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Contamination of human whole casein by whey proteins

Quantitative aspects

by

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Résumé

La caséine entière humaine préparée par précipitation du lait à pH acide est toujours contaminée par des protéines du lactosérum (Nagasawa et al., *1974). Ceci conduit à un fractionnement difficile et peut entraîner une surestimation du contenu caséique du lait humain. La quantification des principales protéines du lactosérum présentes : Lactoferrine (LF), Immunoglobulines sécrétoires (sIgA), Sérum Albumine (SA), a été réalisée immunologiquement sur des caséines entières humaines préparées à partir d'un même lait de mélange par des techniques différentes : précipitation acide, fractionnement par le sulfate d'ammonium (caséine* $[NH_4]_2SO_4$*), tamisage moléculaire sur 4 gels différents (Sephacryl S200 ; Ultrogel AcA 54 ; Bio-Gels P100 et A-0.5m). Seules les sIgA et la LF étaient présentes dans la caséine acide (69 et 32 mg/g, respectivement). Les mêmes contaminants étaient détectés dans la caséine* $(NH_4)_2SO_4$*, mais à des taux très nettement inférieurs (10 et 4 mg/g, respectivement). Les fractions caséiques obtenues par tamisage moléculaire contenaient de 20 à 25 % de protéines du lactosérum. Cependant, cette contamination étant essentiellement due aux sIgA dans la fraction issue du Sephacryl S200, l'utilisation d'un tel support suivi d'un fractionnement au sulfate d'ammonium peut permettre l'obtention d'une caséine dépourvue de protéines du lactosérum.*

Mots clés

Caséine humaine - Protéines du lactosérum - Filtration sur gels - Dosages immunologiques.

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Summary

Human whole casein is always contaminated by whey proteins when prepared by acid precipitation (Nagasawa *et al.*, 1974). This leads to difficult fractionation and may lead to over-estimation of the casein content of human milk. Quantification of the main contaminant whey proteins : Lactoferrin (LF), Secretory Immunoglobulins A (sIgA), Serum Albumin (SA) was carried out immunologically in human whole caseins prepared from the same milk by acid precipitation, ammonium sulphate fractionation ($[NH_4]_2SO_4$ casein) and molecular sieving on 4 different gels (Sephacryl S200, Ultrogel AcA 54, Bio-Gels P100 and A-0.5m). Only sIgA and LF (69 and 32 mg/g, respectively) were found in acid casein. The same contaminants were detected, at much lower levels, in $(NH_4)_2SO_4$ casein (10 and 4 mg/g, respectively). The 4 casein fractions obtained by molecular sieving contained 20 to 25 % (v/v) whey proteins. However this contamination was due almost only to sIgA in the Sephacryl S200 fraction. The use of this last technique followed by ammonium sulphate fractionation should give an almost whey protein-free casein.

Key words

Human casein - Human whey proteins - Molecular sieving - Immunological determinations.

INTRODUCTION

Human whole casein, prepared by acidification to pH 4.2-4.7, is contamined by whey proteins, as shown by Nagasawa *et al.* (1974). The authors detected immunologically the occurrence of 2.5 % (w/w) LF and minor amounts of SA in casein prepared by repeated isoelectric precipitation. As we suspected the occurrence of higher contamination, we have performed immunological determinations of sIgA, LF and SA in acid casein prepared from fresh pooled human milk. Attemps to prepare whole casein with less contaminating whey proteins were made from the same pooled milk by ammonium sulphate fractionation (Brignon & Ribadeau Dumas, 1982), and molecular sieving on 4 different types of gels. This last technique took advantage of the aggregation of caseins into large heteropolymers under non-dissociating conditions (Schmidt, 1982). The contamination of the different casein fractions thus obtained was assessed by polyacrylamide gel electrophoresis in the presence of Sodium Dodecyl Sulphate (SDS-PAGE) and by measuring their sIgA, LF and SA contents by radial immunodiffusion.

