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THE CHROMATOGRAPHIC PURIFICATION OF HUMAN KAPPA-CASEIN*

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SUMMARY

The fractionation of human casein was shown to be more difficult than that of caseins from ruminants. As human casein is digested by rennin, although less satisfactorily than with other caseins already studied, the purification of a κ-like fraction was attempted.

Fractionations obtained by precipitation or chromatography gave rise to rennin-sensitive components. Two κ-like fractions could be characterized by chromatography on DEAE-cellulose in the presence of dissociating agents.

INTRODUCTION

Starch gel urea electrophoresis of human casein, in the presence of mercaptoethanol, showed it to have a very complicated composition[1]. Though isoelectric human casein was found to be heterogeneous some time ago[2], very few attempts at fractionation have been made and purified fractions corresponding to the α-, β- or κ-components of cow casein have not been obtained until now.

The digestion of human casein by rennin (EC 3.4.4.3), which has been investigated by ALAIS AND JOLLÈS[3], suggested the presence of a component similar to the κ-fraction of bovine casein which is the specific substrate of this enzyme. Preliminary fractionation experiments by chromatography on ion-exchange columns[4] allowed these authors to prepare a fraction rich in the κ-component from human casein. More recently, MALPRESS AND SEID-AKHAVAN[5] have also chromatographed human casein and found two main fractions: one of them was precipitated by calcium and the other was rennin-sensitive; they were similar to bovine α- and κ-caseins but their electrophoretic behaviour was different and indicated that they were always heterogeneous. More recently, NAGASAWA *et al.*[6] have published the results of an incomplete fractionation of human casein; their data were different from those of the

* 21st Communication on caseins; 20th communication, J. JOLLÈS, P. JOLLÈS AND C. ALAIS, *nature*, 222 (1969) 668.

above mentioned authors and did not mention the presence of a calcium-sensitive component.

This paper is concerned with the fractionation of human casein, the main aim being the isolation of a rennin-sensitive component. As the precipitation methods most commonly used in the study of bovine casein only gave poor results with human casein, chromatography on anion exchange columns in the presence of dissociating agents was tried.

EXPERIMENTAL

Human casein was prepared from fresh pooled milk. As previously reported[1,3], its preparation was more difficult than that of casein from ruminants; thus our previously described method was employed[1].

The following precipitation procedures used for the preparation of cow κ-casein were tried in the case of human casein: (1) precipitation by calcium chloride and ethanol, according to McKENZIE AND WAKE[7]; (2) precipitation by sulphuric acid in the presence of urea, according to ZITTLE AND CUSTER[8].

The chromatographic fractionations were done on DEAE-cellulose columns, at pH 7, either by a salt gradient or by stepwise elution. Because of the strong tendency of casein components to form complexes, a dissociating agent must be added in a fairly high concentration. We used an imidazole buffer containing 3.3 *M* urea[9] and a phosphate buffer containing 20 % dimethylformamide[10].

Starch gel electrophoresis in a horizontal cell was performed according to WAKE AND BALDWIN[11] with a Tris–borate buffer solution, pH 9.3, containing 4.5 *M* urea and 0.03 *M* mercaptoethanol.

The rennin sensitivity was determined by the action of rennin (0.7 μg crystalline rennin/ml) on 0.5 % casein fractions at pH 6.7 and 25°; the release of non-protein nitrogenous substances (NPN) was determined in the 12 % trichloracetic acid (TCA) filtrate[12].

RESULTS

Rennin sensitivity of human casein

We prepared twelve lots of human casein from twelve different pooled milk samples (1–2 l) and found that the rennin sensitivity varied considerably from one batch to another. Table I indicates that three lots were not digested at all and three others only to a very slight extent (about 0.3 % NPN). Six lots released NPN in quantities lower or about the same as with bovine casein. We also observed that two lots were highly soluble in 12 % TCA.

In the following fractionation experiments, we only used batches Nos. 4 and 9; they had a rather low solubility in TCA and gave a good release of NPN which postulated the existence of a κ-like component. The electrophoretic patterns are presented in Fig. 1.

