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DETERMINATION OF NATIVE AND DENATURED MILK PROTEINS BY HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

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SUMMARY

High-performance size exclusion chromatography on a TSK 3000 SW column was used to separate and quantify the individual milk proteins in their native and/or denatured state. Quantitation of the individual native whey proteins within ±10% was achieved.

The denatured proteins were dissociated into their monomer units by using sodium dodecyl sulphate and β-mercaptoethanol. A good separation of the whey protein monomers was achieved and the quantitation of the individual whey proteins within ±10% was possible.

Under these conditions, α_{s1}- and β-casein were eluted as one peak, whereas κ-casein and γ-casein were eluted in the same position as β-lactoglobulin and α-lactalbumin respectively. A method for calculating the areas and hence the concentration of the individual proteins in a mixture of casein and whey proteins is described.

INTRODUCTION

The size, electrophoretic charge and immune response of the various milk proteins is affected by heat treatment[1,2]. Therefore, the methods of size exclusion chromatography[3], ion exchange chromatography[4] and electrophoresis[5], which are often used to separate native proteins cannot be used to separate and quantify the heat-denatured proteins which are present in processed milk samples.

Denatured proteins can be dissociated into their monomer subunits by the combined use of sodium dodecylsulphate (SDS) and β-mercaptoethanol (β-ME)[7]. After SDS treatment, the protein molecules have a net negative charge which is constant per unit mass[8]. The SDS-treated protein molecules therefore migrate in polyacrylamide gel electrophoresis on the basis of their molecular size and not their charge[9,10].

The molecular weight (MW) of the individual milk protein monomers ranges from 11,500 to 67,000[11]. The MW of the major milk protein subunits, *viz.* α_{s_1}-casein, β-casein, β-lactoglobulin and α-lactalbumin lie between 14,000 and 24,000[11]. The initial work on polyacrylamide gradient gel electrophoresis showed that the separation of these subunits was possible, but quantitation of the individual proteins could not be achieved because the proteins separated in very close bands, the gradient gel

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varied in thickness and staining and destaining of the protein bands was not reproducible.

The availability of rigid macroporous silica, *viz*. Micropak TSK 3000 SW[12,13] has enabled the separation of proteins by high-performance liquid chromatography (HPLC). A linear relationship between the retention volume and log MW of proteins between 10,000 and 100,000 was observed[13]. Thus, the MW of the unknown protein and hence its identity can be established in this MW range.

The present paper describes the use of a TSK 3000 SW column for determining the individual proteins in their native and/or denatured state in milk or its products.

MATERIAL AND METHODS

Chromatography

Liquid chromatography was performed with a twin-headed reciprocating pump, Model 750/04, a filter-type selectable wavelength (280 nm) UV monitor (750/11) and an autosampler (750/33) with a fixed-volume loop, all from Applied Chromatography Systems, Luton, U.K. The TSK 3000 SW column (600 × 7.5 mm) was from Toya Soda, Tokyo, Japan. The output from the UV monitor was recorded by an Omniscribe two-pen recorder. The data from the UV monitor was also computed and integrated with a Trilab Model II computer with video display unit and printer (Trivector Scientific, Sandy, U.K.).

Materials

Purified proteins were obtained from Sigma, Poole, U.K., except α-lactalbumin, which was purchased from BDH, Poole, U.K. All reagents used for making buffers were either AnalaR or "purified-for-biochemical" grade (BDH). The buffers were prepared with freshly double-glass-distilled water and were filtered through a 0.45-μm filter by means of an all-glass Millipore filtration unit (Millipore, U.K.).

Whole casein was prepared from raw bulk skimmed milk by isoelectric precipitation with hydrochloric acid at pH 4.6. The filtrate left after the precipitation of casein was used for separating the whey proteins on the column. A sample of purified α_{s_1}-casein and β-casein was kindly provided by Dr. A. T. Andrews, NIRD, Shinfield, U.K.

Separation and quantitation of native proteins

The purified proteins, 100 μl of each (2.5 mg/ml, except α-lactalbumin, 1.0 mg/ml), dispersed in 0.1 *M* phosphate buffer, containing 0.05 *M* sodium chloride and 0.02% sodium azide pH 6.8, were applied to the column. The same buffer at a flow-rate of 0.4 ml/min was used as the mobile phase.

Retention time and area under each peak were determined by the computing integrator. The areas of the overlapping peaks were determined by selecting the integrating parameters so that perpendiculars were drawn from the beginning and the end of each peak to the base line. One ml of a stock solution of each, 625 mg β-lactoglobulin, 250 mg α-lactalbumin, 235 mg γ-globulin and 187.5 mg bovine serum albumin in the phosphate buffer made up to 25.0 ml, was placed in capped vials and deep-frozen. This stock solution was used to quantify a mixture of individual proteins by comparison with single individual proteins. The mixture was subsequently used

as a standard whey protein mixture to calibrate the column and to calculate "response factors" for individual proteins.

