

The Method of Aschaffenburg and Drewry for the Crystallization of β -Lactoglobulin and α -Lactalbumin. 1. Electrophoresis of Fractions and the Calcium²⁺/Ethylenebis (Oxyethylenitrilo) Tetraacetic Acid Shift of α -Lactalbumin

MARVIN P. THOMPSON and DOROTHY P. BROWER
 United States Department of Agriculture
 Agricultural Research Service
 Eastern Regional Research Center¹
 600 E. Mermaid Lane
 Philadelphia, PA 19118

ABSTRACT

β -Lactoglobulin and α -Lactalbumin were isolated and crystallized by the classical method of Aschaffenburg and Drewry, and the purity of the crystals was assessed by native PAGE and SDS-PAGE. Although β -lactoglobulin was free of contaminants, α -lactalbumin was still contaminated after five recrystallization steps. Yields of crystals in this study were lower than reported by Aschaffenburg and Drewry. Further, it was observed that α -lactalbumin undergoes a Ca²⁺/Ca²⁺-free, dependent electrophoretic shift characteristic of many calcium-binding proteins.

INTRODUCTION

Together, β -lactoglobulin (β -LG) and α -lactalbumin (α -LA) compose about 80% of the whey proteins of bovine milk. Reports of the crystallization of these proteins go back nearly a century, making them among the earliest proteins to be crystallized (7). Over the past 30 yr a number of methods have been reported for the isolation of α -LA, all of which involve acid precipitation in the presence of ammonium sulfate (1, 7, 8, 9, 10) or chromatographic methods (2, 4, 19). Chromatographic methods (i.e., Sephadex G-100 and DEAE-Sephadex A50) served to supply purified baboon α -LA upon which the crystallography of the protein was performed and reported (2, 20). With this method (19), no acidification or ammonium sulfate precipitation was performed

(other than isoelectric precipitation of the caseins). In retrospect, this method was significant because α -La, later shown to bind Ca²⁺ (3, 13), was not subjected to low pH (i.e., 2.0), where it would likely release at least a portion of the bound Ca. Thus, the protein upon which crystallography was performed contained one strongly bound Ca²⁺ in a novel binding loop (20).

Similarly, β -LG has been prepared by a number of methods (17). Most methods rely on the insolubility of β -LG at pH 5.2 in the absence of salt. Unlike α -LA, which modifies galactosyl transferase in the lactose synthase system, the specific biological role(s) of β -LG is not known. However, Farrell and Thompson (6) have demonstrated that it inhibits phosphatase activity, a possible role confirmed by Jasinska et al. (15) for milk alkaline phosphatase.

This study began as a result of obtaining α -LA and β -LG from bovine milk as molecular weight standards for SDS-PAGE. To our knowledge, an evaluation of the 30-yr-old method of Aschaffenburg and Drewry (1) had not been reported using zonal electrophoresis. Thus, in this paper we report the electrophoretic behavior of whey protein fractions obtained during the isolation of α -LA and β -LG and the yield of these purified proteins. In a subsequent paper we will report the morphology of their crystals.

MATERIALS AND METHODS

Source of Milk

Freshly drawn pooled milk from Holstein cows was obtained from a local dairy farm in 8 and 22-L lots. No attempt was made to phenotype β -LG for the separate A and B variants.

Preparative Procedures

The β -LG and α -LA were isolated exactly as described by Aschaffenburg and Drewry (1) (Figure 1). Precipitates were separated by filtration on Whatman 2V filter paper except during the crystallization of α -LA and β -LG where centrifugation was used. Crystallization of β -Lg was performed as described. The α -La was crystallized from fraction P4, which was dissolved at pH 6.6 with the aid of 1 N NH_4OH . Saturated $(\text{NH}_4)_2\text{SO}_4$, adjusted to pH 6.6 with NH_4OH , was added slowly by a peristaltic pump. The "discolored precipitates", which formed at 40 and 50% of $(\text{NH}_4)_2\text{SO}_4$ saturation, were removed by centrifugation. Crystallization of the protein occurred freely if the pH was maintained at $6.6 \pm .2$.

Electrophoresis

The procedures for native and SDS-PAGE (16) were as described by Thompson et al. (21). Proteins were analyzed for their ability to undergo the $\text{Ca}^{2+}/\text{Ca}^{2+}$ -free, dependent electrophoretic shift (PAGE) by the following

method. Approximately .2 mg of protein was dissolved in running buffer (21). To one sample was added sufficient CaCl_2 to make the solution 10 mM Ca^{2+} . To another sample, sufficient EGTA ([ethylenebis (oxyethylenetriolo)] tetraacetic acid) was added to make the solution 10 mM. The proteins were electrophoresed in adjacent lanes.

