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Improved Method for the Preparation of Crystalline β -Lactoglobulin and α -Lactalbumin from Cow's Milk

By R. ASCHAFFENBURG AND J. DREWRY

Chemistry Department, National Institute for Research in Dairying, University of Reading

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Crystalline β -lactoglobulin is usually prepared from cow's milk by modifications of the method of Palmer (1934), based on exhaustive dialysis of the 'lactalbumin' fraction isolated by various salting-out procedures. A full description of this type of preparation has recently been given by Larson & Jenness (1955), who list earlier references. The 'lactalbumin' fraction contains another major whey protein, α -lactalbumin, which can be isolated from the β -lactoglobulin mother liquor (Gordon & Semmett, 1953; Gordon, Semmett & Ziegler, 1954; Gordon & Ziegler, 1955).

It has been noted (Gordon & Ziegler, 1955) that 'incomplete removal of β -lactoglobulin appears to hinder the subsequent crystallization of α -lactalbumin', and we have found that the presence of α -lactalbumin tends to interfere with the formation of β -lactoglobulin crystals, and to affect their purity adversely. Furthermore, the lengthy process of crystallizing the β -lactoglobulin is time-wasting when preparation of the α -lactalbumin is the sole objective. A clean separation of the two proteins at an early stage in their preparation would thus provide obvious advantages. Zweig & Block (1954) attempted segregation of the proteins after precipitation with ferric chloride, but achieved only partial separation and a much lower yield of α -lactalbumin than that obtainable by the improved procedure of Gordon *et al.* (1954).

The present paper, which forms part of a general study of whey proteins, describes a simple method for the early and practically complete separation of β -lactoglobulin from α -lactalbumin, and for the

crystallization of the separated proteins by steps which can be carried out simultaneously or independently with a considerable saving in time. The milks of individual cows are now known to contain either a mixture ($\beta_{1,2}$) of two different β -lactoglobulins, or only one (β_1) or the other (β_2) of these (Aschaffenburg & Drewry, 1955). The method is equally applicable to such milks and to their mixture in bulk milk, except for minor modifications during the final stages of the preparation of crystalline β_2 -lactoglobulin.

EXPERIMENTAL

Milk. All samples were of fresh, whole milk from cows of the Institute herds. Fractionation of 2–10 l. of the uncooled milk was started within a few hours of milking.

Reagents. All chemicals used in the fractionation procedures were of analytical reagent quality. This avoids loss of time through re-working, which was found to be necessary when less pure reagents were employed. Distilled water was used in all operations, as the presence of heavy metal ions in tap water led to the formation of discoloured protein precipitates.

Dialysis. This was done in narrow bags made from seamless Visking cellulose tubing by tying one end into a double knot, filling the bag, and securing the other end in the same manner. By leaving no air-space, undesirable increases in volume were reduced to a minimum. Although pressure inside the bags may increase considerably, no bursts have occurred with narrow tubing (not exceeding $\frac{3}{4}$ in. inflated diameter). If necessary, more than one narrow bag was filled rather than one of larger diameter. The bags were made to rotate during dialysis.

pH. This was determined with a glass electrode and a Pye Universal pH meter. All values refer to a temperature of 20°.

Nitrogen. Determinations were made by the semi-micro Kjeldahl method of Rowland (1938), with a selenium catalyst. Heating was continued for 1 hr. after the digests had cleared.

Working temperature. Unless specifically stated, all operations were done at room temperature (approx. 18°). In our experience, this does not affect the preparations adversely, and has, in fact, distinct advantages during the crystallization of β -lactoglobulin which, in confirmation of Palmer (1934), was found to be easier and quicker at room temperature than at that of the cold room.

Paper electrophoresis. This convenient method was used widely during the development stages to test the efficacy of the various fractionation steps. At each step, the proteins remaining in solution were salted out, and concentrated solutions of these as well as of the protein precipitated at the particular stage were analysed to obtain a complementary picture. All concentrates were prepared for electrophoresis by dialysis in seamless Visking cellulose tubing of $\frac{3}{8}$ in. inflated diameter against running tap water. Prolonged dialysis was unnecessary, a minimum time of 90 min. being sufficient to obtain satisfactory electrophoretic resolution. All runs were made in an apparatus of the type described by Flynn & de Mayo (1951), applying 230 v for 16 hr. at room temperature, on Whatman no. 3MM paper at pH 8.6 in barbitone buffer, *I* 0.05. The test samples were applied on the cathodic side, 7.5 cm. from the apex. Bromophenol blue was used for staining, and electrophoretic diagrams were obtained by direct scanning of the untreated strips. As will be discussed elsewhere, these diagrams are not suitable for quantitative evaluation of the concentration and distribution of whey proteins.

