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Aqueous Two-Phase Partitioning of Milk Proteins

Application to Human Protein C Secreted in Pig Milk

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ABSTRACT

Milk of transgenic pigs secreting recombinant human Protein C (rHPC) was used as a model system to determine the utility of aqueous two-phase extraction systems (ATPS) for the initial step in the purification of proteins from milk. The major challenges in purification of recombinant proteins from milk are removal of casein micelles (that foul processing equipment) and elimination of the host milk proteins from the final product. When milk was partitioned in ATPS composed of polyethylene glycol (PEG) and ammonium sulfate (AS), the phases were clarified and most of the caseins precipitated at the interphase. The partition coefficients of the major milk proteins and rHPC were dependent upon the molecular weight of the PEG used in the ATPS. Higher-partition coefficients of the major whey proteins, β-lactoglobulin, and α-lactalbumin were observed in ATPS made up of lower molecular-weight PEG (1000 or 1450) as compared to systems using higher molecular-weight PEG. Lowering the pH of the ATPS from 7.5 to 6.0 resulted in increased precipitation of the caseins and decreased their concentration in both phases. rHPC had a partition coefficient of 0.04 in a system composed of AS and PEG 1450. The rHPC in pig milk was shown to be highly heterogenous by two-dimensional gel electrophoresis. The heterogeneity was owing to inefficient proteolytic processing of the single chain to the heterodimeric form and differences in glycosylation and other post-translational processing. Differential partitioning of the

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multiple forms of purified rHPC in the ATPS was not observed. rHPC after processing in ATPS was recovered in a clear phase free of most major milk proteins. ATPS are useful as the initial processing step in the purification of recombinant proteins from milk because clarification and enrichment is combined in a single step.

Index Entries: Protein C; expression; recombinant animal; milk; aqueous two-phase extraction; purification.

Abbreviations: rHPC, recombinant human protein C; ATPS, aqueous two-phase extraction system; PEG, polyethylene glycol; AS, ammonium sulfate; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; CBB-G, Coomassie Brilliant Blue G; IEF, isoelectric focusing; SC, single chain; HC, heavy chain; LC, light chain; ALB, serum albumin; CS, caseins; BLG, β-lactoglobulin; ALA, α-lactalbumin.

INTRODUCTION

Aqueous two-phase extraction systems (ATPS) are suitable for the separation of complex mixtures including those containing particulates *(1–3)*. ATPS should be useful as the initial step in the isolation of proteins expressed in milk because this complex mixture is composed of lipids, protein micelles, soluble proteins, and carbohydrates. Expression of foreign proteins in the milk of animals is an effective way to produce large amounts of proteins that are ordinarily found in low concentrations from natural sources. Protein C is an anticoagulant found at low concentrations in human plasma. Recombinant human protein C (rHPC) has been expressed in the milk of transgenic pigs to produce adequate amounts for therapeutic uses *(4)*. A purification method has been developed that uses polyethylene glycol (PEG) to precipitate rHPC along with the caseins (major micelle proteins) in the first processing step *(5)*. Additional steps to dissociate rHPC from the caseins and remove the caseins were necessary. A significant challenge in the purification of expressed proteins from the milk of host animals is the initial processing of this complex feedstock.

ATPS have been used to purify the proteins from the whey (acid-soluble) fraction of milk. Chen *(6)* used a PEG-potassium phosphate ATPS at pH 4.5 to separate fat from the protein fraction in cheese whey. A continuous contactor was used with PEG-potassium phosphate ATPS for the large-scale separation of whey proteins *(7)*. ATPS based on PEG-hydroxypropylstarch that included the addition of the hydrophobic ligand PEG-palmitate, were used to separate α-lactalbumin and β-lactoglobulin from bovine whey *(8)*. PEG precipitation has been combined with ATPS, using hydroxypropylstarch and PEG-palmitate, for the large-scale separation of

whey proteins (9). Addition of the polyscaccharides pectin, gum arabic, or arabinogalactan to milk results in a two-phase system, one phase a concentrated solution or gel of milk proteins and the other a concentrated polysaccharide phase (10). Total milk proteins could be concentrated 5–12-fold by formation of these two-phase systems from milk (11).

The major challenges in the processing of milk for the recovery of recombinant proteins are the removal of the casein micelles and elimination of the host milk proteins. The major milk proteins besides the caseins are serum albumin, β-lactoglobulin, and α-lactalbumin. Because these proteins are quantitatively the major milk proteins, we have used gel electrophoresis to measure their partition coefficients and removal from milk during ATPS. We used rHPC as a model protein for the use of ATPS in the initial processing of a recombinant protein in milk. In this article, we have identified suitable ATPS for milk partitioning and determined the influence of these variables on the milk proteins and rHPC recovery.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), β-lactoglobulin, α-lactalbumin, and PEG (1000, 1450, 3350, and 8000 molecular weight; Sigma Chemical, St. Louis, MO). Twice-crystallized acrylamide, bisacrylamide, ampholytes, and Coomassie Brilliant Blue G-250 (CBB-G) were from Serva Biochemicals (Westbury, NY). Protein standards were from New England Biolabs (Beverly, MA). The production of transgenic swine, collection of milk, and purification of rHPC were described in Drohan et al. (5).