Fractionation of human casein by precipitation

Fractionation with calcium and ethanol was attempted with human casein in 4 % solution. The first stages occurred as expected (precipitation by calcium chloride

TABLE I

SENSITIVITY OF HUMAN CASEIN TO RENNIN: LIBERATION OF NON-PROTEIN NITROGEN (NPN) CONTAINING SUBSTANCES

Casein		*NPN*[a]	
Lot No.	*Solution mg N/ml*	*Before rennin action*	*Increase after 60 min*
1	2.47	2.46	0.35
2	0.921	1.35	0
3	1.88	2.11	0.92
4	2.45	1.97	0.90
5	2.21	5.04	0
6	2.38	8.60	1.38
7	2.02	1.80	0.57
8	2.27	20.35	0.27
9	1.93	1.36	1.5
10	2.31	3.14	0.37
11	2.40	3.91	0
12	1.16	4.24	0.87

[a] Percentage of total nitrogen; rennin 0.7 μg/ml, pH 6.7.

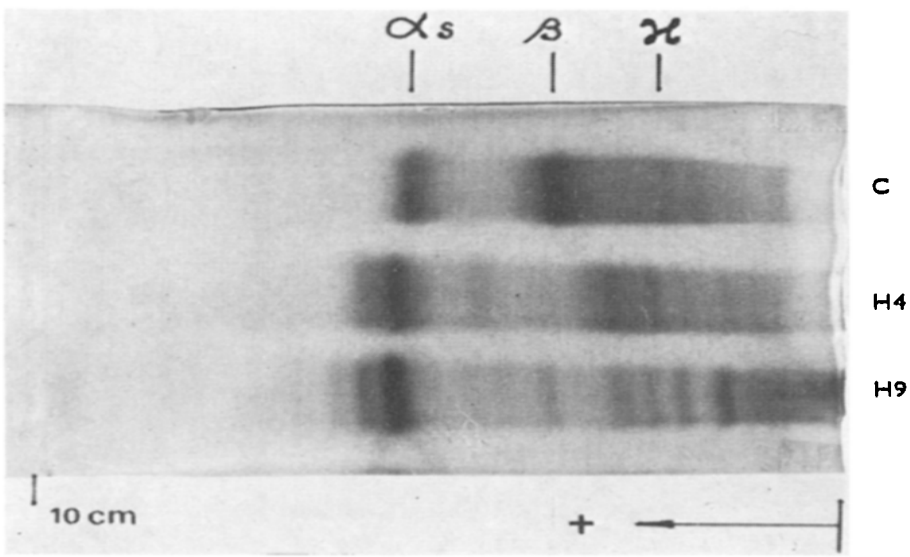

Fig. 1. Starch gel electrophoresis of human (H) and cow (C) caseins; (0.76 *M* Tris–citric acid buffer, pH 8.6, in the presence of 7 *M* urea; 15 h; 15 V/cm in the presence of mercaptoethanol).

and then sodium sulphate to give fractions a and b). However, no immediate precipitation was observed when ethanol was added in the presence of ammonium acetate; at this stage bovine casein gives the characteristic rubbery precipitate of the κ-fraction. The human casein solution floculated slowly at room temperature; the precipitate was separated by centrifugation, washed with 50 % ethanol and dried under vacuum (fraction d).

The four separated fractions representing 92 % of the treated human casein were found in the following proportions (calculated as % recovered substances):

Fraction (a): precipitated by calcium 49.7 %;
Fraction (b): soluble in sodium sulphate 4.4 %;
Fraction (c): soluble in ethanol 36.3 %;
Fraction (d): precipitated by ethanol 9.5 %.

Fractions (a) and (b) do not release any NPN when digested by rennin; fraction (c) releases a small quantity. However, fraction (d) is strongly attacked by rennin; the amount of NPN is 9.4 %, the solution becomes turbid but floculation does not occur. Electrophoresis revealed that all these fractions are heterogeneous and, in particular, the rennin-sensitive component (d) gives rise to several bands.

In the case of bovine casein, a simplified method with sulphuric acid and urea can be used to obtain the κ-rich fraction[8]. With human casein, the precipitations occurred in the ordinary way. We separated fraction (a) by precipitation with sulphuric acid at pH 1.4, from the soluble fraction (b). The former was not digested by rennin; the latter was strongly attacked with a release of 7.3 % of the total nitrogen as NPN, but the electrophoretic pattern remained heterogeneous. An attempt to purify (b) by ethanol treatment gave a solution which was insoluble in water at pH 6.7 and in the electrophoretic buffer solutions.