Dissociation of denatured proteins

The proteins were dispersed in 0.1 *M* sodium phosphate buffer, containing 5%

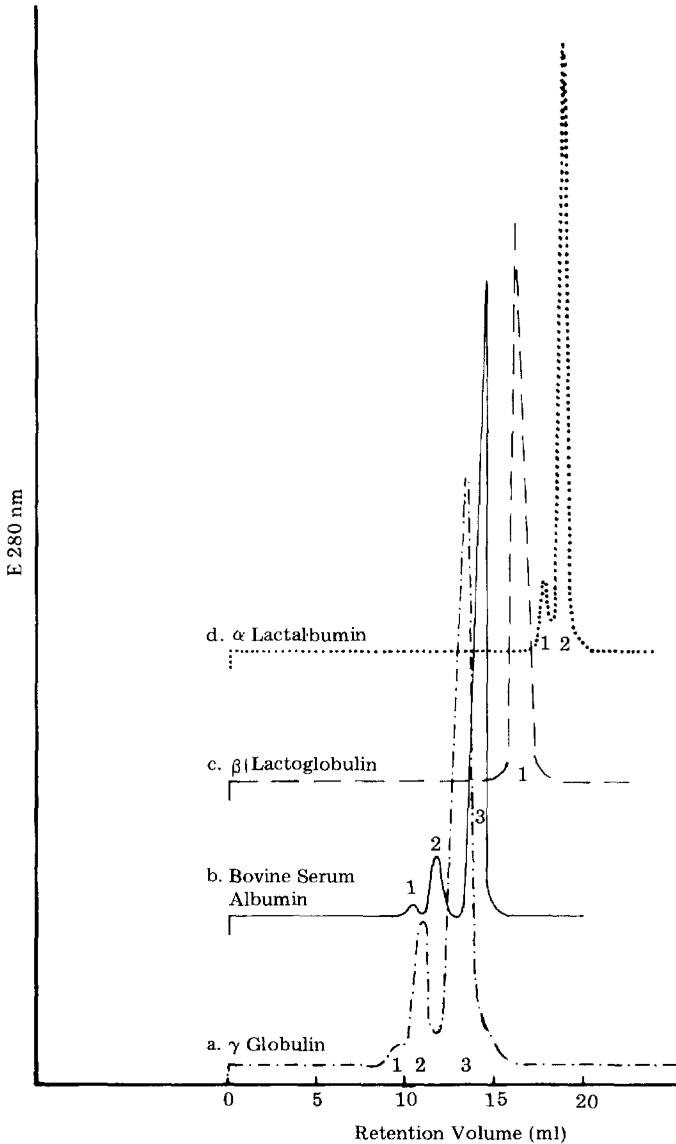

Fig. 1. Elution curves of standard proteins separated individually on a TSK 3000 SW column (see text for details). (a) γ-Globulin: 1 = IgM, 2 = IgA, 3 = IgG. (b) Bovine serum albumin: 1 = tetramer, 2 = dimer, 3 = monomer. (c) 1 = β-Lactoglobulin. (d) α-Lactalbumin: 1 = dimer or impurity, 2 = α-lactalbumin.

