



The Effects of Heating, Alone or in the Presence of Calcium or Lactose, on Calcium Binding to Milk Proteins

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ABSTRACT

The binding of calcium by different milk proteins and the effects of heat were investigated. The influences of calcium and lactose were also investigated. It was shown that the caseins of cow's milk have a high calcium binding capacity, when treated with calcium at 25°C for 30 min, pH 7.00, due to the high number of calcium binding sites. The order of binding capacities is α_{s_1} -casein > β -casein > κ -casein. For whey proteins the order is β -lactoglobulin (β -lg) > α -lactalbumin (α -la). Lactose, in general, when present at 25°C, did not appreciably affect the calcium binding of the major milk proteins. Heating of whole casein at 80 or 95°C for 30 min caused conformational changes of the protein resulting in an increase in its calcium binding ability. The presence of lactose during heating with or without calcium reduced its calcium binding. The calcium binding of α_{s_1} -casein was not affected by heating, throughout the temperature range studied, either in the presence or in the absence of calcium, an indication that α_{s_1} -casein may undergo physical changes at high temperatures, but the accessibility and/or the number of calcium binding

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centres are not increased. β -Casein, when heated with calcium, was subject to conformational changes which were reflected in changes in the degree of calcium-binding. Heating of β -lg in the presence of calcium did not much affect the amount of calcium bound to it. However, in the absence of calcium an increase of 21.5% in calcium binding was observed after heating at 80°C and a decrease of 40.0% after heating at 95°C, compared to unheated samples.

INTRODUCTION

The detrimental effect of calcium ions on the stability of evaporated milk has been recognised since the beginning of this century (Sommer and Hart, 1919). Similarly, the stabilizing effect of casein-whey protein interactions against precipitation by heat has also been recognised for many years (Trautman & Swanson, 1958, 1959; Trautman, 1958; Beeby, 1966; Kannan & Jenness, 1961; Morr, 1965, 1967, 1969).

Forewarming of milk before evaporation is considered to be one of the vital factors affecting the stability of milk during processing and storage of the finished product (Deysner *et al.*, 1929; Rose, 1962; Hartman & Swanson, 1962; Sweetsur & White, 1974). How forewarming affects the stability of evaporated milk, is a question which is not yet fully answered.

The extent of the protein-protein interaction brought about by heating milk, as in forewarming, is affected by the intensity of heat treatment, the length of time, pH, the presence of divalent ions, mainly calcium and lactose.

Ntalianas (1959), using the solubility of trichloroacetic acid-protein derivatives in alcohol as an indication of protein-protein interaction, observed that calcium exerts some effect on these interactions. However, he did not carry out any quantitative determination on the amount of calcium bound to milk proteins and the influence of different parameters on the ability of proteins to bind calcium. In general, while the sensitivity of milk proteins to calcium ions has been the basis of many investigations (Zittle *et al.*, 1956; Ho & Waugh, 1965; Waugh *et al.*, 1970; Dickson & Perkins, 1971; Fox & Hoynes, 1975; Holt *et al.*, 1975; Imade *et al.*, 1977), the influence of calcium, lactose and heat on casein-whey protein interactions has not received enough attention.

The objective of this work was to study the heat-induced changes in major milk proteins, with special reference to the influence of calcium and lactose. These changes were investigated by studying the changes in their calcium binding and their electrophoretograms under various experimental conditions. In a subsequent work the interactions of milk proteins with special reference to the influence of calcium, lactose and heat will be reported.

MATERIALS AND METHODS

The preparation of whole casein

Fresh evening bulk milk, exclusively from British Friesian cows, was collected, brought to the laboratory and immediately separated at 40°C. To four litres of skimmed milk, diluted with three times the volume of water, a mixed solution of 0.5M HCl and 0.5M CaCl₂ was added dropwise with effective stirring until the pH had fallen to 4.6. The temperature during precipitation was kept between 33 and 40°C. Stirring was continued for another 30 min and the precipitate then left to settle. The supernatant was decanted and the residual suspension filtered through a Buchner funnel using a Whatman filter paper No. 50 placed over a No. 31 paper. The precipitate was washed by decantation *in situ* with distilled water until no protein could be detected in the supernatant when equal volumes of 20% TCA acid and filtrate were mixed. After washing, the casein was dispersed in one litre of distilled water and then heated in a water bath at 70°C for 30 min to destroy a proteolytic enzyme, which co-precipitates with casein and might hydrolyse it (Warner, 1944; Shahami, 1966; Kaminogawa *et al.*, 1972). The casein suspension was dialysed at 2–4°C, under constant stirring, three times against a dilute solution (1/50M) of HCl and finally distilled water to obtain a calcium-free product. At the end of dialysis the casein was freeze-dried and then ground to a fine powder. It was washed three times with peroxide-free petroleum ether to remove lipids.

