre-treatment of whey (heating) and by storage at 4 °C. Overall it can be concluded that separation of proteins by ionic CGA is driven mainly by electrostatic interactions however conformational features will finally determine the selectivity of the separation with competitive adsorption having also an effect.

Keywords: Colloidal gas aphrons; Whey proteins; Ionic surfactants; *z*-Potential; Fluorescence; Protein–surfactant interactions; Competitive adsorption

1. Introduction

Colloidal gas aphrons (CGA) are microbubbles (10–100 μm) created by intense stirring of a surfactant solution. These microbubbles as first described by Sebba [1] are composed of a surfactant film and a third surfactant layer (Fig. 1). Further works on their characterisation and elucidation of their structure support this structure [2] however there are no conclusive evidence yet that confirm the multi-layer structure. CGA have been characterised for different types of surfactants, i.e., ionic and nonionic and found that they possess very attractive features particularly for applications in bioseparations: (i) large interfacial area due to their small sizes; (ii) relative stability; once generated and stirring is stopped CGA separate within minutes into two phases without mechanical aid; (iii) their surface properties and hence selectivity of adsorption can be modified by changing the type of surfactant; this is a very important feature which holds regardless the structure.

During the last decade there has been growing interest on the application of CGA to the recovery of proteins. Research efforts on this particular application are still at an early stage as most of the comprehensive studies are limited to pure protein solutions. Jauregi and co-workers [3–5] and Noble and Varley [6] have carried out detailed lab-scale recovery of proteins from single protein model solutions using CGA generated from sodium bis-(2-ethyl hexyl) sulfosuccinate (AOT). Ionic surfactants have

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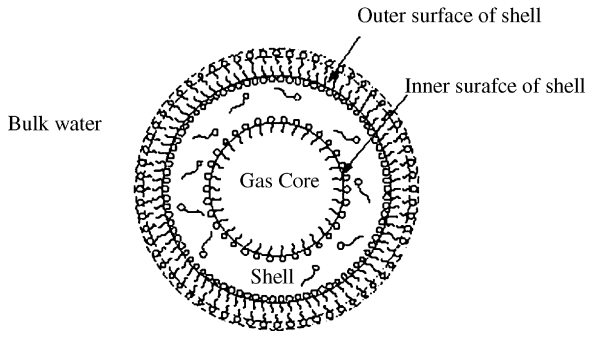

Fig. 1. Proposed structure of CGA by Sebba [1].

shown higher protein enrichment and recovery than nonionic surfactants [5]. A couple of studies have been reported on the selective recovery of a protein from a crude extract using the nonionic surfactant Triton X-114 [7,8] and separation of proteins from whey using ionic CGA [9,10] and nonionic CGA [11]. These studies demonstrate the potential of CGA for protein recovery however there is a lack of understanding of the mechanism of the separation and particularly as to what determines the selectivity of the separation.

In the present work a detailed study on the mechanism of separation of proteins from a complex mixture such as whey using CGA generated from ionic surfactants is presented. Whey is an ideal complex mixture to investigate this given the number of proteins with wide range of physicochemical properties such as size, charge and hydrophobicity (Table 1). The major proteins have acidic isoelectric points whilst the minor proteins which also possess additional biological functionalities, have basic isoelectric points. Therefore the separation of these proteins could be carried out based on differences in surface charge such as by oppositely charge ion exchangers or by ionic CGA. The purpose of the study is to investigate the factors affecting selectivity of the separation of proteins with CGA generated with ionic surfactants and elucidate a general mechanism. The study is based on initial work carried out by the authors following two different separation strategies: (1) separation of minor whey proteins by anionic CGA, i.e., CGA generated from an anionic surfactant AOT [9] and separation of major whey proteins using cationic CGA, i.e., CGA generated from a cationic surfactant, cetyltrimethylammonium bromide (CTAB) [10]. In these works it was demonstrated that CGA are analogous to ion exchangers in terms of the separation principle and therefore the selectivity can be manipulated by changing the type of surfactant, pH and ionic strength. Yet from these studies it is concluded that the selectivity is not solely determined by electrostatic interactions between protein molecules and surfactant molecules in the CGA phase. Here a further insight into the mechanism of the separation is taken by investigating surfactant–protein interactions using zeta potential and fluorescence techniques and also protein–protein interactions are investigated to account for competitive protein adsorption effects. This together with the results on separation leads to an improved understanding of forces driving the separation and a general mechanism of separation of proteins using ionic CGA is drawn.

Table 1
Comparison of protein separation performance between fresh rennet whey and whey powder concentrate (WPC 75)

Protein	Fresh rennet whey (%)	WPC 75 Volac (%)
Lf–Lp	5.5 ± 0.1	14.6 ± 1.0
BSA	10.9 ± 0.2	13.3 ± 7.5
Ig	1.8 ± 0.5	11.8 ± 3.7
β-Lg	86.9 ± 0.6	36.2 ± 4.8
α-La	7.5 ± 0.7	19.5 ± 3.3

CGA separation was conducted at pH 8 and $M_{CTAB}/M_{TP} = 0.3$. Results are expressed in protein recovery in the aphron phase.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade. Glacial acetic acid, anhydrous sodium acetate, sodium dihydrogen orthophosphate dihydrate, di-sodium hydrogen orthophosphate anhydrous and sodium chloride were supplied by BDH (UK). Chymax® was purchased from CHR Hansen. Methanol and isopropanol were purchased from Rectapur™ (Prolabo, Merck). Acrylamide/bis-acrylamide (29:1) solution, β-mercaptoethanol, bromophenol blue, cetyltrimethylammonium bromide, chrome azurol S (CAS), ferric oxide, glycine, methylene blue, sodium dodecyl sulphate, sulfosalicylic acid, temed, trizma® base, trichloroacetic acid, low range molecular weight marker M3913 (6500–66,000 Da), protein standards (α-lactalbumin, β-lactoglobulin, BSA, lactoferrin and lactoperoxidase from bovine milk) were obtained from Sigma Chemical Company. The cheesecloth Miracoth™ was purchased from Calbiochem® (Prolabo, Merck). The laboratory mixer (SL2T) fitted with a four bladed impeller ($D = 30$ mm) surrounded by a high shear screen and with a digital readout impeller speed was supplied by Silverson Ltd. (Waterside, Bucks, UK). SDS–PAGE was performed using an Atto® Dual Mini Slab electrophoresis kit (gel size = 90 mm × 80 mm × 1 mm thick) and a power supply PS 304 Minipac II Apelex supplied by Genetic Research Instrumentation, Dunmow, Essex, UK. Zeta potential was measured using a ZetaMaster (Malvern Instruments Ltd.). Fluorescence was measured with Perkin-Elmer LS5 fluorimeter. The spectrophotometer Ultrospec 1100 pro was purchased from Amersham Pharmacia Biotech (Biochrom Ltd., Cambridge, UK).

