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Separation of the Components of α-Casein
II. The Preparation of α_3-Casein[1]

A method is described for preparing α_3-casein from α-casein involving the use of calcium chloride and ultracentrifugation. It was homogeneous by electrophoresis and sedimentation and contained 0.35% phosphorus, 1.2% sialic acid, and 0.75% hexose. The properties and composition of α_3-casein are discussed in relation to the other components of α-casein.

INTRODUCTION

In the first paper (1) of this series, a method was described for the separation of the principal electrophoretic component of the α-casein fraction, designated α_1-casein. The method of separating α_1-casein was based in part on precipitating it with calcium chloride somewhat as Waugh and von Hippel (2) used for the separation of their S-casein fraction. The portion of the α-casein fraction soluble in calcium chloride was found to contain at least two casein components which are designated α_2- and α_3-casein based on their respective decreasing electrophoretic mobilities at pH 2.35 of 4.6 and 3.1.

The present communication describes the purification and properties of the α-casein component with the mobility of 3.1 at pH 2.35, designated α_3-casein. This casein has some of the properties of the κ-casein fraction of Waugh and von Hippel (2) which they considered to be responsible for stabilizing the micelles of the casein complex.

MATERIALS AND METHODS

The α-casein was prepared from an acid-extracted casein by a modification of the Warner method, as described by McMeekin, Hipp, and Groves (1). Sialic acid was determined by the resorcinol–hydrochloric acid method of Svennerholm (3). We are indebted to Dr. K. Schmid for a quantity of α_1-acid glycoprotein (4) which was used as a standard for the sialic acid determinations. Protein-bound hexose was determined by the use of the orcinol–sulfuric acid reagent, as described by Winzler (5). Sedimentation studies were made using the Spinco[3] Model E ultracentrifuge at 59,780 r.p.m. with 12-mm. Kel-F cells in the An-D analytical rotor. The reported sedimentation values were not corrected for the partial specific volume of the protein or the viscosity of the buffer solution. For preparative work, the Spinco model L ultracentrifuge with the No. 40 rotor at 40,000 r.p.m. was employed. The electrophoretic experiments were performed in a Tiselius apparatus, and mobilities were calculated as described by Warner (6).

EXPERIMENTAL

PREPARATION OF α_3-CASEIN

α_3-Casein was prepared from the fraction of α-casein (Fig. 1*B*) that is soluble in 0.2 *M* calcium chloride. α-Casein was dissolved with dilute sodium hydroxide to give a 2.0% solution at pH 7.0. The solution was made to 0.2 *M* $CaCl_2$ by the slow addition, with stirring, of 1 *M* $CaCl_2$, the pH being kept at 7.0 by the addition of 0.1 *M* NaOH. After standing overnight in the cold, the precipi-

[1] Presented at the 136th Meeting of the American Chemical Society, Atlantic City, N. J., September, 1959.

[2] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[3] Mention of products in this paper does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

tated α_1-casein fraction was removed at room temperature by centrifugation at 3500 $\times$ g for 20 min. The portion soluble in calcium chloride, amounting to about 20% of the total protein, contains at least two electrophoretic components designated α_2- and α_3-casein, as shown by the electrophoretic pattern B in Fig. 1. The removal of calcium chloride from the soluble portion was accomplished by dialysis of the solution after acidifying to a pH of about 3.5. After removal of the calcium chloride, the protein was recovered by lyophilizing the contents of the cellophane bag. All subsequent fractions obtained by centrifugation were processed in the same manner. It was then found that the α_3-casein component could be separated by high-speed centrifugation. This was accomplished by centrifuging under refrigeration a 1% solution at pH 7.0 of the calcium-soluble fraction of α-casein, containing 0.2 M sodium chloride, at 40,000 r.p.m. for 5 hr. The distribution of the protein and the phosphorus content between the pellet and the material in the bottom and top half of the centrifuge tube is shown in Fig. 2. Under these conditions, α_3-casein is concentrated in the separated pellet, as is shown by the electrophoretic pattern C in Fig. 1. The electrophoretic pattern D in Fig. 1 shows the concentration of α_2-casein or λ-casein in the top half of the solution in agreement with the results of Long *et al.* (7) under similar experimental conditions. Our preparation, however, contained a fast-moving electrophoretic component which was not resolved by sedimentation at pH 7.0.

