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A New Protein with a Particular Thermoprecipitability in Bovine Milk (39053)

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Some abnormal proteins in the plasma are known to precipitate temperature dependently. The term cryoglobulin was proposed for cold-precipitable proteins (1), and among proteins exhibiting the opposite type of thermolability are pyroglobulin and Bence–Jones protein (2). During the purification of free secretory component from bovine milk, we discovered a protein with a particular thermoprecipitability which is different from that of the proteins cited above.

Materials and methods. Milk fresh from cows was kindly provided by Biken's Laboratory Co., Uji, Japan, and utilized after being freed from fats and insoluble matters by ultracentrifugation at 45,000*g* for 1 hr in a Spinco Model L ultracentrifuge. The isolation of free secretory component was carried out according to the procedure of Mach (3).

Antisera directed against whole colostral proteins, IgG or secretory IgA were prepared by injecting two or three times into rabbits the corresponding antigen emulsified in complete Freund's adjuvant. The IgG and secretory IgA were purified as described elsewhere (4). Immunoelectrophoretic analysis was carried out according to the Scheidegger's micromethod (5).

Molecular weight was estimated by thin-layer gel filtration on Sephadex G-200 superfine gel equilibrated with phosphate buffered saline, pH 7.0, and by electrophoresis in polyacrylamide gel containing 0.1% sodium dodecylsulfate and 0.5 *M* urea (6). Amido Black 10B was used for staining. Protein concentration was determined according to Lowry's procedure (7), with a calibration curve for bovine serum albumin.

Results and discussion. The milk whey was precipitated at 4° with 50% saturated ammonium sulfate. The resulting precipitate was dissolved, dialysed against 0.01 *M* phosphate buffer, pH 7.4, and applied to a DEAE-cellulose column equilibrated with the same buffer. The proteins which were not retained in the column were collected, concentrated and found to be precipitable at the warmness of the palm of the hand. The precipitate again disappeared when it was returned to the room temperature of 15°. Such an event is shown in Fig. 1. The precipitate began to appear at 25° and did not disappear at any higher temperature. Thus, the thermal property of this protein differed from that of Bence–Jones protein or of plasma pyroglobulin. The protein was tentatively termed 'milk pyroglobulin'. The effect of pH on the precipitation was examined by adjusting pH with 0.1 *M* acetate buffer (for pH 4.0 and 5.0), 0.1 *M* phosphate buffer (for pH 6.0, 7.0 and 8.0), and 0.05 *M* Tris–HCl buffer (for pH 8.6 and 9.0), and it was found that the precipitation took place at pH ranges 5.0 through 8.6. The precipitation was also inhibited in the presence of 0.1% sodium dodecylsulfate, 5 *M* guanidine hydrochloride, 0.5% bovine serum albumin or 0.5% bovine α-lactalbumin. Unlike other inhibitors, the inhibition of the precipitation by the albumins did not take place immediately on addition of the albumins, but was completed after 1–2 days incubation at 4° depending on the concentration of the milk pyroglobulin. Bovine IgG from colostrum did not have the same effect.

For the purification of the protein, the precipitate formed at 37° was centrifuged down at 3000 rpm for 15 min at the same temperature and dissolved in cold phosphate-buffered saline, pH 7.0. The procedure was repeated twice. The purified protein was precipitable at a temperature which was dependent on the concentration, as shown in Table I. The higher the concentration, the lower was the temperature at which precipitation occurred. The preparation of the milk pyroglobulin formed a single arc of precipitation at β to γ regions on immunoelectro-

