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Note

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Fractionation of the whey proteins and casein of cow's milk on Sephadex DEAE A-50 and A-25

After casein, the second characteristic group of cow's milk proteins is the whey proteins. In spite of their low proportion of the total amount of cow's milk proteins (about 25%), the whey proteins have important biological significance. Whey proteins are modified by their dependence on various factors, *e.g.*, breed of cow, kind of fodder and season. Compared with casein, the whey proteins are more labile to heat treatment and hence the extent of their change in composition can be readily used for assaying the intensity of the heat treatment received by the milk during processing.

The study of the individual casein fractions is important for the characterization of cow's milk from different breeds. The changes in the composition of the individual casein fractions make it possible to follow various factors that affect their formation, as for the whey proteins mentioned above. The changes in the composition of casein or paracasein fractions can also be detected during technological milk treatment and in the course of cheese processing.

There are reasons why the study of the whey proteins and casein of cow's milk is of wide interest. Various changes in both protein groups can be clearly distinguished only when good separation methods are available. Of the methods previously used for the separation of both groups of cow's milk proteins, electrophoresis in starch or polyacrylamide gels has been successfully applied[1]. We tried to devise another suitable method for obtaining the individual protein fractions in higher amounts and to use them for further investigations.

Experimental and results

Materials. Raw milk was supplied by the Laktos Milk Plant, Radlice, near Prague, Czechoslovakia, and Sephadex DEAE A-25 and A-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydrolyzed starch was prepared according to ref. 2, and Amido Black 10B was obtained from Merck, Darmstadt, G.F.R. Folin reagent was prepared in our laboratory[3]. Other chemicals used were supplied by Lachema, Brno, Czechoslovakia.

Preparation of whey and casein. A 20-ml volume of 10% acetic acid was added to 100 ml of skimmed milk. After standing the mixture for 10 min, 20 ml of 1 *N* sodium acetate solution were added. Precipitated casein was centrifuged, and the supernatant liquid (whey) was retained for further treatment. Casein in the centrifuge bottle was washed twice with 100 ml of distilled water and then used for separation purposes.

Chromatographic fractionation of whey proteins on Sephadex DEAE A-50. For the sample preparation, 50 ml of the supernatant liquid obtained after casein centrifugation (whey) were concentrated to a volume of 6-8 ml by means of coarse, dry Sephadex

G-25[4]. Concentrated whey was applied to the chromatographic bed of Sephadex DEAE A-50 for the resolution of the individual protein fractions.

The fractionation was carried out as follows. A 12-g amount of Sephadex DEAE A-50 was allowed to swell in 300 ml of phosphate buffer of pH 6.5 containing 0.1 *M* NaCl and the swollen Sephadex was packed into a K 25/45 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The whole volume of concentrated whey was applied to the prepared Sephadex bed and then subjected to gradient elution with 200 ml of 0.1 *M* NaCl and 200 ml of 0.7 *M* NaCl, both prepared in 0.1 *M* phosphate buffer of pH 6.5. The flow-rate was 40 ml/h. The eluate fractions were collected at 10-min

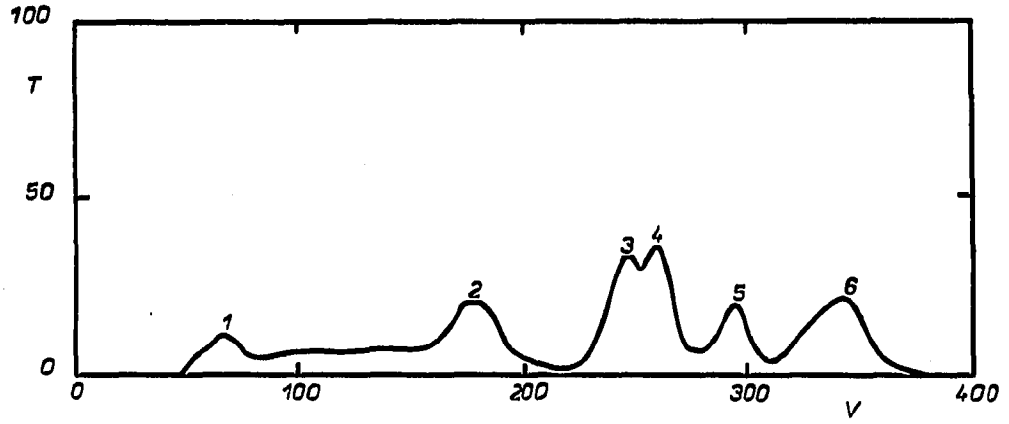

Fig. 1. Fractionation of whey proteins on Sephadex DEAE A-50. Bed dimension corresponding to 12 g of dry Sephadex DEAE A-50 in a K 25/45 column. Gradient elution with 200 ml of 0.1 *M* NaCl and 200 ml of 0.7 *M* NaCl (both prepared in 0.1 *M* phosphate buffer of pH 6.5) at a flow-rate of 40 ml/h. *T* = transmittance at 254 nm; *V* = elution volume (ml). 1 = Immunoglobulins, first fraction (IgG1); 2 = immunoglobulins, second fraction (IgG2); 3 = serum albumin and α-lactalbumin (SA+LA); 4 = α-lactalbumin (LA); 5 = β-lactoglobulin, first fraction (LG1); 6 = β-lactoglobulin, second fraction (LG2).

