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Note

Fractionation of casein micelles and whey protein aggregates on Sephacryl S-1000

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Gel permeation chromatography has become a useful biochemical tool with dextran-, polyacrylamide- and agarose-based gel beads being very effective in the separation of proteins with molecular weights ranging towards an upper limit of *ca.* $4 \cdot 10^7$ for a 2% agarose gel. Increases in the size of the proteins to be separated are concomitant with a decrease in structural strength of the gel matrix. A product based on etched glass beads has been used for larger protein particles[1], but it needs to be pretreated with polyethylene glycol[2] or silicone[3] to prevent protein adsorption. A new dextran derivative, Sephacryl S-1000, has been produced which should be capable of separating particles up to 300 nm in diameter, equivalent to a globular protein molecular weight of $3 \cdot 10^8$, with a decreased possibility of protein adsorption to the matrix.

The casein micelles of bovine milk range in size from 10 or 15 nm (the micellar subunit) to 700 nm although the bulk of them are less than 150 nm in diameter[4] and can be fractionated using the controlled-pore glass support. The present study reports the application of Sephacryl S-1000 to the fractionation of casein micelles and its use in the fractionation of the high-molecular weight particles of denaturated whey protein that are generated when milk is heated to temperatures close to 100°C[5]. In a previous study[5] it was found that when milk was heated the whey proteins formed small aggregates on the surface of the casein micelles and that these could be separated from the caseins by dispersing the casein micelles and chromatographing the dispersion on Sepharose 4B. The whey protein aggegates eluted at the void volume and electron microscopy indicated that they were often greater than 100 nm in length. It was reasoned that Sephacryl S-1000 would thus be capable of fractionating these aggregates.

EXPERIMENTAL

Skim milk was obtained on two separate occasions from the Product Development Centre of the New Zealand Dairy Research Institute. The pH of some skim milk samples was adjusted by the addition of 50–300 μl of 0.1 *M* hydrochloric acid or sodium hydroxide solution; the samples were mixed and held for 30 min prior to pH determination. These were heated in a bath of boiling water for 10 min and were held for a further 30 min at room temperature before the pH values were measured.

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All reagents were analytical-reagent grade, except imidazole which was reagent grade, and the water was purified by reverse osmosis followed by treatment in a Milli-Q apparatus (Millipore).

Column chromatography

Samples (1.5 ml) of unheated or heated skim milk were chromatographed on a 360 × 26 mm column of Sephacryl S-1000 (Pharmacia) at a flow-rate of 40 ml/h in simulated milk ultrafiltrate (SMUF) at pH 6.70 at room temperature (20°C)[6]. The column effluent was collected in 4-ml fractions and 1-ml aliquots of these were diluted 1:1 with 0.2% sodium dodecyl sulphate (SDS) in dilute sodium chloride solution to give a transparent solution. The absorbances of these mixtures were measured at 280 nm and these were taken as indicative of the protein contents of the solutions. The turbidities of a selection of effluent fractions from the unheated milk sample were measured at selected weavelengths between 350 and 800 nm using a Zeiss PMQII spectrophotometer. The average radius of the light-scattering particles in each fraction was determined from the slope of the log plots of absorbance *versus* wavelength[7].

Aliquots (0.75 ml) of heated and unheated milk samples were also mixed 1:1 with 1% SDS solution and the mixture chromatographed on Sephacryl S-1000 in an SDS buffer (1 g SDS, 2 g imidazole, 3 g sodium chloride made to 1 l at pH 7.0). The absorbances of the effluent fractions (6 ml) were determined at 280 nm.

Electrophoresis in polyacrylamide gels was carried out by standard methods[8].

RESULTS AND DISCUSSION

Gel permeation chromatography of skim milk on Sephacryl S-1000 allowed the separation of differently sized casein micelles in SMUF buffer (Fig. 1A). In some

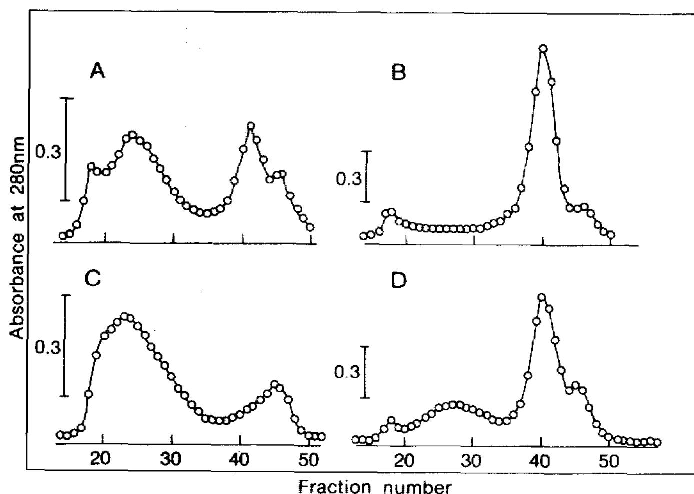

Fig. 1. Elution profiles of milk samples chromatographed on Sephacryl S-1000. (A) pasteurized skim milk in SMUF buffer; (B) pasteurized skim milk in SDS buffer; (C) heated skim milk in SMUF buffer; (D) heated skim milk in SDS buffer. The flow-rate was 40 ml/h at room temperature (*ca.* 20°C) and 4-ml fractions were collected.

respects Sephacryl S-1000 resembles the controlled-pore glass supports described earlier (*e.g.* refs. 4 and 9). When skim milk was chromatographed in SDS buffer (Fig. 1B) most of the 280-nm absorbance was in a single peak which corresponded to monomeric protein molecules. A small void volume peak was present near fraction 18.

