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Changes of Size Distribution and Surface Area of Protein Particles in Milk by Application of the Freeze-Fracturing Technique

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ABSTRACT

Freeze-fracturing was applied to study the effect of heat treatment and calcium ion addition, prior to heating to 92°C for 15 s, on the microstructure and size distribution of protein particles in milk.

Protein particles become visible and their distribution and surface area are changed by calcium addition and heat treatment. Protein particles are disintegrated upon heating into smaller size units, while addition of calcium ions prior to heating promotes large particle formation and retards whey protein denaturation via a shielding effect.

INTRODUCTION

Many electron microscopic techniques are used to study the physical properties of dairy products. These are correlated with the submicroscopic structure of constituents, their distribution and their interaction (Rüegg *et al.*, 1980; Hermansson & Buchheim, 1981; Kalab, 1981; Buchheim, 1982).

Freeze-fracturing is one of the techniques which has been extensively used in various studies of the microstructure and size distribution of protein particles in milk and its products (Thomas *et al.*, 1980; Buchheim, 1981, 1982; Omar & Buchheim, 1982; Omar, 1985, 1987, 1988). This technique may yield a more natural illustration of object structure and the protein particles become visible with less artifacts (Buchheim, 1982).

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During milk processing, changes occur in protein particles with heat treatment or by additional ingredients (Rüegg & Blanc, 1978; Smietana, 1979; Gupta & Ganguli, 1980; Munyua & Larsson, 1980; Yoshikawa *et al.*, 1982; Omar, 1985; Omar & Smietana, 1986).

The purpose of this study is to evaluate the changes in size distribution and surface area of protein particles in heated milk and in milk enriched with calcium ions prior to heating, by the application of the freeze-fracturing technique.

MATERIALS AND METHODS

Milk was obtained from the bulk milking of the herd of the Federal Dairy Research Centre, FRG, Kiel, and it was divided into three portions. Calcium chloride at the level of 3·6 mM was added to the first portion followed by heating to 92°C for 15 s, the second sample was heated to the same temperature and time; the third one remained as a control. Trials were conducted in triplicate.

Electron microscopy

Freeze-fracturing was applied to produce replicas of the milk samples for electron microscopy. Milk fixation was achieved by adding 1% of glutaraldehyde to the milk samples, mixing and leaving for 30 min. A small portion (2–3 mm^3) of milk was mounted on specimen holders using glycerol at 33% v/v as an intermediate layer for increasing mechanical stability. The specimens were quickly frozen by immersion into melting Freon 22 (−160°C) and stored under liquid nitrogen. Freeze-fracturing was carried out in a BALZERS BA 360 M unit at an object temperature of −120°C. For replication, the freshly freeze-cleaved surface was immediately shadowed with 2 nm platinum/carbon under an angle of 45° and further stabilized by 20 nm of pure carbon. The replicas were floated onto distilled water and then transferred to 5% sodium hypochlorite for approximately 2 h and passed again to distilled water. Fat was removed by a short treatment in pure acetone. Electron microscopy was carried out with a Siemens ELMISKOP I at 80 Kv. Micrographs were made at a 2000-fold magnification followed by a 3-fold enlargement. The size distribution of protein particles in milk was calculated from 12 micrographs obtained from different fields of the specimens. The particles of the final prints were counted and grouped into size classes with a range of 16·5 nm; particles in each range were assigned the mean diameter of the range. The measured distributions were converted to the real number frequencies and the relative surface area (Thomas *et al.*,

1980; Omar & Buchheim, 1983). The different fields of each sample were compared with the Chi-square distribution test as various numbers of degrees of freedom at the 5% level of significance (Lothar, 1970).

Undenatured whey protein index (WPNI)

The level of WPNI in milligrams per gram of total solids was determined according to the method described by ADMI (1971).

