Extraction of $\beta$-Lactoglobulin from Bovine Milk by Affinity Counter-Current Distribution in Aqueous Two-Phase System

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ABSTRACT

The counter-current distribution pattern of milk and whey $\alpha$-lactalbumin and $\beta$-lactoglobulin in a two-phase system containing 7% (wt/wt) Dextran T-500 and 5% (wt/wt) polyethylene glycol 6000 was investigated. Both proteins showed similar distribution profiles and were clearly separated from the main bulk of milk protein. The use of polyethylene glycol bound to a hydrophobic ligand (palmitate polyethylene glycol) enhanced the affinity of both proteins for the upper phase rich in polyethylene glycol when assayed in the presence of 5 mM EDTA. This hydrophobic binding ability of $\alpha$-lactalbumin, but not of $\beta$-lactoglobulin, was strongly sensitive to the presence of Ca$^{2+}$. Thus, a counter-current distribution of whey in the presence of palmitate-polyethylene glycol and Ca$^{2+}$ allowed a single extraction of $\beta$-lactoglobulin, which was apparently free of detectable contamination, as assessed by SDS-PAGE.

(Key words: partitioning, aqueous two-phase, $\beta$-lactoglobulin, $\alpha$-lactalbumin)

Abbreviation key: CCCD = centrifugal counter-current distribution, CCD = counter-current distribution, G = partition ratio, PEG = polyethylene glycol.

INTRODUCTION

The separation of macromolecules by extraction on counter-current distribution (CCD) using aqueous two-phase systems composed of dextran and polyethylene glycol (PEG) is well documented (2, 6, 21). These systems usually include a salt that generates an electrical potential across the interface between the phases. The separation of the biomaterial depends on differences in the net charge of the substances and on their surface properties, which account for partition of the macromolecules between the two phases. In these two-phase systems, dextran is preferentially confined to the lower phase and PEG to the upper phase. Hence, if a specific ligand were covalently bound to one of these polymers, a substance that binds to the ligand should show high affinity for the phase containing the ligand-polymer (affinity partitioning) (6, 21).

The CCD method was adapted by Albertsson (2) for use with aqueous two-phase systems to carry out multiple partition of particles or macromolecules. The counter-current procedure is achieved by keeping the bottom phases (and interfaces) stationary while the top PEG-rich phases are moved by rotation to carry out each transfer. After each transfer, the phases are vigorously shaken and allowed to separate, and a new transfer step is then initiated (20).

To our knowledge, there are no previous studies dealing with the application of this two-phase system to the isolation of undenatured proteins from milk or its whey. However, CCD in Dextran-PEG two-phase systems could be a useful single procedure for extracting these proteins.

The objective of the present work was to study the distribution of the major whey proteins, $\alpha$-lactalbumin and $\beta$-lactoglobulin (7), in a counter-current procedure using phase partitioning. These two proteins showed very similar partition behavior in a two-phase system containing 7% (wt/wt) Dextran T-500 (Pharmacia, Uppsala, Sweden) and 5% (wt/wt) PEG
In order to separate them, we took advantage of the fact that the α-lactalbumin binding to hydrophobic ligands is Ca\(^{2+}\)-sensitive (11, 18). Thus, the results obtained show that hydrophobic affinity using palmitate-PEG in the presence of Ca\(^{2+}\) could be a useful single procedure for extracting and isolating β-lactoglobulin from cow’s milk whey.

**MATERIALS AND METHODS**

**Samples**

Fresh bovine milk was obtained from healthy Holstein animals collected at the Quiiez dairy farm of Zaragoza, Spain. The milk used in phase partitioning experiments was kept at 4°C (2 to 3 h) without any physical or chemical treatment. Whey was obtained from fresh skim milk by ultrafiltration through Diaflo H1P100 (Amicon, Danvers, MA), supplying proteins below 100 kDa. For electrophoresis, whey was adjusted its pH to 4.6 by addition of concentrated acetic acid and removal of precipitated casein.

Purified α-lactalbumin and β-lactoglobulin were purchased from Sigma Chemical Co., St. Louis, MO (L-538 and L-7880).