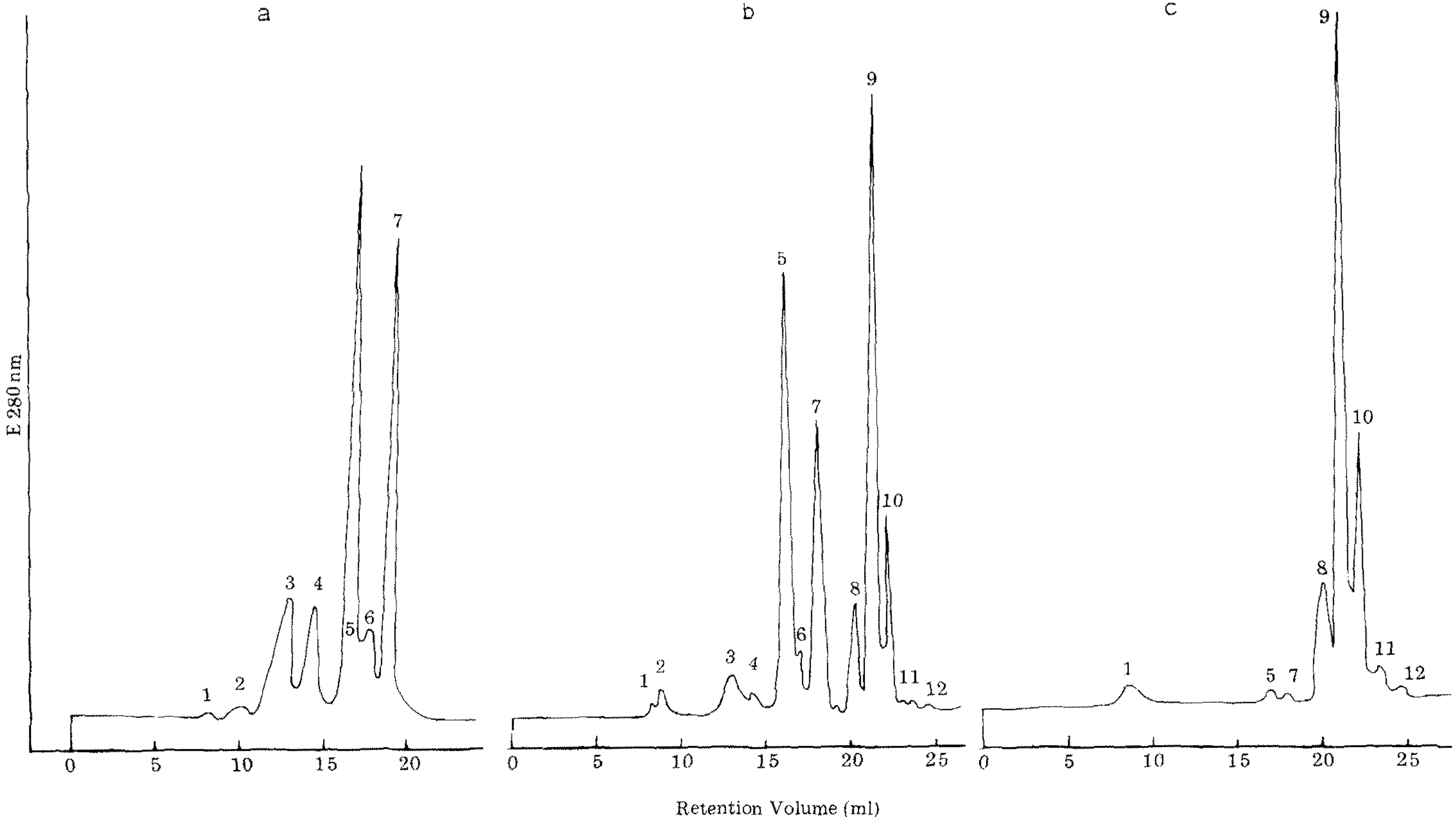

Fig. 2. Elution curves of proteins separated on a TSK 3000 SW column. (a) A mixture of γ-globulin, bovine serum albumin, β-lactoglobulin and α-lactalbumin (see text for details). 1 and 2 contain IgM, IgA bovine serum albumin tetramer and dimer, 3 = IgG, 4 = bovine serum albumin, 5 = β-lactoglobulin, 6 = same as peak 1 in Fig. 1d, 7 = α-lactalbumin, 8 12 = *unidentified*; (*b*) raw whey, a filtrate obtained after acid precipitation of casein from raw skimmed milk, identity of peaks same as in a; (c) whey similarly obtained but from heat-treated (85°C for 30 min) skimmed milk, identity of peaks same as in a.

SDS and 1% β-ME pH 6.8. The maximum degree of dissociation was observed when the above solution of proteins was heated at 85°C for 15 min. For separating dissociated proteins 0.1 *M* phosphate buffer, containing 0.1% SDS, was used for the mobile phase at a flow-rate of 0.4 ml/min.

RESULTS AND DISCUSSION

Separation of native proteins

The elution profile of the standard proteins applied individually to the column is presented in Fig. 1. The identities of the individual peaks were established by comparing the calculated MW of the peaks, based on their retention volumes and the known MW of the proteins.

γ-Globulin gave three peaks. Milk contains three major immunoglobulins IgG (MW 163,000), IgA (400,000) and IgM ($>1 \cdot 10^6$)[11]. The first peak (about 3% of total area) therefore corresponds to IgM, the second peak (15% of the area) to IgA and the third peak (82% of the area) to IgG. The first peak of bovine serum albumin (3% of total area) represents its tetramer, the middle peak (12% of the area) its dimer and the last peak (85% of the area) its monomer (Fig. 1b). β-Lactoglobulin was eluted as a single peak (Fig. 1c). α-Lactalbumin, on chromatography, gave two peaks. The first peak (about 12% of the total area) was calculated to be of approximately 28,000 MW. The second peak corresponds to the main α-lactalbumin peak (Fig. 1d). It appears that the first peak is either an impurity or a dimer of α-lactalbumin. The identity of this peak will be discussed in a later section.