The electrophoretic (protein) standards for SDS-PAGE were α -LA (bovine, 14 kdal), β -LG (bovine, 18 kdal), ovalbumin (43 kdal), and serum albumin (SA) (bovine, 67 kdal).

RESULTS AND DISCUSSION

Method

Figure 1 is a flow diagram showing all the essential features of the protocol of Aschaffenburg and Drewry (1) for the preparation of crystalline β -LG and α -LA. Only one exception to the procedure was noted. Aschaffenburg and Drewry reported that the pH of fraction F1 dropped to 2.0 when concentrated HCl was added at a rate of 1 ml/100 ml of filtrate. Such is not the case, for in using this procedure for

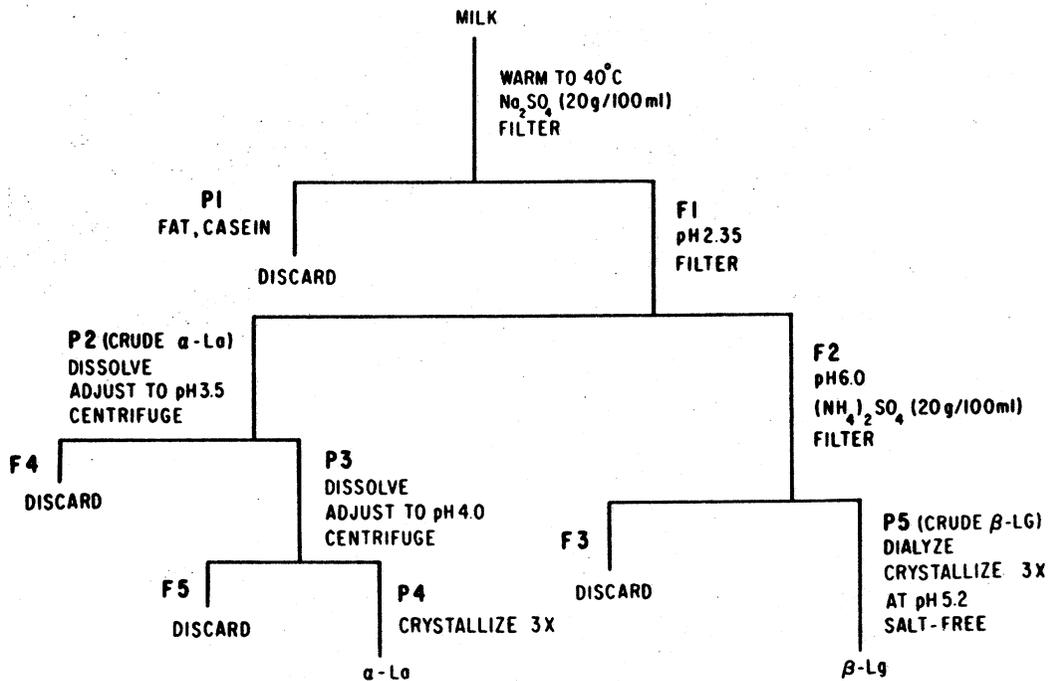


Figure 1. Flow diagram of the method of Aschaffenburg and Drewry (1) for the isolation and crystallization of β -lactoglobulin (β -LG) and α -lactalbumin (α -LA).

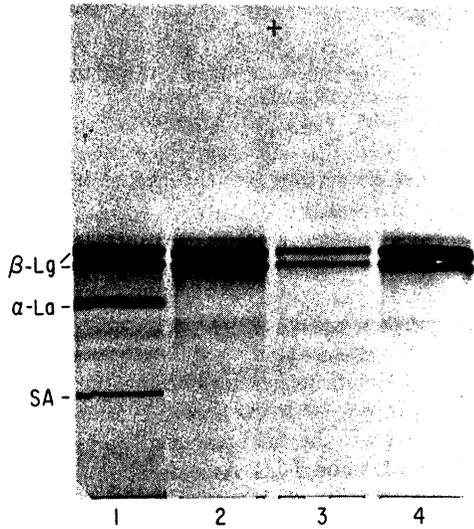


Figure 2. The PAGE of fractions F1, F2, F3 and P5 in lanes 1 to 4, respectively. Protein concentration was approximately 50 μ g/slot. β -LG = β -Lactoglobulin, α -LA = α -lactalbumin, SA = serum albumin.

several preparations, we have never observed the pH, at the above rate of addition, to fall below 2.35, nor did we make any attempt to