**Detection and Quantification of Proteins**

Samples collected from centrifugal CCD (CCCD) chambers were lyophilized and dissolved in .75 ml of 50 mM sodium phosphate pH 7.5. Then 4 ml of these solutions or of the whey prepared as stated was incubated with 50 μl of 30% mercaptoethanol and 50 ml of 45% SDS at 100°C for 5 min; SDS-PAGE was carried out in 10 to 15% PhastGel® gradient (Pharmacia PhastSystem®, Pharmacia). Staining was carried out by the optimized silver method for SDS-PAGE gradient gel media (PhastGel®), and densitometric tracing was performed on an LKB Ultrascan XL laser densitometer (Pharmacia).

β-Lactoglobulin and α-lactalbumin were determined by quantitative immunoelectrophoresis according to Laurell (12). Plates were prepared with 1% of agarose (Serva Feinbiochemica GmbH and Co., Heidelberg, Germany) in .05 M veronal buffer pH 8.2, containing an appropriate quantity of specific antisera obtained as described by Piñeiro et al. (17). Protein assay was made by the Bradford method (5).

**Two-Phase System**

The systems were prepared from stock solutions of the polymers in water; 40% (wt/wt) PEG 6000 (Serva) and 20% (wt/wt) Dextran (relative molecular mass = 500,000, Pharmacia) prepared as previously described (21). The polymer solutions were weighed and mixed to give the final concentrations described herein. When batch experiments were carried out, 5.0 g of two-phase system were prepared and mixed by 20 inversions. Phase separation was accelerated by centrifugation at 1000 × g for 1 min. All operations, both the batch and CCD experiments, were conducted at 0 to 4°C.

**CCCD**

We constructed the CCCD machine following the invention described by Akerlund (1) (this work contains a detailed scheme of the machine). With this device, the time for the separation of the two phases is shortened by centrifugation. The apparatus contains 60 chambers arranged in a circle, allowing transfer of the upper phases to the lower phases. The mixed two-phase system (1.55 ml) was loaded in chambers 3 to 59. The composition of the two-phase system was Dextran 7% (wt/wt), PEG 5% (wt/wt), 2 mM EDTA, and 10 mM sodium phosphate pH 7.0. An 8.0-g two-phase system was prepared with this composition together with 3.5 g of milk or whey. Aliquots (1.55 ml) of this mixture were then loaded in chambers 0 to 2. Shaking and centrifugation times were 45 and 30 s, respectively, and 57 transfers were performed. After the run, the systems were transformed into one phase by the addition of 1.55 ml of 50 mM sodium phosphate pH 7.0 to each cavity. The fractions were then collected and analyzed.

**RESULTS**

By comparing the partition of the total milk protein with that of commercial pure proteins, the usefulness of phase partitioning for α-lactalbumin and β-lactoglobulin isolation was evaluated. Different polymer and salt concentrations were first investigated to find the op-
Figure 1 shows the CCCD diagrams obtained for α-lactalbumin, β-lactoglobulin, and total protein from milk (Figure 1a) and whey (Figure 1b) partitioned in the system. In the case of CCCD of milk (Figure 1a), a large portion of the protein bulk appeared in the last 20 chambers. Caseins were electrophoretically detected in this peak (data not shown), whereas α-lactalbumin and β-lactoglobulin were found close together between chambers 20 to 30, coinciding with the first half of another protein (Figure 1a) peak appearing around chamber 30. Both α-lactalbumin and β-lactoglobulin showed similar distribution profiles in the CCCD diagrams obtained from milk (Figure 1a) or whey (Figure 1b). However, in the case of whey, the position of both proteins coincided with the main peak of protein, located between chambers 20 and 30 (Figure 1b).

The distribution of a molecule in a two-phase system is represented by the partition ratio (G). The G value is defined as the percentage of the molecule in the mobile part of the system (upper phase in CCD) divided by the percentage in the stationary part (lower phase). From the position of the peaks in the CCCD diagram, the G value can be calculated using the equation \( G = i/(n - i) \), where \( i \) is the number of the chamber to which the peak has moved, and \( n \) is the number of transfers (10). Thus, the G values of the α-lactalbumin and β-lactoglobulin were calculated from the position of the peaks in the CCCD diagram (Figure 1a and b). Thus, the estimated G values of the α-lactalbumin (1.0 and .9) and β-lactoglobulin (.7 and .6) from milk and whey, respectively, were similar in each case. At the middle position of the run, a difference of the G value of .1 means about 2 chambers of difference. In our experiments (n ≥3), the position of peaks varied in ±1 chamber.

