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Selective extraction of native β-lactoglobulin from whey

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ABSTRACT

A method is proposed for the selective extraction from whey of β-lactoglobulin, the major allergenic component of cow milk. It represents an application of subunit exchange chromatography, which exploits the tendency of β-lactoglobulin to undergo reversible association–dissociation equilibria at slightly acidic pH values. Immobilized A and B β-lactoglobulin subunits are capable of interacting in a highly specific and reversible way with the soluble protein and can be used to extract A and B β-lactoglobulin from whey under conditions that favour subunit association. The soluble protein retained by the immobilized protein can be recovered under conditions that promote dissociation into subunits. The subunit exchange column, if coupled to an anion-exchange column, allows the complete deproteinization of whey. All the whey proteins are extracted in their native form and are of high purity; their functional properties are intact even after lyophilization. This important characteristic and the possibility of removing β-lactoglobulin selectively render the proposed method a potentially powerful tool in the processing of whey to produce human food products for specific end-uses such as hypoallergenic milk formulas.

INTRODUCTION

In recent years, the attitude towards whey proteins has changed and they are no longer considered as waste products, but rather as valuable nutrients that can be used by the food industry. However, this use is dependent on, among other factors, the possibility of tailoring whey proteins for specific end-uses and recovering them with intact functional properties [1]. Their use in the baby-food industry, for example, for the production of hypoallergenic infant milk formulas, requires the selective removal of β-lactoglobulin, the major allergenic component of cow milk [2].

β-Lactoglobulin, which represents half of whey proteins, occurs in the form of two genetic variants, A and B, plus a minor variant, C, of different electrophoretic mobility; it is a dimer of relative molecular mass 36 000. At pH <3.7 and >5.4, the two main genetic variants dissociate reversibly into single polypeptide chains; the extent of dissociation increases with increasing temperature. Between pH 3.7 and 5.2 in the cold, the native dimeric molecule undergoes a reversible tetramerization which in the A variant is maximum in the pH range 4.40–4.65; the tendency to tetramerize is significantly less marked with the B variant [3–8]. In mixtures of A and B β-lacto-globulin all molecules can aggregate with three types of bonds of different strengths and mixed A–B tetramers are also formed [9].

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The occurrence of association–dissociation phenomena in β-lactoglobulin suggested the application of subunit exchange chromatography for its selective extraction from whey. This is a bioaffinity chromatographic method which exploits the fact that immobilized subunits of polymeric and self-associating proteins retain the capacity to interact in a very specific and reversible way with soluble subunits of the same or of a homologous protein. Hence, under experimental conditions where the soluble protein is in self-association equilibrium, subunits are exchanged between the liquid and the solid phase and part of the protein, that was initially in solution, is bound to the matrix. The amount of matrix-bound polymer thus formed is governed by the law of mass action and depends on several parameters, *i.e.*, on the concentration of immobilized and soluble protein, on the association constants in the liquid and in the solid phase and on the stoichiometry of the association reaction [10–12]. In principle, therefore, one may achieve purification of the polymerizing protein in two steps by changing the state of association in an appropriate way. In the first, the protein is extracted from the medium and bound to the immobilized subunits under conditions that favour polymer formation. In the second step, the specifically bound protein is eluted under conditions that promote dissociation of the polymer into subunits [13–15].

The data reported in this paper show that subunit exchange chromatography can be employed for the selective extraction of A and B β-lactoglobulin from whey. Moreover, the experimental conditions used in the bioaffinity column are such that other whey proteins can be recovered easily in a concentrated from by means of an anion-exchange column. A major advantage of the proposed method is that all the proteins are obtained in their native form and hence are amenable to a variety of uses in the food industry.