Quantitation of native whey proteins

A "response factor" for the individual proteins was calculated by dividing the concentration of proteins by the area obtained for the major peak of the individual proteins.

An aliquot of 100 μl of the 10-fold diluted stock solution was chromatographed (Fig. 2a). The area under each major peak was determined and the concentration of the proteins was calculated by using the response factor obtained for the individual proteins. The calculated mean composition of the individual proteins was in close agreement with the actual values. (Table I)

The separation profiles of whey from raw skimmed milk and from heat-treated skimmed milk (heated at 85°C for 30 min) are presented in Fig. 2b and c. Peaks 1 and 2 consist of proteins of MW $> 2 \cdot 10^5$, probably comprising lipoprotein particles, IgM, bovine serum albumin tetramer, etc. Peaks 3, 4, 5 and 7 represent IgG, bovine serum albumin, β-lactoglobulin and α-lactalbumin, respectively. Peak 6 had the same retention volume, and the ratio of the areas between peaks 6 and 7 was the same as that obtained when purified α-lactalbumin was chromatographed (Fig. 1d). The identity of peaks 8–12 are not established, but they are probably due to the presence of proteose-peptones and other low-MW organic compounds known to be present in whey.

The elution profile of whey proteins obtained in this study is superior to that reported earlier[14,15]. Diosady[15] *et al.* used two Synchropack GPC 100 columns (250 × 4 mm), whereas Bican and Blanc[14] used a TSK 3000 SW column (300 × 7.5 mm) at a flow-rate of 1 ml/min. Since a 600 × 7.5 mm column at a flow-rate of 0.4 ml/min

TABLE I

ACTUAL AND DETERMINED COMPOSITION OF A PURIFIED PROTEIN MIXTURE AS NATIVE AND DENATURED PROTEINS

Protein	*Actual composition*		*Determined as native proteins*			*Determined as denatured proteins*		
	Concentration (mg/ml)	*Concentration (%, w/w)*	*Concentration (mg/ml)*		*Concentration (%, w/w)*	*Concentration (mg/ml)*		*Concentration (%, w/w)*
			*Mean**	*S.D.***		*Mean**	*S.D.***	
γ-Globulin	0.94	18.0	0.86	0.05	17.6	0.85	0.09	16.8
Bovine serum albumin	0.75	14.5	0.73	0.06	14.9	0.84	0.10	16.6
β-Lacto-globulin	2.50	48.2	2.35	0.12	48.1	2.35	0.10	46.4
α-Lactal-bumin	1.000	19.3	0.95	0.06	19.4	1.02	0.06	20.2

* Mean of ten determinations.

** This indicates that 95% of results can be expected to be between 2 × S.D.

was used in this study, a higher number of theoretical plates was obtained, resulting in a better resolution.

Practically all of the γ-globulin, serum albumin, β-lactoglobulin and α-lactalbumin was denatured when skimmed milk was heated at 85°C for 30 min. (Fig. 2c). The separation profile of whey ex pasteurised skimmed milk (72°C for 15 sec) was very similar to raw skimmed milk. This suggests that proteins are not denatured during this pasteurisation temperature. This technique, thus, would be useful in assessing the extent of denaturation of individual whey proteins.

Separation of denatured whey proteins

The elution profile of dissociated standard proteins is shown in Fig. 3. The first peak (5% of the total area) in the bovine serum albumin profile is due to a dimer not fully dissociated into its monomers, and the second is the main serum albumin peak (Fig. 3a). The first peak (5% of the total area) in the γ-globulin profile is due to undissociated globulin, the second peak (65% of the area) is due to the heavy-chain (50,000 MW) fraction, and the third peak (about 30% of the area) is that of the light-chain (23,000 MW) fraction of γ-globulin (Fig. 3b). The first peak in Fig. 3c is due to β-lactoglobulin monomer.

Denatured α-lactalbumin gave one peak with a small shoulder (Fig. 3d), whereas two peaks were observed when it was separated in its native state. The shoulder is due to aggregated SDS molecules, as discussed later. If the first peak (28,000 MW) observed in the native separation of α-lactalbumin (Fig. 1d) was due to the presence of proteins other than α-lactalbumin, it should have given a different peak after dissociation. The fact that only one peak of α-lactalbumin was observed after dissociation suggests that a portion of the α-lactalbumin exists as a dimer in the native state and that it dissociates into its monomers on treatment with SDS and β-ME. However, further work is required to confirm this proposition.