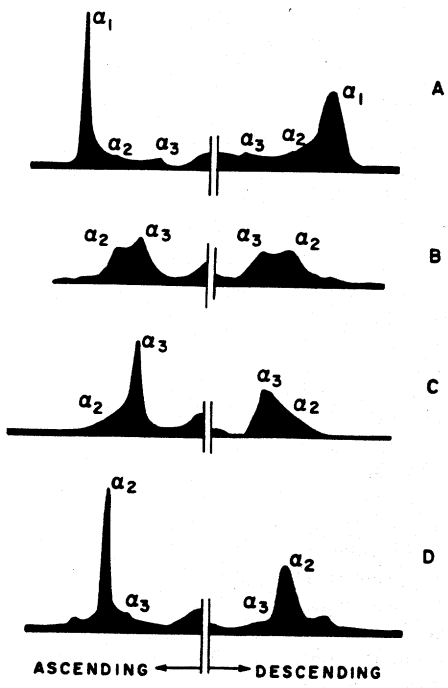

FIG. 1. Electrophoretic patterns obtained in HCl–NaCl buffer at pH 2.35 with an ionic strength of 0.1; protein concentration about 1% at a field strength of 4.70–4.92 v./cm. for 3 hr. A, α-Casein; B, α-casein soluble in 0.2 M $CaCl_2$ at pH 7; C, pellet formed on centrifugation of B, α_3 fraction; D, top half of solution on centrifugation of B, α_2 fraction.

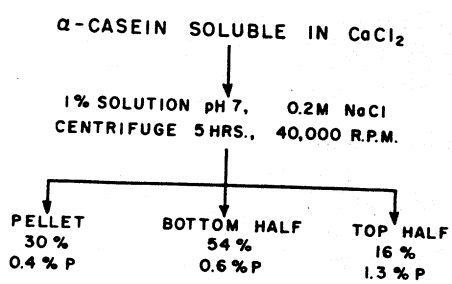

FIG. 2. Fractionation by ultracentrifugation of α-casein components soluble in $CaCl_2$.

Further purification of the α_3-casein could not be attained efficiently by recentrifugation of the pellet under the same conditions. However, it was found by changing the salt concentration that further purification could be accomplished. Pure α_3-casein is obtained when the pellet that is formed by centrifugation in the presence of 0.2 M salt is recentrifuged twice in the absence of salt. The purity of the α_3-casein is based on the removal of α_2-casein, which is the fast-moving electrophoretic component and slow-sedimenting component, as illustrated in Fig. 3. It was also found that α_3-casein could not be resolved into more than one component by starch gel electrophoresis at pH's 8.6 and 3.1 in the presence and absence of 7 M urea.

PROPERTIES

The S_{20} value of the α_3-casein component increased from 8 to 16 as the impurity, α_2-casein, was removed. Also, with purification,

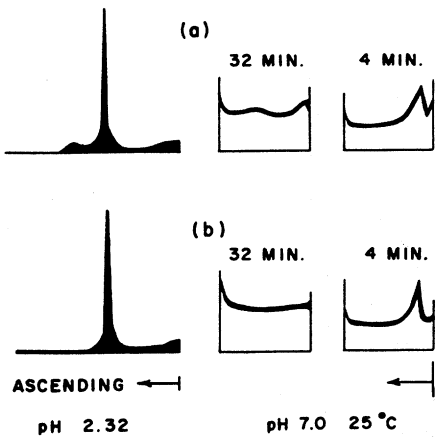

FIG. 3. Electrophoretic and sedimentation patterns before and after final purification of α_3-casein. Electrophoretic patterns obtained as given in Fig. 1. Sedimentation patterns in 0.1 ionic strength phosphate buffer (16) pH 7.0 at 25°. (*a*) Results on pellet obtained by centrifugation in salt, $S_{20} = 1.3$ and 12.4; (*b*) results on pellet after final purification by centrifugation in absence of salt, $S_{20} = 23.1$.