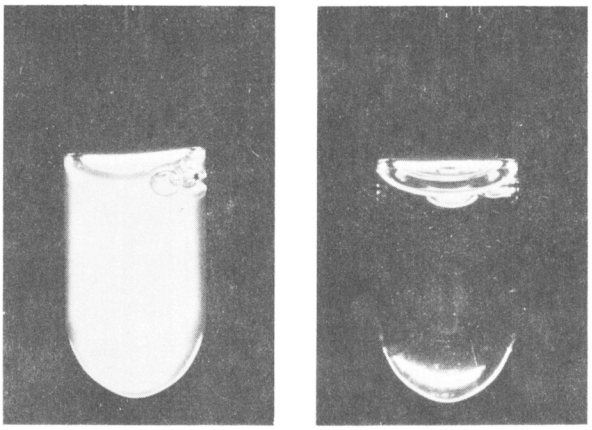

FIG. 1. The passed-through fraction from DEAE-cellulose equilibrated with 0.01 *M* phosphate buffer, pH 7.4, heated at 37° (A) and after recooling to 15° (B).

TABLE I. EFFECT OF TEMPERATURE ON THE PRECIPITATION OF THE MILK PYROGLOBULIN AT VARIOUS CONCENTRATIONS.[a]

Temperature (°C)	Concentration (mg/ml)							
	10	1.0	0.50	0.25	0.12	0.062	0.031	0.015
4	−	−	−	−	−	−	−	−
10	+	+	±	−	−	−	−	−
15	+	+	+	±	−	−	−	−
20	+	+	+	+	±	−	−	−
37	+	+	+	+	+	+	+	−

[a] The sign + indicates the appearance of a cloudy precipitate, the sign ± the appearance of opalescence, and the sign − no change.

phoretic analysis with antiwhole colostral proteins serum, whereas it did not form any precipitation arc with antisecretory IgA serum which contains antibodies directed against α-chain and secretory component. The results are shown in Fig. 2. There was also no precipitin reaction with anti-IgG serum containing anti-γ-chain and anti-L-chain antibodies. Such findings indicated that the preparation contained asingle gamma-globulin which was different from immunoglobulins or free secretory component.

The protein was next submitted to SDS-polyacrylamide gel electrophoresis and thin-layer gel filtration for the estimation of molecular weight. As shown in Fig. 3, two major bands were revealed in SDS-polyacrylamide gel electrophoresis, the molecular weight being estimated at 19,000 and 10,000, respectively. The proteins in both bands were found precipitable when the gel was dipped into saline heated at 45°, to eliminate SDS and urea. When both proteins were allowed to react with antiwhole colostral proteins serum in agarose gel, they formed a single precipitin arc which is completely fused (Fig. 4). These results indicate that both proteins possess heat-precipitability and identical antigenic determinants despite the twofold different molecular weights. Thin-layer gel filtration on Sephadex G-200 superfine gel gave, however, a molecular weight of 60,000, as shown in Fig. 5. The mixture of the protein with bovine serum albumin, incubated at 4° for 2 days, gave rise to a new spot which was observed neither in bovine serum albumin nor in the purified protein, and the migration distance corresponded to that of a molecule with a molecular weight of 20,000. The analysis of such a mixture by SDS-polyacrylamide gel electrophoresis revealed a superimposed pattern which included that of both proteins. This is shown in Fig. 6. In light of these findings it is possible that the protein was depolymerized in the presence of bovine serum albumin and was dissociated into the subunit molecules.