2.2. CGA generation

Colloidal gas aphrons were created by stirring 400 ml of buffered AOT solutions (0.025 mol/l CH_3COOH/CH_3COO^- for pH 3, 4 and 5, 0.025 mol/l, 0.020 mol/l and 0.010 mol/l Na_2HPO_4/NaH_2PO_4 for pH 6, 7 and 8, respectively) and CTAB buffered solutions (0.1 mol/l $CH_3CO_2H/NaCH_3CO_2$ for pH 4, 0.015 mol/l Na_2HPO_4/NaH_2PO_4 for pH 6, and 0.0625 mol/l Tris–HCl for pH8) at 8000 rpm for 10 min at room temperature using a high-speed impeller as described previously by Jauregi et al. [3].

2.3. Whey preparation

Sweet whey was prepared with fresh whole pasteurised and standardised milk. The milk was heated up to 37 °C. Then 1 ml of a 0.1% (v/v) of commercial rennet (Chymax®) solution was added to the milk and stirred gently for 5 min. Casein coagulation was performed for 1 h. The curd was then cut in order to drain the lactoserum. Incubation was prolonged for another 30 min after which the whey was filtered using cheesecloth. Remaining protein particles and fat were eliminated by centrifugation for 30 min at 10,000 rpm and 4 °C. Whey was then filtered through Whatman® paper no. 4. Whey was stored at −18 °C and used in subsequent experiments.

2.4. Batch recovery of proteins using ionic CGA

CGA are generated as described above and once stirring is stopped liquid within the gas–liquid dispersion starts to drain which results in a CGA top phase and liquid bottom phase. When half of the initial volume of liquid drains a given volume of aphrons is added to a beaker containing a given volume of whey. The mixture is stirred gently for 5 min using a magnetic stirrer and then allowed to separate for 1 min. The liquid phase is removed by pipette and the aphrons are collapsed by intense stirring for about 10–30 min at room temperature. In experiments with CGA generated with CTAB (CGA (CTAB)) a precipitate is formed in the CGA phase and therefore the liquid from CGA phase is allowed to drain completely by storing the mixture at 4 °C for approximately 4 h. This results in two distinct fractions being separated, the CGA phase resulting in a white wet precipitate and the liquid phase. Surfactant and protein determination were carried out on the liquid phase. Protein concentration was determined by SDS–PAGE and image analysis [9] and surfactants concentration were determined following colorimetric assays as described by Fuda et al. [9] for AOT and Fuda et al. [10] for CTAB.

2.5. Study of protein–surfactant interactions

Interactions between CGA and whey proteins were followed by analysing the intrinsic fluorescence and zeta potential of individual whey proteins in the absence and presence of AOT. The influence of main operating parameters such as pH, ionic strength and surfactant to protein mass ratio (MAOT/MTP) on the interactions was also investigated.

(a) z-Potential: effect of surfactant on protein overall surface charge as a measure of strength of interaction.
(b) Fluorescence: effect of surfactant on protein conformation.

2.5.1. Zeta potential

Electrophoretic mobility was measured at 25 °C using a Zeta-Master (Malvern Instruments Ltd.). Protein samples were prepared as a 2 μmol/l for α-La, β-Lg and BSA and 0.3 μmol/l for Lf and Lp in buffered solutions (0.1 mol/l CH_3COOH/CH_3COO^- for pH 4, 0.015 mol/l Na_2HPO_4/NaH_2PO_4 for pH 6, and 0.0625 mol/l Tris–HCl for pH 8). Solutions were incubated with increasing concentration of AOT in a 3.5 ml final volume, for 2–3 h at room temperature. The effect of increasing ionic strength on protein–AOT complex charge was investigated for each condition in the presence of 0.2 mol/l NaCl. Measurements were performed in triplicates for two samples. Zeta potential was determined from the electrophoretic mobility using the Smoluchowski equation [12]:

$$\zeta = \frac{u_E \eta}{\varepsilon} \quad (1)$$

where u_E is the electro-osmotic mobility, ε the permittivity of the dielectric, η the viscosity and ζ the zeta potential.

2.5.2. Fluorescence spectroscopy

The binding of AOT to the individual whey proteins and its effect on protein conformation is measured by the change in the intrinsic fluorescence intensity of protein tryptophan residues before (F_0) and after (F) the addition of surfactant. To a 4 ml cuvette appropriate amount of AOT and whey protein (β-Lg, α-La, BSA, Lf or Lp) were added and diluted to 3.5 ml with buffered solutions (0.1 mol/l CH_3COOH/CH_3COO^- for pH 4, 0.015 mol/l Na_2HPO_4/NaH_2PO_4 for pH 6, and 0.0625 mol/l Tris–HCl for pH8). Solutions were incubated for at least 2 h and fluorescence intensity was measured using a Perkin-Elmer LS300 spectrofluorimeter. Protein concentrations were 2 μmol/l for α-La, β-Lg and BSA and 0.3 μmol/l for Lf and Lp in buffered solutions. Fluorescence intensities of β-Lg and α-La were obtained at excitation and emission wavelength of $\lambda_{ex} = 280$ nm and $\lambda_{em} = 335$ nm (slit 5/2.5). Fluorescence intensities of BSA were obtained at excitation and emission wavelength of $\lambda_{ex} = 280$ nm and $\lambda_{em} = 342$ nm (slit 15/2.5). Fluorescence intensities of Lf were obtained at excitation and emission wavelength of $\lambda_{ex} = 284$ nm and $\lambda_{em} = 348$ nm (slit 2.5/10). Fluorescence intensities of Lp were obtained at excitation and emission wavelength of $\lambda_{ex} = 287$ nm and $\lambda_{em} = 342$ nm (slit 2.5/10). Experiments were performed in duplicates.