the solubility of the α_3-casein decreased in both acid and alkali. The electrophoretically pure α_3-casein gave a value of $S_{20} = 23.1$ *S* at pH 7.0 at 25° and at 2°. In acid solution, the sedimentation constant was very large, as indicated by the boundary moving out of the cell before maximum speed was attained. At pH 11.8, α_3-casein gave a number of sedimenting components with lower S_{20} values, and electrophoresis at pH 11.8 also showed a number of components to be present and the ascending and descending patterns were not in agreement. Electrophoretic patterns at pH 10.1 of α_3-casein which had been exposed to pH 11.8 at low temperature showed heterogeneity, indicating that under these conditions α_3-casein is unstable, since this preparation gave only one electrophoretic component at pH 10.1 with a mobility of −8.9 before making to pH 11.8. The nature of this change has not been determined.

The composition of α_3-casein compared with that of α_1-casein (1) is given in Table I, and some properties of α_3- compared with α_1-casein and λ-casein from the data of Long *et al.* (7) are given in Table II. The data clearly showed that α_3-casein is a separate and distinct component of α-casein.

The electrophoretic data in Fig. 4 suggest that the isoelectric point of α_3-casein is probably lower than α_1-casein. The insolubility of α_3-casein between pH 3.0 and 6.0 prevents the determination of this point more accurately. The decrease in net charge, as is demonstrated by the mobility values of α_3-casein compared with α_1-casein, in both acid and alkaline solutions is of interest since it reflects differences in the net number

TABLE I

COMPOSITION[a] OF α_3- AND α_1-CASEIN

	Per cent	
	α_3	α_1
N	14.6	14.1
P	0.35	0.85
Ash[b]	1.70	2.35
True ash[c]	0.89	0.40
Hexose	0.75	0.00
Sialic acid	1.21	0.00

[a] On a moisture-free basis.

[b] Determined after adding magnesium acetate and corrected for magnesium oxide.

[c] Attributed to inorganic contamination. The value given was obtained by subtracting the theoretical ash of phosphorus pentoxide formed from the casein from the determined ash.

TABLE II

COMPARISON OF SOME PROPERTIES OF α_3-, α_1- AND λ-CASEIN

	Casein		
	α_3	α_1	λ[a]
Stability to $CaCl_2$	Yes	No	Yes
Clotting with rennin (Ca present)	Yes	—[d]	?
S_{20}, pH 7.0, 25°[b]	23.1	3.0	1.1
Mobility, pH 2.3[c]	3.6	6.9	4.6[e]

[a] Data as reported by Long (7) referred to in our nomenclature as α_2-casein.

[b] At 2° the values for α_3- and α_1-casein are unchanged.

[c] At 0.1 ionic strength in HCl–NaCl buffer on descending boundary.

[d] α_1-Casein-insoluble.

[e] Value for α_2 in the presence of α_3 and the fast-moving component calculated from the descending boundary, shown in Fig. 1*D*. Value of 5.2 obtained on a purified sample.

of negatively and positively charged groups in the molecule. These differences should be reflected in the polar amino acid content of these casein components.

The ultraviolet absorption curves in Fig. 5 show that the aromatic amino acid composition of α_3- and α_1-casein is significantly different. The difference in the specific extinction coefficient of 1.56 for α_3-casein compared with 1.05 for α_1-casein and 1.025 for α-casein (8) is of sufficient magnitude so that gross errors in a calculated protein concentration from the absorption data at 278 mμ would occur if the α-casein fraction contained varying amounts of α_3-casein. Since the specific extinction coefficient of α-casein and its major component, α_1-casein, are about the same, and α_3-casein is higher, then α-casein must contain a component with a lower specific extinction coefficient than α_1-casein.