EXPERIMENTAL

Chemicals

The different β-lactoglobulins, *i.e.*, the genetic species A and B and their mixture, were crystallized products from Sigma (St. Louis, MO, U.S.A.). Whey was obtained from fresh cow milk (Centrale del Latte, Rome, Italy) after precipitation of casein at pH 4.65. Milk and a sodium acetate buffer of ionic strength, (I) 0.1 M at pH 4.65 were mixed in equal volumes and stirred for 15 min at room temperature while maintaining the pH constant by addition of 1 M acetic acid in small amounts. The precipitated casein was removed by centrifugation for 10 min at 15 000 g in a Sorvall centrifuge and the supernatant (whey) was dialysed against the same acetate buffer. After dialysis, whey was clarified by filtration through a Millipore filter.

Equipment

Gel electrophoresis experiments were performed according to Davis [16]. Sedimentation velocity experiments were carried out in a Spinco Model E ultracentrifuge equipped with an RTIC unit at 52 000 rpm and 10°C; the sedimentation coefficients were corrected to $s_{20,w}$ (sedimentation coefficient corrected to the value it would have in a solvent with the viscosity and density of water at 20°C) by standard procedures.

Protein immobilization

The mixture of A and B β-lactoglobulin was immobilized under different experimental conditions (such as pH of immobilization and coupling time, extent of matrix activation, concentration of added protein). After the coupling step, the matrix was washed with coupling buffer; subsequently, any non-covalently bound protein was removed with a dissociating buffer at acidic pH, namely 0.1 *M* NaCl–HCl at pH 2.0. The concentration of immobilized protein was determined spectrophotometrically by using the specific absorbance ($A_{1\,\mathrm{cm}}^{1\%}$) of 9.6 at 278 mm [5]. The turbidity of the material was minimized with the use of protein-free gel in the reference beam.

Determination of the associating capacity of the immobilized protein

The capacity of immobilized β-lactoglobulin to bind the protein in solution was measured by frontal analysis of the eluate from a thermostated chromatographic column containing *ca.* 5 ml of immobilized protein and equilibrated with associating buffer (0.1 *M* sodium acetate buffer, pH 4.65) at a constant flow-rate of about 10 ml/h. β-Lactoglobulin solutions in the same buffer were percolated through the column until a steady state was reached, *i.e.*, until the absorbance of the effluent was the same as that of the in-flowing solution. The effluent absorbance was monitored at 278 nm with a Gilford 2000 spectrophotometer equipped with a flow cell. The elution volume of the protein, V, was calculated by constructing the equivalent sharp boundary [10]. The column was freed from the retained protein with dissociating buffer, 0.1 *M* NaCl–HCl (pH 2.0), unless stated otherwise. The same buffer was used to measure the void volume of the column, V_0.

RESULTS AND DISCUSSION

Application of subunit exchange chromatography to purified β-lactoglobulin

The effects of the pH of immobilization, extent of matrix activation and concentration of added protein, on the amount of A and B β-lactoglobulin immobilized per millilitre of added gel are shown in Table I. It is worth pointing out that pH values above 7.0 were avoided owing to the slow denaturation processes that take place under these pH conditions [4].

β-Lactoglobulin immobilized under all the conditions given in Table I is stable for several months when kept at pH 4.65 in the presence of 0.2% sodium azide. The

TABLE I

IMMOBILIZATION OF A AND B β-LACTOGLOBULIN ON SEPHAROSE 4B

Coupling reaction carried out using 0.05 *M* phosphate buffer; coupling time, 18 h at 6°C.

Sample No.	pH of immobilization	mg BrCN/ml Sepharose	mg β-lactoglobulin added/ml Sepharose	mg β-lactoglobulin immobilized/ml Sepharose
1	6.0	50	8	1.5
2	6.7	50	8	3.8
3	6.7	100	14	4.8
4	6.7	50	14	4.1

leakage of the protein from the matrix was found to correspond to about 2% of the immobilized material 8 months after the coupling reaction.