MATERIALS AND METHODS

Fresh pooled human milk, provided by Lactarium de Paris, was defatted by 2 successive centrifugations at 3,200 g for 20 min, Phenyl

Methane Sulfonyl Fluoride (PMSF, Boehringer, Mannheim, WG) was added (1mM final concentration) to avoid eventual proteolysis, and 5 ml aliquots were kept at — 20° C. All the experiments were performed with the same milk (the casein fraction of which is represented on fig. 1, n° 1). Acid casein was prepared by lowering the pH of skim milk to 4.6 with 1N HCl, dialyzing against water and centrifuging. The casein precipitate was washed 3 times with water, redissolved in water to pH 7.0 with 1N NaOH. A second cycle of precipitation, centrifugation, washing was then carried out followed by redissolution and freeze-drying.

Purified LF, SA and β-casein were obtained as described previously (Brignon & Ribadeau Dumas, 1982 ; Chtourou *et al.*, 1984). The method of Steinbuch & Audran (1969) was used for preparing sIgA from human colostral whey, with the following modifications. Precipitation by caprylic acid was carried out in 0.06 M Na acetate, pH 5.0 instead of 4.6. After centrifugation, the supernatant was brought to pH 5.7 and thoroughly dialyzed against 0.015 M Na acetate, pH 5.7. It was then mixed with DEAE-cellulose equilibrated in the same buffer. Batchwise desorption of sIgA was performed as described (Steinbuch & Audran, 1969). They were finally purified by Sephacryl S300 chromatography in 0.05 M Tris-HCl, 0.3 M NaCl, pH 8.2.

Immunsera against the purified proteins (LF, sIgA, SA and β-casein) were obtained in rabbits. The technique of Mancini *et al.* (1965) was used to quantify these proteins by radial immunodiffusion.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed according to Laemmli (1970) in 15 % acrylamide gels. Samples at 2 % (w/v) in water were diluted with a volume of denaturing solution (4 % SDS, w/v ; 1 % 2-mercaptoethanol, v/v), incubated 1 hour at room temperature prior to electrophoresis.

All gel filtrations were performed at room temperature on the same column (100 cm × 1.5 cm I.D.) with the same buffer (0.05 M Tris-HCl, 1 M NaCl, pH 8.5). Flow rates were from 8 to 30 ml/h. The absorbance was recorded at 280 nm and the effluent was collected in 5 ml fractions. Fractions corresponding to each peak were thoroughly dialyzed against water and freeze-dried. Dextran blue 2000 (Pharmacia, Fine Chemical AB., Uppsala, Sweden) was used for measuring column void volume. The following molecular sieving gels were employed : Sephacryl S200 (Mw 5,000 to 250,000 ; Pharmacia) Bio-Gel P100 minus, 400 mesh and Bio-Gel A-0.5 m (Mw 5,000 to 100,000 and 10,000 to 500,000, respectively ; Biorad, Richmond, Cal.) ; Ultrogel AcA 54 (Mw 5,000 to 70,000; LKB, Bromma, Sweden).

RESULTS AND DISCUSSION

Fig. 1 shows the pattern usually obtained with human whole casein on SDS-PAGE. It clearly shows the whey protein contaminants. We

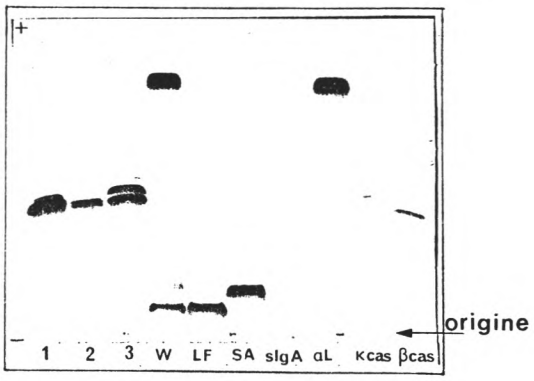

fig. 1

SDS-PAGE pattern of human milk proteins. 1, 2, 3 : Human whole caseins prepared by acid precipitation from 3 different milks. W : human whey ; LF : human lactoferrin ; SA : human serum albumin ; sIgA : secretory immunoglobulins A ; α-L : human α-lactalbumin ; χ-cas : human χ-casein ; β-cas : human β-casein.