Fractionation of human casein by column chromatography on DEAE-cellulose in the presence of urea

As fractionation on DEAE-cellulose with an imidazole–HCl buffer, pH 7.0, containing 4.5 *M* urea and a NaCl gradient gave good results with bovine casein, this method was applied to human casein. Three attempts were made, each with some modification, in order to improve the separation. Fig. 2A and Table II summarize

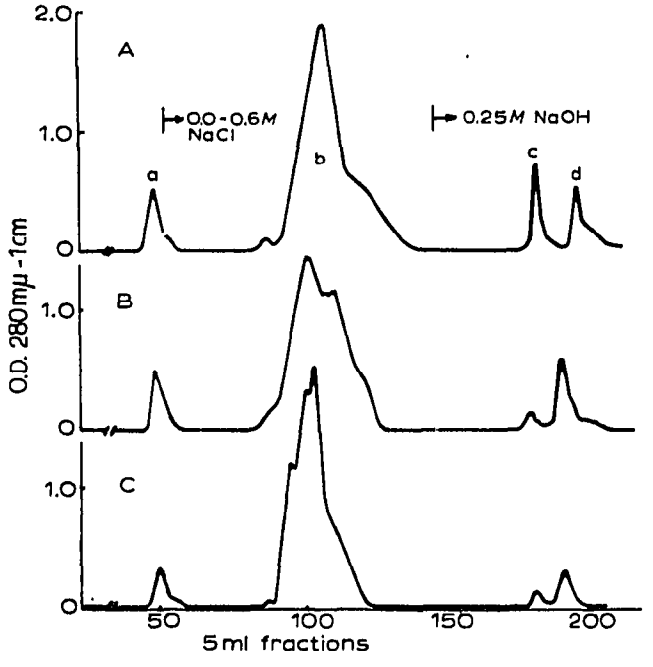

Fig. 2. Chromatograms obtained on DEAE-cellulose (50 × 3 cm column) of: (A) = 1 g human casein; (B, C) chromatograms of peak (b); 0.02 *M* imidazole–3.3 *M* urea buffer, pH 7.

TABLE II

FRACTIONATION OF HUMAN CASEIN ON DEAE-CELLULOSE COLUMNS IN THE PRESENCE OF UREA[a]

Fraction	*Chromatogram A (1 g human casein)*		*Chromatogram B (0.73 g fraction b of A)*	*Chromatogram C (0.60 g fraction b of B)*		*Chromatogram D (0.15 g fractions c and d of A + B + C)*	
	Yield	*NPN*	*Yield*	*Yield*	*NPN*	*Yield*	*NPN*
a	8		6	4		0	
b	81	(1) 0.5 (2) 1.5	87	92	(1) 1.5 (2) 6.0 (3) 10.2	85	1.5
c	6	7.3	1	0.7		9	15.6
d	5	0	6	3.3		6	0.4
Total yield	92		95	90		70	

[a] The yields of the fractions are expressed as % of total dry substances eluted from the column (after dialysis). NPN = Non-protein nitrogen liberated by rennin (0.7 μg/ml) at pH 6.7 expressed as % of total nitrogen. Fractions a are insoluble at pH 6.7; fractions b were subdivided into 2 or 3 parts according to their pattern (Fig. 2).

the results of the third experiment. A minor fraction (a) was eluted with the void volume; it contained a substance insoluble at pH 7.0 in the absence of urea. Over the course of the NaCl gradient, a very small peak appeared first (less than 1 % of the eluted substances) followed by a wide asymmetrical peak which could be divided into 2 parts; the first part which was the more abundant, was hardly digested by rennin, while from the second part more NPN could be obtained than from whole human casein. About one-tenth of the casein was not eluted by the NaCl gradient. NaOH permitted the recovery of two well-separated fractions; the first (c) was still neutral and strongly rennin-sensitive, the solution becoming opalescent; the second (d) was alkaline and not attacked by rennin at pH 6.7.

The substances corresponding to the large peak eluted in the gradient (b) were chromatographed twice more (see Figs. 2B and 2C and Table II). Any further satisfactory fractionation was unobtainable and we did not try higher urea concentrations in case they caused irreversible denaturation.