2.6. Measurement of changes in whey composition upon storage

Whey was stored in 40 ml fractions at 4 °C and −18 °C. Samples kept at −18 °C were defrosted overnight at 4 °C before use. After 0, 7, and 14 days of storage whey pH and composition were determined and direct precipitation experiments were conducted at various CTAB to protein mass ratios. Results were compared to control experiments performed at day 0 with fresh whey.

3. Results and discussion

3.1. Separation of proteins with CGA (AOT)

The separation strategy followed with CGA (AOT) is described in Fig. 2 with Lf–Lp being the target products. Results obtained in the separation of proteins with CGA (AOT) at varying pH (Fig. A.1-Appendix) and varying ionic strength [9] indicate that electrostatic interactions are the driving force of

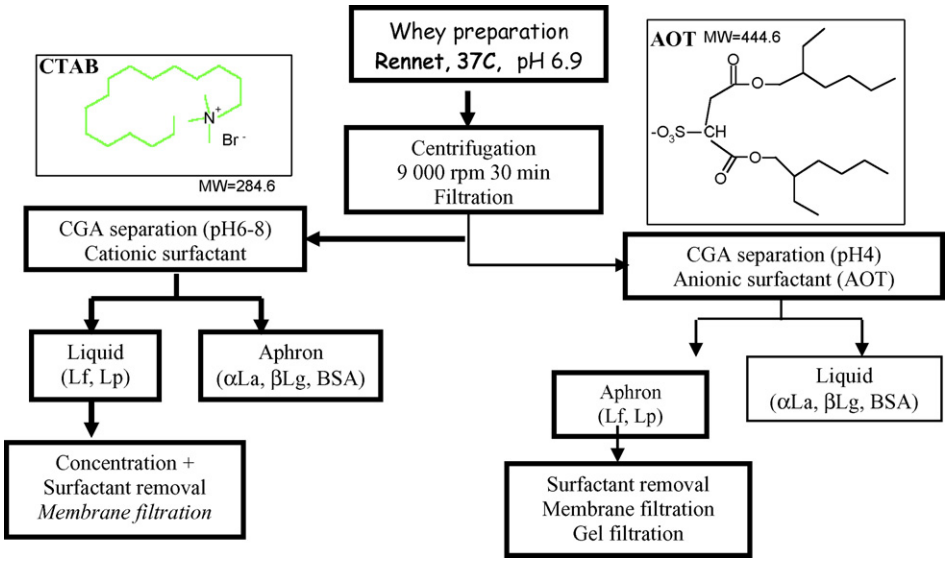

Fig. 2. Schematic diagram of the two separation strategies followed with the cationic surfactant CTAB and anionic surfactant AOT.

the separation. Maximum recovery of Lf–Lp were obtained at the lowest pH and ionic strength whilst maximum purity was obtained at the highest pH and ionic strength. Therefore best separation is obtained at conditions that favour strong electrostatic interactions between these proteins and AOT molecules in the CGA (Fig. A.2-Appendix). At such conditions purity was low as other major proteins such as β-Lg, which accounts for 50% of total protein, are even more strongly charged than target proteins (Fig. A.2-Appendix). In order to establish what determines the selectivity of the separation a further insight is taken into individual protein–surfactant interactions and the effect of competitive adsorption among the minor and major proteins is also considered.

3.1.1. Competitive adsorption

To explain the role of protein competition on the selectivity the effect of β-Lg on the separation of the Lf–Lp fraction was investigated using model mixtures: (i) binary mixture, composed of lactoferrin and lactoperoxidase (Lf–Lp) and (ii) ternary mixture composed of lactoferrin, lactoperoxidase and β-lactoglobulin (Lf–Lp–β-Lg). The strong dependence of recovery of Lf–Lp fraction on pH is clearly observed in both mixtures (Fig. 3) with the highest recoveries (82%) being achieved at low pHs where the difference in charge between proteins and CGA is the largest and lower recoveries are achieved at basic pHs, e.g., 37% at pH 8. Recovery of Lf–Lp is lower when mixed with β-Lg than in the pure mixture over all pH range which shows clearly that there is a competitive effect. Interestingly the strongest competitive effect is observed at pH 5. At this pH, according to surface charge characteristics of these proteins (Fig. 2), similar recovery of Lf–Lp to that obtained at lower pHs should be expected as observed in the pure mixture (Fig. 3) and moreover separation of β-Lg into CGA phase should not be favoured as it is strongly negatively charged. Thus it can be concluded that the adsorption of Lf–Lp to CGA is reduced by the dominant β-Lg molecules in solution and although electrostatic interactions seem to be the driving force of the separation the selectivity cannot be completely explained based on differences in surface charge. In order to confirm this further interactions between individual proteins and AOT molecules were investigated by *z*-potential and fluorescence measurements.

3.1.2. Protein–surfactant interactions

In the present section the strength of interaction between individual whey proteins and AOT is studied in more detail using single protein solutions. Changes induced on zeta potential and Trp fluorescence upon binding of AOT to Lf, Lp and β-Lg were used to assess the magnitude of interaction of these proteins and CGA. Similar patterns of interaction were found between both minor whey proteins (Lf and Lp) and AOT molecules and the major whey proteins (β-Lg) and AOT molecules, according to the zeta potential measurements (Figs. 4 and 5). The positive charges of Lf, Lp and β-Lg were significantly reduced upon addition of AOT at pH 4 whilst as expected, the negative charges

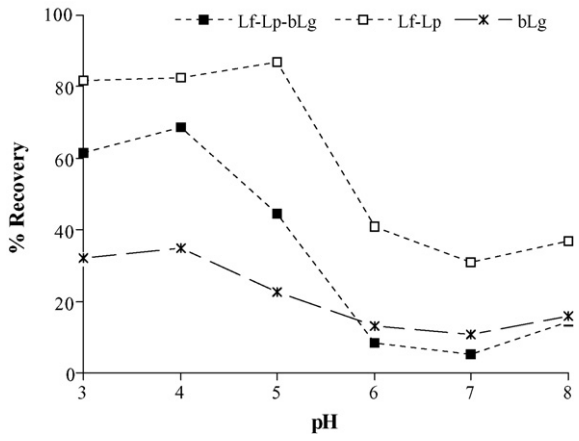

Fig. 3. Recovery of the Lf-Lp fraction from (■) Lf-Lp-βLg mixture and (□) Lf-Lp mixture (✻) Recovery of βLg from Lf-Lp-βLg mixture. Experiments were performed at [NaCl] = 0 mol/l.