RESULTS AND DISCUSSION

Figures 1–3 illustrate the appearance of the protein particles in the milk specimens. Protein particles were observed to be essentially spherical in shape with smooth surface and to be composed of a large number of small subunits. The real number frequencies of the particles in different micrographs in every sample were compared with each other by means of the (χ^{-2}) test in order to assess whether the specimens were correctly prepared and measured. No significant difference was found in the size distribution derived from various micrographs of each sample. It can be argued, from the statistical analysis, that both the preparation of specimens and the population method give results which can be triplicated.

The size distribution of the protein particles in milk varied on average from 16·5 nm to more than 165 nm in diameter. Protein particles with diameters less than 16·5 nm and up to 300 nm were detected but statistically were not significant. This result is in agreement with those of Schmidt *et al.* (1973), who interpreted the size distribution of protein particle aggregates, in native bovine milk exhibiting considerable polydispersity ranging from 20–300 nm, as being composed of a large number of more or less spherical subunits ranging from 5 to 20 nm in diameter.

Tables 1 and 2 and Figs 4–7 show the effect of added $CaCl_2$ on the size distribution and the surface area of protein particles and, for comparison, show the effect of heat treatment. There is essentially a difference between the two treatments in size distribution and surface area, suggesting that calcium affects mainly the enlargement of the relative surface area and the absolute particle size distribution of milk proteins. In this study, milk samples subjected to heat treatment only to 92°C for 15 s, showed a relatively distinct distribution of particle size. Protein particles disintegrated upon heating lead to splitting of the large particles into smaller sizes. This could be explained by the report of Rüegg & Blanc (1978), who observed that heating of milk caused an increase in the number of free submicelles from disintegrated casein micelles. The data also indicate that milk treated with

Fig. 1. Electron micrograph of protein particles in bulk milk fixed in 1% glutaraldehyde solution for 30 min, cryoprotected with 33% glycerol. (Original magnification × 60 000). C: casein micelles; W: whey phase.

3·6 mM of $CaCl_2$, followed by heating to 92°C for 15 s, gave an increase in the number of large particles, this being due to the interaction of calcium ions and protein particles in milk.

Munyua & Larsson (1980) found that addition of calcium ions to the native skim milk promotes micelle formation and enlargment by the interaction of the micelles with one another. Thus the real distribution of protein particles is reduced due to the distortion of calcium bridges by the heating effect and leads to a shift in the size and dispersion of the protein particles to a smaller size (Gupta & Ganguli, 1980). Calcium ion addition to milk therefore has a promoting effect on the heat-treatment via a shielding