A very small peak, corresponding to 11,000 MW, was observed each time a protein was chromatographed after dissociation. A peak having the same retention volume and the same area was also observed when the dissociating buffer, containing SDS and β-ME but no protein, was chromatographed. It appears that this peak was due to aggregated SDS molecules, which scatter light. This peak appeared as a trailing peak following the α-lactalbumin peak. The area of this peak was determined and was subtracted from the area of the "α-lactalbumin" peak to determine the actual area due to α-lactalbumin. The last peak shown in the bovine serum albumin profile (Fig. 3) was due to β-ME[12], since a similar peak was obtained when a mobile phase containing only β-ME was applied to the column. This peak was observed in all chromatograms but is not drawn.

The stock purified whey protein mixture, which was used in the native protein chromatography, was denatured by heating at 85°C for 30 min and then dissociated and separated (Fig. 4a). The first peak was due to undissociated γ-globulin and bovine serum albumin. Peaks 2 to 7 correspond to bovine serum albumin, the γ-globulin heavy-chain and light-chain fraction, β-lactoglobulin, α-lactalbumin and SDS aggregated molecules, respectively.

When the column was new, the two peaks of bovine serum albumin and the γ-globulin heavy-chain fraction were separated so that the areas under the individual peaks could be integrated separately (Fig. 4a). The integrator parameters were se-