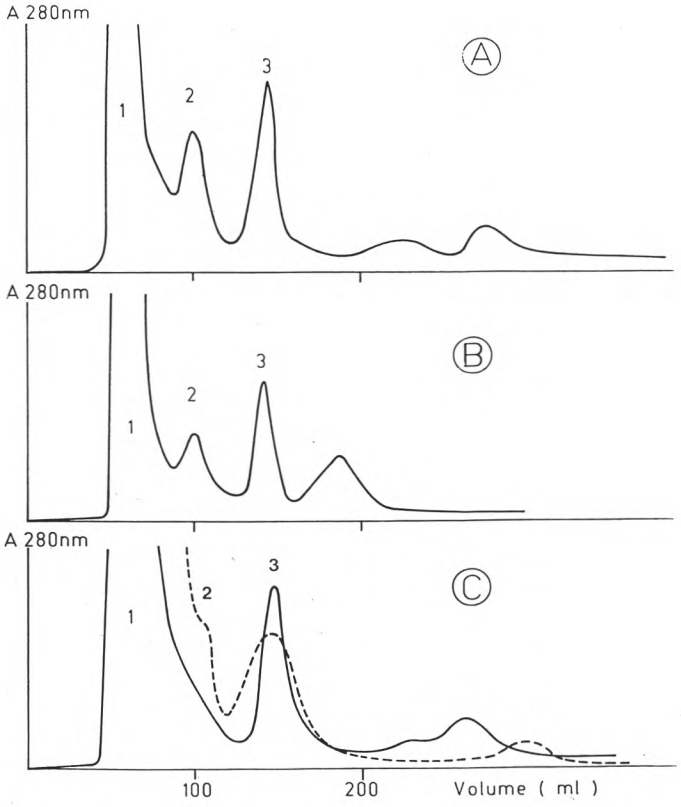

fig. 2

Elution profiles of 5 ml defatted human milk from A, Sephacryl S200 ; B, Bio-Gel A-0.5 m ; C, Ultrogel AcA 54 —— and Bio-Gel P100 — —. Flow rate : 30 ml/h. In each case the last fraction does not contain any protein.

have recently developed a method for fractionating milk proteins by gradient ammonium sulphate precipitation (Brignon & Ribadeau Dumas, 1982). It has been used here in steps instead of gradient for preparing whole casein. However the yield was somewhat low and it was observed that fractionation was influenced by the degree of proteolysis in milk. Then a search for another technique was undertaken. Molecular sieving could theor etically allow the separation of caseins from the other milk proteins as they form co-aggregates (micelles) of high molecular weight in milk (Schmidt, 1982) whereas the main human whey proteins have the following molecular weights : LF 80,000 ; SA 66,500 ; α-lactalbumin 14,100 ; sIgA 267,000 (heavy chains 65,000 ; light chains 25,000 ; secretory piece 71,000 ; J chain 16,000). However it was necessary to perform chromatography at high ionic strength in order to eliminate ionic interactions (protein-protein and protein-gel) which affected the resolution. In these conditions casein micelles dissociate into submicelles (Schmidt, 1982), but these are still co-aggregates of molecular weights much larger than sIgA.

