SHORT COMMUNICATIONS

Preparative-scale fractionation of bovine, caprine and ovine whey proteins by gel permeation chromatography

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The whey proteins of the cow, goat and sheep have previously been fractionated on an analytical scale by reversed-phase HPLC (De Frutos *et al.* 1992), anion-exchange FPLC (Andrews *et al.* 1985; Manji *et al.* 1985; Laezza *et al.* 1991) and gel permeation FPLC (Andrews *et al.* 1985; Hill & Kakuda, 1990). Anion-exchange and gel permeation FPLC can readily be scaled up for laboratory preparation of whey protein fractions. There is some indication, however, that anion-exchange FPLC does not give complete separation of β-lactoglobulin and α-lactalbumin from the other minor whey protein fractions (Girardet *et al.* 1989).

In previous work it has been shown that gel permeation FPLC gives a satisfactory fractionation of the whey proteins of the cow (Law *et al.* 1993), goat (Law & Brown, 1994) and sheep (Law, 1995). In this paper we describe a scaled-up method of gel permeation that can be used for fairly rapid preparation or purification of four main whey protein fractions from the milks of these species.

MATERIALS AND METHODS

Milk samples

Samples were collected from the bulk milk of Friesian cows, British Saanen goats and Friesland ewes at the Institute. The milks were skimmed by centrifugation at 1000 ***g*** for 30 min, and adjusted to pH 4·6 at 20 °C by the addition of 1 M-HCl. The supernatant was passed through Whatman no. 42 filter paper, and whole whey protein was obtained by dialysing against deionized water for 72 h, and freeze drying.

Gel permeation chromatography

Whey protein was dissolved in 100 mM-Tris-HCl–0·5 M-NaCl buffer, pH 7·0 at a concentration of ~ 20 mg ml^{-1} and passed through a 0·45 μm filter. Samples containing between 80 and 400 mg whey proteins were fractionated by gel permeation chromatography at 20 °C on a column (26 × 950 mm, bed volume 504 ml) of Superdex 75 prep grade (Pharmacia Biotech, St Albans, AL1 3AW, UK) connected to a Pharmacia FPLC system. Buffer was passed through the column at

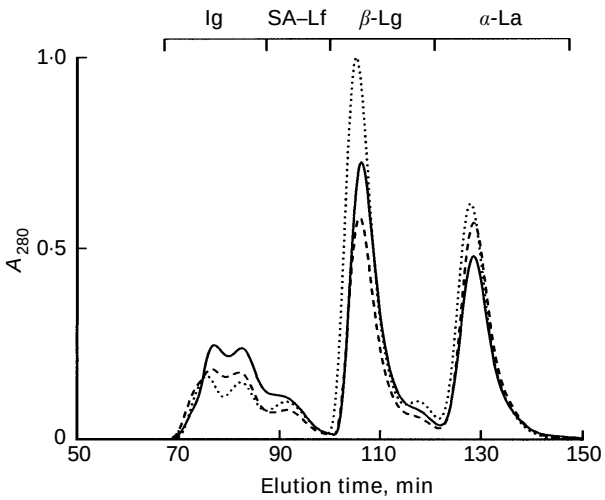

Fig. 1. Elution profiles obtained by gel permeation chromatography of bovine, caprine and ovine whey proteins on a column (26 × 950 mm, bed volume 504 ml) of Superdex 75 prep grade in 100 mM-Tris-HCl–0·5 M-NaCl buffer, pH 7·0. Whey proteins (80 mg) were applied to the column and eluted at a flow rate of 2·5 ml min^{-1}. ·····, Cow; – – – –, goat; ——, sheep. Four main fractions were obtained by pooling all of the protein-containing eluate, as shown by the divisions at the top of the Figure. Ig, immunoglobulins IgM and IgG; SA–Lf, serum albumin and lactoferrin; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

a flow rate of 2·5 ml min^{-1} using either a High Precision P-500 pump or a P-1 peristaltic pump (Pharmacia Biotech). One column volume of buffer was passed through to ensure complete elution of absorbing material, and 5·0 ml fractions of eluate were collected. The absorbance of the eluate was monitored at 280 nm, and all of the protein-containing eluate was pooled as indicated below to give four main fractions. Quantitative information was obtained by collecting absorbance measurements on a Dell computer using PC1000 Software (Thermo Separation Products, Stone, ST15 0HH, UK), and concentrations of the individual whey proteins were calculated using absorbance coefficients as described by Law *et al.* (1993). Pooled fractions were dialysed against deionized water for 72 h and freeze dried.