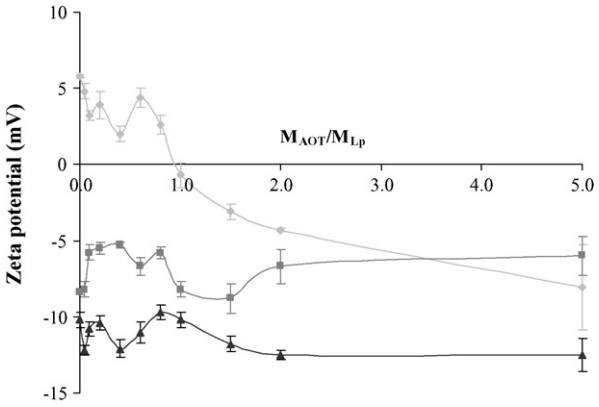

Fig. 4. Zeta potential of Lp-AOT complex as a function of mass ratio of AOT to Lp (M_{AOT}/M_{Lp}) measured at varying pH: 0.1 mol/l sodium acetate buffer pH 4 (♦), 0.015 mol/l sodium phosphate pH 6 (■) and 0.0625 mol/l Tris/HCl pH 8 (▲).

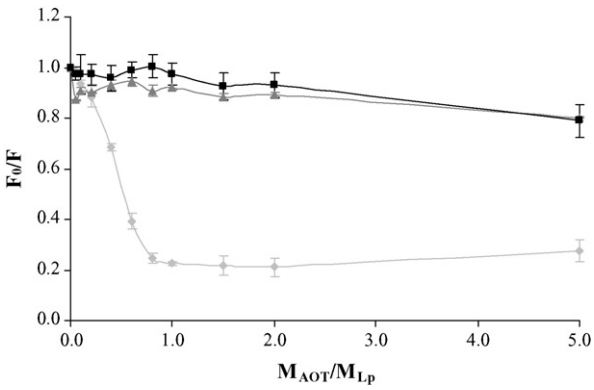

Fig. 6. Intrinsic fluorescence intensity of Lp before (F_0) and after (F) the addition of AOT as a function of the mass ratio of AOT to Lp (M_{AOT}/M_{Lp}) measured at varying pH: 0.1 mol/l sodium acetate buffer pH 4 (♦), 0.015 mol/l sodium phosphate pH 6 (▲) and 0.0625 mol/l Tris/HCl pH 8 (■).

of the proteins at pH 6 and 8 remained almost constant upon addition of the negatively charged AOT. On the other hand, a considerable enhancement of Trp fluorescence, i.e., reduction in F_0/F was observed on AOT addition at pH 4, while no significant changes were detected at pH 6 and 8 for all proteins (Figs. 6 and 7). However at pH 4 a deeper drop of F_0/F was observed for Lf (from 1 to 0.3) and Lp (from 1 to 0.2) than for β-Lg (from 1 to 0.6) at equivalent M_{AOT}/M_{TP}. This suggests that the interaction between AOT molecules and Lf, Lp is stronger than between AOT and β-Lg molecules which results in significant conformational changes and this could explain the better separation obtained for Lf–Lp than for β-Lg at pH 4 (Fig. 3). The addition of 0.4 mol/l NaCl results in a significant decrease in the magnitude of both zeta potential and fluorescence measurements for all proteins (results not shown here). This shows an attenuation of the interaction between AOT and whey proteins, and further reinforces the concept that electrostatic interactions play an important role in the binding of AOT to proteins. Therefore Lf, Lp and β-Lg interact similarly with AOT head groups. These interactions can be correlated with the separation behaviour of proteins into the aphron phase (surfactant-rich phase). At pH 4 all the proteins are positively charged and will establish attractive electrostatic interactions with the anionic AOT molecules. Under these conditions there is no selective distinction between the proteins for the binding sites on AOT. Moreover protein competition causes a reduction in the selectivity of separation. Therefore following this approach only poor selectivity of separation could be achieved under optimal conditions thus the reverse separation strategy was investigated with CGA (CTAB) in which the major proteins become the target proteins (Fig. 2).

3.2. Separation of proteins with CGA (CTAB)

Results obtained following this separation strategy (Fig. A.3-Appendix) support once more the initial hypothesis that main forces driving the separation are electrostatic and that CGA act in that respect as ion exchangers At optimum conditions such as, pH 8, low ionic strength, and $M_{CTAB}/M_{Tp} = 0.4$ β-Lg was selectively recovered in the CGA and in the form of an insoluble precipitate [10]. This leads to improve drainage of liquid within the CGA phase which contains 'contaminant' proteins and thus higher selectivity is achieved. Interestingly both BSA and α-La

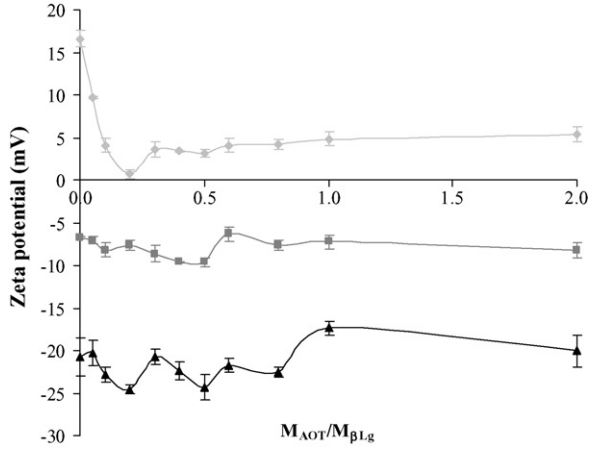

Fig. 5. Zeta potential of βLg-AOT complex as a function of mass ratio of AOT to βLg ($M_{AOT}/M_{\beta Lg}$) measured at varying pH: 0.1 mol/l sodium acetate buffer pH 4 (♦), 0.015 mol/l sodium phosphate pH 6 (■) and 0.0625 mol/l Tris/HCl pH 8 (▲).