Aqueous Two-Phase Partitioning and Ammonium Sulfate Precipitation

Milk from transgenic swine and purified proteins were partitioned in ATPS using the methods described previously (12). The ATPS were expressed as mass fraction (g of salt or polymer per 100 g of phase system) to allow comparison to previously measured phase diagrams (13). The ATPS was made up before the milk was added. The systems were made by weighing the salt, concentrated polymer solution, and sufficient water to a total of 9 g. The ATPS was mixed to dissolve the salt and the pH was adjusted using concentrated NaOH or HCI. To conserve the amount of material used, 0.9 mL of this system was added to 0.1 mL of milk or protein solution and the solution was mixed by rocking for 20 min at an ambient temperature. Samples were centrifuged (ambient temperature) for 5 min (12,000*g*) to separate the phases. The top phase was carefully removed by aspiration and the bottom phase by puncturing the bottom of

the tube with a push pin and draining it into a clean tube. The interphase (floating precipitate material between phases) was carefully avoided during the collection of the other phases.

Precipitation of proteins was accomplished by mixing an AS stock solution (mass fraction of 40%, pH 7.5) to transgenic swine milk in a ratio of 0.25 mL of milk per mL of solution. After mixing, the pH was checked and adjusted to the final value. Solutions were placed on ice for 30 min, then centrifuged for 5 min (12,000*g*).

The collected ATPS phases and AS supernatants were dialyzed using a microdialyzer apparatus (Life Technologies, Inc., Gaithersburg, MD). Samples for gel analysis were dialyzed against a volume fraction of 0.1% SDS for 4 h at ambient temperature and then lyophilized. Samples for enzymatic activity were dialyzed against 0.05 mol/L TRIS pH 7.4 at 4° for 2 h.

Electrophoresis and Western Blotting

One-dimensional sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) were composed of either a volume fraction of 15% acrylamide or volume fractions of 10% to 20% acrylamide (linear gradient). The gels were run on an SE 600 vertical-slab gel unit (Hoefer Scientific, San Francisco, CA) using the buffer systems of Lammeli *(14)* as modified by Fling and Gregerson *(15)*. The system was maintained at 12°C and run at 100V to 110V for 1800 V · h). Gels were either stained with silver using the method of Oakley et al. *(16)* as modified by Edwards et al. *(17)* or with Coomassie Brilliant Blue G-250 (CBB-G), *(18)*. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using the method of Towbin et al. *(19)*. The membranes were then developed using a sheep anti-human Protein C antibody (Affinity Biologicals, Ontario, Canada) as previously described *(20)*.

Measurement of Partition Coefficients Using Gel Electrophoresis

Samples of dialyzed top and bottom phases were run on SDS-PAGE and stained using CBB-G *(18)*. Stained gels were imaged and analyzed using the whole-band program of the BioImage Visage 110 system (Bedford, MA). The relative amount of the major milk proteins present in the top and bottom phases was determined by comparing the intensity of the bands stained with CBB-G. Proteins were identified by their molecular weight relative to standard proteins. Although the values derived from this method are subject to the complication of the presence of other proteins with similar molecular weights, the relative intensities of the bands are useful to estimate the efficiency of the purification step.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed using the Iso-Dalt system *(21)*. The first dimension was an isoelectric focusing (IEF) gel

containing 9 mol/L urea, a volume fraction of 3.3% total acrylamide, a volume fraction of 1.1% pH 5.0–7.0 ampholytes, a volume fraction of 1.7% pH 3.0–10.0 ampholytes, and a volume fraction of 2.0% (v/v) Nonidet P-40. Samples were diluted with SDS-solubilization buffer containing volume fractions of 2% SDS, 1% dithiothreitol, 10% glycerol, and 0.05 mol/L 2-(cylohexylamino) ethane sulfonic acid. The IEF gel was focused at 800 V for 20 h. The second-dimension gel was an SDS-PAGE with a volume fraction of 10% to 20% acrylamide (linear gradient) gel as previously described. The gels were run for 1800 V · h (12°C).

Amidolytic Activity Assay of rHPC

After Protein C was activated by Protac (American Diagnostica, Greenwich, CT) for 10 min at ambient temperature, the amidolytic activity was determined by the hydrolysis of a tripeptide derivative, Glu-Pro-Arg-*p*-nitroanilide (S-2366, Chromogenix, Molndal, Sweden) as previously described *(20)*.