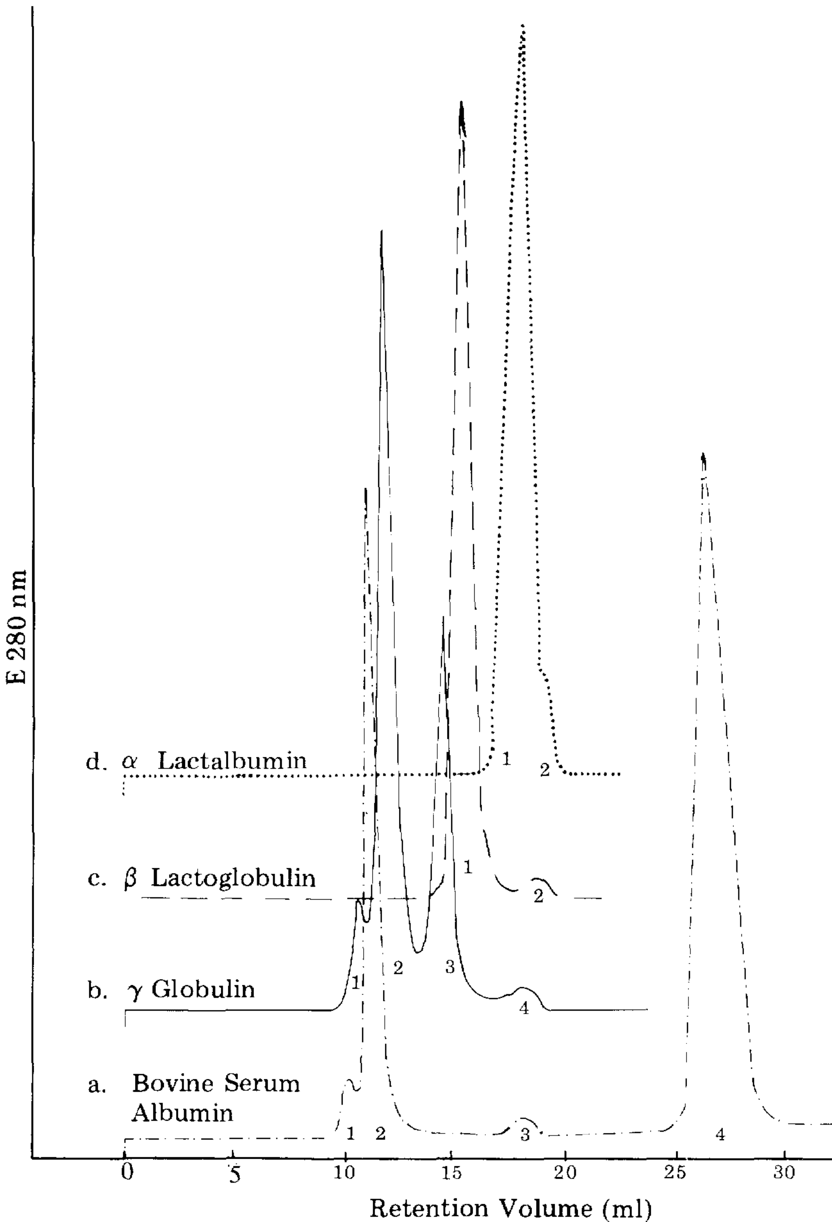

Fig. 3. Elution curves of standard dissociated proteins on a TSK 3000 SW column (see text for details). (a) Bovine serum albumin: 1-undissociated bovine serum albumin polymer, 2 = bovine serum albumin, 3 = aggregated SDS molecules, 4 = β-ME; (b) γ-globulin: 1 = undissociated γ-globulin, 2 = heavy-chain, 3 = light-chain fraction of γ-globulin, 4 = SDS aggregated molecules; (c) β-lactoglobulin: 1 = β-lactoglobulin monomer, 2 = aggregated SDS molecules; (d) α-lactalbumin: 1 = α-lactalbumin, 2 = aggregated SDS molecules.

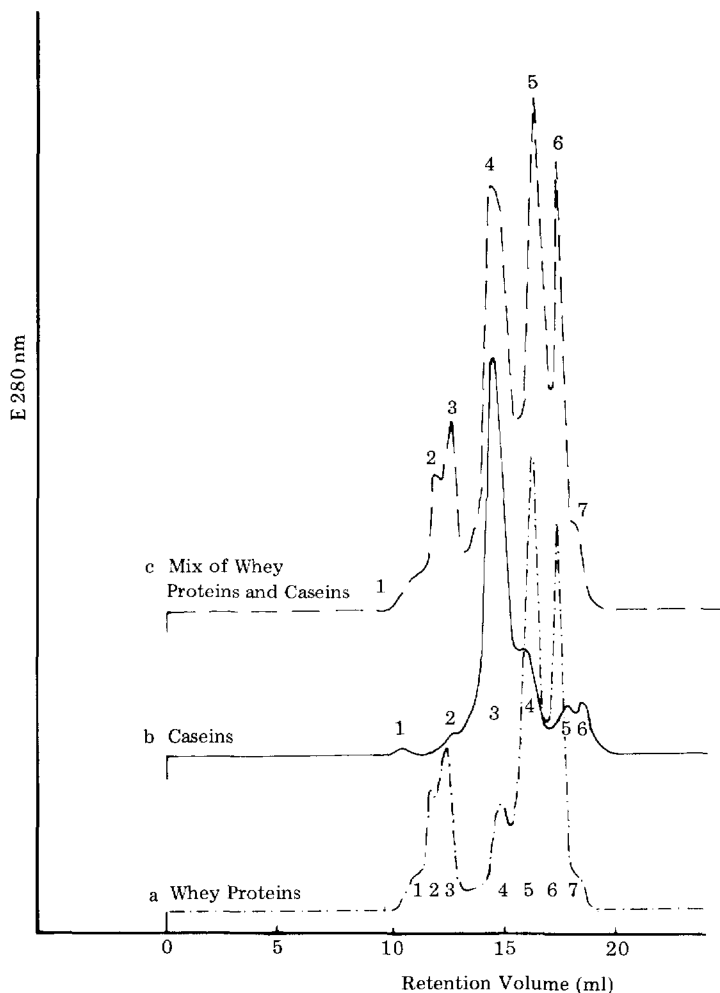

Fig. 4. Elution curves of dissociated protein samples separated on a TSK 3000 SW column. (a) Same mixture of proteins as in Fig. 2a: 1 = undissociated γ-globulin and bovine serum albumin, 2 = bovine serum albumin, 3 and 4 = heavy- and light-chain fractions of γ-globulin, 5 = β-lactoglobulin monomer, 6 = α-lactalbumin, 7 = aggregated SDS molecules; (b) whole casein: 1 and 2 = undissociated casein, 3 = $\alpha_{s\Gamma}$ and β-casein, 4 = κ-casein, 5 = γ-casein, 6 = aggregated SDS molecules; (c) mixture of (a) and (b): 1 = high-molecular-weight proteins, 2 = bovine serum albumin, 3 = γ-globulin heavy-chain fraction, 4 = $\alpha_{s\Gamma}$ and β-casein and light-chain fraction, 5 = β-lactoglobulin monomer and κ-casein, 6 = α-lactalbumin and γ-casein, 7 = aggregated SDS molecules.

lected so that perpendiculars were drawn from the start and end of the peaks to the base line, and the areas within each peak were determined. Continuous use of the column resulted in the fusion of the two peaks, because of a decreased separating efficiency of the column. The column could be restored to its original efficiency by topping it with the 3000 SW gel and washing it first with 20% methanol in water, then distilled water, and finally the mobile phase.