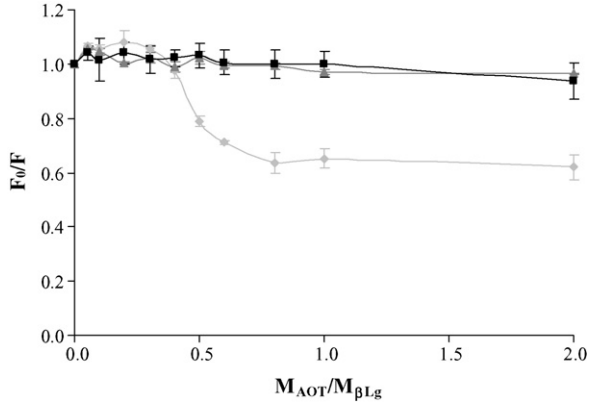

Fig. 7. Intrinsic fluorescence intensity of βLg before (F_0) and after (F) the addition of AOT as a function of the mass ratio of AOT to βLg ($M_{AOT}/M_{\beta Lg}$) measured at varying pH: 0.1 mol/l sodium acetate buffer pH 4 (♦), 0.015 mol/l sodium phosphate pH 6 (▲) and 0.0625 mol/l Tris/HCl pH 8 (■).

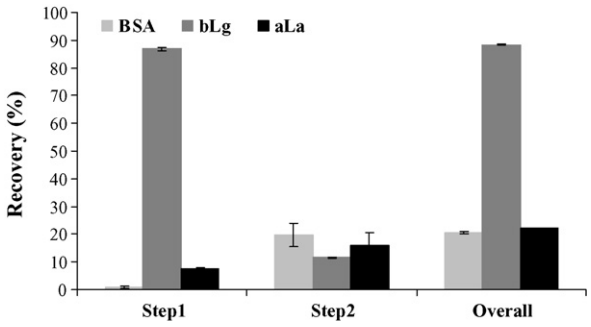

Fig. 8. Comparison of BSA ( ), βLg ( ) and αLa ( ) recovery from whey after two step CGA separation.

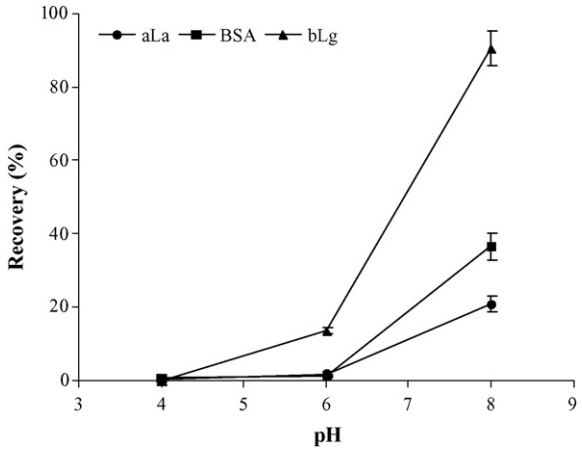

Fig. 9. Recovery of αLa (•), BSA (■) and βLg (▲) from binary protein mixtures containing αLa + βLg and BSA + βLg.

were found mostly in the liquid phase despite having surface charge characteristics similar to those of β-Lg (Fig. 2). Further work was carried out in order to assess if this was partly due to a competitive effect as found in the CGA separation with AOT (see above).

3.2.1. Competitive adsorption between β-Lg, α-La and BSA

A two-step separation was carried out in order to remove in the first step most of the β-Lg and in this way reduce any competitive effect and increase recovery of α-La and BSA in the CGA phase. A slight increase in the recovery of BSA (20%) and α-La (22%) in the second step after prior removal of β-Lg (Fig. 8) suggests that there is some competitive effect. Further work was carried out with model mixtures in order to assess better the effect of competitive interaction on the selectivity of the separation. Experiments were performed with single (BSA, α-La or β-Lg), binary (BSA–α-La, BSA–β-Lg, α-La–β-Lg) and ternary (α-La–BSA–β-Lg) protein solutions prepared with pure commercial proteins. Experiments conducted with single and binary solutions containing only BSA and/or α-La did not lead to any precipitation and less than 15% recovery of each of these proteins was achieved at optimum conditions which favour electrostatic interactions. With single, binary and ternary solutions containing β-Lg a precipitate was formed in the aphron phase at conditions favouring electrostatic interactions, i.e., pH 6 and 8; almost 90% β-Lg and up to 40% BSA and 35% α-La were recovered at pH 8 (Fig. 9). These results clearly show that protein–protein interactions occur between albumins and