Fig. 2 shows the elution patterns obtained with the different molecular sieves. For all of them the exclusion volume, determined with Dextran Blue, was close to 55 ml. In each case fraction 1 contained mainly the caseins, as it can be seen on SDS-PAGE (Fig. 3). In human milk, β-casein amounts to at least 90 % of whole casein (Chtourou

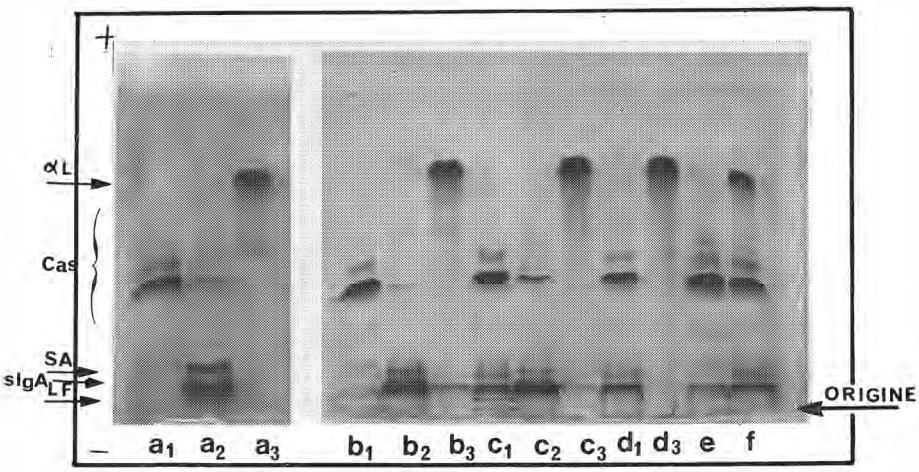

fig.3

SDS-PAGE of the different fractions obtained by gel filtration

Fractions 1,2 and 3 obtained from : Sephacryl S200 (a_1, a_2, a_3), Bio-Gel A-0,5 m (b_1, b_2, b_3), Ultrogel AcA54 (c_1, c_2, c_3), Bio-Gel P100 (d_1, d_3). Isoelectric casein (e). Undiluted human milk used for all experiments (f).

et al., 1984). Fraction 3 was α-lactalbumin whereas fraction 2 contained the other whey proteins.

α-lactalbumin was not determined in fractions 1 since its absence was shown by SDS-PAGE (Fig. 3). LF, sIgA and SA were determined by radial immunodiffusion in each fraction 1, as well as in acid casein and in 2 preparations of "$(NH_4)_2SO_4$ caseins" (table 1). It appears that these two preparations contained only small amounts of sIgA and LF, with traces of SA. Contamination of acid casein by sIgA and LF was higher. Fractions 1 from Ultrogel AcA 54 and Bio-Gel P100 had quite similar compositions. They contained ca. 24 % whey proteins (sIgA, LF and SA).

TABLE 1

Contents in sIgA, LF and SA of the different whole human casein preparations.

Preparation		sIgA (mg/g)	LF (mg/g)	SA (mg/g)	sIgA + LF + SA (g/100 g)
Acid casein		69.0	32.5	traces	10.1
$(NH_4)_2SO_4$ casein	1*	10.0	7.5	traces	1.7
	2*	10.0	0.3	traces	1.0
Fraction 1 (Sephacryl S-200)	1*	242.0	1.4	traces	24.3
	2*	192.0	1.4	traces	19.3
Fraction 1 (Bio-Gel A-0.5 m)		187.0	7.6	3.0	19.7
Fraction 1 (Ultrogel AcA54)		142.0	58.0	37.0	23.7
Fraction 1 (Bio-Gel P100)		140	71.2	35.0	24.6

* 2 different preparations.

Although fractions 1 from Sephacryl S200 and Bio-Gel A-0.5m had not very different compositions, the former appeared to be of more interest as it only contained traces of SA and minute amounts of LF. However sIgA were present at a fairly high level (table 1). The best separation on this molecular sieve was obtained at a flow rate of 30 ml/h.

From immunological determinations the starting human milk contained 3.75, 1.39, 1.31 and 0.33 g/l of β-casein, sIgA, LF and SA respectively. This indicates that most sIgA were present in fraction 1

from Sephacryl S200. Nevertheless we think that this molecular sieve is interesting for preparing human whole casein free from whey proteins. Indeed we have shown previously (Brignon & Ribadeau-Dumas, 1982) that sIgA and human caseins can be easily separated by ammonium sulphate fractionation in a single step.

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