Fig. 3 shows the starch gel electrophoretic patterns of the substances eluted in

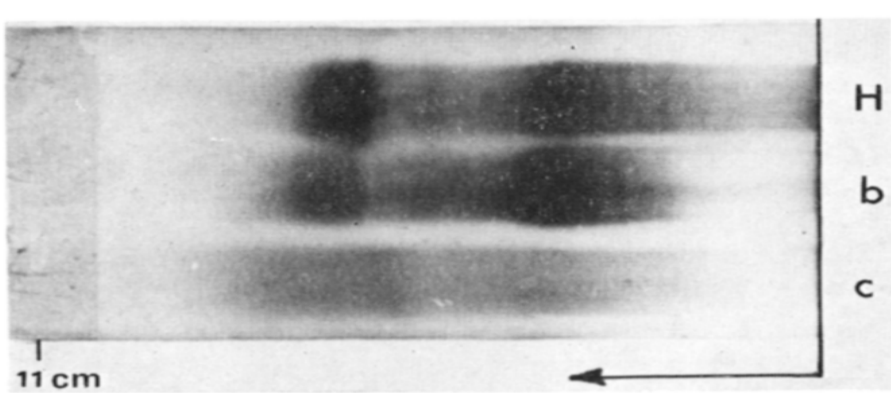

Fig. 3. Starch gel electrophoresis in the presence of mercaptoethanol, of human casein (H) and of the fractions obtained during chromatography on DEAE-cellulose reported in Fig. 2 (b and c); for conditions, see Fig. 1.

the chromatogram in Fig. 2B. The fraction eluted by NaOH, which is not completely soluble in water at pH 6.7, seems to have been partly denatured.

Table II shows that the three parts observed in the large peak of the chromatogram (C) are attacked by rennin in different ways. The third part (tubes No. 106–120) contains a protein fraction which is highly rennin-sensitive (10.2 % of NPN released) and which becomes milky; it represents about 6 % of the original human casein.

A fourth chromatogram (D) was performed with a mixture of fractions (c) and (d) eluted by NaOH. Surprisingly most of this substance was eluted by the NaCl gradient; it was not easily attacked by rennin. Only one-tenth of the substance was eluted by NaOH and was neutral; this fraction (D-c) was highly rennin-sensitive as shown in Table II.

In conclusion, we were able to separate two human κ-like fractions with different chromatographic behaviour.

Fractionation of human casein by column chromatography on DEAE-cellulose in the presence of dimethylformamide

Dimethylformamide has a dissociating effect on protein complexes; it has already been used in the fractionation of bovine casein with stepwise elution by NaCl[10]. The method was applied to human casein; Figs. 4 and 5 and Table III present the results of a chromatogram. In spite of a final elution by NaOH, only 77 % of the substances put on the column were recovered. Table III also shows that important differences in the absorbancy of each component at 280 mμ must exist.

Components with a low electrophoretic mobility were separated in fraction (a) which was not attacked by rennin, while fractions (c) and (d) eluted by 0.15 and 0.25 *M* NaCl were rennin-sensitive. It was shown that they mainly contained the most mobile components electrophoretically. However, it was the alkaline fraction (g) which gave the highest release of NPN with rennin; this human casein fraction did not appear to contain any fast moving components, but only slow moving components in a blurred zone. As in previous experiments, rennin-sensitive fractions were found in 2 different zones of the chromatogram; and furthermore, their composition as determined by electrophoresis was different.

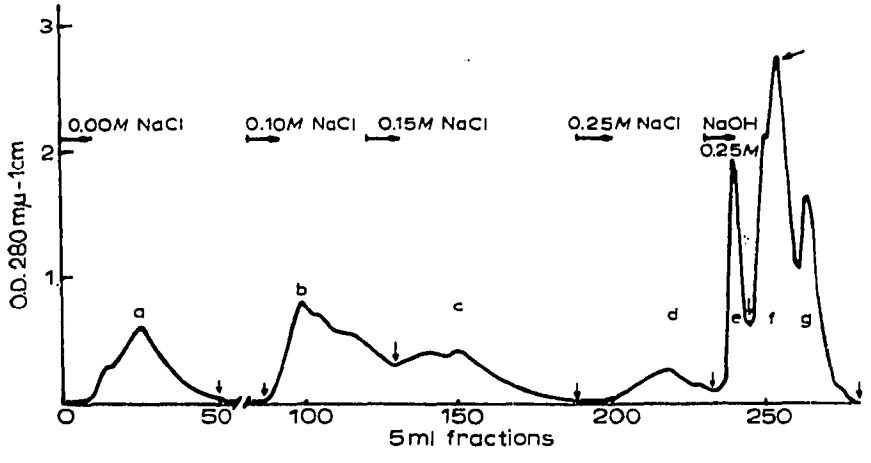

Fig. 4. Chromatography on DEAE-cellulose (36 × 2 cm column) of 1 g human casein; 0.02 *M* phosphate–0.2 *M* boric acid buffer, pH 7, with 20 % dimethylformamide.

