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HEAT DENATURATION OF WHEY PROTEINS COMPARATIVE STUDIES WITH PHYSICAL AND IMMUNOLOGICAL METHODS

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Résumé

DENATURATION THERMIQUE DES PROTÉINES DU LACTOSÉRUM. ETUDE COMPARATIVE ENTRE LES METHODES PHYSIQUES ET IMMUNOLOGIQUES. - Des échantillons d'un même lot de lait ou de lactosérum ont été soumis à des traitements thermiques variés ; les cinétiques du pourcentage de dénaturation de la β -lactoglobuline et l' α -lactalbumine ont été établies par des méthodes immunologiques (immunodiffusion radiale avec des antisérums spécifiques) et physiques (filtration sur gel de Sephadex G100, électrophorèse en gel de polyacrylamide) afin d'évaluer les possibilités et limites de ces techniques et d'expliquer les divergences rencontrées dans la littérature. De très bons résultats sont obtenus par filtration sur G100 et immunodiffusion radiale, mais cette dernière est plus rapide et plus quantitative. L'électrophorèse sur gel de polyacrylamide ne convient pas pour les études quantitatives : le pic de la β -lactoglobuline B est fortement contaminé par des polymères de la β -lactoglobuline A, et le pic de l' α -lactalbumine par des polymères des β -lactoglobulines A et B.

The ability of bovine milk to withstand high processing temperatures is a very important characteristic since without this the processing of milk (pasteurization, sterilization, dehydration) would be impossible. Knowledge of the effect of heat treatment on individual milk proteins is thus of importance in understanding the changes in the properties of milk which occur during treatment.

Caseins are remarkably heat-stable : bovine Na-caseinate in water withstands heating at 140° C for >60 min (pH 6.7) before coagulation occurs, whilst whole bovine milk typically coagulates after 20 min at 140° C. The non-casein proteins of milk, on the other hand, are

less heat-stable, undergoing complete denaturation in 10 min at 90 °C (reviewed by Fox and Morrissey, 1977).

While considerable information on heat-induced denaturation and interaction of whey protein and casein components of milk has been acquired by numerous investigators using different milk protein systems under a variety of experimental conditions, the results have often conflicted both qualitatively and quantitatively. For example, various whey proteins were shown by Larson and Roller (1955) to be heat-denatured at different rates : the immune-globulin (Ig) fraction is denatured first, followed by serum albumin which,

although not a milk-protein *per se*, is always present in bovine milk. β -lactoglobulin (β -Lg) is less rapidly affected by the same heat treatment while α -lactalbumin (α -La) is the most resistant. Aurand *et al.* (1963) reported that the effect of heat on stability in increasing order was Ig, α -La, serum albumin, β -Lg. Neither group include kinetic data on the rates of denaturation. Gough and Jenness (1962) found the reaction kinetics of β -Lg denaturation to be first order, while Dupont (1965) and more recently Lyster (1970) report second order kinetics.

The heat denaturation of whey proteins has long been studied by several techniques, i.e., electrophoresis (Larson and Roller, 1955; Aurand *et al.*, 1963; Nielsen *et al.*, 1973; Lee *et al.*, 1975; Adams *et al.*, 1976; Hansen and Melo, 1977) determination of residual protein content by microkjeldahl analysis of nitrogen (Gough and Jenness, 1962; Kenkare *et al.*, 1964) direct spectrophotometric measurements (Townend and Gyuricsek, 1974; Takase *et al.*, 1976) or using Folin reagent (Elsazly *et al.*, 1978), ^{131}I labelling (Wiechen and Knopp, 1974), immunodiffusion analysis (Lyster, 1970) starch gel electrophoresis and sedimentation velocity (Sawyer, 1968, 1971) polarographic measurements (Dupont, 1965; Mc Kenzie *et al.*, 1971) gel filtration chromatography (Morr and Josephson, 1968; Elfagm and Wheelock, 1977, 1978 a and b), micro-complement fixation test (Baer *et al.*, 1976). The kinetics observed in immunodiffusion technique (Lyster, 1970) or in G 100 filtration chromatography (Elfagm and Wheelock, 1977) for β -Lg denaturation after heating milk were in particular so different that it was therefore of interest to do a comparative study: thus samples of a single batch of milk or whey were subjected to various heat treatments and the rates of denaturation of whey proteins evaluated by both immunological (radial immunodiffusion) and physical methods (G 100 filtration chromatography and polyacrylamide gel electrophoresis). With this approach we hoped to assess on the utility and limitation of these techniques for studies of heat induced whey protein denaturation and to clarify some of the conflicting results.

