SIALIC ACID IN MILK

III. Sialic Acid in Normal Milk and in the Fractions of Milk Proteins obtained by Diethylaminoethyl-cellulose column

By

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The sialic acid content in individual milk was reported in the previous paper (8). In this experiment, an attempt was made to determine the sialic acid content in whey protein and some casein components. Mainly, anion-exchange cellulose, Diethylaminoethyl (DEAE) was used for the fractionation of skimmilk and casein.

Material and Methods

Sample: Individual milk and bulked milk were obtained form the Hokkaido University farm and kept at 3–4°C until skimming. The cooled individual milk and bulked milk were skimmed by centrifugation at 2,000 rpm for 15 minutes at room temperature. The skimmed portion was pipetted into a beaker after removing the cream layer by means of a spatula.

Acid casein and whey: Acid casein and whey from the skimmilk were obtained by adjusting 2 ml samples to pH 4.6 (with 5 ml of 1 M acetic acid-sodium acetate buffer of pH 4.6) in conical centrifuge tubes. Then acid casein and whey was used for the determination of the sialic acid.

Precipitated protein and serum containing non-protein nitrogen soluble in 12% trichloroacetic acid (TCA): To the skimmilk in the conical centrifuge tubes TCA was added to attain a final concentration of 12% and the mixture was held for 30 minutes at room temperature. Then the precipitated protein and supernatant liquid were separated by centrifugation.

Lactoglobulin and lactalbumin: The procedure for isolation of lactoglobulin and lactalbumin was accomplished by the method of ROWLAND (15).

β-lactoglobulin: This whey protein was isolated by the method of ASCHAFENBURG and DREWRY (1).

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α_s-, β- and κ-casein: These caseins were kindly supplied by Mr. K. SATO (16) and Dr. S. YOSHIDA (24).

Preparation of the sample for chromatography: Ten percent solution of tetrasodiumethylenediamine tetra-acetate (ETA) was added dropwise under gentle agitation to the skimmilk in order to give a final ETA-concentration of 1%. The mixture was dialyzed in cold for 48 hours against a large quantity of 0.02 M phosphate buffer at pH 7.0. The buffer was agitated with a magnetic stirrer and changed every 12 hours.

Acid casein for chromatography was prepared for the same original skimmilk. The casein precipitated at pH 4.6 was washed four times with distilled water and redissolved. This procedure was repeated three times. The final preparation was redissolved with 0.1 N NaOH, raising the pH, with constant agitation. Then the casein solution (ca. 2%) adjusted to pH 7.0, and was dialyzed by a procedure similar to that used in the dialysis of the skimmilk.

Chromatography on DEAE-cellulose column: The method followed the procedure (stepwise elution) of YAGUCHI et al. (22) with a modification; a hydrostatic head of about 120 cm was applied, instead of nitrogen pressure, to keep the flow rate of 800 ml per hour. The effluents were read on a Hitachi spectrophotometer EPU-2 A 280 mμ. After that the effluents belonging to the same fraction were mixed and 30 ml of the mixed effluents was dialyzed in cold for 24 hours against a large quantity of distilled water to remove inorganic ions. The distilled water was agitated with a magnetic stirrer and changed twice.

Determination of sialic acid content: The samples, containing 50–200 μg of sialic acid, were hydrolyzed in small tubes having glass stoppers in 10 ml of 0.1 N sulfuric acid at 80°C for 60 minutes. Then the sialic acid content in the hydrolyzates was determined with SVENNERHOLM's resin method (17), which was reexamined previously (8). The ratio of sialic acid content to protein content ($N \times 6.38$) was calculated and expressed in percentage.

Determination of nitrogen content: Micro-Kjeldahl method was used. Protein content was then calculated by multiplying the nitrogen content by the factor of 6.38.

