Separation of the Bovine Colostrum M-1 Glycoprotein into Two Components

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1. Two glycoproteins were isolated from the M-1 acid glycoprotein fraction of bovine colostrum. 2. The lighter glycoprotein had a molecular weight of 7200, contained about 28.4% of carbohydrate, and had an absorption maximum at 275 nm. The heavier glycoprotein had a molecular weight of 12000, contained 39.0% of carbohydrate, and had no absorption maxima in the 240–300 nm. range of the spectrum. 3. The carbohydrate moiety of both glycoproteins was removable from the polypeptide moiety under the conditions of the β -elimination reaction. 4. Periodate oxidation experiments showed that sialic acid was linked to galactose in both proteins.

The M-1 group of glycoproteins from bovine colostrum whey has previously been purified by DEAE- and CM-cellulose chromatography. This fraction was shown to contain small amounts of serum orosomucoid and large quantities of a low-molecular-weight protein containing large amounts of carbohydrate (Bezkorovainy, 1965). The colostrum-specific glycoprotein was apparently homogeneous by moving-boundary-electrophoresis and ultracentrifugation criteria but it contained several *N*-terminal residues. Gel-electrophoresis results were inconclusive. It was concluded that this protein was actually a mixture of very closely related molecular entities, differing from each other by a few amino acid residues (Bezkorovainy, 1967).

More extensive investigation of the properties of the colostrum M-1 glycoprotein revealed that it was separable, on the basis of size, into two closely related glycoproteins. This paper describes the physical and chemical properties of these two colostrum glycoproteins.

MATERIALS AND METHODS

Colostrum. Colostrum was collected from individual Holstein animals and represented a pool of the first two milkings after parturition. Octanoic acid (1 ml./U.S. gallon) was added to the colostrum as preservative. Clear wheys for protein isolation were prepared from the colostrum as described by Bezkorovainy (1965).

Proteins. Orosomucoid was isolated from human serum as described by Bezkorovainy (1965), and was used in many of the experiments described below as a reference substance. Colostrum immune globulin was isolated from colostrum whey by the method of Smith (1946). This protein was isolated from the colostrum sample used for the isolation of

the glycoproteins to test for possible degradative processes taking place in the colostrum (e.g. bacterial action or auto-digestion).

Neuraminidase. The enzyme was of the same origin and was used under the same conditions as that described by Bezkorovainy (1967).

Chromatography and gel-filtration media. DEAE- and CM-cellulose were purchased from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). They were coarse grade and their capacities were 0.9 and 0.7 m-equiv./g. respectively. Gel-filtration media were purchased from Pharmacia Corp. (New York, N.Y., U.S.A.). Sephadex G-75 was medium grade, with a water-regain capacity of 7.5 ± 0.5 g./g. Sephadex G-25 was coarse grade with a water-regain value of 2.5 ± 0.2 g./g. Conditions under which the gel-filtration experiments were performed are given in the legends of the appropriate figures.

Separation of the colostrum M-1 glycoprotein fraction into two components. Bezkorovainy (1965, 1967) described the purification of the low-molecular-weight M-1 glycoprotein of bovine colostrum by the use of DEAE- and CM-cellulose, Sephadex G-100 and DEAE-Sephadex columns in that order. The Lowry protein test (Lowry, Rosebrough, Farr & Randall, 1951) was used to monitor the columns, and single peaks were always observed in the last two purification steps. It was, however, subsequently observed that if, in the penultimate gel-filtration step, extinctions at 280 nm. were measured in parallel with the Lowry protein determination in the fractions eluted, the two elution diagrams were not superimposable. Chromatography on Sephadex G-75 gave similar results. These results suggested that the protein previously studied (Bezkorovainy, 1967) was not homogeneous and was possibly separable, on the basis of size, into two fractions by chromatography on Sephadex G-75.