The associating capacity of the immobilized samples was measured as described under Experimental. A typical experiment in 0.1 *M* acetate buffer (pH 4.65) at 8°C is shown in Fig. 1. The establishment of a reversible association–dissociation equilibrium between the liquid and the solid phase results in the specific binding of soluble β-lactoglobulin to the immobilized subunits and hence in the retardation of the elution of β-lactoglobulin (V) with respect to an inert protein (V_0). The difference in elution volume, $\Delta V = V - V_0$ (Fig. 1), is related to the amount of protein bound to the matrix under steady-state conditions, $[\bar{Y}]$, by the expression $[\bar{Y}] = \Delta Vc$, where c is the steady-state concentration of the protein in solution. It is useful to define the quantity $[\bar{Y}]/R - X_t$, where $R - X_t$ is the amount of immobilized monomer, which may be called the binding number as its limiting value at infinite concentration of soluble protein equals $n-1$, n being the degree of polymerization [11].

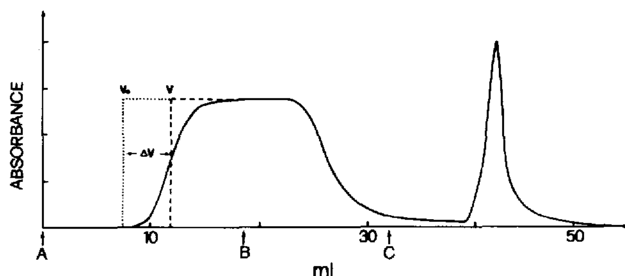


Fig. 1. Elution profile of A and B β-lactoglobulin from a column containing Sepharose-bound A and B β-lactoglobulin. The protein in the solid phase was 4.8 mg/ml of settled gel and the bed volume was 5.0 ml. The column was equilibrated with 0.1 *M* acetate buffer (pH 4.65) at 8°C. Arrows indicate the application of (A) the β-lactoglobulin solution at 2 mg/ml, (B) equilibrating buffer and (C) 0.1 M NaCl–HCl dissociating buffer (pH 2.0). Dashed lines represent the position of the hypersharp front of the boundaries of β-lactoglobulin (V) and of a non-interacting protein (V_0).

β-Lactoglobulin is probably immobilized on the matrix as the monomer of relative molecular mass of 18 kilodalton, as the coupling reaction is expected to shift the association–dissociation equilibrium of the native dimeric species [4,7] towards monomer formation. In line with this expectation, no traces of protein were found in the eluate on extensive washing of the immobilized protein with dissociating buffer after the coupling reaction.

Table II reports the results obtained in experiments of this kind in which solutions of A and B β-lactoglobulin at a concentration of 2 mg/ml in acetate buffer of $I = 0.1$ *M* were allowed to interact with the same protein mixture immobilized under the conditions detailed in Table I. The associating capacity of the Sepharose-bound protein varies as a function of the coupling conditions. In particular, a change in the immobilization pH from 6.7 to 6.0 (samples 1 and 2) and a high extent of matrix activation (samples 3 and 4) decrease the associating capacity of the immobilized protein; in the latter instance the effect is probably due to multi-point attachment to the matrix.

TABLE II

AMOUNT OF β-LACTOGLOBULIN RETAINED ON A COLUMN OF IMMOBILIZED A AND B β-LACTOGLOBULIN

A column (5 ml) was saturated with β-lactoglobulin or whey at a concentration of 2 mg/ml in 0.1 M acetate buffer (pH 4.65 or 6.0) at the indicated temperatures; elution with 0.1 M NaCl–HCl (pH 2.0).