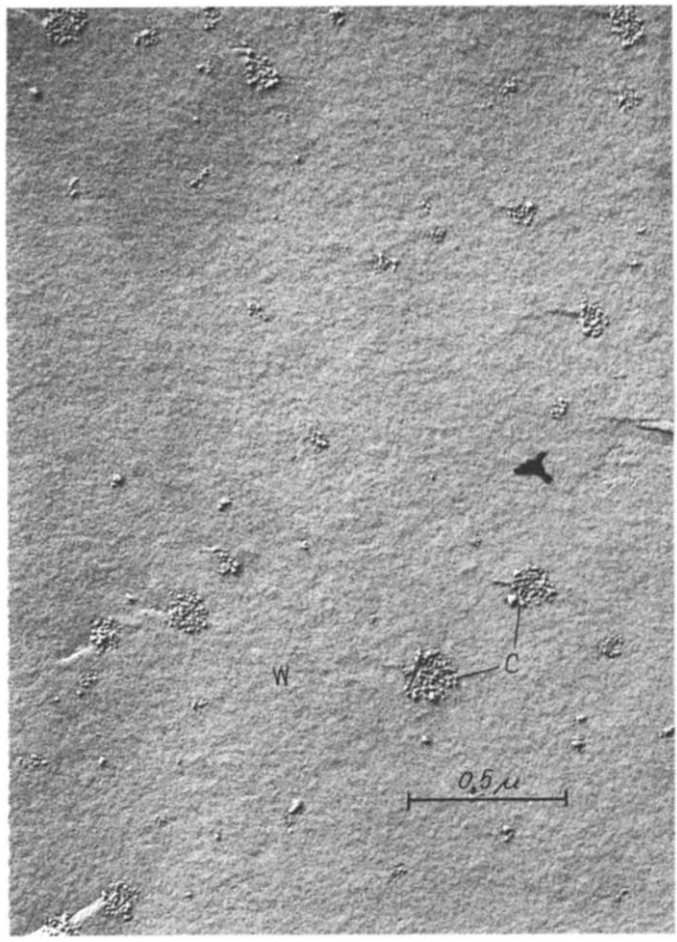

Fig. 2. Electron micrograph of protein particles in milk heated to 92°C for 15 s, fixed in 1% glutaraldehyde solution for 30 min, cryoprotected with 33% glycerol. (Original magnification × 60 000). C: casein micelles; W: whey phase.

effect and enhances interaction between the protein particles (Yoshikawa *et al.* 1982; Mehaja & Cheryan, 1983).

To obtain a clear interpretation of the real distribution and the surface area of the protein particles, the average number obtained from milk protein was calculated for all specimens together and combined into three classes as shown in Figs 8 and 9. Also proportional changes of the real number frequencies and relative surface areas resulting from heating and from calcium ion addition were extrapolated as shown in Figs 10 and 11.

There was a tendency of the small particles of 16·5 to 33 nm in diameter to decrease by 34·33% and large particles of 49·5 to 99 and more than 99 nm in

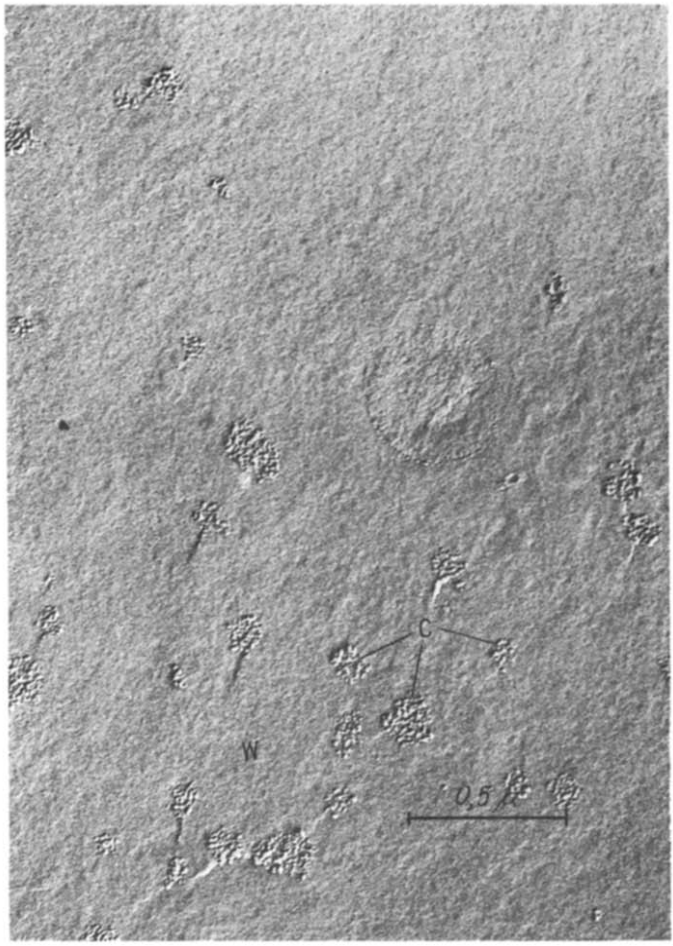

Fig. 3. Electron micrograph of protein particles in milk enriched with 3·6 mM $CaCl_2$, heated to 92°C for 15 s, fixed in 1% glutaraldehyde solution for 30 min, cryoprotected with 33% glycerol. (Original magnification ×60 000). C: casein micelles; W: whey phase.

diameter to increase by 18% and 16·4%, respectively, as soon as calcium was added to the milk prior to the heating, compared with heated milk only.