The procedure that was finally adopted for the isolation of the colostrum M-1 glycoproteins was as follows. (1) Dialysed colostrum whey from 4l. of colostrum was passed through a DEAE-cellulose column as described by Bezkorovainy (1965). The size of the DEAE-cellulose

Fig. 1. Purification of the 275nm.-positive and-negative glycoproteins by repeated gel filtration on Sephadex G-75 columns (2.2 cm. diam. $\times$ 40 cm.). Each fraction was 1.5 ml., and the rate of flow was 3 ml./hr. The solvent was 0.05M-sodium cacodylate-0.1M-NaCl buffer, pH 7. The amount applied to the column represented by (a) was 323 mg., and the recoveries were 32.7 mg. for fractions 25-40, 216.0 mg. for fractions 41-56 and 41.0 mg. for fractions 57-70. The amounts applied to the columns represented by (b), (c), (d) and (e) were 323, 165, 269 and 58 mg. respectively, and the recoveries indicated in the respective figures by the bars were 136, 58, 190 and 45 mg. respectively. $\times$, Lowry-reaction-positive material; $\circ$, E_{280} ; $\bullet$, orcinol-positive material (hexose).

column was, however, increased to accommodate the larger quantity of whey. The column (4 cm. diam. $\times$ 45 cm.) was packed under a pressure of 10 lb./in.². (2) The DEAE-cellulose fraction containing the M-1 glycoproteins was chromatographed on a CM-cellulose column as described by Bezkorovainy (1965). (3) The break-through fraction from the CM-cellulose experiment was repeatedly chromatographed on Sephadex G-75 as shown in Figs. 1(a) to 1(e).

The rechromatography process was stopped when the elution diagrams constructed from the Lowry reaction, extinction at 280 nm., and the orcinol (hexose) reaction were superimposable (as in Figs. 1d and 1e). Two colostrum M-1 glycoproteins were thus obtained in yields of 140 and 58 mg. of the heavier (Fig. 1d) and the lighter (Fig. 1e) components respectively from the starting colostrum volume of 4 l.

Chemicals. NaBH₄ was obtained from Metal Hydrides

Inc. (Beverly, Mass., U.S.A.). It was labelled 98% pure. Amino acids and dinitrophenylated amino acids were purchased from Mann Laboratories (New York, N.Y., U.S.A.). All other chemicals were purchased from Fischer Laboratories (Chicago, Ill., U.S.A.) and all were of reagent grade.

Physical measurements. Ultracentrifugal analyses were done in a Spinco model E apparatus at 20° and 59780 rev./min. in bucket-type synthetic-boundary cells. Sedimentation constants were calculated by conventional concentration-sedimentation coefficient plots (Schachman, 1957).

Diffusion-constant determinations were performed in a Spinco model H apparatus at 4° at several protein concentrations in the 2 ml. cells. Proteins were dialysed against the appropriate buffers before the analysis. The diffusion coefficients were evaluated from the interference-fringe patterns (Kodak contour projector) by the method of Svensson (1951). Since no dependence of the diffusion coefficients on protein concentrations was noted, all values obtained for an individual protein fraction at different concentrations were averaged and reported as $D_{20,w}^0$ values.

Moving-boundary electrophoresis was done in a Perkin-Elmer model 238 instrument at 4° in the standard-size cell. Proteins were dialysed against the appropriate buffers before use. Analyses were done at protein concentrations of 0.7% in sodium barbital, pH 8.6, and sodium acetate buffers, pH 4.5 (*I* 0.1 in both cases), at a current of 10 ma.

Ultraviolet-absorption spectra and individual wavelength readings in both the u.v. range and the visible range were measured in a Zeiss PMQ-II spectrophotometer. Partial specific volumes of all proteins studied were computed from composition results by the method of Schachman (1957). Molecular weights were calculated from the sedimentation and diffusion results by the Svedberg equation.

Unless otherwise noted, all physical measurements were performed in 0.5 M-sodium cacodylate-0.1 M-NaCl buffer, pH 7. Its density was 1.0057 g./ml. and its relative viscosity at 25° was 1.030.

Analytical procedures. Hexoses, sialic acid, hexosamine and protein were determined by the orcinol, thiobarbituric acid, Ehrlich-reagent and the Lowry-reagent methods respectively, as described by Bezkorovainy (1965). Amino acid analyses were done in a Spinco model 120 apparatus by the method of Moore, Spackman & Stein (1958). The 'long' column was permitted to run for 240 min. to ensure the elution of glucosamine and galactosamine. All hydrolysates were prepared by heating 3-4 mg. of the protein with 3 ml. of peroxide-free constant-boiling HCl (distilled from a solution of FeSO₄ in 6 M-HCl) at 110° for 24 hr. under N₂ in a sealed tube. The hydrolysates were then repeatedly freeze-dried to remove the HCl. Glucosamine and galactosamine were calculated by using the constant for leucine. Corrections for losses of amino acids during hydrolysis due to the presence of large quantities of carbohydrate were made by the method of Anderson, Hoffman & Meyer (1965). All amino acid results reported are averages of at least two analyses. Tryptophan was determined by the method of Bencze & Schmid (1957).