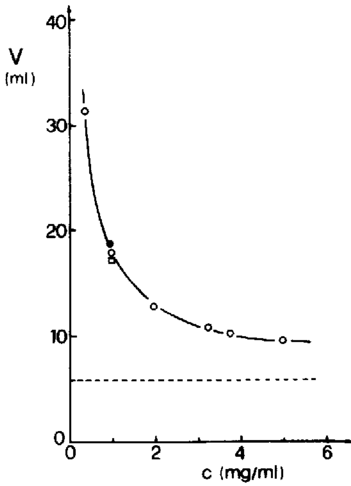
Solution	Immobilized protein[a]	mg bound β-lactoglobulin/mg immobilized protein[b]		
		8°C		23°C, pH 6.0
		pH 4.65	pH 6.0	
A and B β-lactoglobulin	1	0.13	–	–
	2	0.30	0.23	–
	3	0.22	0.32	–
	4	0.44	0.36	–
Whey	2	0.31	0.21	–
	3	0.19	0.37	0.23[c]
	4	0.53	–	0.41[c]

[a] Samples have the same numbering as in Table I.
[b] Amount of protein recovered after application of dissociating buffer.
[c] Protein eluted with 0.2 M acetate buffer (pH 3.6).

As mentioned above, the law of mass action requires the value of $[\bar{Y}]$, and therefore the elution volume V, to depend on the concentations of soluble and immobilized protein and on the association constants in solution and on the solid phase. The dependence of V and $[\bar{Y}]/R-X_t$ on the steady-state concentration of soluble protein was measured for sample 4 and is depicted in Figs. 2 and 3, respectively. Fig. 2 shows the significant increase in elution volume when the concentration of soluble protein is low (<2 mg/ml), a typical feature of subunit exchange chromatography. Fig. 3 shows that the data are consistent with a dimerization constant of 250 dl/g both in solution and on the solid phase. It also shows that at high protein concentrations (above 4 mg/ml), although dimer formation is still predominant, higher polymers are formed. In line with these findings, sedimentation velocity experiments carried out with the same A and B β-lactoglobulin mixture at a protein concentration of 5 mg/ml yielded a single peak with $s_{20,w}$ = 2.9S, a value which corresponds to the weight-average relative molecular mass (34 kilodalton) calculated with the dimerization constant given above using a partial specific volume $\bar{V}$ = 0.750 and f/f_0 = 1.2.

Selective extraction of β-lactoglobulin from whey

The high capacity and affinity of immobilized A and B β-lactoglobulin for the protein in solution can be exploited for its selective extraction from whey. Whey, in the associating buffer 0.1 M acetate (pH 4.65), was percolated through a column of immobilized A and B β-lactoglobulin equilibrated with the same buffer in sufficient volume to ensure the establishment of a steady state (Fig. 4, top). The elution pattern shows two distinct sigmoidal profiles: the first (at V_0) corresponds to the elution of all



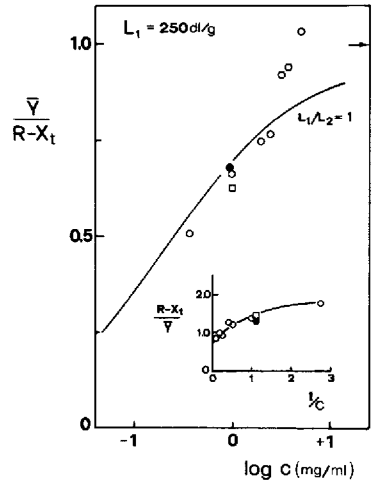


Fig. 2. Elution volume of A and B β-lactoglobulin from a column containing Sepharose-bound A and B β-lactoglobulin as a function of protein concentration in solution. ○ = Mixture of both genetic variants; ● = variant A; □ = variant B. The protein in the solid phase was 4.1 mg/ml and the bed volume 5.0 ml. The column was equilibrated with 0.1 *M* acetate buffer (pH 4.65) at 8°C. The dashed line represents the elution volume (V_0) of a non-interacting protein.