Other methods

SDS-PAGE was carried out as described previously (Law *et al.* 1993). Anion-exchange FPLC was as described by Andrews *et al.* (1985), but with an increased elution volume of 35 ml.

Results and discussion

The elution profiles obtained by gel permeation chromatography of ~ 80 mg whey proteins are given in Fig. 1. Some small molecular mass peptides eluted at ~ 170 min (not shown in Fig. 1) but later eluting materials were non-protein. The profiles were similar to those obtained previously with an analytical Superdex 75 HR 10/30 column for fractionation of whey proteins of the cow (Law *et al.* 1993), goat (Law & Brown, 1994) and sheep (Law, 1995). The elution profile for bovine whey proteins also resembled that obtained by lengthier methods of gel permeation chromatography on Sephadex G-100 or G-75 (Armstrong *et al.* 1970).

The four main whey protein fractions shown in Fig. 1 were examined by SDS-

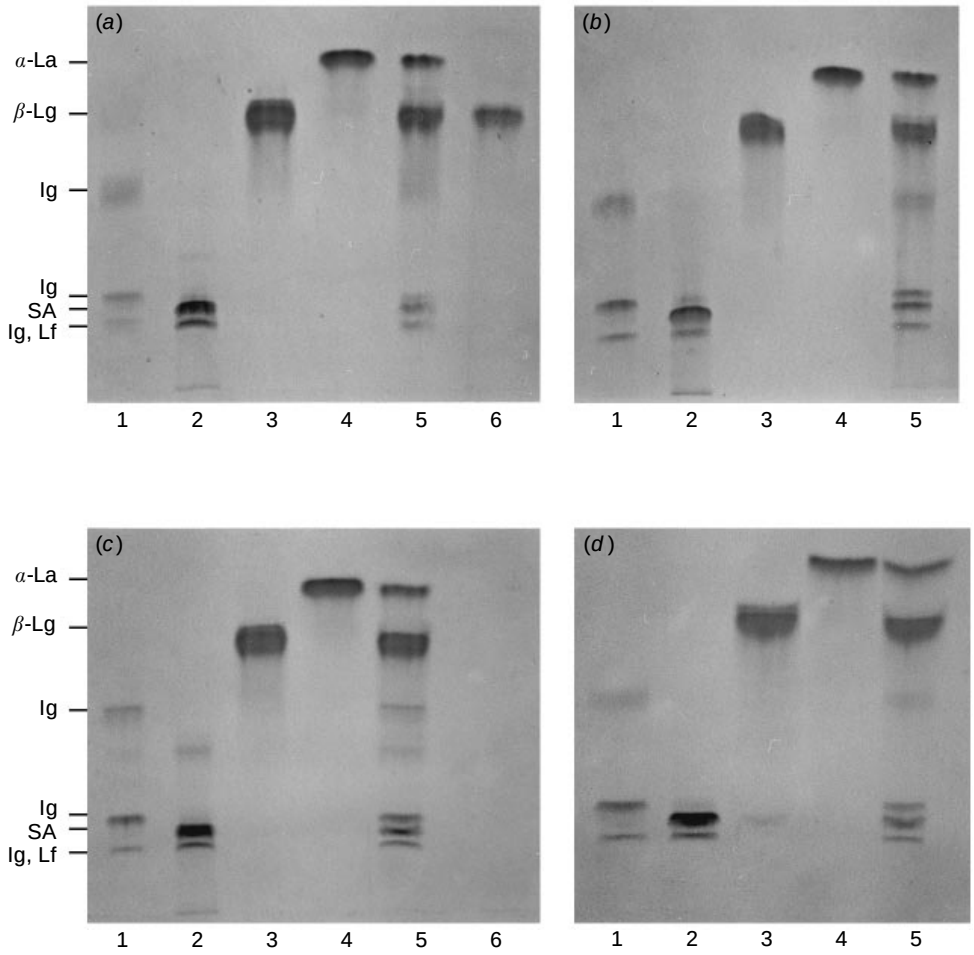

Fig. 2. SDS-PAGE patterns on homogeneous gels (200 g/l) for fractions obtained by gel permeation chromatography of whey proteins on a column of Superdex 75 prep grade. (*a*) Cow, (*b*) goat and (*c*) sheep as shown in Fig. 1 (gel permeation, 80 mg sample loading); (*d*) goat as shown in Fig. 4 (gel permeation, 400 mg sample loading). Lane 1, immunoglobulins; lane 2, serum albumin and lactoferrin; lane 3, β-lactoglobulin; lane 4, α-lactalbumin; lane 5, whole whey proteins; (*a*) lane 6, shoulder on β-lactoglobulin peak at ~ 118 min (see Fig. 1). α-La, α-lactalbumin; β-Lg, β-lactoglobulin; Ig, immunoglobulins IgM and IgG; SA, serum albumin; Lf, lactoferrin.