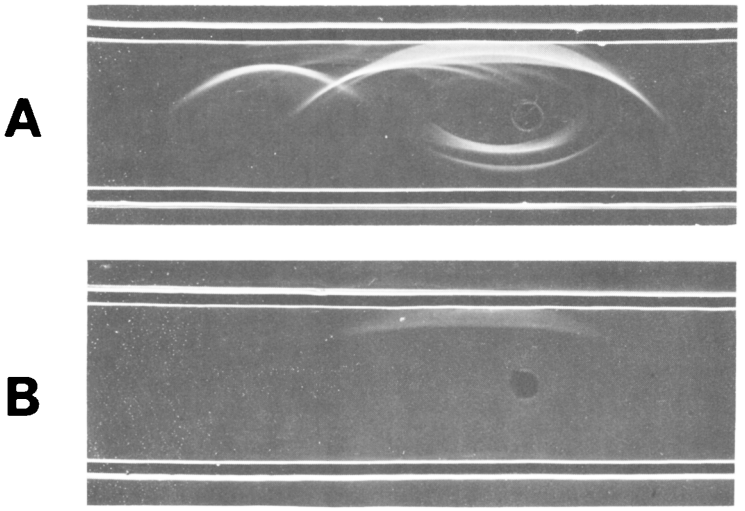

FIG. 2. Immunoelectrophoretic analysis of the milk pyroglobulin. (A), whole colostral whey; (B), the purified pyroglobulin. In upper and lower troughs of each plate, antiwhole colostral proteins serum and antisecretory IgA serum, respectively. The analysis was carried out at 4°. Anode is to the left.

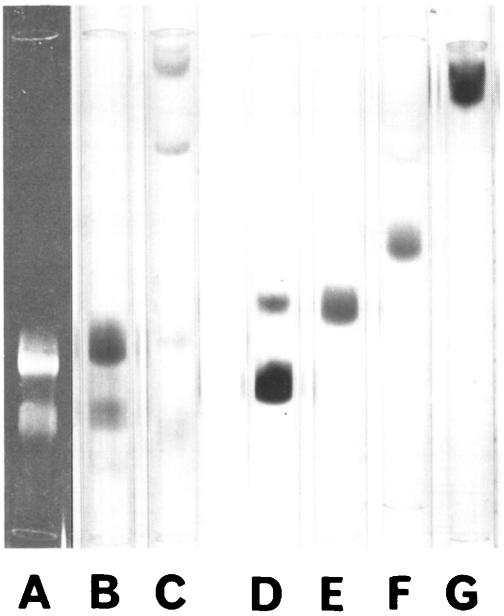

FIG. 3. Electrophoretic patterns obtained on 10% polyacrylamide gels, in 0.1 *M* phosphate buffer, pH 7.2, containing 0.1% SDS and 0.5 *M* urea. In (A) and (B), the purified pyroglobulin heated in saline and stained, respectively; in (C) milk proteins not retained on DEAE-cellulose equilibrated with 0.01 *M* phosphate buffer, pH 7.4; in (D) through (G) cytochrome C, chymotrypsinogen, egg albumin and IgG as reference proteins.

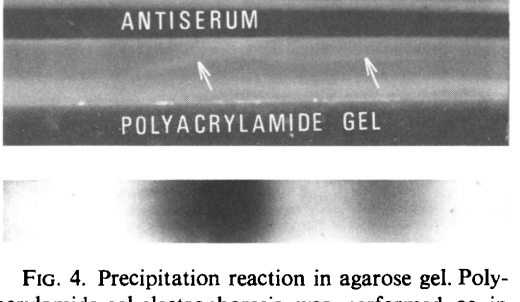

FIG. 4. Precipitation reaction in agarose gel. Polyacrylamide gel electrophoresis was performed as in Fig. 3 and the gel was embedded in 1.2% agarose gel after being dipped in saline at 45° for 2 hr. The antiwhole colostral proteins serum was poured into the trough cut parallel to the gel, and allowed to react with the proteins separated in the gel. Stained gel is shown below the figure of precipitation reaction.

A protein with the same properties was isolated from another individual sample and also from pooled samples from eight cows, suggesting that the protein is generally present in bovine milk. The yield of the protein was 4.4 mg from 100 ml of the pooled milk whey.