Ultracentrifugation of Pig Milk

Skim pig milk was centrifuged at 158,000*g* for 45 min at ambient temperature (Airfuge, Beckman Instruments, Palo Alto, CA). The supernatant was removed and used as the micelle-free (whey) fraction. The pellet was washed by suspension in a volume of TRIS-buffered saline (0.05 mol/L TRIS, 0.15 mol/L NaCl, pH 7.5) equal to the original volume of milk and centrifuged as previously described. The washed pellet was then suspended in TRIS-buffered saline equal to the original volume of milk and used as the micelle fraction.

Size Exclusion Chromatography

Size-exclusion chromatography (SEC) was performed using a Biosep SEC-S3000 column (300 × 7.8 mm; Phenomenex, Torrance, CA). The buffer was 0.1 mol/L sodium phosphate pH 7.2 with a flow rate of 1.0 mL/min at ambient temperature. Samples of top and bottom phase (0.2 mL) containing rHPC were injected and the single-peak area measured. Protein concentration was determined by measurement of a standard curve using rHPC.

RESULTS

Heterogeneity of rHPC Expressed in Pig Milk

rHPC purified *(20)* from the milk of transgenic pigs was found to be heterogeneous because of proteolytic processing and post-translation modifications, including glycosylation and gamma carboxylation *(5,20)*. We used two-dimensional gel electrophoresis to examine the major forms of rHPC (Fig. 1A) and observed massive charge and size heterogeneity.

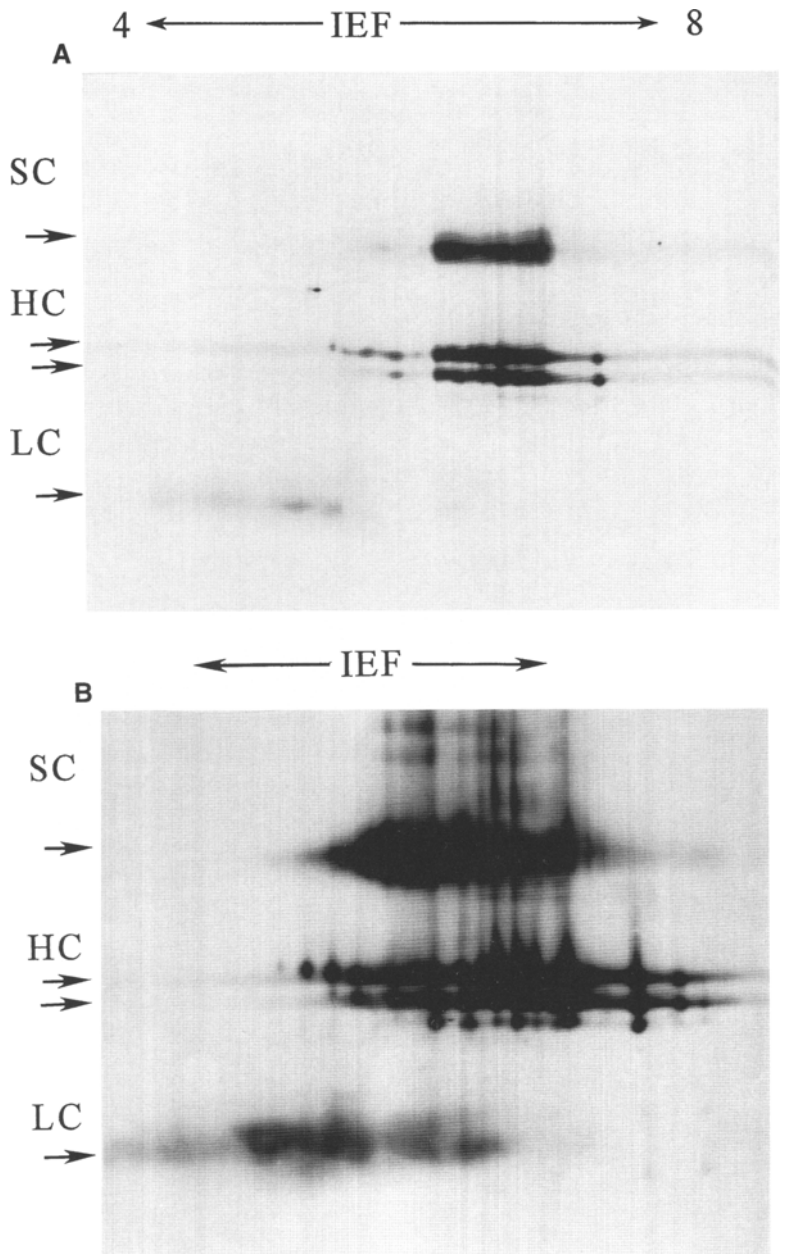

Fig. 1. **(A)** Two-dimensional gel electrophoresis of rHPC. Two-dimension gel electrophoresis and silver staining of rHPC was performed as described in the text. Isoelectric-focusing dimension is indicated (IEF). The single chains (SC) and the two major glycoforms of heavy chain (HC) and light chain (LC) of rHPC are indicated. The HC and LC forms of rHPC result from the proteolytic processing of the SC form. **(B)** Western blot of gel shown in Fig. 1A.