In the course of this experiment, undenatured whey protein (WPNI) was measured as being 10·23 mg/g in bulk milk and was reduced to 8·77 mg/g in milk enriched with calcium chloride followed by heating and reduced to 6·30 mg/g in heated milk only. This means that heating the milk reduced the WPNI by 36·6% while calcium addition reduced this index by 14·3%. Our studies suggest that calcium ion addition to milk before heating retarded the whey protein denaturation (WPNI) by 22·3%. This could be attributed to the protective action of calcium ions against thermal denaturation of whey

TABLE 1
Relative Size Distribution of Protein Particles in Milk Fixed in 1% Glutaraldehyde Solution for 30 min

Sample		*Size of particles (nm)*											*Total*
		16·5	*33*	*49·5*	*66*	*82·5*	*99*	*115·5*	*132*	*148·5*	*165*	*>165*	
Bulk milk	Particle numbers	784	196	237	142	159	199	65	57	35	41	29	1 944
	Percentage	40·33	10·08	12·19	7·30	8·18	10·24	3·34	2·93	1·80	2·11	1·50	100
Heated milk	Particle numbers	1 237	287	190	152	142	102	86	40	33	33	28	2 330
	Percentage	53·09	12·32	8·15	6·52	6·09	4·38	3·69	1·72	1·42	1·42	1·20	100
Milk + calcium heated	Particle numbers	250	109	96	135	154	106	89	74	45	45	35	1 143
	Percentage	21·87	9·51	8·40	11·81	13·47	9·27	7·79	6·47	3·94	3·94	3·07	100

TABLE 2
Relative Surface Area in Freeze-Fracture Microphotographs of Protein Particles in Fixed Milk

Sample		*Size of particles (nm)*											*Total*
		16·5	*33*	*49·5*	*66*	*82·5*	*99*	*115·5*	*132*	*148·5*	*165*	*>165*	
Bulk milk	Particle surface area	40·33	40·32	109·71	116·80	204·5	368·64	163·66	187·52	145·80	211	291·93	1 881·21
	Percentage	2·14	2·14	5·83	6·27	10·87	19·60	8·70	9·97	7·75	11·22	15·51	100
Heated milk	Particle surface area	53·09	49·28	73·35	104·32	152·25	157·68	180·81	110·08	115·02	142	219·60	1 357·48
	Percentage	3·91	3·63	6·40	7·68	11·22	11·62	13·32	8·11	8·47	10·46	15·18	100
Milk + calcium heated	Particle surface area	21·87	38·16	75·60	188·96	336·75	333·72	381·71	414·08	353·97	394	814·72	3 353·54
	Percentage	0·65	1·14	2·25	5·63	10·04	9·95	11·38	12·35	10·56	11·75	24·29	100

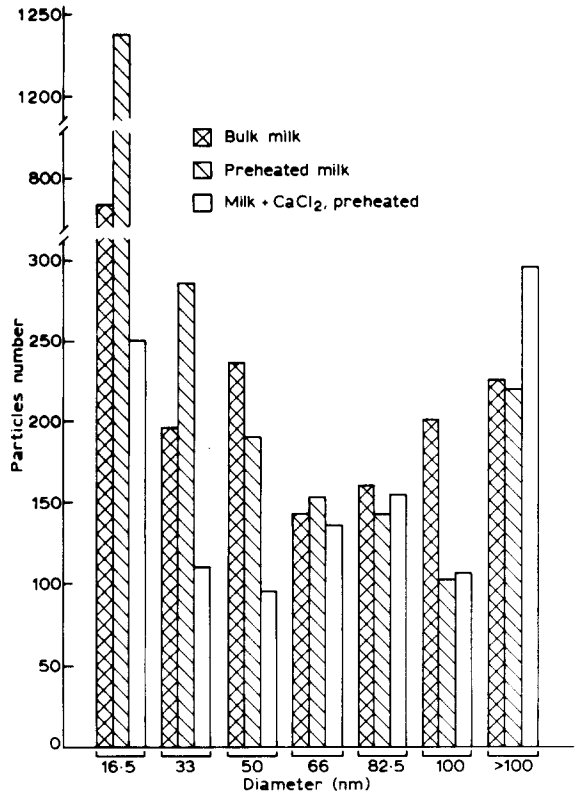

Fig. 4. Histograms of absolute particle size distribution of protein in fixed milk. Absolute numbers of particles on 12 microphotographs. (*c.* 24 × 30 cm) of magnification of 60 000.