Dinitrophenylation of glycoproteins for *N*-terminal group analysis was done at 40° for 2 hr. at pH 8 in an automatic pH-stat. The pH was maintained by titration with 0.1 M-NaOH. Hydrolysis and paper chromatography of the DNP-amino acids was performed as described by Fraenkel-Conrat, Harris & Levy (1955).

Qualitative analysis of hexoses was performed by

hydrolysis with dil. H₂SO₄ and paper chromatography as described by Bezkorovainy (1967). Alternatively, hydrolysis was performed with 2 M-HCl at 100° for 2 hr. under N₂. The identification of hexosamines was performed by a modification of the method of Bychkov & Kolesnikova (1966). The proteins were hydrolysed and the hexosamines were degraded by ninhydrin as described by the above authors. However, before paper chromatography, the sample was successively treated with Dowex 1 (X8; Cl⁻ form), Dowex 50 (X8; H⁺ form) and freeze-dried. Semi-quantitative estimation of the arabinose and lyxose formed was done by elution of the spots with acetic acid and colorimetry, as described by Bezkorovainy (1963).

Periodate oxidation. Proteins were dissolved in 0.5 M-sodium acetate buffer, pH 4.6, to give a final protein concentration of 7.0 mg./ml. Portions (2 ml.) of such solutions were mixed with 2 ml. of 0.1 M-sodium metaperiodate, and the reaction mixtures, along with controls without the periodate, were kept at 4° for 2 and 24 hr. in the dark. The reaction was terminated by the addition of 0.2 ml. of ethylene glycol. The samples were then dialysed and freeze-dried.

Alkali-borohydride treatment of proteins. Glycoproteins were treated with alkali and borohydride for various times by a modification of the method of Horowitz & Murty (1967). The protein was dissolved in 5 ml. of water, and 0.1 ml. of 5 M-NaOH and 57 mg. of NaBH₄ were added to the solution. The protein concentration was 10 mg./ml. The reaction mixture was kept under N₂ at 37° for 16-43 hr., after which the borohydride was destroyed by acidification to pH 5-6. The reaction mixture was then dialysed for 24 hr. in the cold with continuous agitation against 40 vol. of water. The diffusate was immediately freeze-dried, whereas the dialysis residue was once more dialysed against water under the same conditions and then freeze-dried.

RESULTS

Absorption spectra of colostrum glycoproteins. The u.v.-absorption spectra of two colostrum M-1 glycoproteins, whose isolation is represented by Fig. 1, is shown in Fig. 2. The fraction emerging

Fig. 2. Ultraviolet-absorption spectra of the 275 nm.-positive material at pH 7 (2.5 mg./ml., curve C) and at pH 13 (1.25 mg./ml., curve A), and of the 275 nm.-negative material at pH 7 (curve D) and pH 13 (curve B), both at concentrations of 2.5 mg./ml.

Fig. 3. Ultracentrifugal analysis of the 275nm.-negative (upper pattern), and the 275nm.-positive (lower pattern) glycoproteins at concentrations of 10 mg./ml. The picture was taken 96 min. after the start of the run at a bar angle of 60°. A synthetic-boundary cell was used.

towards the end of the elution pattern in Fig. 1(a) showed a typical u.v.-absorption spectrum with a maximum at 275nm. at pH 7. In 0.1M-sodium hydroxide, however, a shift of the maximum to 292nm. was observed. The fraction eluted closer to the break-through volume, on the other hand, had no absorption maximum either at pH 7 or at pH 13. The two types of glycoproteins are subsequently referred to as the 275nm.-positive and the 275nm.-negative glycoproteins respectively.

Physical parameters. The physical homogeneity of the 275nm.-positive and -negative proteins was established by ultracentrifugal and moving-boundary-electrophoresis criteria. Both the 275nm.-positive and the 275nm.-negative glycoproteins showed single components in the ultracentrifuge and by the electrophoresis procedure at pH 8.6 and 4.5. Representative patterns are shown in Figs. 3 and 4. The stationary boundary seen in Fig. 4 represents the so-called δ -peak, which was observed with all proteins studied by moving-boundary electrophoresis in this laboratory.