Table 1
Partition Coefficient of Purified rHPC in ATPS[a]

System mass fraction	Partition Coefficient
15% PEG 1000, 14% AS, pH 7 5	0 64 ± 0 18 (4)
15% PEG 1450, 14% AS, pH 7 5	0 04 ± 0 02 (4)

[a] The partition coefficient of rHPC was determined by size-exclusion chromatography as described in the Materials and Methods section. Partition coefficient was defined as concentration in top phase divided by concentration in bottom phase. The values given are the means ± the standard deviation of the number of determinations in parenthesis.

Western-blot analysis showed that protein spots detected by silver staining were recognized by human Protein C specific antibody (Fig. 1B). The major features observed are the high amount of the single-chain (SC) form owing to inefficient cleavage and the two major glycosylation-charge trains of the heavy chain (HC). The light chain (LC) of rHPC has a shift to more acidic isoelectric forms as compared to the heavy chain. The SC form was localized in the same pH range as the HC suggesting that the isoelectric points of the native two-chain molecules are defined by the charge of the HC.

Selection of ATPS and Influence of PEG Molecular Weight

A number of ATPS for partitioning of milk were screened with two criteria. The first criterion was that the phases were clarified and the second was that added pure protein C partitioned mainly to one phase. The ATPS systems composed of AS and PEG precipitated the casein micelles at the interphase region as a firm white layer and rHPC found mainly in the bottom phase. The top and bottom phases were easily recovered in a clear form after centrifugation. Because AS and PEG are both efficient protein-precipitating agents, we chose low concentrations for initial experiments.

We further examined the influence of the molecular weight of PEG on the partition of rHPC. Pure rHPC was partitioned in ATPS composed of AS and PEG with different molecular weights. Analysis by SDS-PAGE and SEC indicated that the partitioning of rHPC was strongly influenced by the molecular weight of the PEG used in the ATPS. When PEG of molecular weight of 1450 or greater was used in the ATPS, rHPC was almost exclusively in the bottom phase, whereas systems using PEG 1000 had a significant amount of rHPC in the top phase (Table 1). Partition coefficients are defined as the concentration of solute in the top (polymer-rich) phase divided by the concentration in the bottom (salt-rich) phase. When analyzed by SDS-PAGE, a significant difference in the partitioning of the SC, LC, and the major glycoforms of the HC of rHPC was not observed (data not shown).

When milk was added to the ATPS, a firm white layer of precipitated material was present at the interphase region after centrifugation. The white layer was made up of precipitated caseins and other proteins. When systems composed of higher molecular weight PEG (3350 or 8000) were used, the major milk proteins were almost exclusively in the bottom phase and very little of the milk proteins (including rHPC) partitioned to the top phase (results not shown). Proteins not present in either phase are assumed to be precipitated. Greater numbers and amounts of proteins were found in the top phases in ATPS using lower molecular-weight PEG of 1000 and 1450 (Fig. 2A, lanes 3 and 4, respectively). Partitioning of milk in ATPS composed of lower molecular-weight PEG (1000 and 1450) resulted in a greater differential separation of the major milk proteins when compared to ATPS composed of higher molecular-mass PEG 8000 in which most of the proteins were present in the bottom phase.

We found that approx 40–50% of the caseins remained in the bottom phase (Fig. 2A, lane 9) of ATPS (composed of mass fractions of 14% AS and 15% PEG 1450, pH 7.5) compared to the levels in the milk added to the system (Fig. 2A, lane 2). The bottom phase was clear, indicating partial dissociation of the caseins from the micelles. The partition coefficients of the major whey proteins were measured from CBB-G stained gels (Table 2). Albumin was present mainly in the bottom phase. β-Lactoglobulin and α-lactalbumin were present in higher concentrations in the top phase.

