EVIDENCE FOR AN IONIC BINDING OF LIPOPROTEIN LIPASE TO HEPARIN.

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SUMMARY. The interaction between lipoprotein lipase from bovine milk and heparin has been studied. A specific, ionic binding of the enzyme to an agarose gel containing covalently bound heparin was demonstrated. This binding was exploited for the purification of the enzyme from skim milk by affinity chromatography on a column of heparin-substituted agarose. The purified enzyme had a higher specific activity (about 10,000 units per mg protein) than any other preparation of lipoprotein lipase described so far.

Lipoprotein lipase (LPL, EC 3,1,1,3) hydrolyzes plasma lipoprotein triglycerides, thereby making their fatty acids available to the tissues (1,2). Numerous reports in the literature indicate a functional relation between this enzyme and heparin or heparin-like glycosaminoglycans. The enzyme can be released from its extracellular sites by heparin or by any of several other polyanions, both in vivo and in vitro (2). Evidence compatible with the association in vivo of LPL with a heparin-like glycosaminoglycan was obtained by Olivecrona and Lindahl (3) who found appreciable amounts of heparan sulfate in an enzyme preparation from bovine milk. Furthermore, Korn reported that LPL from chicken adipose tissue was partially inactivated on incubation with a bacterial heparinase (4). The activity of the enzyme is often enhanced by heparin in low concentrations (5,6,7). This has been ascribed to an increased binding of the enzyme to its substrate (6,8) and/or an allosteric effect of heparin on the enzyme (7). Conversely, the LPL of post-heparin plasma becomes less stable on removal of heparin by passage over an anion exchange column (9).

Although the observations cited above strongly suggest that heparin can

interact with LPL, no direct evidence for such an interaction has as yet been reported. The present data demonstrate a strong and reversible ionic binding of LPL to heparin. We have also purified the enzyme extensively by affinity chromatography on a column of heparin covalently bound to an agarose gel.

MATERIALS AND METHODS.

Analytical methods: The assay for LPL was based on that described by Robinson (2). A complete mixture consisted of: 0.5 ml of 1.35 M Tris-HCl pH 8.1; 224 mg of bovine serum albumin in 1.2 ml of Krebs-Ringer phosphate buffer pH 8.1; 0.1 ml of heparin solution (20 IU/ml); 0.8 ml of human serum dialyzed against 0.16 M NaCl; 0.1 ml of Intralipid (Vitrum, Stockholm, Sweden, 10% lipid emulsion); and 2 ml of enzyme solution. Duplicate 1 ml aliquots were withdrawn at 0 min and after 60 min incubation at 37° and the free fatty acid contents were determined by titration (10). One enzyme unit corresponds to the release of one μmol of fatty acid per hour.

Protein was determined by the method of Lowry et al (11) with bovine serum albumin as the standard.

Ion exchangers: Heparin was covalently bound to an agarose gel (Sepharose 4B, Pharmacia Fine Chemicals, Uppsala, Sweden) activated by treatment with cyanogen bromide (12). The details of the preparation procedure, as well as the properties of the batch used (Hep.II-Seph.4B-IV, in the following denoted "heparin-Sepharose") will be described elsewhere by one of us (Iverius). A reference gel ("ethanolamine-Sepharose") was prepared by treating activated agarose with ethanolamine. CM-Sephadex C50, SE-Sephadex C50, and DEAE-Sephadex A50 were purchased from Pharmacia Fine Chemicals.

Preparation of soluble enzyme from milk: Fresh, unpasteurized bovine milk was centrifuged and the skim milk was recovered. LPL was released from the casein micelles (13) by dialysis at 4° against several changes of 0.02 M sodium citrate in 0.1 M sodium chloride, pH 7.0, and then against 0.005 M sodium barbital in 0.157 M sodium chloride, pH 7.4. The dialyzed skim milk

was centrifuged at 37,000 x g for 90 min to yield a clear solution containing about 95% of the original LPL activity of the skim milk (equivalent to about 65% of the total lipoprotein lipase activity of whole milk).

Binding of LPL to ion exchangers: Duplicate samples of dialyzed skim milk (1 ml, corresponding to 125 enzyme units) were mixed with equal volumes of ion exchangers, equilibrated with 0.005 M sodium barbital in 0.157 M sodium chloride, pH 7.4. Each tube was kept at 4° with frequent swirling for 2 hours and was then centrifuged. The enzyme activity and protein content of the supernatants were determined.

Affinity chromatography of LPL: A sample of dialyzed skim milk was applied to a column of heparin-Sepharose, and eluted with a linear gradient of sodium chloride, as described in detail in the legend to fig. 1.