The fractionation of whole casein

The separation of α_{s1} -casein

α_{s1} -Casein was separated from the whole casein by a combination of the first part of the method proposed by Thompson and Kiddy (1964) (the DEAE cellulose-urea-chromatographic purification step was omitted) and the method of Tripathi and Gherke (1970), slightly modified. (Step 1 was twice repeated, and the precipitate, consisting mainly of α_{s1} - and κ -caseins, was washed 6–8 times with cold (2°C) water.)

Separation of β -casein

For the fractionation of β -casein the method of Aschaffenburg (1963) was applied with some minor modifications. The product was further purified by treatment with calcium phosphate gel as described by Green (1969).

Separation of κ -casein

κ -Casein was prepared from whole casein by the urea-sulphuric acid method of Zittle and Custer (1963). The ethanol precipitation step was

omitted, because κ -casein was further purified by treatment with calcium phosphate gel as described by Green (1969).

At the final stage of separation, the suspensions of α_{s1} -, β - and κ -caseins were dialysed three times against distilled water until the dialysate was free from chlorides. They were freeze-dried, powdered, further dried over P_2O_5 under vacuum and stored, when not in use, at -20°C , in brown air-tight bottles.

Separation of whey proteins

Whey proteins β -lactoglobulin (β -lg) and α -lactalbumin (α -la) were prepared, from the whey resulting from precipitation of casein at its isoelectric point, following the method of Armstrong *et al.* (1967).

All batches of protein were checked for calcium (a) by the atomic absorption method and (b) compleximetrically (Ntailianas & Whitney, 1963), and for purity by polyacrylamide gel (PAG) electrophoresis. All of them were free from calcium and free from other proteins. The moisture of all protein samples was within the range 0.5–0.8%. No corrections were introduced for this low moisture content in subsequent calculations.

A full account of the methods for preparation and purification of proteins is given by Pappas (1979).

Experimental solutions

All volumetric apparatus used was of borosilicate glass and class A. All chemicals used were 'Pro analysi' grade. Glass-distilled water was used throughout.

Standard calcium chloride solution

A weighed amount of CaCO_3 was dissolved in a minimum volume of dilute HCl to give a clear solution. Sodium barbital was added to make its final concentration 30mM. The pH of the solution was carefully adjusted to 7.00 ± 0.05 with dilute NaOH or HCl, as required. A digital Orion pH meter, Model 801A (Orion Research Inc., Cambridge, Mass., USA) was used. A few drops of toluene were added as a preservative (Warner, 1944). The concentration of calcium of this solution was determined using potassium permanganate after precipitation of calcium as oxalate (Vogel, 1962). This solution was diluted quantitatively with 30mM sodium barbital buffer pH 7.00 to the required experimental calcium concentrations (3, 10, 15 and 20 mM).

Standard di-sodium di-hydrogen ethylenediamine tetra-acetate ($\text{Na}_2\text{H}_2\text{EDTA}$) solution

$\text{Na}_2\text{H}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (12.060 g) was dissolved in water (12 pellets of NaOH

were added to assist solution (Ntailianas & Whitney, 1963)) and then 1.00 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ was added. This solution was diluted to 2 litres with distilled water and standardised against the standard CaCl_2 solution using Eriochrome black T indicator (Vogel, 1962).

Sodium barbital buffer 30 mM, pH 7.0

Sodium barbital (5,5-diethyl-barbituric acid disodium salt) was dissolved in distilled water to make a final concentration of 30 mM. The pH of the solution was adjusted to $\text{pH } 7.00 \pm 0.05$ with dilute HCl or NaOH, as required.

Protein solutions

Proteins and lactose were generally used at concentrations close to those found in milk (Jenness, 1970). However, where the expected calcium binding capacity was low, as for κ -casein (Imade *et al.*, 1977), or when the protein concentration in milk is very low, as for α -la (0.07%), the protein concentrations used were higher than those found in milk. The concentrations (w/v) used in these experiments were: whole casein 2.50%, α_{s1} -casein 1.37%, β -casein 0.62%, κ -casein 0.75%, β -lg 0.30%, α -la 0.30%. Lactose monohydrate was used at 5.00%.

