The Occurrence of a Heparin-like Glycosaminoglycan in Bovine Milk and its Possible Association with Lipoprotein Lipase

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Heparin-like glycosaminoglycans occur in considerable quantities in many tissues; however, no physiological role has so far been conclusively assigned to these compounds.[1] Numerous observations have indicated a functional relationship between heparin and the enzyme lipoprotein lipase (EC 3.1.1.3.). Injection of heparin causes liberation of the enzyme into the circulating blood,[2] and it has been suggested that part of the injected heparin binds to the enzyme.[3] Removal of heparin from such a preparation results in loss of enzymic activity.[4] Korn [5] has shown that lipoprotein lipase from chicken adipose tissue is partly inactivated by treatment with heparinase and has suggested that a heparin-like polysaccharide may form an integral part of the enzyme. This hypothesis is actualized by the findings described in the present paper, which indicate that an endogenous heparin-like glycosaminoglycan and lipoprotein lipase are both concentrated in the same fraction of bovine milk.

Fractionation of milk proteins. The preparation of a lipoprotein lipase-rich fraction from milk is based on the procedure of Korn.[6] Fresh unpasteurized milk obtained from a commercial dairy was separated into skim milk and cream (50 % fat) by centrifugation. The cream was washed twice with water, chilled to 4°C and churned with the aid of an electrical kitchen whisk. The resulting buttermilk was filtered through cheesecloth, lyophilized and then extracted with 0.025 M ammonia. The extract was centrifuged in the Spinco Ti 50 rotor at 50 000 rpm for 1 h. Three layers were obtained: a pellet, a water-clear solution and a white, fatty material that floated. The clear, intermediate layer was lyophilized to yield a white powder, freely soluble in distilled water.

An acetone-dried protein fraction was prepared from skim milk by precipitation with 20 volumes of acetone at room temperature. After 1 h in the cold room the precipitate was collected by filtration at 4°C, and was then further extracted with 25 volumes of acetone and finally with 20 volumes of freshly distilled diethyl ether.

Isolation of glycosaminoglycans. Samples of milk protein ranging from 0.3 to 1.6 g were digested with papain, essentially following the procedure of Fransson and Rodén.[7] After 24 h each digest was passed through a Celite pad which was subsequently extracted with 2 M KCl. The combined filtrates and extracts were diluted to 0.1 M with respect to chloride and then fractionated by chromatography on DEAE-cellulose. Following the application of samples, the columns (1 × 3 cm, previously equilibrated with 0.05 M HCl) were washed with 5 ml of 0.05 M HCl and then eluted with 5 ml each of 0.5 M, 1.0 M, and 2.0 M NaCl in 0.05 M HCl, in the order mentioned. Uronic acid-containing fractions were dialyzed and lyophilized.

Analytical methods. Uronic acid was determined by the carbazole method.[8] Glucosamine: galactosamine ratios were determined by gas liquid chromatography [9] or by means of an amino acid analyzer following hydrolysis of polysaccharide in 4 M HCl for 14 h at 100°C. Hexosamine residues with sulfated or unsubstituted amino groups were estimated as described previously.[10] Protein was determined by the Lowry method.[11] Electrophoresis of glycosaminoglycans was carried out using strips of cellulose acetate in 0.1 M barium acetate [12] or 0.1 M HCl,[13] according to the methods of Wessler.

Lipoprotein lipase was assayed as described by Robinson.[14] Intralipid (AB Vitrum, Stockholm) was used as fat substrate and human serum for "activation" of the substrate. All assays were carried out in duplicate. One enzyme unit corresponds to 1 μmole of fatty acid liberated per hour.

Results. The lipolytic activity of the skim milk and the buttermilk preparations is shown in Table 1. Lipoprotein lipase is characterized by its requirement for plasma lipoproteins to "activate" the fat substrate.[2] In the present experiments the lipolytic activity of whole milk as measured in the absence of serum was about 30 % of that observed with "activated" substrate (Table 1), the latter assay accounting for the total lipase content. About 80 % of the total lipase and practically all of the lipase which did not require "acti-

Table 1. Lipolytic activity of some fractions from bovine milk.