Results

As shown in the results presented in Table 1, the sialic acid was observed not only in casein but also in whey protein and serum. About two-thirds of the total sialic acid content were contained in milk protein, which may be a protein-bound sialic acid, and on the other hand, one-third of it was found in 12% TCA-soluble serum. The latter was almost dialyzed in 24 hours at room temperature. The sialic acid content in casein, whey protein, TCA-precipitated

TABLE 1. Sialic acid content in skimmilk, casein, whey protein, TCA-precipitated protein, and TCA-soluble serum of normal milk

	μg sialic acid per milliliter of skimmilk	
	Bulked milk	Individual milk (Holstein)
Skimmilk	158.0	152.5
Acid casein	89.0	88.0
Whey protein and serum	68.5	63.8
TCA-precipitated protein	104.5	101.5
TCA-soluble fraction	53.5	50.5

protein and TCA-serum of the bulked and individual milk showed similar values. The sialic acid content in fractions of whey proteins is given in Table 2. Acid whey used was a soluble portion at pH 4.6, which shows a high percentage of the sialic acid (1.33%). But this may be considered because dialyzable sialic acid is included in acid whey as mentioned above.

TABLE 2. Sialic acid content in fractions of whey proteins

	Sialic acid per protein %
Acid whey	1.33
Whey protein precipitated with 0.5 saturation $(NH_4)_2SO_4$ (lactoglobulin)	0.78
Whey protein precipitated with saturation $(NH_4)_2SO_4$ (lactalbumin)	0.72
Proteose-peptone	0.70
β-lactoglobulin	0.09

Whey proteins precipitated with 0.5 saturation $(NH_4)_2SO_4$ and saturation $(NH_4)_2SO_4$ are called lactoglobulin and lactalbumin, respectively. Neither of these are pure protein. The sialic acid contained in both of them was not dialyzed. Hence they may probably be protein-bound sialic acid. On the other hand, β-lactoglobulin was almost pure on an electrophoresis pattern, though it was not a crystalized. It also contained sialic acid as shown Table 2.

Eighty milliliters of dialyzed skimmilk containing 1.66 g protein was applied on DEAE colum (6.5 by 5.5 cm) and eluted with 18 different buffers. The effluent diagram obtained is shown in Figure 1. Each buffer yielded a corresponding peak and formed 18 fractions. These fractions were designated with

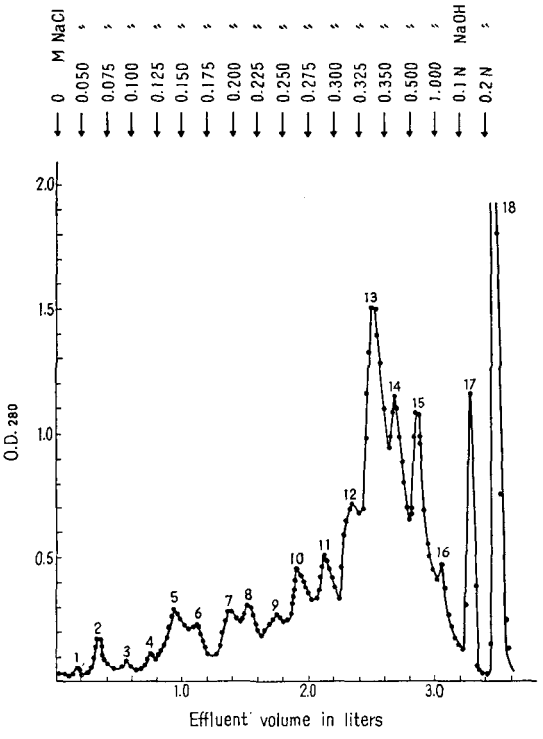

Fig. 1. Effluent diagram of proteins of skimmilk; 80 ml of dialyzed skimmilk (1.66 g of protein) was applied to DEAE column (6.5 by 5.5 cm). 20 ml each of the eluate was collected at a flow rate of 800ml/hour. Vertical arrows show the points of change of eluting buffers added to the column.

numerals from 1 to 18. The recovery of protein and sialic acid content in the skimmilk applied on DEAE column was 92.77% and 89.90%, respectively. The contents of protein and sialic acid in the fractions shown in Figure 1 are presented in Table 3, and the yield is expressed in percentage of the total material recovered. From the sesults in Figure 1 and Table 3, the largest peak, fraction 13, comprised of 18.36% of the total proteins of the skimmlk applied on the column, and followed by fraction 18. However the highest sialic acid content was included in the peak, fraction 18. In other words, fraction 18 contained not only a higher protein content but also the highest sialic acid content. On the other hand, fraction 11 and 10 contained a higher sialic acid content as compared with the protein content in other fractions. Therefore, the highest percentage of the sialic acid per protein was found in fraction 18 (0.77%), and second fraction 11 (0.66%). The sialic acid in fraction 1, 2,