Fig. 3. Binding of A and B β-lactoglobulin in solution to a column containing Sepharose-bound A and B β-lactoglobulin as a function of the protein concentration in solution. The ordinates correspond to the binding number, *i.e.*, to the amount of soluble protein bound under steady-state conditions to the matrix, $\bar{Y}$, divided by the amount of immobilized monomer, $R - X_t$. The continuous curve was calculated according to eqns. 5 and 6 in ref. 10. Experimental conditions and symbols as in Fig. 2.

inert whey proteins and the second to the elution of β-lactoglobulin. After clearing the column with the equilibrating acetate buffer, the specifically bound protein was eluted by application of the dissociating buffer. The amount of eluted protein closely corresponds to that expected on the basis of the amount of whey applied to the column. The inset in Fig. 4 shows the quality of the separation achieved. Table II summarizes the results obtained with the samples described in Table I.

Fig. 4 (bottom) shows an experiment carried out at 20°C and pH 6.0. These conditions were tested because they allow complete deproteinization of whey to be achieved simply by applying the inert proteins that are eluted in the void volume of the subunit affinity column onto an ion-exchange column (see below). The elution profile is very similar to that obtained at pH 4.65, indicating that the subunit affinity column is efficient also at higher pH. In this experiment elution of the specifically bound β-lactoglobulin was attempted at pH 3.6, where the matrix is more stable than at pH 2.0; the recovery of the protein corresponds to about 80% of the column capacity.

Deproteinization of whey

Complete deproteinization of whey can be obtained easily by associating a DEAE-cellulose column [4,17,18] to the subunit exchange column. To this end, whey in 0.1 *M* acetate buffer (pH 6.0) was percolated through the column containing

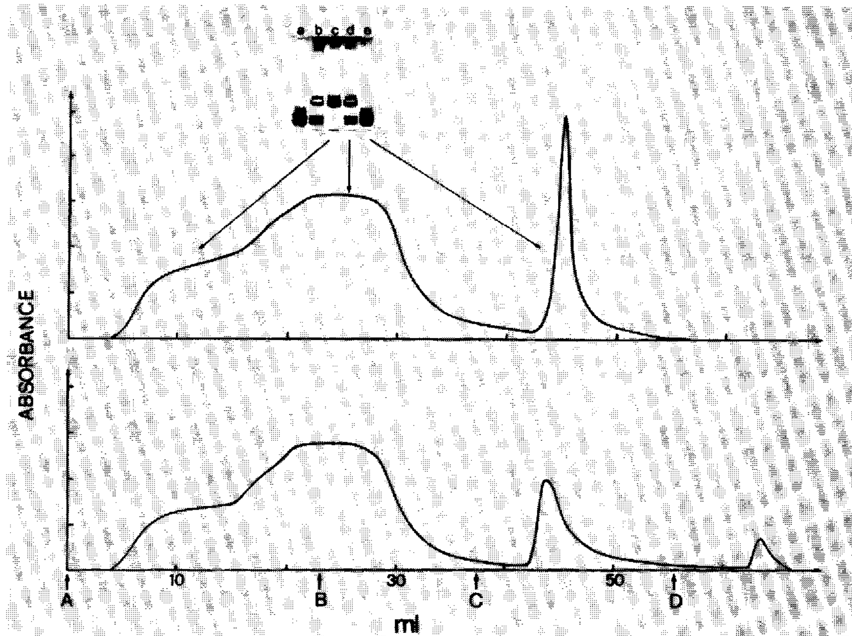


Fig. 4. Selective extraction of A and B β-lactoglobulin from whey with a column containing Sepharose-bound A and B β-lactoglobulin. The protein in the solid phase was 4.1 mg/ml and the bed volume was 5 ml. Bottom: the column was equilibrated with 0.1 *M* acetate buffer (pH 6.0) at 23°C. (A) Application of whey at 2 mg/ml in the same buffer; (B) application of equilibrating buffer; (C) application 0.2 *M* acetate dissociating buffer (pH 3.6); (D) application 0.1 *M* NaCl–HCl dissociating buffer (pH 2.0). Top: the column was equilibrated with 0.1 *M* acetate buffer (pH 4.65) at 8°C. Application of whey at 2 mg/ml in the same buffer, application of equilibrating buffer, application of 0.1 *M* NaCl–HCl dissociating buffer (pH 2.0) as indicated for the bottom part by A, B and C, respectively. The inset shows the polyacrylamide gel electrophoresis patterns [16] of (a) commercial A and B β-lactoglobulin, (b) the whey solution applied and (c–e) the fractions indicated on the elution profile.