The partitioning of rHPC found in milk was evaluated by immunoblotting. The majority of the rHPC in milk was detected in the bottom phase (Fig. 2B, lanes 8 and 9). The ATPS composed with PEG 1000 had significantly higher amounts of rHPC in the top phase (Fig. 2B, lane 3) compared to a system composed of PEG 1450 (Fig. 2B, lane 4). The partition coefficient obtained with milk were the same as those obtained using purified rHPC in the ATPS (Table 1). To determine if rHPC recovered was functional, we measured the amidolytic activity of rHPC in the top and bottom phase of the ATPS composed of AS and PEG 1450 at pH 7.5 (Table 3). Recovery of the rHPC activity was higher in the bottom phase, confirming the results obtained with purified rHPC.

Influence of Ammonium Sulfate and PEG Concentration

Increasing the concentration of AS and PEG in the ATPS results in a greater difference in composition between the top and bottom phases (the length of the tie-line in the phase diagram increases). The increased difference in composition between the top and bottom phases results in more extreme partition coefficients. Increased concentrations of salt and polymer cause precipitation if the proteins reach the limits of their solubility in the phases. We used AS-PEG 1000 concentrations (with mass fractions of 14.0

and 15.0%, respectively) that give the shortest tie-line length in the phase diagram for this system *(13)*. Lower concentrations of AS and PEG than this will fall in the one-phase region of the phase diagram. Partitioning pig milk in ATPS containing higher concentrations of AS and PEG 1000 (with mass fractions of 16.0 and 18.5%, respectively) resulted in a bottom phase that was free of caseins but did not contain detectable rHPC activity in the phases. This indicates that rHPC and the caseins are both close to their solubility limits at the AS and PEG concentration used in the ATPS.

Because AS is an efficient protein precipitating agent, we measured the recovery of rHPC in single-phase AS systems. The results in Table 3 show that the activity of rHPC decreases rapidly when AS concentrations increase over a mass fraction of 20%. Running these samples on SDS-PAGE and staining with CBB-G indicated that the caseins precipitated when the concentration of AS was greater than a mass fraction of 20%. The results in Table 3 are based on the enzymatic activity of the micelle-free supernatant being 100%. The supernatant of milk with lower concentrations of AS had higher enzymatic activity compared to that of the micelle-free supernatant. The increased activity could be owing to increased dissociation of rHPC from the micelles or removal of inhibitor substances from the milk by the AS.

Influence of pH

The pH of the ATPS can influence the solubility of the protein in the phases and also change the partition coefficient by changing the protein charge. A commonly used method to remove caseins from milk is isoelectric precipitation. Lowering the pH of skim cow milk to 4.6 results in the precipitation of caseins, whereas the major whey proteins remain in solution *(22)*. The solubility of the pig caseins should also be decreased by lowering the pH. When an ATPS made up with mass fractions of 15% PEG 1450 and 14% AS at pH 6.0 was used to partition pig milk, the bottom phase contained only low concentrations of caseins (Fig. 3A, lane 2). However, the bottom phase of the pH 6.0 system did not contain higher concentrations of the whey proteins, α-lactalbumin and β-lactoglobulin, compared to the bottom phase of the corresponding system at pH 7.5 (lower partition coefficients in Table 2). The activity of rHPC in the bottom phase of the pH 6.0 system was lower indicating that the solubility of rHPC in the bottom phase also decreased (Table 3).

All of the separations presented so far used a single extraction. In developing a process for the isolation of rHPC, multiple extraction and precipitation steps could be used. One scheme used was to partition milk in ATPS using AS and PEG 1450 at pH 7.5 and collecting the bottom phase. Acidification to pH 6.0 followed by centrifugation of the precipitated caseins yielded a bottom phase free of caseins and most of the whey proteins. The SDS-PAGE of the resulting bottom phase of this experiment is shown in Fig. 3A, lane 3.