In view of the physicochemical and immunological characters, the data presented indicate that the milk pyroglobulin is a new protein. It was isolated, in effect, under the conditions where the free secretory compo-

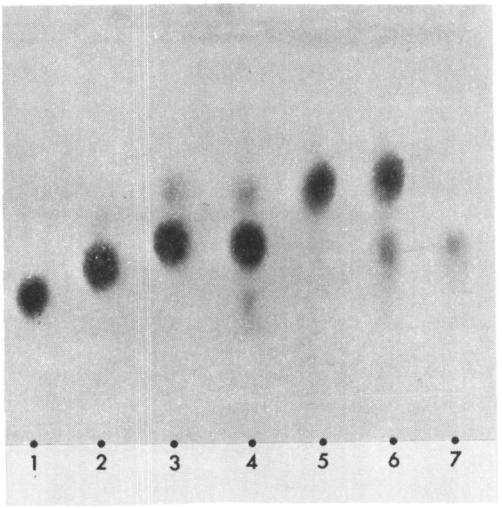

FIG. 5. Thin-layer chromatogram on Sephadex G-200 superfine gel. Ten microliters of samples were applied at the indicated starting points and developed at 10° descending angle for 4 hr at 4°. (1) and (2), chymotrypsinogen and egg albumin of reference, respectively; (3), BSA; (4), the pyroglobulin in 0.5% BSA incubated for 2 days at 4°;(5), IgG; (6) the pyroglobulin in 0.5% IgG incubated for 2 days at 4°; (7) the pyroglobulin only.

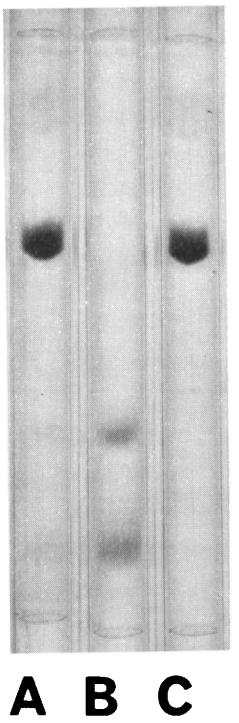

FIG. 6. Electrophoretic patterns obtained on 10% polyacrylamide gels, in 0.1 *M* phosphate buffer, pH 7.2, containing 0.1% SDS and 0.5 *M* urea. In (A), the pyroglobulin in 0.5% BSA incubated for 2 days at 4°; in (B) and (C), the pyroglobulin and BSA, respectively.

nent obtained was contaminated only with small amounts of IgG, but its particular thermal property may have hindered its discovery in the gamma globulin fraction. It is possible that other investigators (3, 8, 9) could have overlooked the protein which precipitated during the purification procedure of the free secretory component or the immunoglobulins. The possibility that it is a degradation product derived from immunoglobulins must reasonably be ruled out, since the protein did not react with antibodies against γ-chain, α-chain and L-chain of immunoglobulins. In addition to the particular thermoprecipitability, the protein possessed a property of being dissociated into subunit molecules. This was also unique in that the dissociation took place in the presence of bovine serum albumin as well as in the presence of sodium dodecylsulfate and urea. Two populations of the dissociated molecules had a molecular weight of 19,000 and 10,000, respectively, and carried identical antigenic determinants. This suggests that the minimal subunit of the protein is probably the molecule with a molecular weight of 10,000. The functional significance of these unique properties is now under investigation together with further studies on the structure.

Summary. A new protein with a particular thermoprecipitability was isolated from bovine milk and tentatively termed milk pyroglobulin. The protein which was soluble at a relatively cold temperature was precipitated by raising the temperature to a certain degree depending on the concentration of the protein. The precipitate disappeared on recooling. This protein had the electrophoretic mobility of gamma globulin but did not carry either antigenic specificities of immunoglobulins or of free secretory component. The molecular weight was estimated to be approximately 60,000 in thin-layer gel filtration on Sephadex G-200 superfine gel,

but the protein appeared to be convertible to molecules with a lower molecular weight of approximately 20,000 in the presence of bovine serum albumin. The presence of the albumin inhibited the thermoprecipitation as did α-lactalbumin but not IgG immunoglobulin from bovine colostrum. In SDS-polyacrylamide gel electrophoresis, the protein was separated into two components having a molecular weight of 19,000 and 10,000, respectively. Both components were thermoprecipitable and carried identical antigenic determinants.

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