immobilized β-lactoglobulin and equilibrated in the same buffer. The inert proteins that elute in the void volume were diluted with an equal volume of distilled water and applied to a DE-52 column equilibrated with the same buffer, *i.e.*, 0.05 *M* sodium acetate (pH 6.0). All the whey proteins bind to the column and can be eluted either all at the same time with 0.1 *M* acetate buffer (pH 4.65) containing 0.2 *M* NaCl, or in a differential way by applying an ionic strength gradient [4,17,18]. The gel electrophoretic patterns in Fig. 5 show the different fractions isolated in the various stages of the procedure. All the proteins obtained can be lyophilized and maintain their functional and physico-chemical properties, in particular their solubility, intact after rehydration.

CONCLUSIONS

The proposed use of a subunit affinity column containing immobilized A and B β-lactoglobulin offers the possibility of extracting both variants selectively from whey, thus permitting the use of the remaining whey proteins for the preparation of

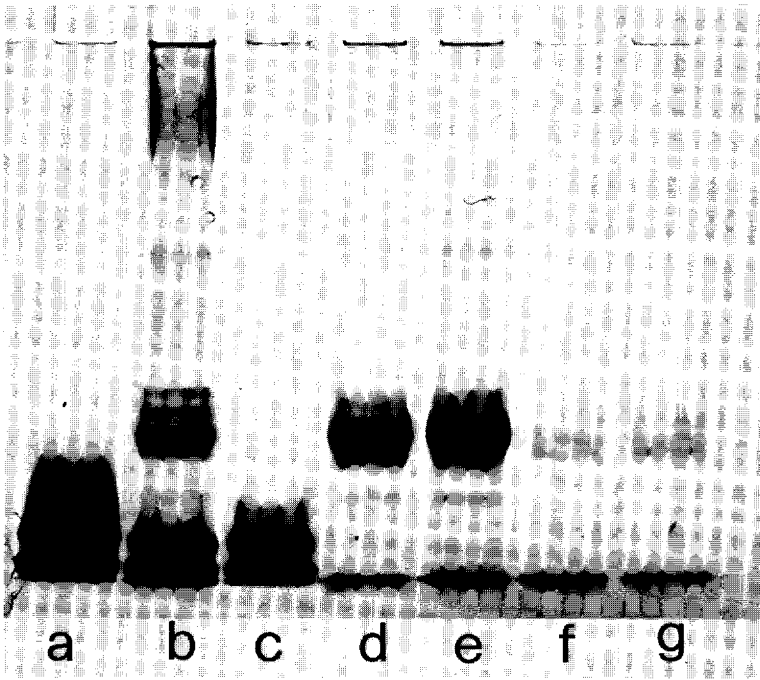


Fig. 5. Polyacrylamide gel electrophoresis patterns of whey proteins purified by the combined use of subunit-exchange and ion-exchange chromatography. (a) Commercial A and B β-lactoglobulin; (b) whey; (c) A and B β-lactoglobulin purified by subunit-exchange chromatography; (d–g) protein fractions eluted from a DEAE-cellulose column as described in the text.

special diet food, *e.g.*, hypoallergenic infant milk formulas. The combined use of the bioaffinity column with a DEAE column allows the complete deproteinization of whey and the recovery of the remaining whey protein in a concentrated form. A further advantage of the proposed method is that all the proteins can be lyophilized and are fully soluble and functional after rehydration.

Immobilized β-lactoglobulin can also be used to extract the protein directly from milk [19].

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