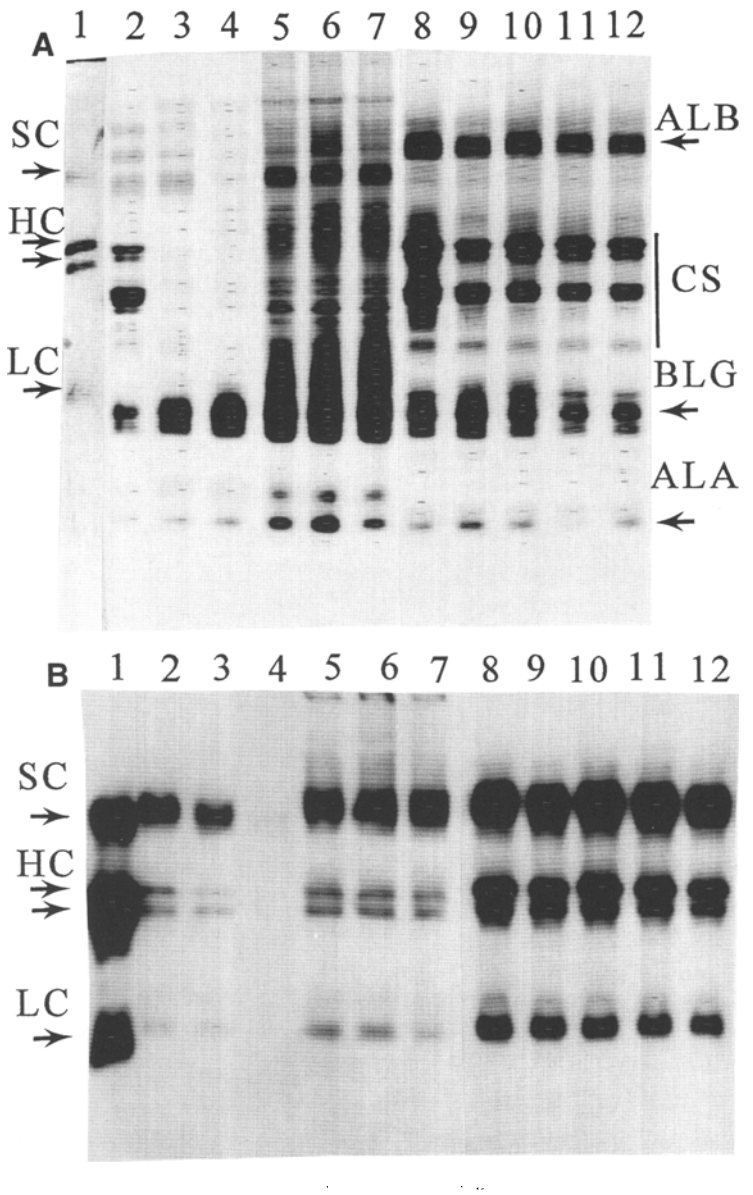

Fig. 2. **(A)** SDS-PAGE of pig's milk partitioned in ATPS. Samples were analyzed on a volume fraction of 15% acrylamide gels and stained with silver as described in text. Lane 1 contains purified rHPC showing single chains (SC), heavy chains (HC), and light chain (LC). Lane 2 contains pig's milk showing the location of albumin (ALB), caseins (CS), β-lactoglobulin (BLG), and α-lactalbumin (ALA). Top phase (lane 3) and bottom phase (lane 8) of milk partitioned in ATPS (mass fractions of 15% PEG 1000 and 14% AS pH 7.5). Top phase (lane 4) and bottom phase (lane 9) of milk partitioned in ATPS (mass fractions of 15% PEG 1450 and 14% AS, pH 7.5). Milk was treated with a volume fraction of 1% Triton X-100 for 30 min then partitioned in ATPS (mass fractions of 15% PEG 1000, 14% AS pH 7.5 and a volume fraction of 0.2% Triton X-100, final concentration). These samples are top phase (lane 5) and bottom phase (lane 10).

Table 2
Partition Coefficients of Pig-Whey Proteins[a]

System mass fraction	Albumin Band	β-Lactoglobulin Band	α-Lactalbumin Band
15% PEG 1000, 14% AS, pH 7 5	0 35 ± 0 06 (3)	3 3 ± 1 0 (3)	1 8 ± 0 4 (3)
15% PEG 1450, 14% AS, pH 7 5	0 28 ± 0 03 (3)	3 8 ± 0 6 (3)	1 7 ± 0 3 (3)
15% PEG 1450, 14% AS, pH 6 0	0.21 ± 0 02 (3)	2 5 ± 0 0 (3)	0 7 ± 0 1 (3)

[a] Relative concentrations of protein bands were measured from SDS-PAGE stained with CBB-G as described in the Materials and Methods section.

Table 3
Recovery of Amidolytic Activity of rHPC in ATPS and AS Supernatants[a]

System mass fraction	**Recovery of Added Activity (%)**
16 7% AS pH 7 5 Supernatant	149 0 ± 28.3 (3)
19 3% AS pH 7 5 Supernatant	130 7 ± 20 8 (3)
21 8% AS pH 7 5 Supernatant	49 0 ± 14 2 (3)
26 8% AS pH 7 5 Supernatant	None Detected
15% PEG 1450, 14% AS, pH 7 5 Top Phase	15 1 ± 4 1 (3)
15% PEG 1450, 14% AS, pH 7 5 Bottom Phase	95 6 ± 17 9 (3)
15% PEG 1450, 14% AS, pH 6 0 Top Phase	None Detected
15% PEG 1450, 14% AS, pH 6.0 Bottom Phase	64 6 ± 12 6 (3)
158,000 x g Supernatant of Pig Skim Milk	100 0

[a] Amidolytic activity of rHPC was measured as described in the Materials and Methods section. Values are means ± the standard deviation of the number of determination in parenthesis. The values are normalized so that the activity of 158,000*g* supernatant was 100%.