Earlier works have described the interactions of PEG-bound fatty acids with β-lactoglobulin (4, 18, 19) and α-lactalbumin (11, 19). We decided to study the usefulness of this hydrophobic affinity partition to separate these proteins. Because palmitate was the polymer ligand that gave the strongest interaction with β-lactoglobulin (19), palmitate-PEG was chosen as the ligand-polymer to be employed.

In order to find the optimal conditions to achieve this hydrophobic affinity partition, the optimal conditions for protein extraction. In batch experiments, the highest milk protein partitioning in the upper phase rich in PEG was achieved in a two-phase system containing 7% (wt/wt) Dextran T-500, 5% (wt/wt) PEG 6000, and 10 mM sodium phosphate pH 7.0. Results show the percentage of the total protein (O), α-lactalbumin (△), and β-lactoglobulin (Δ) in each of two chambers determined after the run. Partition ratio, \( G \), is calculated by the equation \( G = i/(n - i) \) where \( i \) is the number of the chamber to which the peak has moved, and \( n \) is the number of transfers.
effect of different palmitate-PEG concentrations on the partition of commercial pure α-lactalbumin and β-lactoglobulin was first tested by using batches of two-phase systems. These batch experiments were carried out in the presence of 5 mM EDTA, because α-lactalbumin undergoes Ca²⁺-dependent conformational changes (13, 14), and hydrophobic interaction has been proven to be very sensitive to the conformational state of the protein (4, 8, 19). The results obtained are shown in Figure 2. The partition of both proteins is dependent on the palmitate-PEG concentration present in the two-phase system. The affinity for the upper phase drastically increases when the proportion of PEG in the form of palmitate-PEG increases from 0 to a maximum at 6%.

It is noteworthy that palmitate-PEG concentrations above 6% lead to a fall in the protein partition in the upper phase. Because a multistep procedure such as CCCD has a certain accumulative effect on the binding to the specific ligand, the use of the optimal concentrations (6%) found in the batch experiment (Figure 2) could probably lead to a relative decrease of the enhanced partition in the upper phase. Therefore, a concentration of 2% palmitate-PEG was chosen to carry out the CCD experiments. The enhancement of the biomaterial affinity for the upper phase of a two-phase system accounts for a relative displacement of the CCD peak to the right. Consequently, the expected effect of the palmitate-PEG in the CCD of α-lactalbumin and β-lactoglobulin would make them partition closely with the main peak of the bulk of milk protein (caseins). (Figure 1a). Therefore, all of the experiments on affinity partitioning were carried out from whey.

Figure 2. Partition of α-lactalbumin (◊) and β-lactoglobulin (□) as a function of the concentration of palmitate polyethylene glycol (PEG) expressed as the percentage of the total PEG in the system that is replaced by the PEG-ligand. The system composition was as in Figure 1.
Figure 3a shows the effect of palmitate-PEG (in the presence of 5 mM EDTA) in promoting a sharp increase in the G values of α-lactalbumin (G = 3.6) and of β-lactoglobulin (G = 6.2) from whey with respect to those obtained in the absence of the ligand (Figure 1b). Although the observed effect was higher for β-lactoglobulin than for α-lactalbumin, more than 50% of this latter protein was found in the same chambers in which β-lactoglobulin was detected.

When CCCD was carried out in the absence of EDTA, the G value of the peak chamber of α-lactalbumin fell drastically (G = 1.0) and was very similar to that obtained in the absence of palmitate-PEG (Figure 1, a and b). No modification of the G value of the β-lactoglobulin was observed in the absence of EDTA (Figure 3b) with respect to that obtained in its presence (Figure 3a). However, a considerable proportion of the α-lactalbumin was still partitioning with β-lactoglobulin. The addition of 1 mM Ca²⁺ to the system (Figure 3c) led to almost the complete separation of the two proteins; <6% of the α-lactalbumin was copartitioned with the first half of the β-lactoglobulin peak.