The response factor for the individual proteins was calculated as described

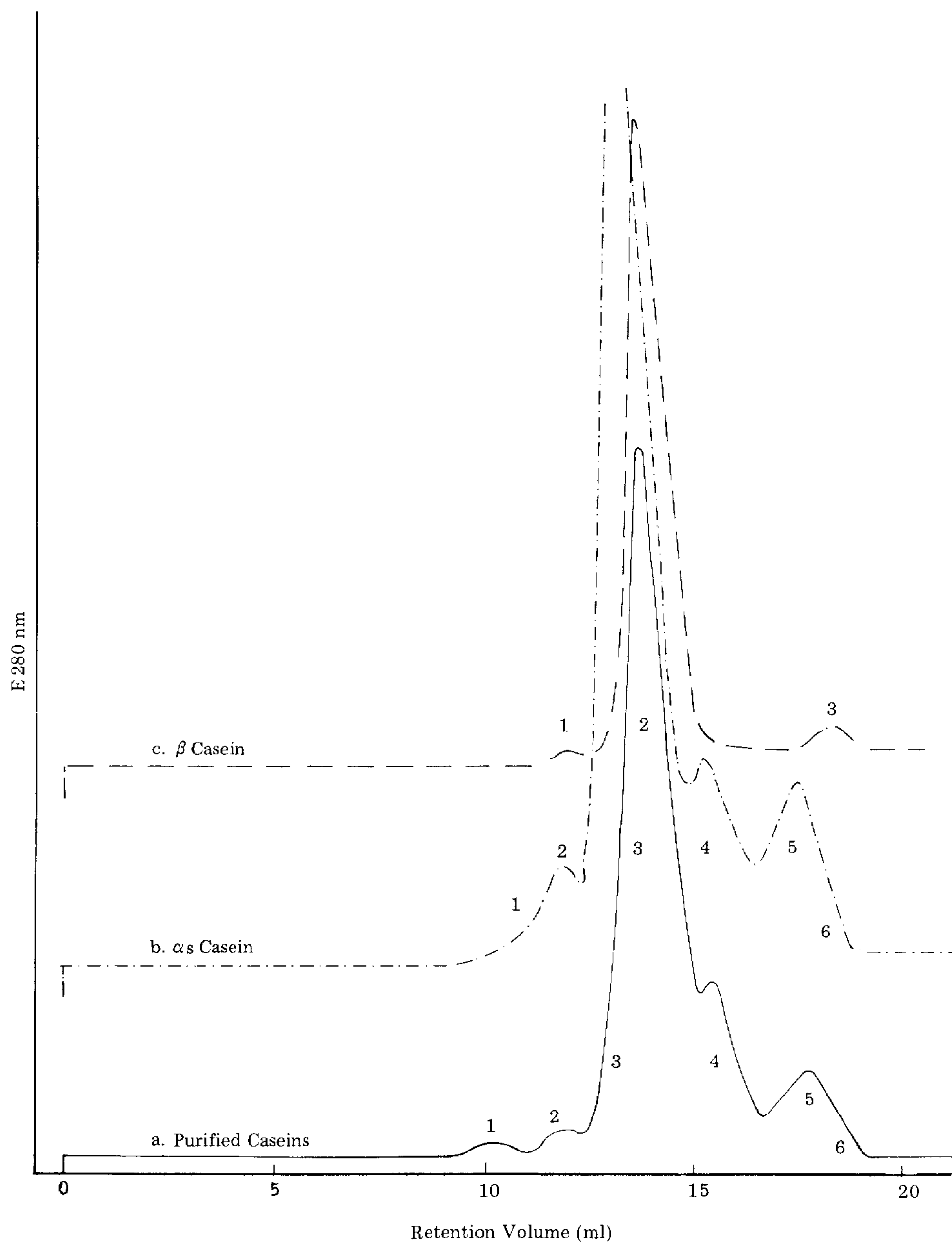

Fig. 5. Elution curves of dissociated caseins on TSK 3000 SW column. (a) Whole caseins: peaks identity same as in Fig. 4b; (b) $\alpha_{s\Gamma}$-casein: peak identity same as above, except peak 3 = $\alpha_{s\Gamma}$-casein only; (c) β-casein: 1 = undissociated casein, 2 = β-casein, 3 = aggregated SDS molecules.

earlier from experiments such as that in Fig. 3. The concentration of the individual proteins from the areas of each peak in Fig. 4a was calculated (Table I). The concentrations of β-lactoglobulin and α-lactalbumin, determined as denatured proteins, were practically the same as determined by the native protein separation (Table I). The values of bovine serum albumin and γ-globulin varied more in the denatured state than the native state. This is, as explained earlier, due to the close proximity of the two peaks.