Material and Methods

Milk and whey

Raw milk was obtained from a local dairy

and the whey was prepared by rennet coagulation (37 °C, 120 min) and centrifugation (3000 g, 15 min).

β -lactoglobulin and α -lactalbumin

These were purified from whey by gel filtration chromatography on a Sephadex G 100 column (90 × 2.5 cm) in Tris-HCl buffer at pH 8.0 (0.1 M Tris, 0.15 M NaCl) containing 0.1 % NaN_3 .

Specific antisera for β -lactoglobulin or α -lactalbumin

Antisera were prepared from rabbits or sheep given a course of injection of antigen in Freund's complete adjuvant, as previously described (Levieux, 1974) for other proteins.

Heat treatment

Milk or whey samples were heated between two glass-plates, spaced by a plastic U frame 2 mm thick, kept immersed in a water bath. 5 ml of milk or whey were injected between the two glasses using a syringe and needle. At the end of the heat treatment the samples were withdrawn by syringe and transferred to pre-cooled tube (ice-water mixture).

Radial immunodiffusion

The technique of Mancini *et al.* (1965) was carried out on glass-plates (7.5 × 11.5 cm) coated with 15 ml of 1.5 % agar (Noble, Difco) containing specific antisera for α -La or β -Lg.

Gel filtration chromatography

Samples were fractionated at room temperature on a Sephadex G 100 (Pharmacia) column (40 × 1.5 cm) with Tris-HCl buffer at pH 8.0 (0.1 M Tris, 0.15 M NaCl containing 0.1 % NaN_3) as eluant. The flow rate was approximately 10 ml/h and the volume of each fraction 0.8 ml. The extinction was measured at 280 nm. No traces of β -Lg in the α -La peak were detectable by radial-immunodiffusion analysis and no α -La was detected in the β -Lg peak. Peak areas were calculated to quantify the heat-induced changes in protein particle size.

Polyacrylamide gel electrophoresis (P.A.G.E.)

The electrophoresis procedure followed that described by Davis (1964). The gels were scanned in a densitometer after destaining and peak areas calculated. Preliminary studies showed that the denaturation of each protein

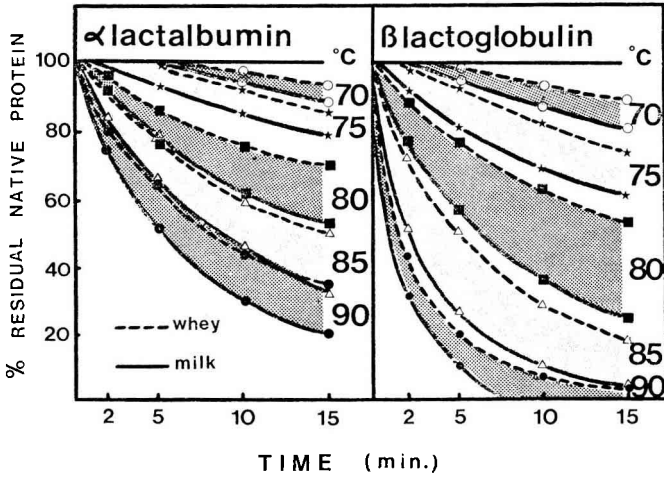


Fig. 1 - Heat denaturation kinetics of α -lactalbumin and β -lactoglobulin determined by radial-immunodiffusion analysis.

in control and heat processed whey cannot be calculated from the difference between the peak areas, as proposed by Hansen and Melo (1977), since the relation between peak area and concentration was not linear. Thus standard reference curves for β -Lg were obtained using increasing dilutions of bovine whey in caprine whey so that the total amount of whey protein in the sample was maintained relatively constant. No standard curves were prepared for α -La since the α -La peak from P.A.G.E. after heat denaturation was found to be heavily contaminated by aggregated β -Lg (as described in results). All samples were centrifuged at 11 000 g for 5 min before gel filtration chromatography or P.A.G.E.