Chromatography of heated milk in SMUF (Fig. 1C) and SDS buffers (Fig. 1D) gave chromatograms with lower absorbance near fraction 40 than the corresponding unheated milks (Fig. 1A and B) and greater absorbance between fractions 20 and 30. The protein material in fraction 42 in the elution pattern shown in Fig. 1A was found to be whey protein by gel electrophoresis and thus it is likely that the observed absorbance changes are caused by similar thermal denaturation and aggregation of the whey protein as that previously noted[5]. From the size estimations made on the casein micelles in the column effluent shown in Fig. 1A, the peak in Fig. 1D

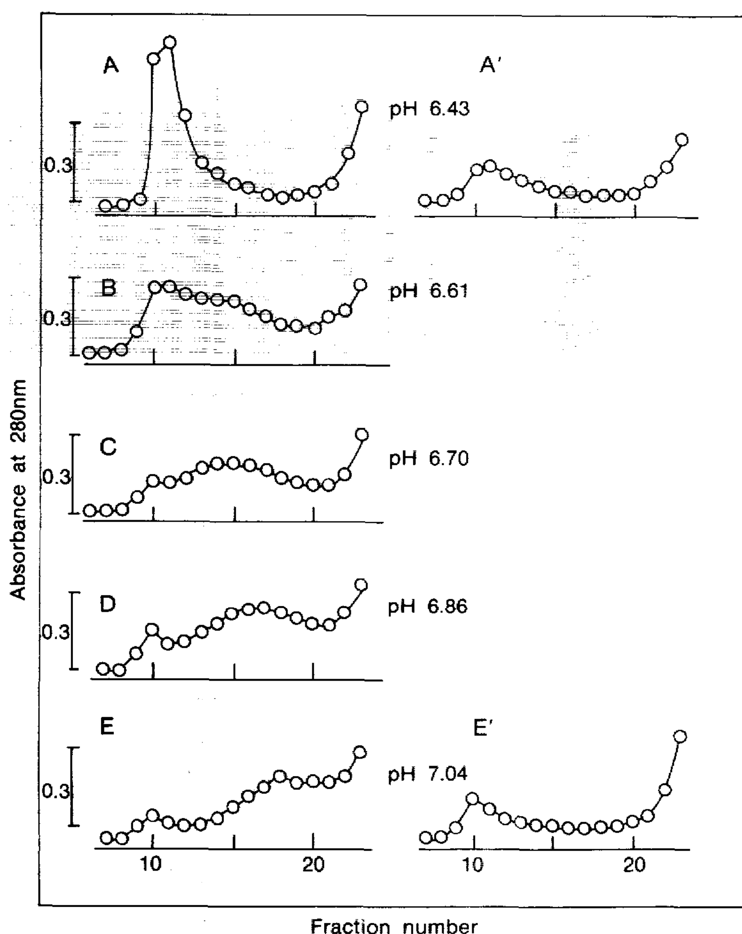

Fig. 2. Elution profiles of heated milk samples chromatographed on Sephacryl S-1000 in SDS buffer. Chromatograms A–E correspond to the samples heated after adjustment to the pH shown. Samples A′ and E′ were samples A and E but 50 μl of 2-mercaptoethanol were included in each sample.

near tube 28 corresponds to particles of *ca.* 35-nm radius; a value consistent with the earlier electron microscopic data[5].

A series of chromatograms were run in which acid or alkali was added to the milk samples prior to their heat treatment. Larger fractions were collected and only the whey protein aggregate portion of the chromatograms are shown (Fig. 2). At pH values close to neutral, the elution profiles were similar to that shown in Fig. 1D. However, after heating at a lower pH, the whey protein aggregates were much larger (Fig. 2A and B).

The reverse effect (Fig. 2E) occurs if the milk pH is raised prior to heating. Addition of 2-mercaptoethanol, a disulphide reducing agent, to the milk after the heat treatment and shortly before the column chromatography virtually eliminated the peaks of whey protein aggregate at both high (Fig. 2E′) and low pH (Fig. 2A′). In a previous study[5] it was shown that pH affected the configuration, as seen by electron microscopy, of the heat-induced whey protein–κ-casein aggregate. It is now clear that the size of these aggregate particles is also strongly influenced by the pH of the milk.

It is clear, therefore, that the technique of column chromatography on Sephacryl S-1000 offers a relatively simple method of measuring the size distribution of milk protein particles with radii between 25 and 75 nm.

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