METHODS OF FRACTIONATION

Isolation of the 'lactalbumins'

Whole milk is warmed to about 40°, and anhydrous Na_2SO_4 (20 g./100 ml.) added with stirring. After the salt has dissolved, and the temperature has fallen below 25°, the mixture is filtered through fluted Whatman no. 4 papers. There is no need to prepare separated milk, since the presence of the milk fat improves the rate of filtration. The filtrate (F1) contains the 'lactalbumins', as shown in Fig. 1a. The precipitate containing the globulins and proteose-peptone components of milk, in addition to the casein and fat, is discarded. If the globulins and proteose-peptone components are required, the casein and fat may first be separated by acid precipitation, and the other components precipitated as above after the pH has been adjusted to 6.8.

Separation of β -lactoglobulin and α -lactalbumin

The volume of filtrate F1 is measured, and conc. HCl (1 ml./100 ml.) added with vigorous stirring. This reduces the pH to approximately 2, causing rapid precipitation of all the protein constituents with the exception of the β -lactoglobulin, as shown in Fig. 1b, c. If the preparation is left to stand at

this stage, Na_2SO_4 tends to crystallize out, and should be redissolved by gentle warming, as a reduction of its concentration affects the subsequent salting-out of the β -lactoglobulin fraction. The precipitate (P2) is best separated from the supernatant (F2) by prolonged centrifuging, but the precipitate does not always pack well. In that case, it must be obtained by filtration through a fluted Whatman no. 4 paper. The separated proteins can now be worked up simultaneously. Their subsequent behaviour suggests that they are unaffected by the

Fig. 1. Electrophoretic pattern of (a) the 'lactalbumins', and of (b) the β -lactoglobulin in the supernatant, and (c) the α -lactalbumin in the precipitate obtained after acidification to pH 2.

treatment at pH 2, a finding in line with the work of Zweig & Block (1954) whose procedure involves a lengthy dialysis under even more acid conditions.

Crystallization of β -lactoglobulin

The volume of supernatant F2 is measured, and the pH brought to about 6 by dropping conc. NH_3 soln. (about 0.6 ml./100 ml.) into the well-stirred solution. The β -lactoglobulin is then precipitated with $(\text{NH}_4)_2\text{SO}_4$ (20 g./100 ml.). After the protein has flocculated, it is filtered by suction through a thin pad of Hyflo Super-Cel deposited on a Whatman no. 1 filter paper. The filtrate is discarded, and the filter cake triturated with a little water to produce a paste just sufficiently fluid to permit transfer to a dialysing bag. It is desirable to reduce the volume of supernatant F2 to about one-eightieth to one-hundredth at this stage. The bag is closed and left stationary in water saturated with toluene. After dialysis overnight, the contents of the bag are filtered by suction through Whatman no. 1 paper to remove the filter aid. The pH of the clear filtrate is adjusted to 5.8, toluene added, and dialysis continued for 24 hr. by rotating the bag in several changes of water. Without rotation, the period of dialysis should be increased to at least 48 hr.

The pH is now reduced to 5.2 with dil. HCl, which causes the solution to turn cloudy. Preparations of β_2 -lactoglobulin may remain almost clear at this stage, and their treatment will be discussed separately. With all other forms of β -lactoglobulin, the protein separates on further dialysis within a few hours in the form of orthorhombic plates or of an oil that can be converted quickly into crystals by seeding. Crystals from bulk milk preparations, and crystals of β_1 - or $\beta_{1,2}$ -lactoglobulin may be used indiscriminately for seeding, as they are isomorphous (Green, North & Aschaffenburg, 1956). Without seeding, crystallization may be delayed, although it has always occurred within 24 hr. Once crystals have formed, most of the protein separates within 24 hr. when, with concentrated solutions, as little as 5–10% of the nitrogenous matter has been found to remain in solution.

According to Larson & Jenness (1955) the first crystalline product obtained by the conventional method should be recrystallized at least four times. This is not necessary with crystals prepared by the present method, since the starting material (F2) is virtually free from proteins other than β -lactoglobulin (cf. Fig. 1b). With preparations made from bulk milk, the yield of crystals (dry wt.) has been 1.3–1.5 g./l. of filtrate F1, i.e. of the same order as that reported by Palmer (1934) of 1.5 g./l. of whey. Similar yields have been obtained in preparing $\beta_{1,2}$ - and β_1 -lactoglobulin.