Rennin Action on α_3-Casein

A 0.26% solution of α_3-casein containing calcium at pH 6.9 was clotted by rennin with a 100:1 ratio of protein to enzyme, and, after 15 min., 16.9% of the total nitrogen was found in the supernatant. This is consistent with the finding of Wake (9) that fractions rich in κ-casein released large amounts of nonprotein nitrogen by rennin action. The values for the specific extinction

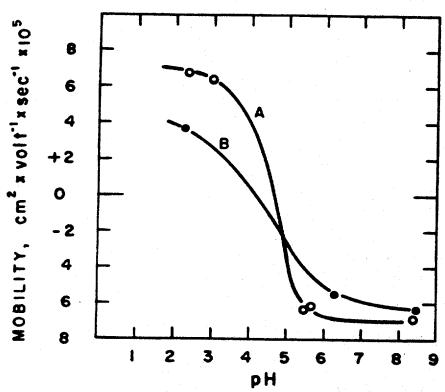

Fig. 4. Effect of pH on mobility of α_3- and α_1-caseins at 1.0°, ionic strength of 0.1 at a protein concentration of 0.5–1%. Veronal buffer at pH 8.5, sodium acetate at 6.3, and HCl–NaCl at pH 2.35. Data for α_1-casein from Ref. (1). *A*, α_1-Casein; *B*, α_3-casein.

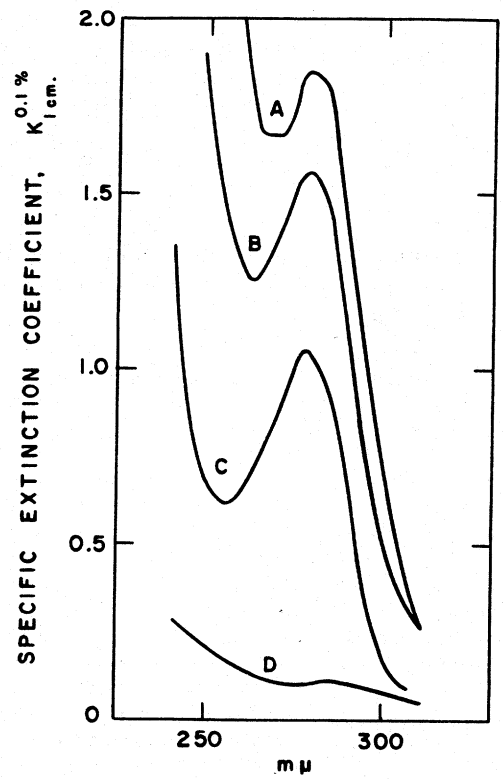

Fig. 5. Ultraviolet absorption curves at pH 6.0–8.0. The specific extinction coefficient is indicated in brackets. *A*, α_3-Paracasein, rennin clot (1.85); *B*, α_3-casein (1.56); *C*, α_1-casein (1.05); *D*, non-rennin-clotted portion of α_3-casein (0.11). The values for *A* and *D* were obtained by calculating the concentration from the determined nitrogen using 14.79 as the per cent nitrogen on an ash-free moisture-free basis, the value found for α_3-casein.

coefficient for the clotted portion was 1.85 and the soluble portion 0.11, as given in Fig. 5, using the nitrogen content of α_3-casein of 14.8% for both portions. The use of this assumed value for the nitrogen content is obviously inaccurate. Nitschmann *et al.* (10) have reported 11.4% and Brunner *et al.* (11) 11.7% for the nitrogen content of the macropeptide isolated from the supernatant obtained by clotting casein with rennin. The lower value for the nitrogen content of the soluble portion would give a value of about 0.085 for the specific absorption constant instead of 0.11. This very low value for the extinction coefficient of the rennin-solubilized α_3-casein is consistent with the find-

ings of Nitschmann *et al.* (12) and Jollès *et al.* (13) that the soluble glycopeptide isolated from the rennin activity on casein contained no aromatic amino acids.