TABLE 3. Percentage yield of protein and sialic acid content of fractions obtained by chromatography

Fraction No.	Protein yield* (%)	Sialic acid yield* (%)	Sialic acid per protein (%)
1	0.59	—	—
2	1.20	—	—
3	0.92	—	—
4	1.01	1.27	0.12
5	4.38	—	—
6	1.87	—	—
7	3.33	2.00	0.21
8	3.22	2.59	0.29
9	3.51	3.18	0.21
10	5.34	9.86	0.51
11	5.39	12.64	0.66
12	84.0	7.11	0.29
13	18.36	6.81	0.18
14	9.29	6.69	0.14
15	9.45	6.86	0.17
16	3.71	—	—
17	3.09	—	—
18	16.94	42.26	0.77

* Yield is expressed as a percentage of the total material recovered.

3, 5, 6, 16 and 17 could not be measured with the resin method. It is, therefore, considered that there was little or no sialic acid in the fractions.

Sixty milliliters of dialyzed casein solution containing 1.2 g protein was applied on DEAE column and eluted with 11 different buffers (buffer No. 8 to 18). The effluent diagram obtained is shown in Figure 2. Each buffer yielded a corresponding peak, forming 11 fractions, and was designated with numerals as used in Figure 1. The effluent diagram shown in Figure 2 was quite similar to that of the same portion shown in Figure 1. But the percentage of the sialic acid in each fraction as given in Table 4 showed a slight lower value than that of the same fraction in the skimmilk shown in Figure 1 (cf. Table 3).

The sialic acid content in whole casein, α_s-casein, β-casein, and κ-casein was given in Table 5, and the effluent diagram of the caseins obtained from DEAE column is shown in Figure 3.

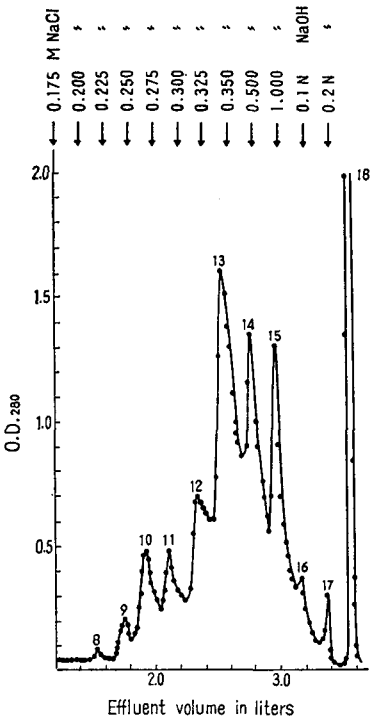

Fig. 2. Effluent diagram of proteins of whole casein; 50 ml of dialyzed casein solution (1.20 g of protein) was applied to DEAE column (6.5 by 5.5 cm). 20 ml each of the eluate was collected at a flow rate of 800 ml/hour. Vertical arrows show the points of change of eluting buffers added to the column.

TABLE 4. Percentage yield of sialic acid of fractions obtained by chromatography of casein*

Fraction No.	8	9	10	11	12	13	14	15	16	17	18
Sialic acid per protein (%)	—	—	0.29	0.34	0.14	0.11	0.14	0.15	—	—	0.68

* Yield is expressed as a percentage of the total material recovered.

TABLE 5. Sialic acid content of casein fractions

	Sialic acid/protein (%)
Whole casein	0.35
α_s-casein	0.10
β-casein	0.11
κ-casein	0.54