Results

Radial immunodiffusion analysis

Results of immunological titration of α -La and β -Lg from milk or whey heated 2, 5, 10 or 15 min at 70 to 90 °C are reported in figure 1. β -Lg is consistently more sensitive to heat denaturation than α -La, and both proteins are more easily heat denatured in whole milk than in whey.

Gel filtration

A typical curve obtained for control whey in G 100 chromatography is reported on figure 2 and the position of α -La and β -Lg determi-

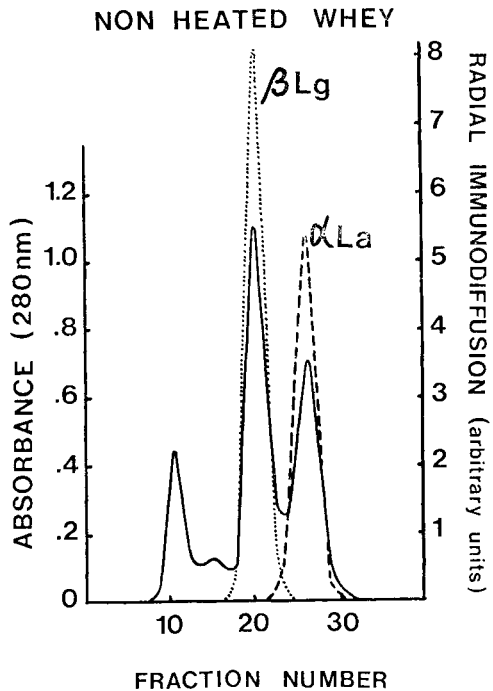


Fig. 2 - G 100 chromatography of whey with determination of α -lactalbumin and β -lactoglobulin position by radial-immunodiffusion analysis.

ned by radial-immunodiffusion analysis is shown. The first peak represents the void volume and contains mainly immunoglobulins. The second small peak, just before β -Lg, is essentially serum albumin.

Some of the more typical results obtained for heated whey are reported on figure 3. The first peak which contains the aggregates of α -La and β -Lg not removed by centrifugation, increases with heating-time and the β -Lg peak decreases more abruptly than α -La peak. The G 100 column allows a very clear demonstration of residual α -La and β -Lg within 5 to 6 hours.

Polyacrylamide gel electrophoresis (P.A.G.E.)

A densitometer scan of non heated whey, with the major peaks indicated is reported on figure 4.

Typical kinetics of heat-induced denaturation of whey proteins is reported in figure 5. As immunodiffusion and chromatographic analysis also reveal, β -Lg undergoes denaturation faster than α -La.

Comparison of figures 4 and 5 shows that β -Lg B is more rapidly denatured than β -Lg A, but quantitative analysis is impossible since the β -Lg B peak, like the α -La peak, is heavily contaminated by polymers of β -Lg. Thus for heated whey, only β -Lg A can be measured accurately by P.A.G.E.

Tables 1 and 2 compare the results obtained with these three techniques for β -Lg, and with the two first techniques for α -La, since P.A.G.E. could not be used to quantitate α -La. There is fairly good agreement. However, radial-immunodiffusion gives slightly higher values consistently, probably because the small aggregates of β -Lg or α -La still reacting with antisera are discriminated against in the physical technique. P.A.G.E. gives also consistently higher values than gel filtration for β -Lg analysis although only β -Lg A (the most resistant form of the two β -Lg) was quantitated instead of total β -Lg after gel filtration.

Discussion

Sensitivity and specificity are characteristics of immunochemical methods, and these properties would seem of particular value for determining the residual native protein in milk after heating. Since the method used is speci-