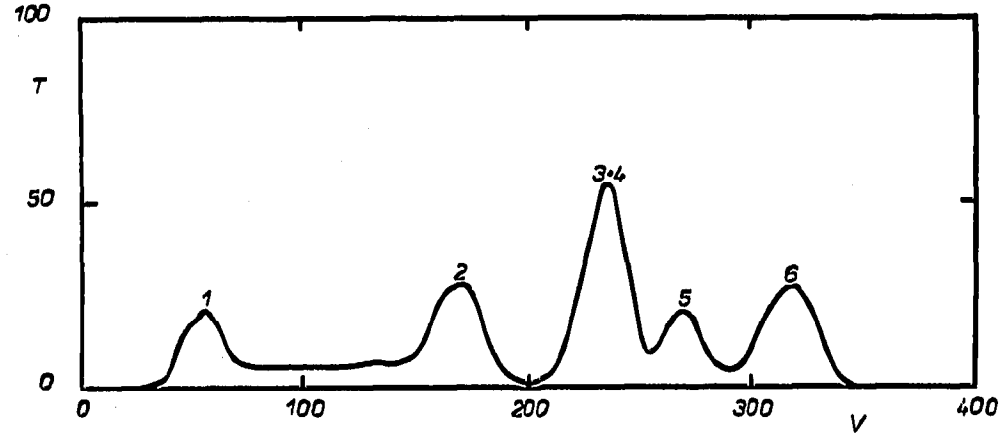

Fig. 2. Fractionation of whey proteins on Sephadex DEAE A-50. Experimental conditions and designation of fractions as in Fig. 1. In some instances fractions 3 and 4 were not separated.

intervals. The protein content of the eluate was determined by continuous absorption measurement at 254 nm on a UV analyzer (Development Workshop of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia) and the fractions were simultaneously treated with Folin reagent[5].

The elution patterns obtained by the absorption measurements (Figs. 1 and 2) were evaluated by the planimetric method and the results were compared with those obtained by starch electrophoresis[1] and its densitometric evaluation (Table I). The identification of the individual fractions after chromatography on Sephadex A-50 was carried out by starch electrophoresis[1].

TABLE I

SEPARATION OF WHEY PROTEINS OF COW'S MILK ON SEPHADEX DEAE A-50

All values are quoted as percentages of the total content of whey proteins.

Protein fraction[a]	*Experiment No.*											
	1			*2*			*3*			*4*		
	C_{SP}[b]	C_P[c]	E[d]	C_{SP}	C_P	E	C_{SP}	C_P	E	C_{SP}	C_P	E
IgG1	10	11		8	8		20	26		12	13	
			18			11			18			21
IgG2	11	13		15	11		14	3		11	3	
SA	20	6	14		4	13		1	12	15	2	11
				34			39					
α-LA	28	20	24		22	32		28	23	28	29	28
β-LG	31	50	44	43	55	44	27	42	47	34	53	40

[a] Symbols for fractions: IgG1 = first fraction of immunoglobulins;
IgG2 = second fraction of immunoglobulins;
SA = serum albumin;
α-LA = α-lactalbumin;
β-LG = β-lactoglobulin.

[b] C_{SP} = planimetric evaluation of chromatographic patterns after spectrophotometric measurements.

[c] C_P = planimetric evaluation of chromatographic patterns after colorimetric determination (Folin reagent).

[d] E = densitometric evaluation after starch electrophoresis.

The main advantage of the proposed method for the fractionation of whey proteins by Sephadex ion-exchange chromatography lies in the fact that immunoglobulins were separated into two distinct fractions, well resolved by both detection methods (spectrophotometry and colorimetry). In some instances, the resolution of serum albumin and lactalbumin was not so successful by the spectrophotometric assessment and the values found for serum albumin were higher than those obtained by electrophoresis. However, these disadvantages were avoided in the colorimetric protein determination with Folin reagent. α-Lactalbumin and serum albumin were present in one (Fig. 2) or two fractions (Fig. 1). We consider that owing to the mixed sample of cow's milk, two genetic variants of α-lactalbumin could be present. Serum albumin was always the component of the first fraction, if separation into two fractions was achieved. By determining the proteins in the eluate by Folin reagent, it is possible to verify the serum albumin fraction separately as well as the supposed genetic variants of α-lactalbumin.