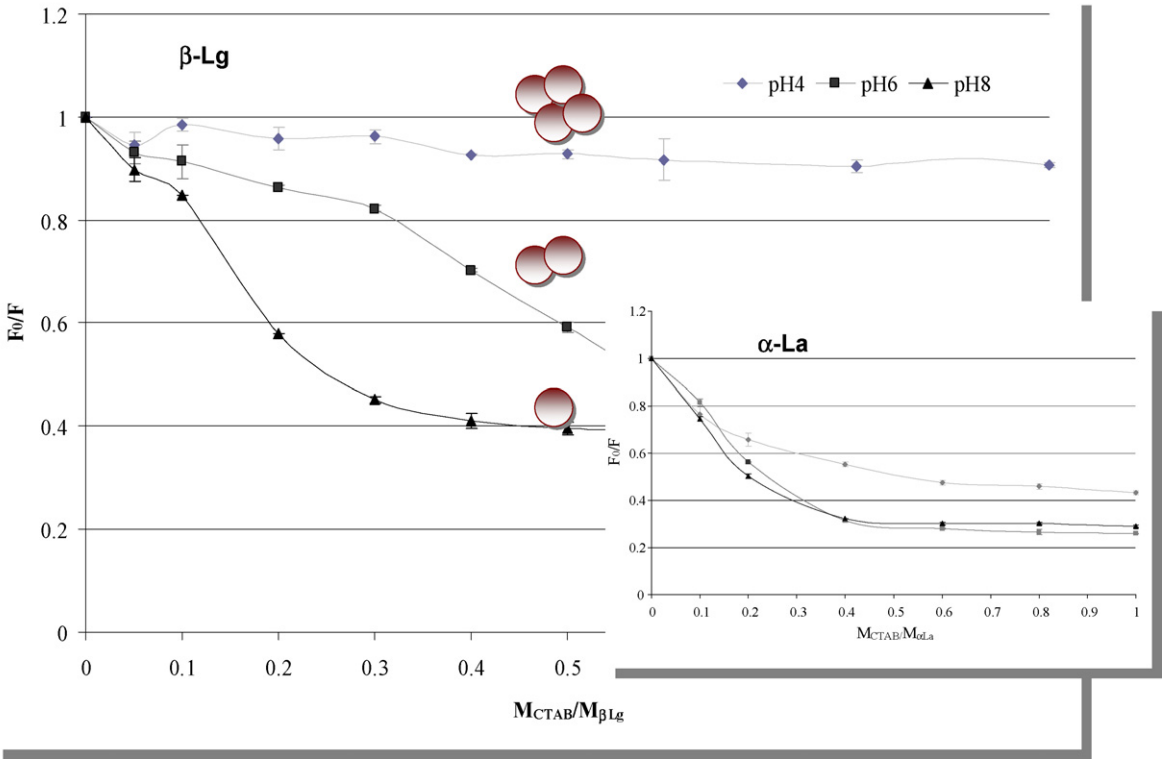

Fig. 10. Comparison of fluorescence intensity patterns followed by αLa and βLg upon addition of CTAB and varying pH.

β-Lg, causing the formation of aggregates; proteins are more susceptible to aggregate in these model solutions than in whey as the lactose and minerals normally stabilising the protein structure are depleted. These results confirm that separation of α-La and BSA into the aphron phase is not exclusively driven by electrostatic interactions as found in a previous study by the authors with CGA (CTAB) [10]. Proteins with similar charges may compete for cationic binding sites on CGA however protein competition explains only partially the poor separation of α-La and BSA as effective recovery does not occur in the absence of β-Lg. Fluorescence measurements for β-Lg, α-La and BSA indicate that protein conformation is also affected by increasing CTAB concentration. The surfactant effect on protein conformation increases with pH as shown by differences in fluorescence emission patterns (Fig. 10). However binding of CTAB to albumins does not differ substantially at pH 6 and 8 and similar binding trends were observed for all pH conditions. Differences observed in the shape of the fluorescence titration curves indicate that these proteins interact differently with the surfactant and could most likely be explained by conformational differences. In fact, BSA and α-La are globular monomeric proteins while bovine β-Lg exists as a dimer of two identical subunits at neutral pH [13]. The sigmoidal shape of the titration curve of β-Lg by CTAB at pH 6 is characteristic of a cooperative interaction between CTAB and β-Lg. This cooperative effect is less noticeable at pH 8 as the dissociation of the dimer is predominant at this pH. A number of studies have reported the effect of pH on β-Lg denaturation, showing that denaturation occurs through an initial dissociation of dimer to monomer followed by a change in the polypeptide chain conformation, and subsequent aggregation [13]. The interaction between CTAB and β-Lg seems to follow this sequence and yields insoluble aggregates which are difficult to re-dissolve with urea, guanidinium hydrochloride, acidic or alkaline treatment. The interaction with the other negatively charged proteins is milder as it does not lead to precipitation. Thus this could explain the differences in selectivity: in a first stage proteins interact similarly with CTAB by electrostatic interactions but the strength of these interactions is dependent on their conformational properties and their tendency to denaturation. In order to prove this further a set of experiments were carried out in which changes in protein conformation were induced prior to separation with CGA.

3.3. Protein conformational changes induced by processing and their effect on selectivity

Various processing treatments during the manufacture can affect the functional properties of whey protein products [14] and hence their separation behaviour. Separation experiments were performed with a 2.5% (w/v) whey protein concentrate sample and results were compared to those obtained with fresh rennet whey. Interestingly at similar pH and M_{CTAB}/M_{TP} no precipitation of β-Lg was observed when whey protein concentrates were used as the starting material, and thus lower separation of β-Lg in the aphron phase ($36.2 \pm 4.8\%$) was achieved (Table 1). Moreover the recovery of the other proteins is slightly higher, which could be due to the presence of aggregates in the sample. The manufacture of whey powders involves pasteurisation. When whey proteins have been heated to a high temperature, they unfold partially, and the hydrophobic amino acid residues normally buried within the native structure are exposed, resulting in an increased reactivity of such groups [14]. Therefore, this once again favours hydrophobic interactions between proteins and CGA generated with ionic surfactants and results in a poor separation as observed previously with fresh whey at high ionic strength. This further confirms that first contact between proteins and surfactant molecules occurs via electrostatic interactions which are important in modulating the first stage of the selectivity of separation; if these interactions are hindered prior to contact with CGA as for example when protein is in denatured "hydrophobic" form, poor separation will be achieved.