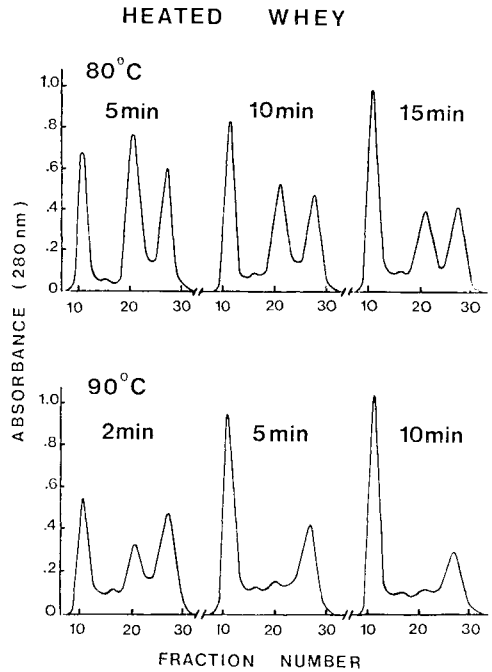


Fig. 3 - G 100 chromatography of heated whey

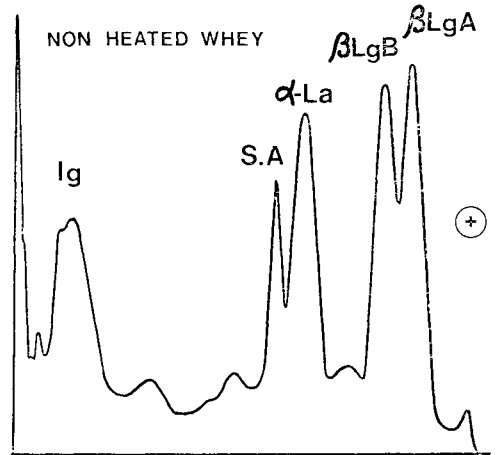


Fig. 4 - Polyacrylamide gel electrophoresis of whey

Table 1 - Heat denaturation of β -lactoglobulin analysed by radial-immunodiffusion, G 100 chromatography and polyacrylamide de gel electrophoresis

Duration of heating (min)	Residual native protein (%)		
	radial immunodiffusion	G 100 chromatography	polyacrylamide gel electrophoresis
<i>Temperature of heating : 80° C</i>			
2	88	84	90
5	76	68	73
10	62	50	54
15	50	40	44
<i>Temperature of heating : 85° C</i>			
2	71	64	66
5	50	43	45
10	20	22	25
15	17	15	18
<i>Temperature of heating : 90° C</i>			
2	42	33	38
5	19	18	18
10	8	8	9
15	3	4	4

Table 2 - Heat denaturation of α -lactalbumin analysed by radial-immunodiffusion and G 100 chromatography

Duration of heating (min)	Residual native protein (%)	
	Radial immunodiffusion	G 100 chromatography
<i>Temperature of heating : 80° C</i>		
2	96	90
5	86	80
10	75	73
15	68	65
<i>Temperature of heating : 85° C</i>		
2	91	88
5	80	72
10	58	55
15	49	47
<i>Temperature of heating : 90° C</i>		
2	80	69
5	65	58
10	44	43
15	34	35

fic, it was unnecessary to fractionate the samples. The techniques are also convenient for processing the large number of samples needed for kinetic measurements, and the sensitivity of the method makes it possible to heat very small volumes of milk.

Luz and Todd (1964), using several immunological methods, suggested that heat denatured protein may still react with antiserum to the native protein. This would invalidate radial-immunodiffusion methods, whose use depends on the ability of the antisera to discri-

Table 3 - Comparison of results obtained by Larson and Rolleri (1955) using moving-boundary electrophoresis, Lyster *et al.* (1974) and the author using radial-immunodiffusion.

References	Residual native protein of milk (%)			
	Temperature of heating during 30 min (° C)			
	70	74	77	82
β -lactoglobulin				
moving-boundary electrophoresis				
Larson and Rolleri (1955)	70	47	32	7
radial immunodiffusion				
Lyster <i>et al.</i> (1974)	81 ^a	59 ^a	38 ^a	14 ^a
Levieux	68 ^b	50 ^b	30 ^b	6 ^b
α -lactalbumin				
moving-boundary electrophoresis				
Larson and Rolleri (1955)	91	68	50	29
radial immunodiffusion				
Lyster <i>et al.</i> (1974)	...	80 ^a	64 ^a	32 ^a
Levieux	82 ^b	72 ^b	62 ^b	31 ^b

a : calculated values ; b : extrapolated values.