	Enzyme units per mg protein					
	Complete assay system		Assay system without serum		Recovery of [a] "LPL" activity %	
	1	2	1	2	1	2
Whole milk [b]	0.64	0.60	0.23	0.14	100	100
Skim milk [b]	0.55	0.51	0.22	0.21	69	67
Buttermilk [b]	0.19	0.38	0.01	0.04	0.5	0.3
Ammonia extract [b] of lyophilized butter milk	$3.9 \pm 1.6(16)$		$0.06 \pm 0.05(5)$		$0.33 \pm 0.22(8)$	

[a] LPL (Lipoprotein lipase) activity is calculated as the difference between values obtained by assays with and without serum, respectively.

[b] Values for whole milk, skim milk, and buttermilk represent two different batches. Values for ammonia extract of lyophilized buttermilk are mean $\pm$ S.D. of values from several batches, with number of batches analyzed given by figures in brackets.

vated" substrate was recovered in the skim milk fraction.

Thus, about ⅓ of the lipoprotein lipase, but little other lipolytic activity had distributed to the cream fraction, in good agreement with the observations of Korn.[6] The enzyme in buttermilk was almost exclusively lipoprotein lipase as judged by its requirement for serum. The lipase of the ammonia extract of lyophilized buttermilk corresponded to about 0.3 % of the total lipoprotein lipase activity of whole milk and had a specific lipoprotein lipase activity about 10 times higher than that of whole milk or skim milk. The reason for the low recovery of lipase activity in this fraction is not known; the enzyme must have been either largely inactivated during the churning or separated with the butter. The sample of buttermilk extract subjected to glycosaminoglycan analysis had a specific activity of 5.2 units/mg protein.

The uronic acid contents [5] of the two milk protein fractions are shown in Table 2. Both the skim milk and the buttermilk preparations contained uronic acid. However, the uronic acid content per mg protein of the latter preparation was about twenty times higher than that of the skim milk preparation. Electrophoresis in barium acetate showed the presence in all three polysaccharide fractions from buttermilk of material which migrated like heparin or heparan sulfate. The 0.5 M fractions also contained a component with the migration rate of hyaluronic acid or der-

Table 2. Uronic acid content of two fractions from bovine milk.

	Uronic acid, μg per 100 mg of protein [a]		
	0.5 M NaCl	1.0 M NaCl	2.0 M NaCl
Acetone-ether-dried preparation from skim milk	2.3	1.0	0.2
Ammonia extract of lyophilized buttermilk	15	59	6

[a] Column headings refer to NaCl concentrations of eluates from DEAE-cellulose.

matan sulfate. Most of the polysaccharide in the 0.5 M fraction was low-sulfated, as demonstrated by electrophoresis in 0.1 M HCl. In this medium the 1.0 and 2.0 M fractions produced spots typical of heparan sulfate, thus considerably retarded compared to commercial heparin. Further analysis of the 1.0 M fraction showed that glucosamine was present in amounts similar to those of uronic acid, whereas galactosamine was found in trace amounts only. Approximately half of the hexosamine residues were *N*-acetylated as shown by the indole reaction after deamination with nitrous acid.[15] It is concluded that the major polysaccharide component of the 1.0 M fraction is a heparin-like glycosaminoglycan, presumably heparan sulfate (heparitin sulfate).[1]

This study was initiated because of the numerous observations in the literature suggesting an interaction between lipoprotein lipase and heparin.[1–5] The results demonstrate, to the best of our knowledge for the first time, that bovine milk contains a low-sulfated heparin-like glycosaminoglycan, presumably heparan sulfate. The concurrent concentrations of lipoprotein lipase and endogenous heparin-like glycosaminoglycan in the same fraction of bovine milk are compatible with the existence of an enzyme-glycosaminoglycan complex. The properties of the glycosaminoglycan as well as its possible association with lipoprotein lipase are being studied further.

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