To summarize the results obtained, very good agreement was reached between the Sephadex ion-exchange chromatography and starch electrophoresis in the determination of the individual whey protein fractions. In addition, the main advantage of the chromatographic method is the resolution of immunoglobulins into two fractions, which has considerable importance for ascertaining the degree of heat treatment of cow's milk because the fraction IgG1 is substantially more sensitive to heat treatment than the fraction IgG2[6]. The heat denaturation of the IgG1 fraction is influenced mainly by the temperature reached rather than the duration of the heat treatment.

Chromatographic fractionation of casein on Sephadex DEAE A-25. For the sample preparation, 25 ml of Tris–citrate–urea buffer of pH 8.6 (Tris 0.05 *M*, citric acid 0.0065 *M*, urea 6 *M*) were added to 5 g of isolated casein, and the mixture was homogenized and allowed to stand overnight. Then 0.5 ml of 2-mercaptoethanol was added, 30–60 min before the sample was subjected to chromatographic separation. Just before the separation, the sample of casein was centrifuged and the supernatant liquid was used for column application.

The fractionation was carried out as follows. A 35-g amount of Sephadex DEAE A-25 was allowed to swell in 250 ml of the buffer of pH 8.6 and the swollen Sephadex was packed into a K 24/45 column. To the Sephadex bed, 5 ml of prepared casein sample was applied and then eluted with 400 ml of 0.5 *M* NaCl in the buffer of pH 8.6 at a flow-rate of 80 ml/h. The fractions were collected at 5-min intervals and the eluate was examined simultaneously by continuous recording of the UV absorption at 254 nm (Fig. 3).

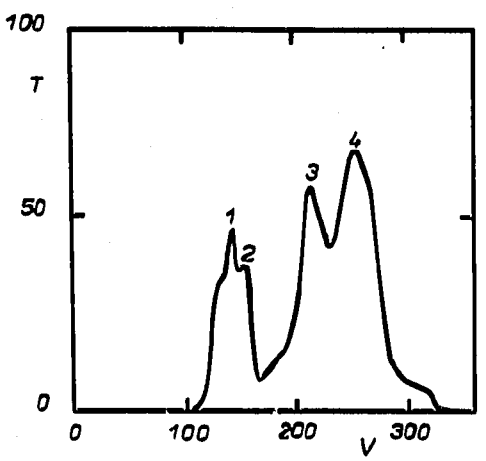

Fig. 3. Fractionation of casein on Sephadex DEAE A-25. Bed dimension corresponding to 35 g of dry Sephadex DEAE A-25 in a K 25/45 Pharmacia column. Elution with 400 ml of 0.5 *M* NaCl in Tris–citrate–urea buffer of pH 8.6 at a flow-rate of 80 ml/h. T = transmittance at 254 nm; V = elution volume (ml). 1 = κ-Casein; 2 = γ-casein; 3 = β-casein; 4 = α_s-casein.

The individual casein fractions were identified by means of starch electrophoresis, and quantitatively determined by the planimetric method from the absorption measurement record. The results obtained by this new method were compared with those from parallel experiments with starch electrophoresis. The comparison of both

methods is shown in Table II. The separation of casein fractions achieved on Sephadex DEAE A-25 is in agreement with that obtained by ROSE *et al.*[7] on DEAE-cellulose, but the time required for separation is substantially decreased when the Sephadex support is used.

TABLE II

SEPARATION OF CASEIN FRACTIONS ON SEPHADEX DEAE A-25

The values are quoted as percentages of the total casein content and were obtained by a planimetric evaluation of the chromatographic patterns or by densitometry (electrophoresis).

Casein	*Experiment No.*							
	1		*2*		*3*		*4*	
	C[a]	*E*[b]	*C*	*E*	*C*	*E*	*C*	*E*
α_{s1}	37	34	42	31	40	31	26	35
α_s-like		12		21		25	29	16
β	41	31	31	29	41	32	28	36
α	15	16	18	14	16	10	12	12
γ	7	7	9	5	3	2	5	1

[a] C = Sephadex DEAE A-25.
[b] E = starch electrophoresis.

It can be concluded that the proposed Sephadex ion-exchange chromatography method gives almost the same resolution of fractions as electrophoretic starch gel separation, with the exception of the separation of the fractions of α_s and α_s-like caseins. The separation of these fractions was not as good when using Sephadex ion-exchange chromatography. Nevertheless, the advantage of this method undoubtedly lies in the possibility of obtaining casein fractions in high yields that can be used for further, more detailed studies. It is possible that another method for the determination of the proteins present in the eluate could give a better resolution of fractions, comparable with the separation of the whey proteins.

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