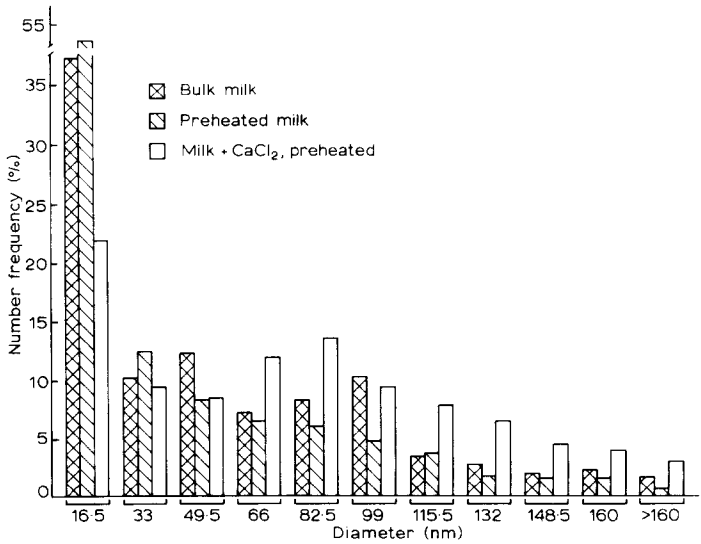

Fig. 5. Histograms of relative particle size distribution of protein in fixed milk.

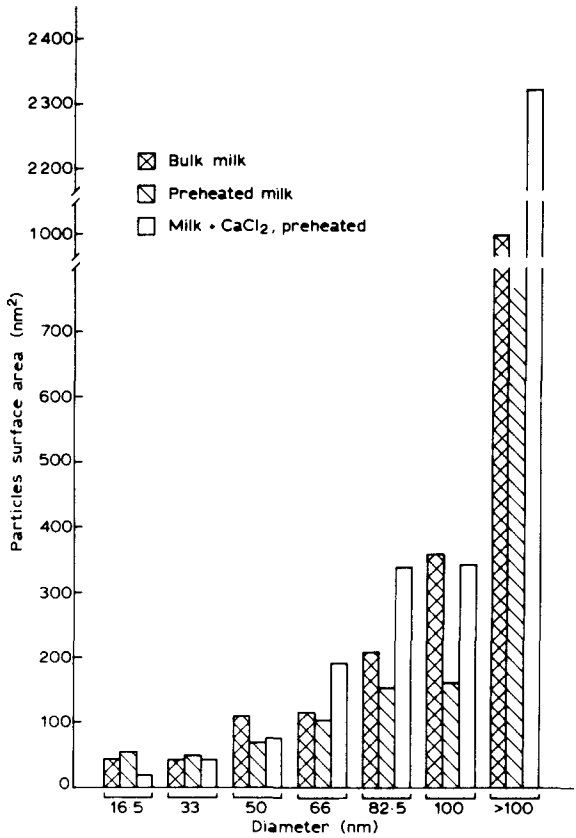

Fig. 6. Histograms of absolute surface area of protein particles in fixed milk absolute area of particles on 12 microphotographs (*c.* 24 × 30 cm) of magnification (*c.* 24 × 30 cm).