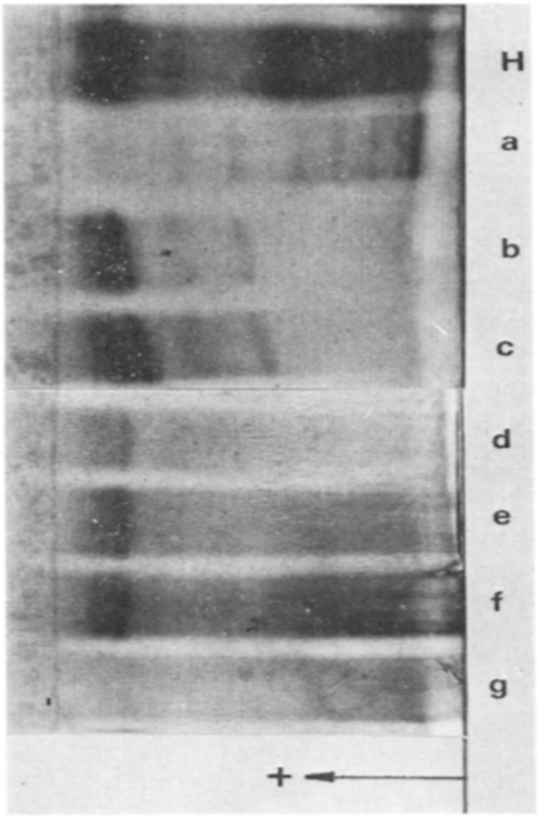

Fig. 5. Starch gel electrophoresis in the presence of mercaptoethanol, of human casein (H) and of fractions obtained during chromatography on DEAE-cellulose reported on in Fig. 4 (a–g); for conditions see Fig. 1.

TABLE III

FRACTIONATION OF HUMAN CASEIN ON DEAE-CELLULOSE COLUMNS IN THE PRESENCE OF DIMETHYLFORMAMIDE

Fractions	*Dry substance*[a] *(after dialysis)*	*% of eluted substances*	*% of total optical density*	*Action of rennin*[b]
a	165	21.5	12	0
b	167	21.7	19	0.9
c	124	16.0	13	4.1
d	73	9.5	6	4.7
e	49	6.4	8	0.3
f	121	15.7	26	1.3
g	71	9.2	16	7.2
Total	770 mg	100	100	

[a] From 1 g human casein.
[b] Non-protein nitrogen liberated by rennin at pH 6.7 expressed as % of total nitrogen.

DISCUSSION

The digestion by rennin and the alteration of the electrophoretic pattern in the presence of mercaptoethanol[1] strongly suggest the existence of a κ-like component

in human casein; its purification, however, seems to be difficult. We cannot at the present time give a precise definition of human κ-casein, but we were able to isolate some fractions which are certainly very rich in a κ-component as they gave rise, after rennin digestion, to an insoluble peptidic 'paracasein' moiety[13] and to a NPN release which is similar or higher than the NPN release from purified bovine and sheep κ-caseins[14] under the same conditions of digestion: human κ-fractions: 7.3 to 15.6 % of total N; bovine κ-casein: 8.5 % of total N; sheep κ-casein: 13.8 % of total N.

As we separated several highly rennin-sensitive human casein fractions, the question arises as to whether there really are different human κ-caseins or only genetic variants. During the fractionation of bovine casein on DEAE-cellulose, κ-casein was eluted by a neutral buffer and it was suggested that the rennin-sensitive alkaline fraction would be a denatured form of bovine κ-casein[9]. In the case of human casein, the situation is not identical; two peaks were eluted by NaOH but the first one was still neutral and it gave rise to a high NPN release.

The hypothesis of the existence of several human κ-caseins could explain the differences among the two κ-fractions previously described by MALPRESS AND SEID-AKHAVAN[5] and by NAGASAWA *et al.*[6]. Although no NPN data were indicated by these authors, it is possible to compare their fractions with those which we have separated by chromatography.

Other fractionation experiments concerning human κ-casein are in progress.

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