Fig. 2 (continued). Milk was treated with a volume fraction of 1% Brij 35 for 30 min then partitioned in ATPS (mass fractions of 15% PEG 1000, 14% AS pH 7.5, and containing a volume fraction of 0.2% Brij 35 final concentration). These samples are top phase (lane 6) and bottom phase (lane 11). Milk was treated with a volume fraction of 1% Tween-20 for 30 min then partitioned in ATPS (mass fractions of 15% PEG 1000, 14% AS pH 7.5, and a volume fraction of 0.2% Tween-20 final concentration). These samples are top phase (lane 7) and bottom phase (lane 12). **(B)** Western blot of gel described in Fig. 2A.

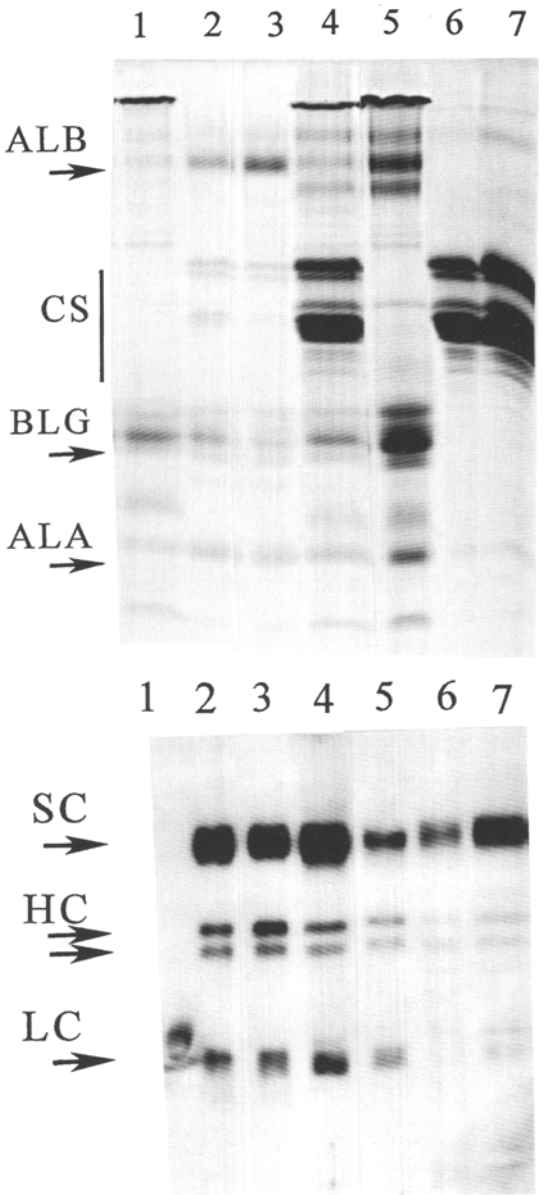

Fig. 3. **(A)** SDS PAGE of micelle and micelle-free fractions and ATPS of pig milk. A gel with a volume fraction of 15% was stained with CBB-G. Samples of a system composed of mass fractions of 15% PEG 1450 and 14% AS, pH 6.0 are top phase (lane 1) and bottom phase (lane 2). Samples of a system composed of mass fractions of 15% PEG 1450 and 14% AS pH 7.5 and bottom phase recovered and adjusted to pH 6.0 and centrifuged, bottom phase (lane 3). Pig's milk (1.2 µL) is shown in lane 4. Samples of the 158,000*g* supernatant (equivalent to 2 µL milk, lane 5) and the washed pellet (equivalent to 2 µL milk lane 6 and equivalent to 4 µL milk, lane 7). **(B)** Western blot of the samples described in Fig. 3A.

Association of rHPC with Casein Micelles and Addition of Non-Ionic Detergents to ATPS

To determine if rHPC is associated with casein micelles, we separated the milk by ultracentrifugation into micelle and micelle-free fractions. Analysis of these fractions by SDS-PAGE confirmed that the micelle fraction (Fig. 3A, lanes 6 and 7, two concentrations) was composed of caseins, and the micelle-free supernatant contained the major whey proteins (Fig. 3A, lanes 5). A Western blot showed that rHPC was also associated with the casein micelles (Fig. 3B, lanes 6 and 7). The rHPC associated with the micelles was enriched in the single-chain form.

Non-ionic detergents (Triton X-100, Brij 35, and Tween-20) were added to milk and ATPS (described in Fig. 2A caption) to determine the effect on the recovery and partitioning of rHPC. The addition of the non-ionic detergents did not increase the recovery of rHPC in the bottom phase (Fig. 2B, lanes 8–12), but did increase the amount of rHPC present in the top phase (Fig. 2B, lanes 3–7). However, addition of the detergents to the ATPS resulted in cloudy phases and increased the amount and number of proteins present in the top phase (Fig. 2A, lanes 5–7).