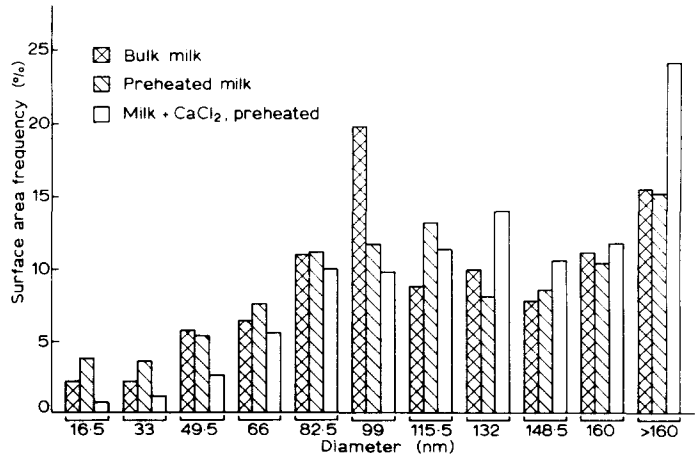

Fig. 7. Histograms of relative surface area of the protein particles in fixed milk.

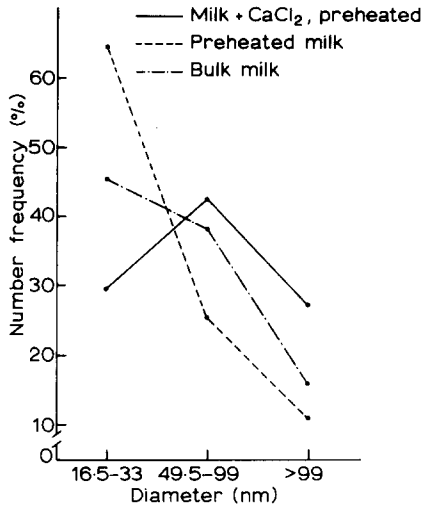

Fig. 8. Average number of relative size distribution of protein particles in milk.

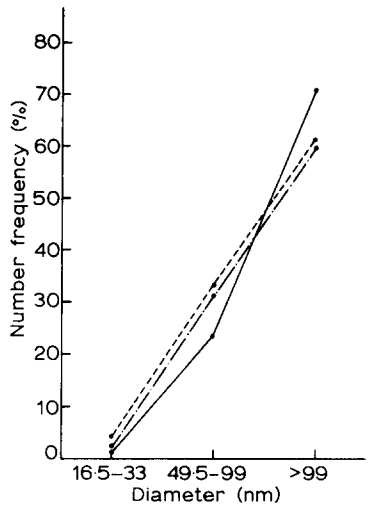

Fig. 9. Average of relative surface area of protein particles in milk.

proteins which causes an integration of whey protein with casein proteins forming a complex resistant to denaturation. Similar results were observed by Smietana (1979) and Omar & Smietana (1986).

Thus it is possible that one important role of calcium addition could be the retardation of the whey protein denaturation and the progressive aggregation of protein particles.

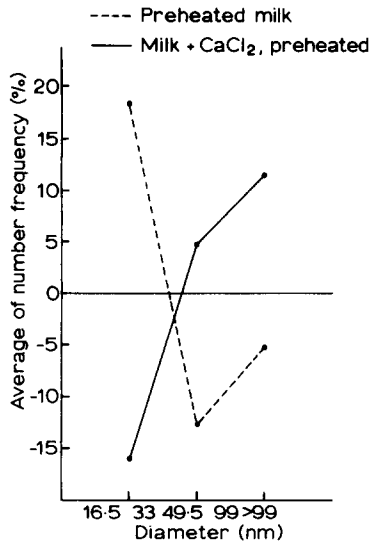

Fig. 10. Proportional changes of real distribution of protein particles in milk.

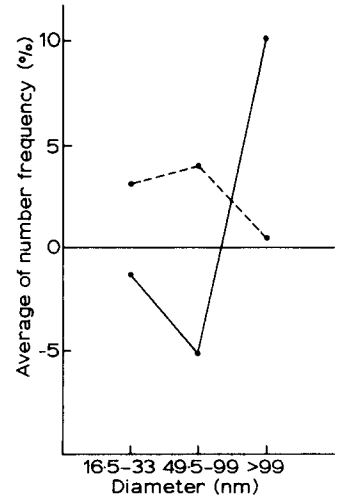

Fig. 11. Proportional changes of surface area of protein particles in milk.

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