PAGE (Fig. 2) and, by comparison with results obtained previously for fractions from an analytical column of Superdex 75 HR (see references above), were identified, in order of elution and decreasing molecular mass, as fraction 1, immunoglobulins; 2, serum albumin and lactoferrin; 3, β-lactoglobulin and 4, α-lactalbumin. In fraction 1, the immunoglobulins in the presence of SDS and 2-mercaptoethanol appeared as three main bands representing the dissociated forms of IgM and IgG. It was not possible to separate serum albumin and lactoferrin in fraction 2 using gel permeation chromatography, because of the similarity of their molecular masses. Previous work (Law *et al.* 1993) showed that the protein in the least mobile band in fraction 2 (Fig. 2, lane 2) was lactoferrin, rather than cross contamination by immunoglobulins. In fraction 3, the small shoulder eluting at ~ 118 min on the right hand side of the main peak was shown by SDS-PAGE to be β-lactoglobulin (Fig. 2*a*, lane 6) and, as discussed previously (Law *et al.* 1993), is believed to be the monomeric form. The

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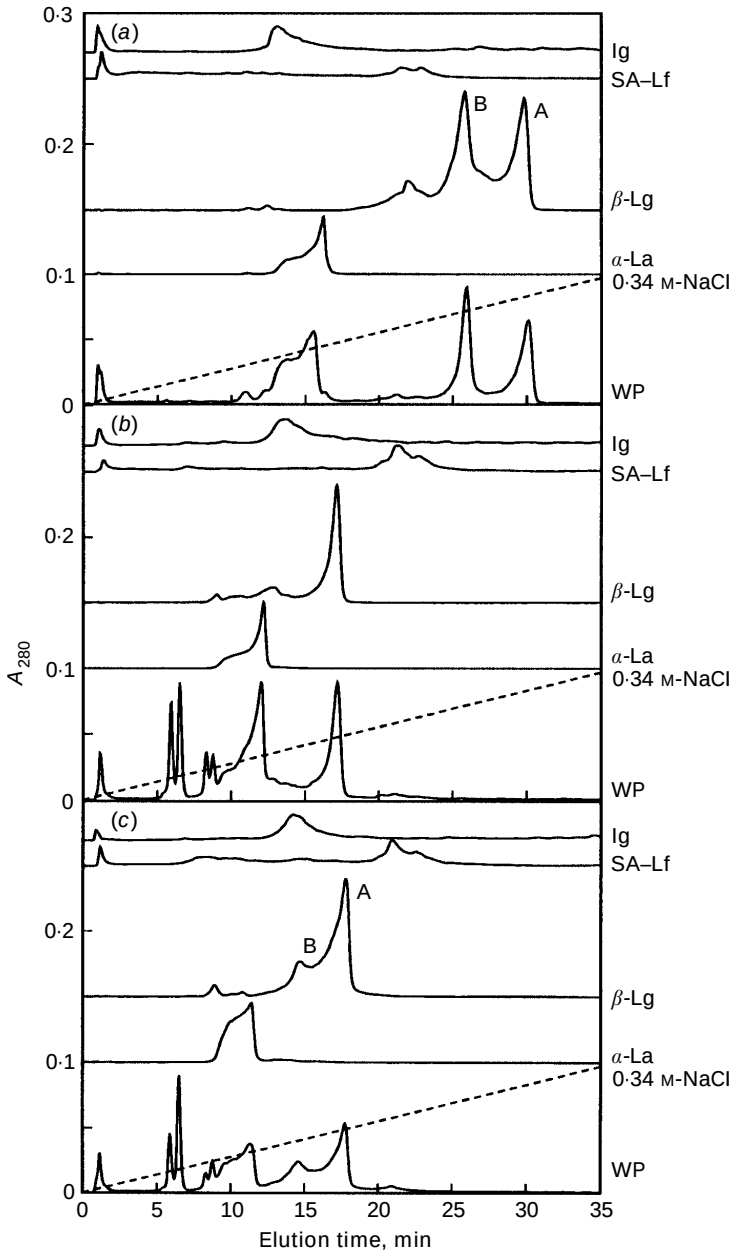