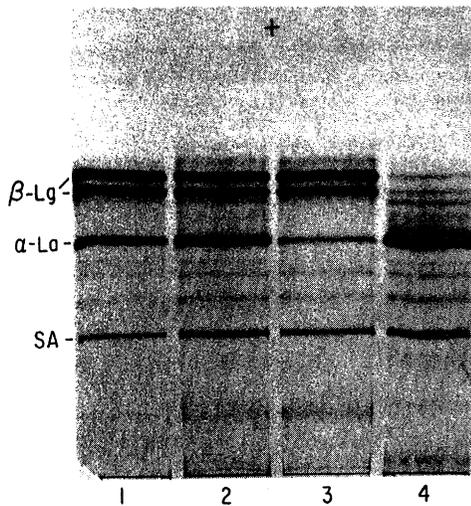


Figure 3. The PAGE of fraction F1, P2, F4, and P3 in lanes 1 to 4, respectively. Protein concentration was approximately 50 μ g/slot. β -LG = β -Lactoglobulin, α -LA = α -lactalbumin, SA = serum albumin.

adjust to pH 2.0. To reduce losses of α -LA during dialysis (1), membranes with a molecular weight retention of 9 kdal were used.

Electrophoresis of Fractions – Native Polyacrylamide Gel Electrophoresis

Figures 2 and 3 show the electrophoretic separation of the essential fractions outlined in Figure 1. Figure 2, lane 1, demonstrates that fraction F1, as expected, contains all of the whey proteins soluble in Na_2SO_4 . Lanes 2 (F2) and 4 (P5) show the progressive purification of β -LG (A and B). Lane 3 (F3) shows that a small amount of β -LG remains soluble in $(\text{NH}_4)_2\text{SO}_4$. The quantity was almost imperceptible, however. Fraction P5 was an excellent starting material for the crystallization of β -LG, because it was free of α -LA and SA, which would, indeed, inhibit crystallization at pH 5.2.

Figure 3 shows the steps of purification of α -LA. As can be observed in lane 2 (P2), there is an enrichment of both α -LA and SA. Most β -LG in P2 is removed when the solution is adjusted to pH 3.5; α -LA precipitates (P3) and β -LG remains soluble (F4). When fraction P3 is dissolved and adjusted to pH 4.0, the remainder of β -LG and SA is soluble (F5, electrophoresis not shown). Fraction P4, the starting material for crystallization, is still far from pure, but

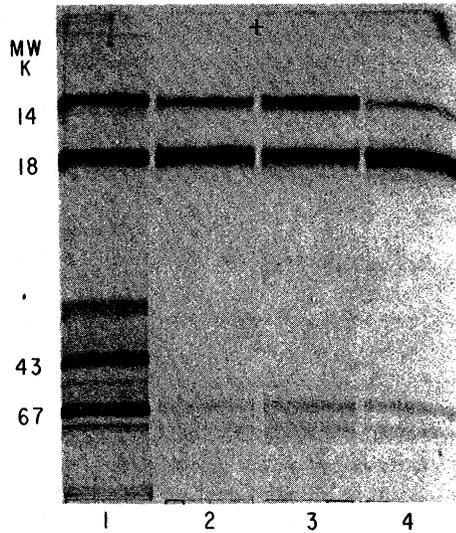


Figure 4. The SDS-PAGE of molecular weight standards, fractions F1, P2, and F4 in lanes 1 to 4, respectively. Protein concentration was approximately 70 μ g/slot, lane 1, and 20 μ g/slot, lanes 2 to 4.

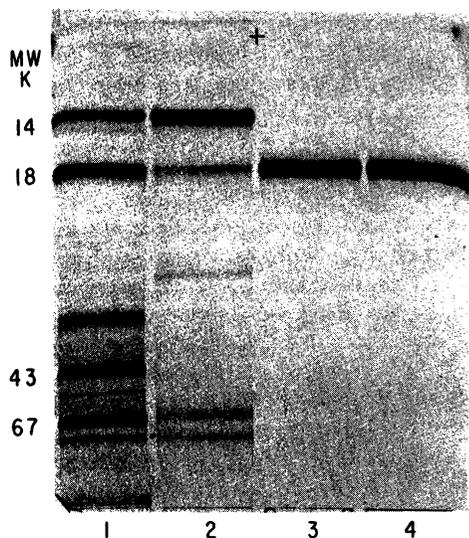


Figure 5. The SDS-PAGE of molecular weight standards, fractions P3, F2, and P5 in lanes 1 to 4, respectively. Protein concentration was approximately 70 $\mu\text{g/slot}$, lane 1, and 20 $\mu\text{g/slot}$, lanes 2 to 4.

crystallization proceeded normally, as will be discussed later.