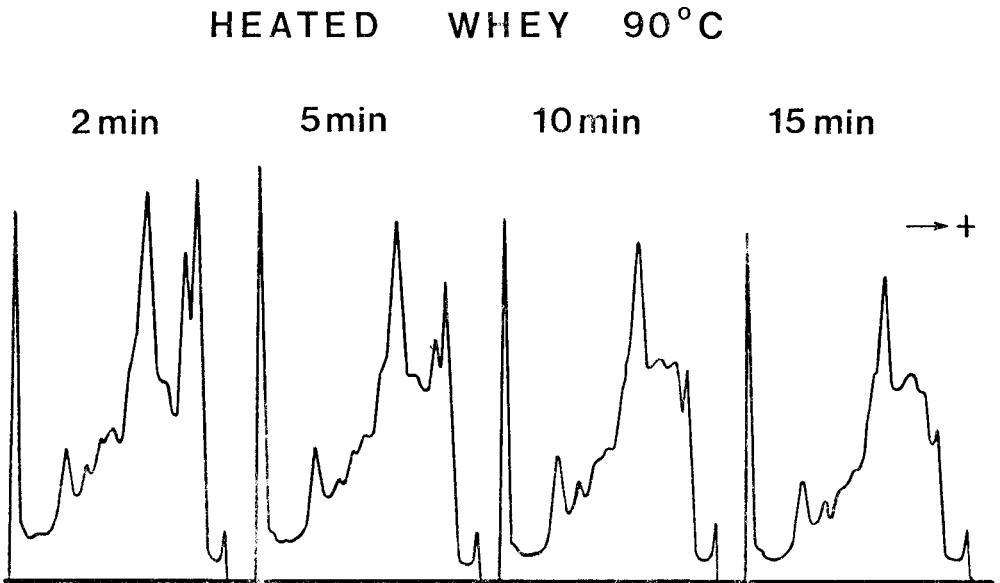


Fig. 5 - Polyacrylamide gel electrophoresis of heated whey

minate between native and heat-denatured protein. Immunodiffusion was therefore applied by Lyster (1970) to mixtures of unheated milk and milk heated at 100° C for 30 min ; this heat treatment should leave less than 1 % of the original whey protein undenatured. The results reported indicate that the antisera was not affected by the presence of denatured protein. According to Lyster, the immunodiffusion method gives consistently higher values than the salt fractionation method (Aschaffenburg and Drewry, 1959) when both methods are used in the same samples. Our results are in good agreement with this observation since we also find consistently higher values in immunodiffusion methods than in the chromatographic or electrophoretic methods. However, in Lyster's paper the agreement between immunodiffusion and salt fractionation was relatively poor : with unheated milk, for example, immunodiffusion gave 3.14 g/l β -Lg while salt fractionation gave 2.62 g/l. The salt-fractionation method was ruled out for this study since one of the limitations of the method is that it does not provide a specific measure of α -La but only of residual albumin, i.e., total albumin less β -Lg, a figure which includes serum albumin.

Table 3 compares the results obtained by Larson and Rolleri (1955), using moving-boundary electrophoresis and the results of Lyster and the author using immunodiffusion. The results of Lyster were calculated from his equation. The results of the author were extrapolated from the graph in figure 1. Our results agree best with those of Larson and Rolleri. Different heating procedures may explain the higher residual native protein values encountered by Lyster : this author heated the skin-milk by immersion of a capillary tube not preheated in a waterbath. Larson and Rolleri allowed five minutes for the milk to come to the water-bath temperature whereas we used glass plates preequilibrated to the temperature of the water-bath.

It is generally accepted that A and B β -Lg contain one cysteine and two cystine residues per 18000 molecular weight monomer (Mc Kenzie, 1967) and it is known that a heat induced interchange disulfide reaction between β -Lg and K-casein occurs near pH 7 before the aggregation of β -Lg itself (Sawyer *et al.*, 1963 ; Mc Kenzie *et al.*, 1971). These findings may explain the higher denaturation of β -Lg in milk compared with whey. Analogous chemical changes and interactions of α -La have