Figure 4 (a and b) shows the electrophoretic densitometric tracing of commercial pure α-lactalbumin and β-lactoglobulin (genetic variant B), respectively. Both proteins can easily be identified as the major components of the densitometric tracing of whey (Figure 4c). The β-lactoglobulin extracted from whey (chambers 50 to 52) by affinity CCCD was entirely located in a major peak; there were no detectable contaminants under the experimental conditions used here (Figure 4d). In this case, the two peaks of the most abundant genetic variant isoforms A and B (3) can be recognized. However α-lactalbumin obtained from CCCD of whey in the experimental conditions of Figure 4c showed some other contaminant proteins, as assessed by SDS-PAGE (data not shown).

**DISCUSSION**

The results obtained show that analytical CCCD of whey, in a two-phase system containing 7% (wt/wt) Dextran, 5% (wt/wt) PEG, 1 mM CaCl₂, 10 mM sodium phosphate, pH 7, with 2% of the total PEG in the form of the

![Figure 4. Densitometric tracing of SDS-PAGE of commercially purified α-lactalbumin and β-lactoglobulin, total whey, and the material recovered from chambers 50 to 52 of Figure 3c.](https://example.com/figure4.png)
palmitoyl derivative, is an adequate technique for extracting \( \beta \)-lactoglobulin. The protein thus obtained appeared to be free of other proteins detectable by SDS-PAGE characterization under the experimental conditions used (Figure 3c).

Under these experimental conditions, the CCCD achieves almost complete separation of the two major whey proteins (\( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin). However, \( \alpha \)-lactalbumin remains partitioned with a \( G \) value close to 1, i.e., in the middle of the run, where the bulk of whey protein appears to be located (Figure 2b and Figure 3c).

The efficiency of this method for purifying \( \beta \)-lactoglobulin is due to the strong hydrophobic binding ability of this protein (4, 8, 9, 19). However, abundant whey proteins, except \( \alpha \)-lactalbumin, do not show this binding ability. However, Ca\( ^{2+} \)-dependent conformational changes (13, 14, 16) of \( \alpha \)-lactalbumin drastically modify its interaction with hydrophobic ligands, as we have shown in this work and as was previously described (11, 19). In this respect, Perez et al. (15) observed that incubation of cow whey with \(^{14}\text{C}\)palmitate did not lead to labeling of \( \alpha \)-lactalbumin. This observation is in agreement with the result described in Figure 3b, in which palmitate-PEG binding was decreased when EDTA was eliminated from the system. The endogeneous Ca\( ^{2+} \) supply from whey could, therefore, account for a conformational change of the \( \alpha \)-lactalbumin, which then decreases the hydrophobic binding. However, the dilution effect of the whey sample in the CCCD two-phase system would make Ca\( ^{2+} \) concentration insufficient to produce the total inhibition of the binding that appears to occur when 1 mM CaCl\(_2\) is present in the system (Figure 3c).

CONCLUSIONS

These results clearly show that hydrophobic affinity partitioning in Dextran-PEG two-phase system could be an effective technique for purifying \( \beta \)-lactoglobulin from whey. In addition, it offers the advantage of making further reextractions of the remaining whey proteins, i.e., by extracting \( \beta \)-lactoglobulin in a Ca\( ^{2+} \)-containing batch two-phase system and by reextracting \( \alpha \)-lactalbumin by a fresh Ca\( ^{2+} \)-chelated phase. Therefore, further studies on the use of less expensive two-phase forming polymers and of large-scale techniques could be justified in order to achieve the purification of undenatured whey proteins in the most convenient way for preparative purposes. In addition, different concentration ratios of Ca\( ^{2+} \) and palmitate-PEG and the use of other hydrophobic ligands should be investigated to find optimized conditions for the extraction of \( \beta \)-lactoglobulin from bovine milk.

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REFERENCES

AFFINITY PARTITIONING OF β-LACTOGLOBULIN