Electrophoresis of Fractions – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Figures 4 and 5 show SDS-PAGE of the essential protein fractions in this study. Clearly, the proteins are readily discernible by molecular weight. Those of interest are α -LA (14 kdal), β -LG (18 kdal), and serum albumin (67 kdal). Figure 4, lane 2, fraction F1 shows all of the whey proteins soluble in Na_2SO_4 , whereas lane 3, P2, shows an enrichment of α -LA over β -LG. Lane 4 demonstrates the solubility (F4) of β -LG at pH 3.5 (see also Figure 3, lane 3). Figure 5 represents the final stages of purification of both α -LA and β -LG. As is noted in lane 2 (P3), α -LA remains contaminated with β -LG and SA, both of which are decreased markedly at fraction P4 (SDS-PAGE not shown). Lanes 3 and 4 represent fractions F2 and P5, respectively, with no detectable contamination of β -LG by other proteins. In PAGE alone, Figure 1, lane 4, fraction P5 is also only slightly contaminated.

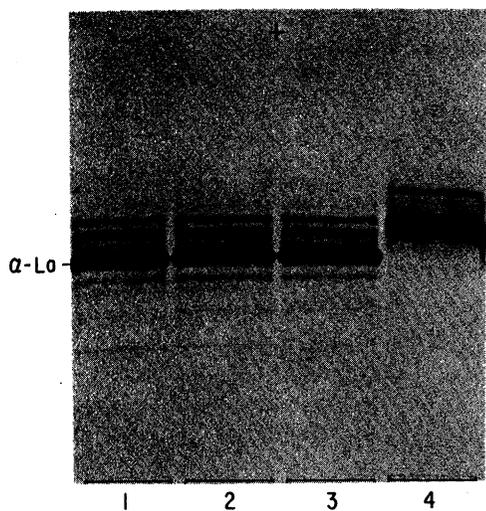


Figure 6. The PAGE of α -LA, first, second, and third crystallizations in the presence of 10 mM Ca^{2+} (lanes 1 to 3); α -lactalbumin (α -LA) (third crystallization) in the presence of 10 mM (lane 4). Protein concentration was approximately 40 $\mu\text{g/slot}$.

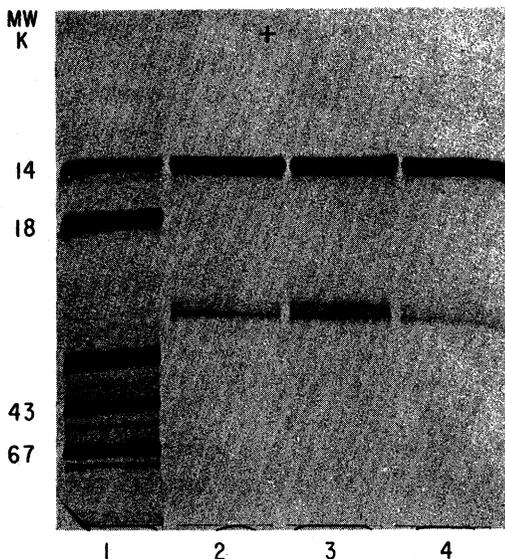


Figure 7. The SDS-PAGE of molecular weight standards and first, second, and third crystallizations of α -lactalbumin in lanes 1 to 4, respectively. Protein concentration was approximately 70 $\mu\text{g/slot}$, lane 1, and 20 $\mu\text{g/slot}$, lanes 2 to 4.

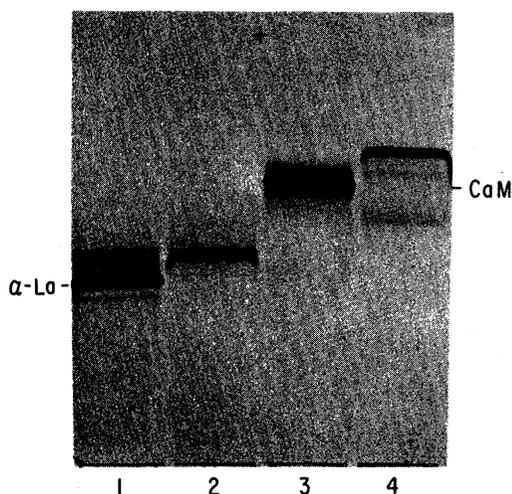


Figure 8. The PAGE of α -lactalbumin (α -LA) in lanes 1 and 2 containing 10 mM Ca^{2+} and 10 mM EGTA, respectively. Lanes 3 and 4 represent calmodulin (CaM) containing 10 mM Ca^{2+} and 10 mM EGTA, respectively. Protein concentration was approximately 60 $\mu\text{g/slot}$.