3.4. Protein conformational changes upon storage and their effect on selectivity

Protein separation experiments were performed with fresh whey and with whey stored for 14 days at 4 °C and at −18 °C in order to study the effect of conformational changes on selectivity of separation. Selectivity of the separation was similar for experiments carried out with fresh whey and whey stored for 14 days at −18 °C however separation carried out with whey stored for 14 days at 4 °C resulted in the formation of a precipitate in the aphron phase containing mainly β-Lg but a larger proportion of BSA than with the other whey samples (Table 2). Thus this variation in the selectivity of precipitation could be due to changes in whey composition occurring during storage at 4 °C during 14 days which will lead to conformational changes. As summarized in Table 2, the major physico-chemical changes measured at this storage conditions are a reduction in pH from 6.7 to 4.3 and a reduction in lactose content as a result of lactose degradation into lactic acid. At this pH α-La is close to its p*I* at which protein structure is prone to destabilisation [15]. BSA structure is also prone to destabilisation around this pH. Therefore both albumins co-precipitate with β-Lg in the presence of a precipitating agent such as CTAB. Several studies have reported the effect of pH on conformational changes of whey proteins. For instance studies reported by Kronman [16] found that under acidic conditions α-La undergoes a trans-conformation to a nonnative state with altered spectroscopic properties. The selective precipitation of α-La at pH around its p*I* (4.2) under gentle heat treatment is governed by the protein–calcium complexation equilibrium [15]. Instability of BSA at acidic pH characterized by a conformational change to a more open form in the pH range 3.6–4.0 has also been reported and at more alkaline conditions BSA becomes more globular [17]. Other studies have reported the stabilizing effect of lactose on whey proteins [18]. In order to establish if the variation in selectivity is due to a pH effect, a lactose effect or a combine effect of these two factors a new set of experiments were carried out with acid whey As shown in Table 2, lactose content and pH remained stable upon storage of acid whey at 4 °C for 14 days. This pH was equal to that observed with rennet whey stored at 4 °C for 14 days. If compared separations obtained with both fresh whey samples it is clear that similar selectivities are obtained despite of the different pH. Thus pH

Table 2
Comparison of separation performance between rennet whey and acid whey

	Rennet whey			Acid whey	
	Fresh	14 days (4 °C)	14 days (−18 °C)	Fresh	14 days
pH	6.7	4.3	6.6	4.7	4.6
Lactose content (w/w, %)	74.5 ± 0.3	65.0 ± 0.1	72.5 ± 0.1	68.0 ± 0.1	64.0 ± 0.1
Protein content (w/w, %)	15.1 ± 0.3	20.2 ± 0.1	15.4 ± 0.1	12.5 ± 0.1	15.5 ± 0.1
Lp activity (mmol/min)	1605	78	1371	1430	442
Protein recovery (%)					
Lf–Lp	5.2 ± 0.3	10.6 ± 1.4	8.9 ± 0.7	9.3 ± 4.1	10.1 ± 0.3
BSA	13.4 ± 1.1	42.6 ± 2.8	18.2 ± 1.8	11.4 ± 0.4	28.3 ± 4.2
Ig	3.8 ± 0.2	11.8 ± 0.5	8.1 ± 0.5	12.7 ± 0.2	6.7 ± 0.2
β-Lg	85.3 ± 2.8	94.0 ± 5.9	76.5 ± 6.1	97.3 ± 1.3	77.3 ± 1.4
α-La	21.2 ± 5.3	56.3 ± 4.1	25.9 ± 2.4	27.3 ± 0.3	39.9 ± 0.8

CGA separation was conducted at pH 8 and $M_{CTAB}/M_{TP} = 0.3$.

alone is not the cause of the variation in selectivity. When compared separations obtained with rennet and acid whey samples stored during 14 days results are also similar. These results indicate that conformational changes and resulting differences in selectivity are mainly due to a stabilising effect of lactose on the albumins, thus reduction in lactose results in increase reactivity of these proteins to surfactant denaturation. Observations from these last experiments proved clearly that preconditioning of the protein, i.e., changes induced in protein conformation, would affect considerably the way in which the protein interacts with CGA. Lander et al. [19] developed the same hypothesis to explain the selective precipitation of plasmid DNA from proteins, RNA, and endotoxin using CTAB solution. Their research lead to the conclusion that the mechanism of selectivity is most likely based upon conformational differences among the several forms of DNA

4. Final conclusions: general mechanism of separation of proteins with ionic surfactants

- First contact between proteins and surfactant molecules in CGA occurs by electrostatic interactions; promoting hydrophobic interactions did result in poor recoveries as shown by a significant decrease in recovery on those experiments carried out at high ionic strength and in those experiments where pre-treatment led to protein denaturation with the subsequent exposure of hydrophobic groups.
- Strength of interaction between protein and surfactant molecules is dependent on the conformational features of the protein and the extent to which these are affected upon interaction with the surfactant.
- Selectivity is enhanced by formation of aggregates and their subsequent flotation into the aphron phase; formation of aggregates maximises drainage which enables effective removal of contaminants into the liquid phase. Poor selectivity with CGA (AOT) partly because separated CGA phase contains also 'contaminant' proteins which although not interacting as strongly with AOT as major proteins as shown by *z*-potential and fluorescence measurements they become trapped in the liquid between the aphrons within the CGA phase.
- A very interesting finding is that the selectivity of the separation and therefore the composition of the recovered fractions can be manipulated by changing the conformation of some of the proteins, i.e., conformation determines selectivity. As observed with experiments performed with CGA generated with AOT, at conditions leading to high recovery, all the whey proteins were in a "stable form" and therefore no such difference in the selectivity of separation were observed. On the contrary when CGA are generated with CTAB, the highest selectivity was achieved at conditions at which β-Lg was susceptible to endure denaturation.
- Protein–protein interaction and competitive adsorption of proteins to CGA has also an effect on the selectivity.