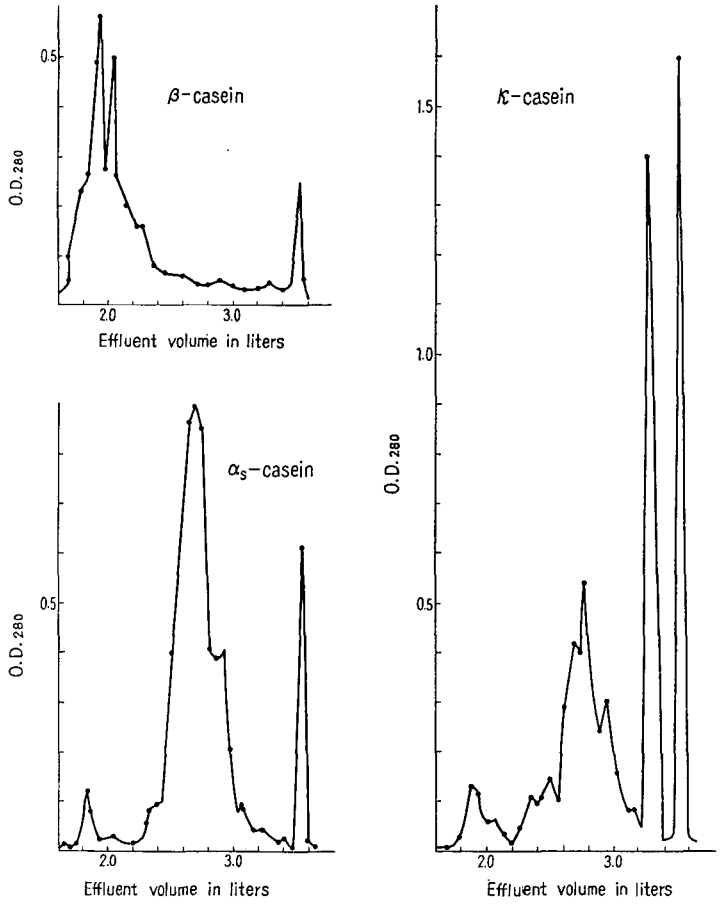

Fig. 3. Effluent diagram of α_s-casein (0.63 g of protein), β-casein (0.45 g of protein) and κ-casein (0.52 g of protein) obtained from DEAE column.

Discussion

From Table 1 and 2, it seems possible that most of the proteinbound sialic acid for the total value is combined with caseins, and some of this acid may be combined with whey proteins.

In the field of milk protien chemistry, most studies on sialic acid have been made in an attempt to focus attention on caseins, especially κ-casein. Therefore, there are some reports concerning the studies on the sialic acid in κ-casein, but there is a surprising lack in the study of sialic acid in whey protein of bovine milk.

MARRIER et al. (9) tried to measure the sialic acid content as an index of the κ-casein content on bovine skimmilk. They reported that κ-casein was the only major fraction containing a significant amount of sialic acid, and heat-coagulable whey proteins contained little or no sialia acid. WAUGH (20) suggested, in his studies on casein interactions and micelle formation, that κ-casein may be contained in whey proteins, which may in turn contribute to the formation of micelles. Some workers also reported that a crystal of ovoalbumin, recrystallized 8–10 times, still contained amino sugars (11), and in the case of blood serum, all proteins except serum albumin contained a trace of sugars that could not be removed (3). So it may be considered that most of the casein and whey proteins contain sialic acid as shown in Table 1, 2 and 3. However the mode of binding of sialic acid with proteins has not been clearly demonstrated as yet.

As shown in Figure 1, milk protein was fractionated into 18 different peaks. YAGUCHI et al. (22) identified each fractions eluted as follows: From the starting point of elution buffer, fractions 1 and 2, fraction 4, fractions 6 and 7, fractions 10 and 11, fractions 12, 13, 14, 15, 16 and 17, and fraction 18 were lactoglobulin, α-lactalbumin, β-lactoglobulin, β-casein, α-casein, and κ-casein, respectively. However, the casein in each fraction (from 9 to 15) was not always pure and did not necessarily present a single peak, except in the case of fractions 14 and 15, on electrophoresis (13). On the other hand, sialic acid was contained in most fractions and the highest acid content was found in fraction 18. YOSHIDA (24) also reported that fraction 18 is probably the κ-casein rich fraction. A point of interest was noted in fraction 10 and 11 which is known to be β-casein. Because these fractions showed a high percentage of sialic acid in spite of the reports that β-casein contained about 0.14–0.23% (9, 10). It is suggested therefore that casein micelle is formed from a complex of α-β-κ-casein and other minor components, and α-κ-casein or β-κ-casein complex interacts strongly with each other. MARIER et al. (9)

reported that the stability of α_s-κ-casein mixture in the precence of calcium appeared to be directly related to the sialic acid content of the mixture, and the observed relation was linear in nature with a 10 to 100% stability and which may be extrapolated to zero stability at 0.06% sialic acid and to 100% stability at 0.37% sialic acid in the test mixture. The value of 0.37% sialic acid is similar to that in normal acid casein (Table 5).