Fig. 3. Elution profiles obtained by anion-exchange FPLC of the whey protein fractions shown in Fig. 1. Freeze-dried whey proteins were dissolved in 20 mM-Tris-HCl buffer, pH 7·0, applied to a Mono Q column and eluted with a 0–0·34 M-NaCl gradient (– – – – –) in this buffer at a flow rate of 1·0 ml min^{-1} according to the method of Andrews *et al.* (1985): (*a*) cow, (*b*) goat and (*c*) sheep. Ig, immunoglobulins IgM and IgG; SA–Lf, serum albumin and lactoferrin; β-Lg, β-lactoglobulin (A, B, genetic variants); α-La, α-lactalbumin; WP, whey proteins.

SDS-PAGE patterns for the whey protein fractions of the cow, goat and sheep were similar to the corresponding patterns obtained previously for fractions from the analytical column (see references above). In particular, there was no evidence of increased cross contamination between the adjacent fractions from the preparative

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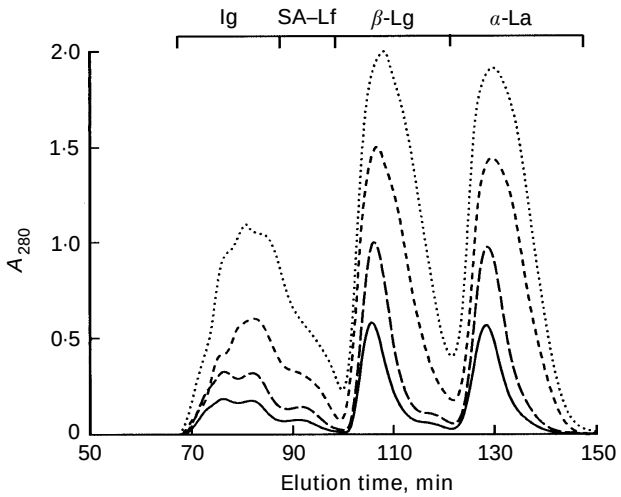

Fig. 4. Elution profiles obtained by gel permeation chromatography with increasing sample loading of caprine whey proteins on a column of Superdex 75 prep grade (conditions as in Fig. 1). The weights of whey proteins applied to the column were ——, 78 mg; – – –, 158 mg; - - - -, 304 mg; · · · ·, 400 mg. Ig, immunoglobulins IgM and IgG; SA–Lf, serum albumin and lactoferrin; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

column, and the major fractions β-lactoglobulin and α-lactalbumin were obtained in pure form.

The elution profiles obtained by anion-exchange FPLC of bovine, caprine and ovine whey protein fractions from the gel permeation column are shown in Fig. 3. Comparison of the elution profiles shows that the material in the void volume was common to fractions 1 and 2 and, if protein, may indicate some cross contamination. The elution profiles of β-lactoglobulin of the cow and the sheep showed two main peaks representing the differently charged A and B genetic variants, whereas that of the goat showed only one major peak. The profiles of β-lactoglobulin also included some smaller peaks with elution times similar to the adjacent fractions, serum albumin–lactoferrin (Fig. 3*a*) and α-lactalbumin (Fig. 3*b*), and may indicate some cross contamination. The high electrophoretic purities of the β-lactoglobulin fractions found on SDS-PAGE (Fig. 2), however, indicate that some of these smaller peaks may be due to an increased proportion of monomer β-lactoglobulin under the conditions used for re-chromatography, as discussed above. The α-lactalbumin fractions, which also appeared electrophoretically pure, show no cross contamination.

The effect of increasing sample loading on the resolution of the whey protein fractions on the preparative column is shown in Fig. 4 for caprine whey proteins. As expected, there was increased broadening of the peaks as the sample weight was increased from about 80 to 400 mg, and there was loss of resolution between fractions 1 and 2. SDS-PAGE of the fractions (Fig. 2*d*), however, showed that even at the highest loading, and including all of the material in the fractions, the degree of cross contamination increased only slightly. The amount of cross contamination could probably also be further reduced by pooling only the eluate in the middle of the main peaks.

Results showed, therefore, that this gel permeation method could be used on a laboratory scale to prepare a composite fraction of serum albumin and lactoferrin, and pure fractions of the immunoglobulins, β-lactoglobulin and α-lactalbumin. Because of improved flow characteristics of the chromatographic material, low

pressure pumps could be used, columns needed re-packing less frequently, and the separation took less than one fifth of the time of previous preparative methods of gel permeation (Armstrong *et al.* 1970).

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