In summary, in order to achieve effective recovery of proteins using ionic CGA first the solutes to be separated need to interact with the surfactant ionic groups and this occurs under conditions favouring electrostatic interactions between protein and surfactant molecules. Secondly, the formed surfactant–protein complexes need to be floated into the aphron phase. According to zeta potential and fluorescence measurements of individual proteins and CTAB the first electrostatic contact occurs for all the proteins, therefore the limiting step seem to be the flotation. In the case of β-Lg flotation occurs because the interaction with CTAB leads to an opening of the protein structure with the subsequent exposure of the protein hydrophobic core. This is followed by nonspecific and cooperative binding at higher surfactant concentrations dominated by hydrophobic forces and causes the precipitation of the molecule. On the other hand for α-La and BSA the interaction with CTAB is not as strong as that between CTAB and β-Lg and no visible denaturation, i.e., precipitation is induced therefore limited flotation occurs hence lower recovery in the aphron phase. This proves that changes in protein conformation and thus modification of protein surface properties upon interaction with CTAB are key factors in the selectivity of separation. This is further proven in the separation of LfLp with CGA (AOT). Interactions between LfLp and AOT

molecules are strong as demonstrated by *z*-potential and fluorescence measurements yet induced conformational changes are not strong enough to cause denaturation and subsequent precipitation which resulted in lower selectivity. Also in this separation was demonstrated that increased drainage, as for instance by increasing ionic strength, leads to higher selectivity since 'contaminant' proteins drain into the liquid phase; highest selectivity is achieved with maximum drainage of CGA when protein precipitates in the CGA (CTAB) phase. From the above observations it can be concluded that CGA based separations are more effective when applied to the recovery of particles than the recovery of soluble compounds. CGA generated from ionic surfactants have been successfully applied to the separation of bacteria, yeast or algae suspensions and fine particles. They have been used efficiently for the recovery of heavy metals, but again, the interaction between the microbubbles and the compound led to the formation of a precipitate that could be floated into the aphron phase.

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Appendix

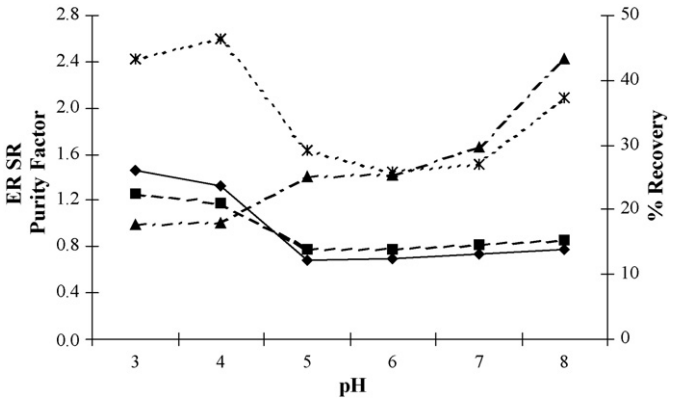

Fig. A.1. Separation ratio (♦) and enrichment ratio (■) and purity factor (▲) and % recovery (✻) of the LF-LP fraction as a function of pH. Experiments were carried out.

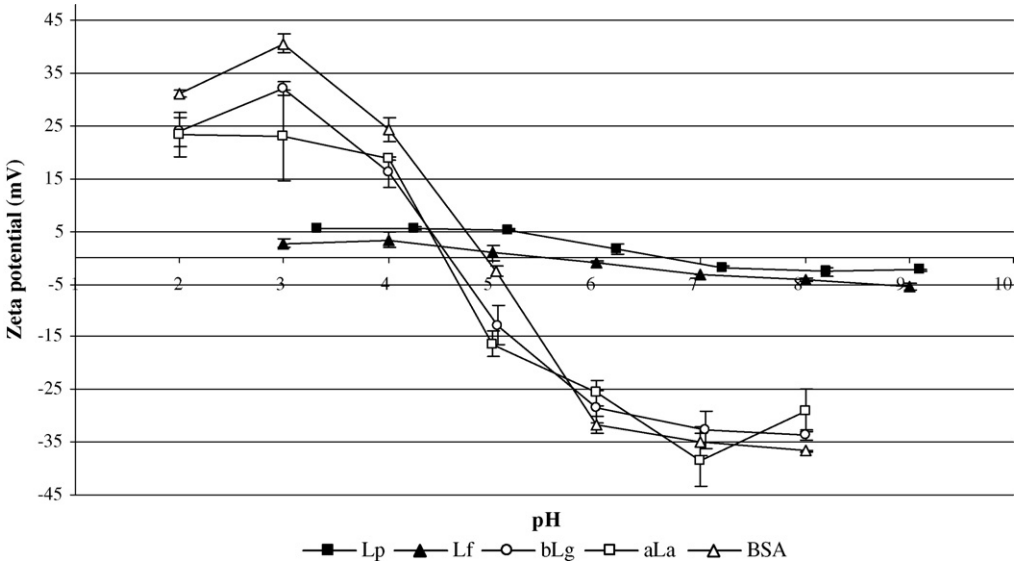

Fig. A.2. Titration curves of whey proteins determined by z-potential measurements at pH 4, IS = 0.1 mol/l (corresponding to 0 mol/l^{-1} NaCl), Cs = 0.84 mmol/l, V_w = 1 ml and V_{CGA} = 20 ml. Adapted from Fuda et al. [9].

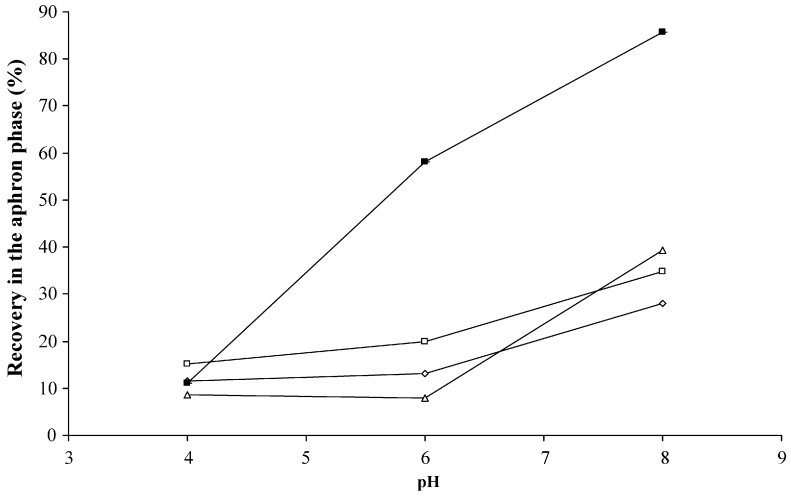

Fig. A.3. Recovery of β-Lg using CGA (CTAB) at surfactant to protein ratio = 0.3 and ionic strength = 0.018 M. β-Lg (■), α-La (□) , Lf Lp (◊), BSA (△). Adapted from Fuda et al. [11].

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