Under the conditions described above for the action of rennin on α_3-casein, over 80% of the sialic acid was found in the soluble portion. The method used for determining sialic acid (3) does not distinguish between free and bound sialic acid. However, Nitschmann and Henzi (12) have isolated a macropeptide from the supernatant of the rennin clot from α-casein containing relatively large amounts of sialic acid, indicating that sialic acid is bound as a glycopeptide. It was found that the calcium-soluble fraction of α-casein contained about the same amount of sialic acid as the α_3-casein isolated from this fraction. Some of the other components of the calcium-soluble fraction of α-casein must therefore also be associated with sialic acid and may also be involved in the action of rennin to yield glycomacropeptides and in the formation of a casein clot.

Stabilization of Casein to Calcium Ions by α_3-Casein

Waugh (14) has shown that there is a casein fraction designated κ-casein that has the property of stabilizing casein micelles in the presence of calcium. He established that micelles form only in the presence of an adequate amount of the κ-casein fraction and that the results depend in a critical fashion on the order of adding the reagents. The κ-casein fraction used by Waugh (14) was stated to be about 90% pure. In our experiments on the stabilizing effect of the pure α_3-casein on α_1-casein in the presence of calcium, it was found that the results were apparently dependent not only on the temperature of the experiment but upon (*a*) the concentration of protein at which the effect was studied and (*b*) the ratio of protein to calcium-ion concentration of the experiment. The concentration of protein at which the stabilizing experiments can be made is limited by the solubility of α_3-casein, namely, about 0.26% at pH 6.9. Experiments on the stabilizing effect of fractions prepared from the calcium-soluble fraction of α-casein showed that components other than α_3-casein also have the property of stabilizing casein micelles in the presence of calcium. Sufficient material for a detailed study of the stabilizing effects of α_3-casein was not available. However, it is of interest to note that the protective action of α_3-casein on the precipitation of α_1-casein by calcium chloride is only about one half as effective as the fraction of α-casein from which it is prepared.

DISCUSSION

The isolation and properties of α_3-casein obtained from the portion of α-casein, soluble in 0.2 *M* calcium chloride, suggest that it is related to the κ-casein fraction prepared from unfractionated casein by Waugh and von Hippel (2) and by Wake (15). α_3-Casein contains 0.35% phosphorus as compared to a phosphorus content of 0.19% in κ-casein, as reported by Waugh (14). The sedimentation constant of impure α_3-casein gave values of S_{20} from 8 to 16 *S* which increased to 23 *S* on purification. Under the same conditions, the sedimentation constant of κ-casein was reported to be 13 *S* (14, 15). However, Waugh (14) reported that at pH 12, κ-casein had a sedimentation constant of 1.37 *S* and a molecular weight of 16,300; and Wake (15) also reported at pH 12 with 0.19 ionic strength phosphate buffer (16) a value of 1.0 *S* and a molecular weight of about 26,000. Although about the same sedimentation constant was obtained by Long *et al.* (7) at pH 6.98 for λ-casein, the phosphorus contents of their λ-casein, Waugh's κ-casein and our α_3-casein differ; and a preliminary amino acid analysis clearly demonstrates that α_3- and α_2-caseins are different proteins. At pH 11.8, α_3-casein is unstable, giving rise to a number of sedimenting peaks of lower S_{20} values. The instability of α_3-casein at pH 11.8 was further shown by the finding that it is no longer electrophoretically homogeneous at pH 10.1 after being exposed to a pH of 11.8.

Both α_3-casein and κ-casein are concerned with stabilizing casein to calcium, though α_3-casein does not appear to be as effective in stabilizing casein as has been found for

κ-casein (14). However, this difference may be only a matter of the method used in determining stabilization. This comparison indicates that α_3-casein and κ-casein are similar, but not identical, and it appears that amino acid analysis of these two caseins would probably be necessary to resolve the question of their identity.

Sullivan (17) has reported that casein fractions, separated by centrifuging skim milk, vary in their sialic acid content and assumed that all the sialic acid is combined with the κ-casein. He calculated that κ-casein contains 2.8% sialic acid. The finding that α_3-casein contains only 1.2% sialic acid indicates that components other than α_3-casein, in the calcium-soluble fraction of α-casein, contain sialic acid, since the entire fraction from which α_3-casein was prepared also contained 1.2% sialic acid.

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