The exact amount of protein(s) was accurately and quickly weighed and dissolved in 15 ml capacity borosilicate, stoppered test tubes in sodium barbital buffer solution. Lactose was added here when it was required. The pH of the solutions was adjusted to 7.00 ± 0.05 with N-NaOH under quick stirring. Finally, more buffer solution was added with a microsyringe to the weight corresponding to 5 ml volume of the solution. The samples were shaken on a continuous horizontal shaker, at a constant speed, until no visible sign of protein was present and then for a further 30 min. In those experiments in which the protein samples were heat-treated in the presence of calcium, the CaCl_2 solution was added to the protein sample at this stage of the experimental procedure before any heat treatment. When heat treatment was carried out in the absence of calcium, calcium was added at the end of heating, after the samples had been cooled to 25°C. Equal volumes (4 or 5 ml) of the protein and calcium chloride solutions of double the required concentration of protein and calcium were mixed together. After mixing, the samples were shaken for another 30 min. Heat treatments were carried out at 25, 80 or 95°C for 30 min by immersing the samples in a thermostatically controlled water bath or at 115°C for 10 min. Heating at 25, 80 or 95°C was done in borosilicate loosely stoppered tubes whereas at 115°C it was done in a small aluminium bath containing high boiling point mineral oil. For heating at 115°C the test tubes with the protein samples were kept hermetically closed with glass stoppers, with a simply constructed device using a high-vacuum silicone grease. After heating, the samples were cooled

to 25°C. Any quantity of water evaporated during heating at 25, 80 or 95°C was replaced at this stage.

The determination of calcium

Calcium concentration in ultrafiltrates was determined compleximetrically against the standard $\text{Na}_2\text{H}_2\text{EDTA}$ solution (Vogel, 1962), whereas in protein solution the method of Ntailianas and Whitney (1963) was followed.

Calcium binding studies

The calcium binding to different milk proteins was studied by applying the continuous ultrafiltration, UF, (diafiltration or equilibrium ultrafiltration) technique, using the multicell micro-ultrafiltration system Model MMC and the UF membranes type DIAFLO® UM10 of 25 mm diameter with a nominal molecular weight cut off of 10 000 (Amicon Corp., Lexington, Mass., USA). A brief description of this technique is given below.

The 10 ml protein samples, of the same calcium concentration, were added to each of the eight cells of the micro-ultrafiltration system. The 800 ml capacity common reservoir was filled with calcium solution of the same calcium concentration as the protein samples. Reservoir and cell compartments were then equally pressurised by nitrogen gas. The system operated under the same stirring speed for all cells. The liquid in the cells, which was continuously discharged through the UF membranes was replaced by an equal volume from the reservoir. Thus, the volume of the sample in the cell remained essentially constant during the run. To find the total calcium binding capacity of the protein(s) the UF experiment allowed binding equilibrium to be reached, i.e. the point where the calcium concentration of the ultrafiltrates was equal to that of the feeding solution. From preliminary experiments it was found that binding equilibrium was established when five times the sample volume of ultrafiltrate (50 ml) had passed through. Therefore the first 50 ml of ultrafiltrates were rejected and the following 5 ml were collected for calcium determination.

The moles of calcium bound to the amount of protein in the sample solution were calculated by the difference of the concentrations of calcium in the protein sample (bound and free calcium) and in the ultrafiltrate.

A full description of the ultrafiltration technique was given by Pappas (1979).

Polyacrylamide gel electrophoresis

A combination of the discontinuous vertical polyacrylamide gel (PAG) electrophoresis method of Kiddy *et al.* (1972) and Sargent (1969) was conducted in tris-glycine electrode buffer pH 8.3–8.4, slightly modified.

When ultrafiltration was completed, 2 ml of the retentate were transferred to a 5 ml volumetric flask. Sucrose (0.4 g) was added to the flask and the mixture diluted to volume with electrode buffer. From this solution, 20 μ l of α_{s_1} -, β -, or whole casein or 10 μ l of κ -casein or 25 μ l of β -lg or α -la were applied to each electrophoresis tube. The type SAE 2761 power unit with a vertical electrophoresis cell of eight tubes capacity (Shandon, London, England) was used.

A constant voltage of 100 V was applied for 1 h 20 min. Gels were stained for 30 min in a solution containing 1% amido black and 7% acetic acid. Destaining was done in 7% acetic acid. Details of the electrophoresis methods applied here are given by Pappas (1979).