Electrophoresis of Crystallized Proteins

Following three times crystallization of β -LG at pH 5.2, there was no visual increase in purity over that demonstrated in Figure 5, lane 4. Thus, no PAGE of the protein is reported.

However, simple recrystallization of α -La does not significantly purify the protein as demonstrated in Figure 6, lanes 1 to 3 and lanes 2 to 4 of Figure 7. Following five to six recrystallizations, there was little improvement in purity of the protein. Many of the contaminating proteins possess β -LA-like properties and probably represent some of the glycosylated α -LA (14). Final purification of α -LA would be best accomplished by ion exchange chromatographic procedures such as reported by Quarfoth and Jenness (19).

α -Lactalbumin and the Calcium²⁺/Ethylenebis (oxyethylenitrilo) tetraacetic Acid Shift

The Ca-modulated protein, calmodulin (CaM), undergoes a Ca^{2+} /EGTA-dependent electrophoretic shift as do most Ca^{2+} -modulated proteins. It has been clearly demonstrated that α -LA strongly binds one Ca^{2+} /molecule (3, 20). As such, it should undergo the same type of shift as CaM in the presence of EGTA (11, 22,

23). Figure 8 (see caption) shows the effects of the addition of EGTA on the electrophoretic behavior of α -LA (lanes 1 and 2) and CaM (lanes 3 and 4) and Figure 6, lane 4. We have determined (not shown) that a minimum of 8 mM EGTA is required to chelate the bound Ca^{2+} in α -LA and cause the complete electrophoretic shift. When Ca^{2+} is chelated from the α -LA "elbow" (20), negative charges of aspartate are exposed resulting in an increased electrophoretic mobility. The electrophoretic shift of α -LA is not as easily observed in lower pH buffers as those described by McLellan (18).

Further, as can be observed in Figure 6, many of the contaminating proteins of α -LA undergo the Ca^{2+} /EGTA-dependent electrophoretic shift, confirming the suggestion that they are, indeed, α -La-like molecules. Neither β -LG nor the caseins undergo this shift, because they do not possess strong Ca^{2+} -binding sites.

Yields of Purified Crystals

Aschaffenburg and Drewry reported yields of α -LA and β -LG to be .38 to .43 g/L and 1.3 to 1.5 g/L of filtrate F1, respectively. Despite extreme care in fractionation, our yields were .30 and 1.0 g, respectively, for α -LA and β -LG based upon the volume of filtrate F1. One apparent reason for this could be that Aschaffenburg and Drewry used milks from either Jersey or Guernsey cattle, whereas our milk supply was from Holstein cattle.

CONCLUSIONS

Although the method of Aschaffenburg and Drewry is 30 yr old, this method clearly produces high quality crystals of β -LG in good yield. Although α -LA crystals are easily prepared, they are not of high purity even after several recrystallizations. Nevertheless, for all practical purposes, such as molecular weight standards and reference proteins, the purity is adequate. Final purification would be accomplished by chromatographic procedures (19).

The Ca^{2+} /EGTA electrophoretic shift is useful in identifying α -LA in mammary tissue extracts and in the wheys of mammals of different species (11, 22, 23). It is also useful for determining the purity of α -LA preparations when contaminating Ca-binding proteins (gly-

cosylated α -LA) or non-Ca-binding proteins migrate with relative mobility (R_m) values similar to α -LA.

Further significance can be ascribed to the electrophoretic shift of α -LA, because most Ca-modulated proteins exhibit similar behavior in the presence or absence of Ca. One can postulate that α -LA is, in fact, a Ca-modulated protein, although there is no unequivocal evidence that Ca^{2+} is necessary for α -LA to function in the lactose synthase system (4, 12). Under physiological conditions, however, it is unlikely that α -LA is not saturated with Ca^{2+} (30 mM in milk). In addition, Ca^{2+} -free α -LA (apoprotein) readily denatures at 37°C (Thompson, unpublished), which provides further evidence that Ca^{2+} stabilizes the protein against thermal denaturation (13) and, therefore, that Ca^{2+} is necessary for lactose synthesis. Based upon numerous reports that most Ca-modulated proteins (i.e., CaM) are multifunctional (5), it seems that α -LA, as well, might modulate other cellular functions in addition to its clearly defined role in lactose synthesis.

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