DISCUSSION

The ATPS composed of AS and low molecular weight PEG are useful for the initial processing step of milk because the phases are clear, and differential partitioning of the milk proteins occurs. In the case of pig milk, β-lactoglobulin and α-lactalbumin partition to the top phase, and albumin partitions strongly to the bottom phase. A large number of proteins present in lower concentrations as observed by bands on SDS-PAGE also show differential partitioning. The partition coefficients of the major proteins in pig milk were strongly influenced by the molecular weight of the PEG used. The lower molecular-weight PEG, 1000 and 1450, had a larger differential partitioning of proteins in the ATPS compared to the higher molecular-weight PEG. ATPS using higher molecular-weight PEG 8000 partitioned most of the proteins to the bottom phase.

We have taken advantage of precipitation by the phase-forming components to remove casein micelles resulting in clarified phases. The concentration of caseins in the phases can be decreased further by either increasing the concentration of AS and PEG or by lowering the pH to 6.0. The effect of increasing the concentration of the phase components or lowering the pH on recovery must be determined for each individual protein product. Acidification of milk to pH 4.6 is an inexpensive method for removing casein micelles, but exposure to low pH is not desirable for a large number of protein products. When we acidified pig milk to pH 4.6,

the resulting supernatant was not clear and a significant amount of the caseins still remained in the supernatant as determined by SDS-PAGE. We used skim pig's milk for these studies but experiments with full-fat cow's milk gave similar results with the addition of a floating layer of lipids above the PEG phase (Cole et al., in preparation).

The purification of rHPC is complicated by its heterogeneity and association with the casein micelles. The mature protein C molecule is the result of a number of processing steps including glycosylation at four sites, modification of nine glutamic acids to γ-carboxyglutamate, β-hydroxylation of an aspartic acid, and proteolytic cleavages *(24)*. Our analysis of purified rHPC by two-dimensional gel electrophoresis, a technique not previously used, revealed the extreme heterogeneity of this protein. Lee et al. *(20)* determined that 30–40% of the rHPC isolated from the milk of transgenic pigs was in the SC form and 10–20% of the rHPC still contained the 24 amino-acid propeptide sequence. They also determined that a portion of the rHPC found in pig's milk had been cleaved at different amino-acid sites resulting in novel amino-termini *(20)*.

The partitioning of rHPC was very sensitive to the molecular weight of the PEG. When the AS concentration was held constant and the molecular weight of the PEG was changed from 1000 to 1450, the partition coefficient of rHPC decreased. Partitioning of the multiple forms of rHPC was not seen in the ATPS. The addition of non-ionic detergents destabilized the micelles as shown by the increased amount and types of proteins extracted into the top phase. The detergents also resulted in increased partitioning of rHPC to the top phase. These results indicate that the addition of non-ionic detergents to the ATPS can increase the dissociation of rHPC from micelles, but the use of the detergents results in cloudy phases and increased amounts of proteins in the phases.

The recovery of rHPC from milk decreased rapidly at AS concentrations above a mass fraction of 20% at pH 7.5. Low concentrations of AS increased the recovery of rHPC activity compared to the micelle-free supernatant. Higher concentrations of AS resulted in the precipitation of both caseins and rHPC. The observation that rHPC and the caseins precipitate at similar concentrations of ammonium sulfate may have several explanations. rHPC could have a lower intrinsic solubility compared to the caseins resulting in precipitation at similar AS concentrations owing to its low concentration. Increased concentrations of AS could enhance hydrophobic interactions between rHPC and the casein micelles resulting in the coprecipitation of rHPC and caseins. The fraction of rHPC associated with casein micelles in milk was enriched in the single-chain form, a form not desired in the final product. The low concentrations of AS that resulted in increased rHPC activity could be explained by the dissociation of rHPC from the micelles or removal of an inhibitor.

The ATPS described in this paper offer an alternative initial step for the purification of recombinant proteins from milk. The ATPS are very rapid which can be important with proteins that are unstable. The separations can be done at ambient temperature and no specialized equipment is needed. The biodegradable salt, citrate can be used with PEG to form ATPS *(23)*. Similar results were obtained in ATPS by substituting sodium citrate for AS in ATPS and partitioning milk from pigs and other species (Cole et al., in preparation). The results obtained from this study of ATPS processing of milk containing rHPC should facilitate the purification of other proteins expressed in milk. We are currently studying the use of these systems for the separation of milk proteins from other species.

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NOTE

The National Institute of Standards and Technology does not endorse any particular brand of product or company. Commercial names are used only for the precise description of experimental materials and procedures.

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