Individual protein composition of the raw and heat-treated whey was determined by this method and was found to be the same and closely similar to the composition of the raw whey determined in its native state. Thus, the quantitation of whey proteins is not affected —whether they are present in either their native and/or denatured state— by the conditions of dissociation and separation used in this study, provided that appropriate response factors are applied.

Separation of caseins

Dissociated whole casein gave five peaks on separation (Fig. 5a). First, two minor peaks (3% of the whole area), eluted MW $> 2 \cdot 10^5$, are likely to be due to undissociated caseins. The third major peak (74% in area) was eluted around MW 28,000–31,000 and would represent α_{s_1}-and β-casein. The fourth (15% in area) and fifth (8%) peaks were eluted around MW 20,000 and MW 11,500–12,000, representing κ-casein and γ-casein, respectively. The purified β-casein sample (Fig. 5c) gave only one peak around MW 27,000–29,000, whereas α_{s_1}-casein (Fig. 5b) gave the same number of peaks and in the same position as the whole casein. The areas of the κ- and γ-casein peaks compared to the main α_{s_1}- and β-casein peak are much smaller in the α_{s_1}-casein sample than in the whole casein. Because the concentrations of α_{s_1}-casein and β-casein solutions applied to the column is not known, the comparison is made only for the ratio of the peak areas. This suggests that the α_{s_1}-casein sample still contained some κ- and γ-caseins as impurities. α_{s_1}-Casein and β-casein, because of their similar molecular weights, do not separate under these conditions. The molecular weights determined in the present study for the various casein fractions after SDS treatment, especially κ-casein, are closer to the actual values based on their amino acid composition than the values reported earlier based on an SDS electrophoretic study[16].

Determination of total individual proteins in a mixture of caseins and whey proteins

Comparison of Figs. 4a and b reveals that κ-casein is eluted with β-lactoglobulin and γ-casein with α-lactalbumin when a mixture of casein and whey proteins are chromatographed after dissociation of the proteins (Fig. 4c). The actual areas due to β-lactoglobulin and α-lactalbumin were calculated by subtracting the areas due to κ-casein and γ-casein from the areas obtained for "β-lactoglobulin" and "α-lactalbumin", respectively in a mixture, as follows:

Measured areas from the purified whole casein separation in Fig. 4b: A = α_{s_1}- + β-casein, B = κ-casein, C = γ-casein + SDS.

Measured areas from the chromatogram of a mixture of caseins and purified whey proteins in Fig. 4c: D = α_{s_1}- + β-casein, E = β-lactoglobulin + κ-casein, F = α-lactalbumin + γ-casein + SDS.

Calculated areas due to casein, lactoglobulin, and lactalbumin in a mixture of

casein and whey proteins: D = casein, E − (D · B)/A = β-lactoglobulin, F − D · (C − G)/A − G = α-lactalbumin, where G = area due to aggregated SDS molecules.

In calculating the areas due to κ- and γ-casein it is assumed that the ratio of κ- and γ-casein to α_{s_1}- plus β-casein is the same in all the samples.

The light-chain fraction of γ-globulin is eluted with the main casein peak. Since the γ-globulin content of milk and its products is very small compared to casein, the contribution of the light-chain fraction to the casein fraction was ignored. However, the contribution of the light-chain fraction to the casein peak can be calculated, if required, by multiplying the area of γ-globulin heavy-chain fraction in a particular sample by the ratio of γ-globulin light-chain to heavy-chain fraction (Fig. 3b), obtained when a purified sample of γ-globulin is chromatographed.

Casein and the stock solution of whey proteins were mixed in a different proportion and the individual whey proteins and casein composition was determined. The calculated values agreed within $\pm 10\%$ of the actual values.

It is assumed in the present study that the composition of caseins with respect to α_{s_1}- and β-, κ-, and γ-casein was the same in all the samples, since the composition of the individual caseins from bulk skim milk samples varied relatively little over a period of a year[17,18]. However, the composition of individual caseins varied with the individual cows, history of the milk, etc.[17,18]. Further work is therefore needed to determine the individual caseins more accurately. HPLC columns, packed with ion-exchange resins, are now available which are likely to separate the individual caseins better than the size-exclusion chromatography column.

The methods presented could be useful for determining the individual protein composition, in its native and/or denatured state, of such milk products as infant milk foods[19], which have undergone heat treatment during processing. The extent of denaturation of the individual whey proteins in any milk product can be determined by this technique as follows. The total individual whey proteins are determined after dissociation and separation of the proteins, as described in Fig. 4. The whey proteins are determined in their native state, as described in Fig. 2, from the filtrate left after precipitating the caseins and denatured whey proteins at pH 4.6. This method can also be used to assess the contamination of one source of proteins by another and for finger-printing proteins in biological and food systems.

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