not been elucidated. Thermodenaturation of β -Lg proceeds via two major steps (Briggs and Hull, 1945 ; Sawyer, 1968). Initially, there is formation of a small aggregate of four monomers which is referred to as the primary denatured form. This aggregation involves sulfhydryl groups and occurs at temperatures above 70 °C. Above this temperature, the rate of formation of the primary denatured form increases sharply to a maximum between 80 and 85 °C. Later, conversion of the small aggregate to a large one ($S_{20} = 29$ S) takes place and this process is referred to as secondary aggregation. This secondary aggregation does not involve sulfhydryl groups and can occur at temperatures lower than those needed for the primary aggregation. While α -La contains no sulfhydryl groups, it does possess 4 disulphide groups/mole, and it is reported that α -La and β -Lg can form intermolecular complexes, presumably via disulphide interchange (Baer *et al.*, 1976 ; Wiechen and Knopp, 1974 ; Elfagn and Wheelock, 1978). These observations account for the limited value of polyacrylamide gel electrophoresis for heat denaturation studies of whey proteins since aggregates of β -Lg have a slower mobility and thus badly contaminate α -La and other fractions. The difference between the results reported by Hansen and Melo (1977) using electrophoresis on U.H.T. processed milk and the Aschaffenburg and Drewry method or precipitation is characteristic : electrophoresis of α -La and β -Lg, respectively showed 67 and 35% of protein remained soluble, whereas protein precipitation gave respectively : 44 and 31%. The discrepancies are essentially in the α -La fraction and, as expected, α -La is higher on electrophoresis. So, only β -Lg A can be electrophoretically quantitated correctly ; however the presents study confirms earlier experiments which indicate that β -Lg B is denatured by heat more rapidly than β -Lg A (Gough and Jenness, 1962 ; Dupont, 1965 ; Sawyer, 1968), and total β -Lg will be overestimated with an accurate estimate only of α -La.

Individual whey protein analysis or electrophoresis of whole milk, as used by Aurand *et al.* (1963) is not really useful since caseins, unaffected by heating, are the major components of the peaks and hide the changes. Although further definition can be accomplished by immunoelectrophoresis, this is only a qualitative approach. The conclusions of these authors on the heat stability i.e. $lg > \alpha$ -La >

serum albumin > β -Lg are not convincing. Morr and Josephson (1968) used P.A.G.E. and gel filtration chromatography to study the effect of calcium, N-ethylmaleimide and casein upon heat-induced whey protein aggregation. Although only qualitative results were reported the plates clearly demonstrate the difficulties of interpretation of P.A.G.E. electrophoresis for whey heated to 90° C for 10 min.

Gel filtration, especially using a G 100 analytical column, is a powerful technique allowing simultaneous direct quantitation of α -La and β -Lg. The results obtained here conflict with the data of Elfagm and Wheelock (1977). However the kinetics reported by these workers are very surprising; for example, the percent residual protein after heating whey at 80 °C for 0, 5, 10 or 15 min for β -Lg or α -La is the same. In some of their studies, the value obtained after 20 min heating is higher than after 5 min. Since gel filtration is generally considered to be a reproducible technique, it is possible that the preparation of whey proteins from the heated samples by ammonium sulphate precipitation, dialysis and freeze-drying may induce variability.

The heat denaturation of isolated β -Lg or α -La is very dependent of protein concentration, buffer pH and molarity, and as Townend and Gyuricsek (1974) point out, the choice of

β -Lg and α -La as models for heat-denaturation of whey is questionable. Hence the frequently conflicting reports. Whey itself must be used although its protein content is less well defined.

Whey protein has the highest biological value of the milk proteins and in recent years, much effort has been devoted to the production of undenatured whey protein concentrates. Whey protein fractions containing α -La have high protein efficient rates and net protein utilization values while fractions containing β -Lg have high net protein utilization values but only moderate protein efficiency value rates (Forsum, 1974). The cross-linking of α -La, however, after drastic processing, is far superior to that β -Lg and, therefore, pure α -La forms a rigid mass during heating, somewhat comparable to cooked eggwhite. Nutritionists believe that high losses of nutritive value occur in heated proteins following cross-linking since highly cross-linked proteins cannot be degraded by digestion enzymes. Immunological and physico-chemical methods are needed for quantitation of residual native protein in processed milk or whey, but the present work indicates that it is important to be aware of the limitations of these methods when the individual protein contents of whey products are assessed.

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Summary

Samples of a single batch of milk or whey were subjected to various heat treatment and the rates of denaturation of whey proteins (β -lactoglobulin and α -lactalbumin) evaluated by both immunological (radial immunodiffusion using specific antisera) and physical methods (G 100 filtration chromatography and polyacrylamide gel electrophoresis) in order to assess the utility and limitation of these techniques, and to clarify some conflicting results previously published. G 100 chromatography and radial immunodiffusion give very good results but the latter is very less time consuming and more quantitative. Polyacrylamide gel electrophoresis must be discarded for quantitative purpose since β -lactoglobulin B peak is heavily contaminated by polymers of β -lactoglobulin A, and α -lactalbumin peak by polymers of β -lactoglobulin A and B.

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