RESULTS AND DISCUSSION

The binding of calcium by different milk proteins

The binding values of calcium by different milk proteins treated at 25°C for 30 min, pH 7.00, with calcium at varying equilibrium-free calcium concentrations, with and without lactose, are presented in Table 1.

Whole casein

Applying the calcium-binding data of Table 1 for whole casein and eqn (1) given by Klotz (1950):

$$\frac{1}{r} = \frac{1}{Kn} \cdot \frac{1}{(\text{Ca}^{2+})} + \frac{1}{n} \quad (1)$$

where

r = the number of moles of calcium bound to 10⁵ g of protein;

K = the intrinsic binding constant;

(Ca^{2+}) = the concentration of free calcium ions in the protein solutions.

The maximum number (n) of moles of calcium bound to 10⁵ g of protein at infinite calcium concentration can be estimated. Thus, plotting $1/r$ against $1/(\text{Ca}^{2+})$, the intercept of the ordinate gives $1/n$ from which n can be calculated. The above equation applies when this plotting gives a linear relationship. However, the data of Table 1 for whole casein deviate from linearity. This may be due to the electrostatic influence of the bound calcium on its subsequent binding and/or the variation in the type and number of calcium binding sites, which become available as the calcium binding builds up. This explanation is in accordance with suggestions given by Klotz (1950).

On the other hand, the binding data of whole casein obtained within the range of calcium concentrations of 10–20 mmoles/litre are in better

TABLE I
The Binding of Calcium by Different Milk Proteins^a

Protein solution	Equilibrium-free calcium concentration (nmoles/litre)					
	3	10	15	20		
	nmoles of calcium bound/litre of protein solution	nmoles of calcium bound/litre of protein solution	nmoles of calcium bound/litre of protein solution	nmoles of calcium bound/litre of protein solution	Moles of calcium bound/10 ⁵ g of protein	Moles of calcium bound/10 ⁵ g of protein
Whole casein, 2.50%	6.01	7.50	7.10	6.65	28.4	26.6
Whole casein, 2.50% + lactose, 5.00%	5.81	7.82	7.56	6.78	30.2	27.1
Change (%)	-3.3	+4.3	+6.5	+1.9		
α_1 -Casein, 1.37%	4.26	4.75	5.76	6.26	42.0	45.7
α_2 -Casein, 1.37% + lactose, 5.00%	—	4.28	5.66	6.28	41.3	45.8
Change (%)	—	-9.8	-1.7	+0.3	—	—
β -Casein, 0.62%	1.25	1.30	1.88	2.15	30.3	34.7
κ -Casein, 0.75%	—	0.78	1.33	1.67	17.7	22.2
κ -Casein, 0.75% + lactose, 5.00%	—	0.75	1.26	1.68	16.8	22.4
Change (%)	—	-3.8	-5.2	+0.6	—	—
β -Lactoglobulin, 0.30%	0.56	0.74	0.98	1.06	32.6	35.3
β -Lactoglobulin, 0.30% + lactose, 5.00%	—	0.73	0.93	1.05	31.0	35.0
Change (%)	—	-1.3	-5.1	-0.9	—	—
α -Lactalbumin, 0.30%	—	0.70	0.80	0.93	26.6	31.0
α -Lactalbumin, 0.30% + lactose, 5.00%	—	0.68	0.74	0.94	24.6	31.3
Change (%)	—	-2.8	-7.5	+1.1	—	—

^a The protein solutions were treated at 25°C for 30 min (a) with calcium at varying equilibrium-free concentrations (the concentrations of calcium in equilibrium with the calcium bound to protein); (b) with and without lactose.

agreement with linearity. Applying eqn (1) gives values of n of 24.1 and 24.7 for whole casein and whole casein + lactose, respectively. Values reported in the literature for whole casein without lactose are $n = 48$ (Chanutin *et al.*, 1942), $n = 22$ (Carr, 1953), $n = 29$ (Zittle *et al.*, 1958), $n = 33-36$ (Tessier & Rose, 1961) and $n = 16$ (Imade *et al.*, 1977). These differences in the value of n may be due to (a) variations in the methods of preparations of proteins; (b) the use of different buffers at different ionic strength, as increases in ionic strength raise the dissociation of calcium caseinates (Chanutin *et al.*, 1942; Klotz, 1950); and (c) variations in the experimental procedures.