The physical parameters of the 275nm.-positive and -negative glycoproteins are given in Table 1. The 275nm.-positive protein is smaller than the 275nm.-negative protein. This is expected from their relative positions in the Sephadex G-75 elution diagram (Fig. 1). Colostrum immune globulin gave physical parameters comparable with those of bovine serum immune globulin (Phelps & Putnam, 1960).

Composition of colostrum M-1 glycoproteins. The amino acid and monosaccharide compositions of the 275nm.-positive and -negative glycoproteins are shown in Table 2. Unusual features of these

Fig. 4. Moving-boundary electrophoresis of the 275nm.-negative glycoprotein. Frames 1, 2 and 3 were seen at pH 4.5 after 33, 49 and 100 min. with an electrophoretic mobility of $-5.7 \times 10^{-5} \text{ cm.}^2/\text{v/sec.}$ Frames 4, 5 and 6 were seen at pH 8.6 after 15, 38 and 92 min. respectively with an electrophoretic mobility of $-8.3 \times 10^{-5} \text{ cm.}^2/\text{v/sec.}$

proteins are the total absence of cystine, the very high glutamic acid, threonine, and proline contents, and the very low contents of basic amino acids. The 275nm.-negative protein contained no tyrosine, arginine or histidine. The isoleucine content of this protein was higher than the leucine content. Both proteins lacked tryptophan.

The hexose:*N*-acetylhexosamine molar ratio was near 5:4 for the 275nm.-negative glycoprotein, and nearly 1:1 for the 275nm.-positive protein. The glucosamine:galactosamine molar ratio was 1:2 for both proteins, as judged by amino acid elution diagrams, assuming that both types of hexosamines are destroyed at equal rates under the hydrolysis conditions used (5.7M-hydrochloric acid at 110° for 24hr.). The sialic acid:hexose molar ratio was 2:3 in both glycoproteins. Relatively large amounts

Table 1. *Physical properties of the 275nm.-positive and -negative colostrum glycoproteins and colostrum immune globulin*

	275nm.- negative glycoprotein	275nm.- positive glycoprotein	Colostrum immune globulin
$S_{20,w}^0$ (s)	1.43	1.09	6.80
$10^{-1} \times D_{20,w}^0$ (cm. ² /sec.)	9.13	11.9	3.73
$\bar{v}$ (ml./g.)	0.685	0.691	0.725*
Mol.wt.	12100	7200	170000

* Assumed to be identical with that of bovine serum immune globulin (Phelps & Putnam, 1960).

Table 2. *Amino acid and carbohydrate composition (in g. of residue/100g.) of the 275nm.-positive and -negative colostrum glycoproteins*

Amino acid	Content (g./100g.)	
	275nm.-negative glycoprotein	275nm.-positive glycoprotein
Lys	4.2	4.0
His	0.0	0.5
Arg	0.0	1.4
Asp	5.1	8.0
Thr	10.8	8.4
Ser	4.6	5.8
Glu	13.6	14.2
Pro	7.2	6.3
Gly	0.7	1.6
Ala	3.6	3.4
CyS	0.0	0.0
Val	3.8	3.9
Met	0.5	0.5
Ile	5.8	4.8
Leu	1.4	4.1
Tyr	0.0	2.7
Phe	0.3	3.0
Trp	0.0	0.0
Carbohydrate		
Hexose	12.4	8.5
N-Acetylhexosamine	12.6	9.7
Sialic acid	14.0	10.2

of sialic acid were always found to react with the Warren reagent without prior hydrolysis of the glycoproteins. This is indicative of the extreme lability of the sialic acid-oligosaccharide bond. Under ideal conditions, the sialic acid:hexose molar ratio may very well be 1:1, however, and the extensive handling of these preparations may result in the removal of part of this component from the glycoprotein.

The nature of the hexoses present in the two glycoproteins was confirmed by hydrolysis with dilute

acid and paper chromatography. Hexose recovery, based on the orcinol reaction, was always better than 85% after such hydrolysis. Both the 275nm.-positive and the 275nm.-negative glycoproteins were found to contain galactose only. Orosomucoid, on the other hand, gave galactose and mannose under identical conditions.