Crystallization of β_2 -lactoglobulin. As mentioned above, solutions of β_2 -lactoglobulin tend to remain

almost clear when the pH is adjusted to 5.2. On further dialysis, they are slow in forming an oil and in crystallizing unaided, and the yield of crystals is usually disappointing. Two factors combine to cause this anomalous behaviour. The concentration of β_2 -lactoglobulin in the milk of cows producing this type tends to be rather low (Aschaffenburg & Drewry, in preparation), and it is, therefore, particularly important to prepare as concentrated a solution as possible of the salted-out protein, and the diamond-shaped crystals formed by spontaneous crystallization have a relatively high solubility. Fortunately, β_2 -lactoglobulin occurs in two forms, the diamond-shaped crystals which are monoclinic, and orthorhombic crystals which are less soluble than the former (Green *et al.* 1956). To obtain β_2 -lactoglobulin in better yield, it is therefore advisable to proceed in the usual manner and to seed the solutions with orthorhombic crystals. If monoclinic crystals are required, they can be obtained with ease by dialysis against water of highly concentrated solutions of the crystals in dil. NaCl.

Crystallization of α -lactalbumin

As shown in Fig. 1c, the precipitate (P2) obtained at pH 2.0 contains the α -lactalbumin together with the blood-serum albumin normally present in milk (Polis, Shmukler & Custer, 1950), two minor whey-protein constituents, and some residual β -lactoglobulin. The precipitate is dissolved in water with the help of dil. NH_3 soln. to one-tenth of the volume of filtrate F1. Any residual β -lactoglobulin is now removed by reducing the pH to 3.5 with dil. HCl, when this protein remains in solution. The centrifuged precipitate is dissolved in one-quarter of the previous volume of dil. NH_3 soln., and dil. HCl added to pH 4.0. At this pH, the α -lactalbumin precipitates slowly, and the sample is best stored in the cold overnight. After warming to room temperature, the precipitate is spun down in the centrifuge. To the separated supernatant, solid $(\text{NH}_4)_2\text{SO}_4$ (11.5 g./100 ml.) is added to bring down additional α -lactalbumin. The supernatant from this precipitate contains the blood-serum albumin, and appears to be a more convenient starting material for the isolation of this protein than the mother liquor obtained after crystallization of β -lactoglobulin by the conventional method which was used by Polis *et al.* (1950).

The α -lactalbumin precipitates are again dissolved in dil. NH_3 soln., and made up approximately to the previous volume. The pH is adjusted to 6.6, and an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ soln., previously brought to the same pH with conc. NH_3 soln., is added under stirring, as recommended by Gordon & Semmett (1953). After removal of the discoloured precipitate by filtration through

Whatman no. 4 paper, the α -lactalbumin is crystallized by their method of further slow addition of saturated $(\text{NH}_4)_2\text{SO}_4$ soln., adding about one-and-a-half times the volume required for half-saturation.

The first crystalline product is usually far from pure, the two minor protein constituents shown in Fig. 1c forming the main contaminants. The faster-moving of these does, in our experience, cling tenaciously to α -lactalbumin, and is difficult to remove even by several paired reprecipitations at pH 4.0 and recrystallizations at pH 6.6 in the manner recommended by Gordon *et al.* (1954). Attempts to remove this contaminant by other fractionation procedures have been unsuccessful. Gordon & Ziegler (1955) have reported a yield of once-recrystallized α -lactalbumin (anhydrous, salt-free basis) of 0.36 g./l. of whey. Our yields (dry wt.) at this stage have varied from 0.38 to 0.43 g./l. of filtrate F1.

Large, well-defined crystals are difficult to obtain by the method described above. They can be prepared with ease by adding saturated $(\text{NH}_4)_2\text{SO}_4$ soln. to the half-saturated filtrate until a faint cloudiness develops, by transferring the solution to a crystallizing dish, and by allowing the protein to crystallize slowly as the $(\text{NH}_4)_2\text{SO}_4$ concentration increases through evaporation. When crystallization is complete, as indicated by the absence of turbidity after addition of saturated $(\text{NH}_4)_2\text{SO}_4$ soln. to a filtered sample, the crystals are taken up in 0.7 saturated $(\text{NH}_4)_2\text{SO}_4$ soln. to dissolve any salt crystals formed during evaporation. Application of this technique has produced three types of α -lactalbumin crystals (Fig. 2). They formed in a haphazard manner when allowed to crystallize spontaneously, and seeding with one type led more often than not to the formation of one of the other types in the evaporating solutions. A photomicrograph of bullet-shaped crystals is to be found in the paper by Zweig & Block (1954).