In experiments using direct ultrafiltration instead of continuous ultrafiltration (diafiltration) and calcium concentrations of 10, 15 and 20 mmoles/litre, the values of n obtained were 22.7 and 22.3 for whole casein without and with lactose, respectively. These differences in n between the direct and continuous UF methods may be explained by the fact that in diafiltration the protein is continuously fed with CaCl_2 solution, so that the casein is almost saturated with calcium. This is not the case in the direct method where the protein is not necessarily saturated with calcium to the same degree. Table 1 shows that whole casein with or without lactose reaches its maximum binding capacity rapidly before the highest concentration of Ca^{2+} is reached, indicating that whole casein shows a high affinity for calcium. It also shows that, above a certain level of CaCl_2 (10 mmoles/litre), the binding capacity tends to decline, presumably due to physical alterations in the casein induced by the bound calcium (Klotz, 1950; Imade *et al.*, 1977; Dalgleish, 1973).

α_{s_1} -Casein

The value of n for α_{s_1} -casein, estimated from their calcium binding data presented in Table 1 is 44.6. Other values reported in the literature are $n = 36.0$ (Dickson & Perkins, 1971), $n = 25.0$ (Imade *et al.*, 1977), $n = 46.5$ (Noble & Waugh, 1965) and $n = 42.3$ (Bingham *et al.*, 1972). These differences may be attributed to the same reasons as for whole casein.

It was observed in these studies that macroscopic precipitation of α_{s_1} -casein did not occur at a calcium concentration of 3 mmoles/litre, but did occur at 10 mmoles/litre. Table 1 shows that binding of calcium to α_{s_1} -casein continued beyond the point of precipitation. This is in agreement with the findings of Dalgleish & Parker (1980). Ho and Waugh (1965), in a brief account of the interaction of calcium with α_{s_1} -casein, as shown by infrared spectroscopy, indicated that calcium binds first with the phosphate groups of this protein and that binding with these groups reaches completion before any precipitation occurs. Taking this into account and the fact that the calcium binding of α_{s_1} -casein in this study continued beyond its precipitation, i.e. when the interaction of calcium with phosphate groups has

reached completion, and that the amount of calcium bound is higher than would be expected if the binding were only to phosphate groups, it is concluded that other groups, possibly carboxylic groups of α_{s_1} -casein, are also involved in the binding of calcium. This is also supported by the fact that dephosphorylated α_{s_1} -casein is still precipitated by calcium (Pepper & Thompson, 1963; Bingham *et al.*, 1972). The maximum theoretical number of moles of calcium bound to one mole of α_{s_1} -casein B (mol. wt 23, 613; Mercier *et al.*, 1972) at pH 7.0 was calculated as 25.4 (Pappas, 1979).

β -Casein

The results for the calcium-binding capacity of β -casein are given in Table 1. The moles of calcium bound to 10^5 g of β -casein were found to be 20.2, 21.0, 30.3 and 34.7 for concentrations of calcium, 3, 10, 15 and 20 moles/litre, respectively. It is seen from these results that the ability of β -casein to bind calcium is lower than that of α_{s_1} -casein for the same calcium concentration.

κ -Casein

The moles of calcium bound to 10^5 g of κ -casein are 10.4, 17.7 and 22.2 for calcium concentration 10, 15 and 20 mmoles/litre, respectively. Dickson & Perkins (1971) found that the number of moles of calcium bound to 10^5 g of κ -casein was 11. The higher values of moles of calcium bound, found in these experiments, may be attributed to the larger concentrations of calcium used in this study, compared to those used by the authors, since the binding of small ions to proteins follows the law of mass action (McLean & Hastings, 1935; Klotz, 1946). This discrepancy may be also attributed to differences in methods and materials used.

β -Lactoglobulin

The moles of calcium bound to 10^5 g of β -lg were found to be 18.6, 24.6, 32.6 and 35.3 for calcium concentrations of 3, 10, 15 and 20 mmoles/litre, respectively. This protein has an appreciable calcium-binding capacity even at low calcium concentration. Since this protein contains no phosphorus (McKenzie, 1971) the calcium must be bound to the free carboxylic groups of aspartic and glutamic acids, which are 11 and 16 per molecule of β -lgA and 10 and 16 for β -lgB, respectively (Braunitzer *et al.*, 1973).

α -Lactalbumin

The moles of calcium bound to 10^5 g of this protein are 23.3, 26.6 and 31.0 for calcium concentrations of 10, 15 and 20 mmoles/litre, respectively. Since this protein contains no phosphorus but 9 and 8 free carboxylic groups of aspartic and glutamic acids per molecule, respectively (Brew *et al.*, 1970), it seems, again that these acids are the calcium binding centres.