If strong interactions between casein components, and micelle formation are brought about by the role of κ-casein, it may be considered that sialic acids exert a causative relationship between the stability and formation of micelles. THOMPSON and PEPPER (18) demonstrated that when sialic acid acid was released by the action of neuraminidase, a loss of stabilizing power (ca. 20%) of κ-casein was observed. EYLAR et al. (4) also found that the electrophoretic mobility and surface charge was changed by the release of *N*-glycolylneuraminic acid and *N*-acetylneuraminic acids. YAGUCHI et al. (23) indicated that a major milk lipase activity was present as part of the κ-casein complex or in the κ-casein itself, and that the κ-casein was eluted in the fractions, which correspond to the same positions as fractions 10, 11 and 12, by DEAE-cellulose column. Some workers (2, 7, 11, 21) reported that κ-casein is also heterogeneous, and ROSE and MARRIER (14) obtained seven peaks from κ-casein by DEAE-cellulose column.

Therefore, it can be readily considered that κ-casein is eluted in several fractions since it is a heterogeneity and exists in milk as a strong complex of α-β-κ-casein. In other words, it is probable that protein associated with various complexes renders the separation of its component proteins particulary difficult. Thus it may be surmized that fraction 10 and 11 probably contain β-κ-casein complex. It is also considered that fraction 18 is not a pure κ-casein component but is a κ-casein rich complex.

The percentage of the sialic acid in the fractions of casein obtained form DEAE-column (Fig. 2 and Table 4) was slightly lower than those in the fractions of skimmilk protein. It seems that some of the sialic acid was isolated from casein in the process of treatment with acid, alcohol and ethanol. GUPTA and GANGULI (6) showed that the sialic acid content in casein extracted with alcohol was lower than that extracted with acetone.

The percentage of the sialic acid of α_s-, β-, and κ-casein shown in Table 5 was 0.10, 0.11 and 0.54%, respectively. The values of sialic acid of α_s-β-, and κ-casein in literature were 0.064–0.145% (5, 6), 0.136–0.23% (9, 10) and 1.16–2.48% (6, 9, 10, 14), respectively. The value of α_s-, and β-casein in Table 5 was similar to those in the literature, but the value of κ-casein was somewhat lower. Perhaps the κ-casein here was not pure and contained

impurities. The effluent diagrams of these casein showed several peaks (Fig. 3), so it may be conjectured that α_s-, β-, and κ-casein used here are heterogeneous. Though WAKE and BALDWIN (19) showed that the casein complex consists of some 20 components in their study using starch gel electrophoresis in the presence of strong urea, the number of components which may exsist in casein have not be clarified.

It may be surmised however that most of the casein components contain sialic acid to some extent and that protein-bound sialic acid contributes to the stability and formation of micelles in milk, and also to the interactions between milk proteins.

Summary

The sialic acid in normal milk was determined and results are summarized as follows:

1. The sialic acid content in bulked milk, casein, whey protein, TCA-precipitated protein and TCA-soluble serum per milliliter of normal milk was 158.0, 89.0, 68.5, 104.5 and 53.5 μg, respectively.

2. The percentage of sialic acid per protein in whole casein, α_s-casein, β-casein, and κ-casein of normal milk was 0.35, 0.10, 0.11 and 0.54, respectively.

3. The percentage of sialic acid per protein in acid whey, lactoglobulin lactalbumin, proteose-peptone and β-lactoglobulin was 1.33, 0.78, 0.72, 0.70 and 0.09, respectively.

4. In 18 protein fractions obtained from normal skimmilk by DEAE-cellulose colum, a higher percentage of sialic acid per protein was contained in fractions 10, 11 and 18, and fraction 18 contained the richest amount of sialic acid.

5. The percentage of sialic acid per protein in the fractions of casein eluted by DEAE-cellulose column showed slightly lower values than those of skimmilk.

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