For a protein, α -lactalbumin has a relatively low molecular weight of about 16 000 (Gordon & Semmett, 1953). This may account for the finding that on ultrafiltration, though not materially on dialysis, the dissolved protein passes through Visking tubing with some ease. We have, in fact, found it possible to prepare crystalline α -lactalbumin from a straight ultrafiltrate of milk.

SUMMARY

1. An improved method for the crystallization of the β -lactoglobulins and α -lactalbumin of cow's milk is described in full. Its main advantage lies in the initial separation of the two types of protein by a simple acidification procedure, thereby permitting simultaneous or independent working-up of the separated fractions.

2. The method is equally applicable to bulk milk, and to the milk of individual cows containing β_1 - or β_2 -lactoglobulin either singly or in combination.

Fig. 2. Different crystal forms of α -lactalbumin ($\times 160$).

3. Yields of β_2 -lactoglobulin may be improved by crystallizing it in the orthorhombic rather than the monoclinic form.

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Connective Tissue Growth Stimulated by Carrageenin

1. THE FORMATION AND REMOVAL OF COLLAGEN

By D. S. JACKSON

Rheumatism Research Centre, University of Manchester

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Although connective tissue growth has been studied intensively by histological methods (for review see Cameron, 1952) and to a lesser degree by electron-optical methods, very few biochemical studies are available in the literature. Kodicek & Loewi (1955) have studied the granulation tissue in regenerating tendon, and Dunphy & Udupa (1955) have reported on a combined biochemical and histological study of connective tissue formation in healing wounds. In neither of these studies was any attempt made to fractionate the collagen into the three types known to exist in developing connective tissue. The paucity of information is probably due to the fact that the amount of new tissue formed in a given period in normal circumstances is small and cannot be separated from pre-existing tissue. Where the new tissue is separable, as for example in healing wounds, the amount of tissue formed is insufficient for detailed biochemical studies. Recently, however, Robertson & Schwartz (1953) have reported a method of producing comparatively large amounts of easily separated connective tissue. This method consisted of injecting a solution of carrageenin subcutaneously into the lower abdominal regions of guinea pigs. Carrageenin is a sulphated polygalactose which is extracted from the seaweed Irish moss (*Chondrus crispus*). Here again, however, no attempt was made to fractionate the collagen.

Quantitatively, collagen is the most important constituent of connective tissue, in which three forms of this protein are known to exist. One form, referred to as acid-soluble collagen, can be extracted either with dilute acetic acid (Zachariades, 1900;

Nageotte, 1927; Leplat, 1933) or by acid buffers (Tustanovskii, 1947). The second form was isolated by Highberger, Gross & Schmitt (1951) from skin by extraction with slightly alkaline phosphate buffers and more recently with neutral salt solution (Gross, Highberger & Schmitt, 1955; Jackson & Fessler, 1955). This fraction has been called 'alkali-soluble collagen' (Harkness, Marko, Muir & Neuberger, 1954) or 'neutral-salt-soluble collagen' (Jackson & Fessler, 1955) and will be referred to in this communication by the latter name. The bulk of the collagen in connective tissue is insoluble but can be rendered soluble by transformation into gelatin.

It has been suggested that acid-soluble collagen is the precursor of insoluble collagen because its concentration in connective tissue decreased with age and because [α - 14 C]glycine was incorporated more rapidly into acid-soluble collagen than into insoluble collagen (Orekhovitch, 1952, 1955). However, Harkness *et al.* (1954) showed that [α - 14 C]glycine is incorporated most rapidly into collagen soluble in neutral salt solution, indicating that this was the true precursor, and further that insoluble collagen need not pass through the acid-soluble stage. It has also been suggested (Bowes, Elliott & Moss, 1953, 1955) that insoluble collagen is a complex of acid-soluble collagen and a mucoprotein of high tyrosine content. That there is no sharp division between acid-soluble and insoluble collagen was indicated by the increased solubility in acid of tendon treated with hyaluronidase (Jackson, 1953).