The identity of the hexosamines was confirmed by, in addition to the amino acid elution diagrams, their degradation to the corresponding pentoses with ninhydrin, followed by paper chromatography. Both the 275nm.-positive and the 275nm.-negative proteins were found to contain glucosamine and galactosamine by this method. Semi-quantitative estimation of arabinose and lyxose seen in these chromatograms indicated a glucosamine:galactosamine ratio of 1:2, in full agreement with the amino acid-analysis results. Orosomucoid, on the other hand, showed the presence of glucosamine only.

Periodate oxidation of colostrum M-1 glycoproteins. Both the 275nm.-positive and the 275nm.-negative glycoproteins were subjected to periodate oxidation in their native and 'desialized' (neuraminidase-treated) forms. Periodate destroyed sialic acid in both the native proteins, but it did not significantly alter the hexose or hexosamine content of these proteins after 2 or 24hr. reaction times. On the other hand, periodate destroyed 95% of the hexose in both the 'desialized' 275nm.-positive and the 'desialized' 275nm.-negative glycoproteins, leaving the hexosamine intact. The loss of hexose after the periodate treatment of the 'desialized' samples was also ascertained by dilute-acid hydrolysis of the affected samples and paper chromatography.

N-Terminal group analysis. N-Terminal group analysis revealed that both the 275nm.-positive and the 275nm.-negative glycoproteins were apparently polydisperse, as had been found with their crude precursor (Bezkorovainy, 1967). However, the amounts of N-terminal amino acids recovered were only 0.2mole/mole of protein for both types of glycoproteins. Low recovery of N-terminal groups has also been observed with K-casein, the only other well-characterized sialic acid-containing glycoprotein of milk and colostrum (Wake, 1959).

Alkali-borohydride treatment of colostrum glycoproteins. Small amounts (30–40mg.) of both the 275nm.-positive and the 275nm.-negative glycoproteins were subjected to conditions bringing about the β -elimination reaction in glycoproteins (Anderson *et al.* 1965). In both cases 80–90% of the carbohydrate and 50% of the protein became diffusible after the alkali-borohydride treatment for 32hr. Orosomucoid, on the other hand, did not show any significant loss in either the total weight or its carbohydrate. The total recovery (in both the diffusible and non-diffusible forms) of hexose, hexosamine and sialic acid after the alkali-borohydride

reaction was 91 ± 9 (S.D.), 55 ± 5 and $80 \pm 14\%$ respectively for both types of glycoproteins. The recovery of hexose and sialic acid may thus be considered to have been nearly quantitative, whereas hexosamine had apparently been partially destroyed by the alkali-borohydride reaction.

A determination of some physical properties of the non-diffusible component of the 275nm.-negative glycoprotein treated with alkali-borohydride revealed the following: $S_{20,w}^0$ 0.53s; $D_{20,w}^0$ 15.0; $\bar{v}$ 0.717; molecular weight 3200. The alkali-borohydride reaction brought about a decrease in the molecular weight of the 275nm.-negative glycoprotein. No such change in molecular weight was observed in alkali-borohydride-treated orosomucoid.

DISCUSSION

The two glycoproteins studied in this work were apparently homogeneous by both ultracentrifugal and moving-boundary-electrophoresis criteria. Their u.v. spectra and molecular weights were their most prominent distinguishing characteristics: the heavier glycoprotein contained no tyrosine and showed no absorption maxima in the 240–300nm. range of the spectrum, whereas the lighter glycoprotein contained tyrosine and exhibited a maximum at 275nm. The shoulder at 280nm. is also typical of the tyrosine absorption spectrum. The hyperchromic shift of the 275nm. maximum to 292nm. in the presence of alkali is characteristic of ionized tyrosine (Bencze & Schmid, 1957).

Alkali-borohydride treatment of the two glycoproteins resulted in the appearance of diffusible carbohydrate material, a decrease in the molecular weight of the non-diffusible material, and a low overall recovery of hexosamine compared with those of sialic acid and hexose. These findings suggest that the oligosaccharide chains of the two glycoproteins are linked to their peptide moieties via an O-glycosidic linkage involving hexosamine. The

elucidation of the exact nature of the hexosamine and the amino acid(s) involved in such a linkage, and the size of the oligosaccharide chains, must await the results of further experiments.

Periodate oxidation experiments demonstrated that sialic acid was linked to galactose in both the 275nm.-positive and the 275nm.-negative glycoproteins. Further, the insensitivity of hexose to periodate oxidation in the native proteins suggests that sialic acid is linked to galactose in the 3-position.

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