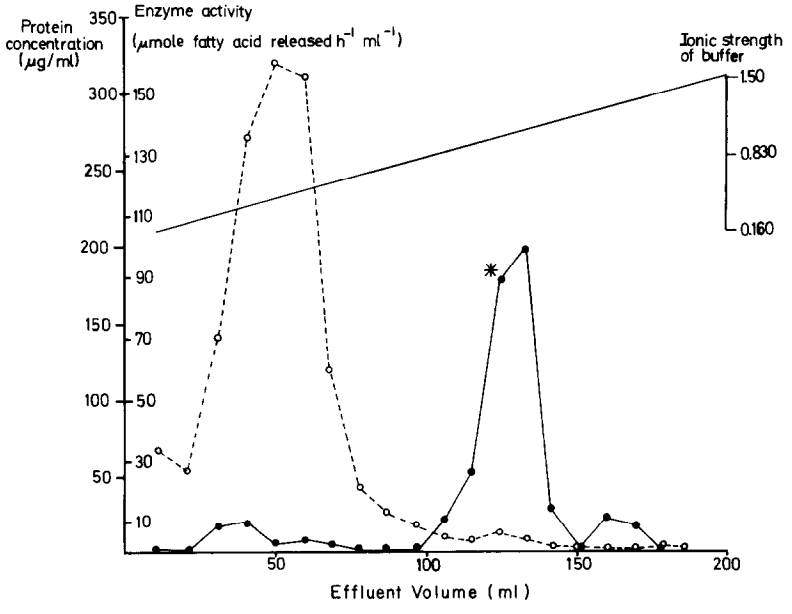

Figure 1. Affinity chromatography of LPL on heparin-Sepharose. A sample (32 ml) of dialyzed skim milk was applied to a column (1x5 cm) of the gel equilibrated with 0.005 M sodium barbital in 0.157 M sodium chloride, pH 7.4. The column was washed with 50 ml of the same buffer and was then eluted with a linear gradient (0.157 M to 1.48 M) of sodium chloride in 0.005 M sodium barbital, pH 7.4. Effluent fractions of 8 to 10 ml were collected and assayed for enzyme activity (●——● ; samples diluted 40-fold prior to assay) and protein (o - - o). The ionic strength of the eluant is indicated by a solid line. The fraction marked * was used as "purified enzyme" for the experiments in table 2.

RESULTS AND DISCUSSION.

Samples of dialyzed skim milk equilibrated with various ion exchangers (CM-, SE-, and DEAE-Sephadex) showed LPL activities higher than those expected (62.5 units/ml) for a uniform distribution of the enzyme added to the equilibration mixtures (table 1). Such high values, also obtained with ethanolamine-Sepharose, probably reflect the exclusion properties of the gels employed. In contrast, after equilibration of the crude enzyme with heparin-Sepharose most of the enzyme activity, but no detectable protein, had disappeared from the solution (table 1). These results suggest a specific binding of LPL to heparin.

The binding of LPL to heparin was turned to advantage for the purification of the enzyme by affinity chromatography of dialyzed skim milk on a column of heparin-Sepharose. Essentially all of the protein applied to the column was eluted at a low ionic strenth (fig. 1). In contrast, the enzyme was eluted as a peak at an ionic strength of 0.83. The peak tube had a specific activity of about 10,000 units per mg protein, thus representing a more than 2,000 fold purification. Fielding has previously purified LPL from post-heparin plasma (14,15). His preparation had a specific activity of 2,345

Table 1. Binding of skim milk LPL to various ion exchangers at pH 7.4 and ionic strength 0.160.

Ion exchanger	Ion exchanged	Enzyme activity in the supernatant (units/ml)	Protein in the supernatant (mg/ml)
Heparin-Sepharose	cation	9	21.2
		10	21.9
Ethanolamine-Sepharose	-	92	21.1
		95	21.3
CM-Sephadex C 50	cation	83	21.9
		91	22.1
SE-Sephadex C 50	cation	93	21.1
		87	20.0
DEAE-Sephadex A 50	anion	106	17.0
		99	17.1

Table 2. Properties of skim milk LPL before and after purification.

Enzyme preparation	Specific activity (units per mg of protein). Assay medium						
	complete	lacking heparin[b]	lacking serum	plus NaCl 0.5 M	plus NaCl 1.0 M	plus protamine 0.5×10^{-4} M[c]	plus protamine 1.0×10^{-4} M[c]
Dialyzed skim milk	4.4	2.4	0	-	-	-	-
Purified enzyme[a]	7400	6400	0	470	0	2600	630

a) Refers to the fraction indicated by an asterisk (*) in figure 1. The specific activity of the peak fraction was about 10,000 units per mg protein.

b) It should be noted that the assay medium lacking exogenous heparin will still contain any glycosaminoglycan present in the serum (Calatroni A., P.V. Donnelly, and N. DiFerrante, J. Clin. Invest., 48, 332 (1969)).

c) The molecular weight of protamine was assumed to be 5,000 (Jirgensons B., Natural Organic Macromolecules, Pergamon Press, Oxford 1962, p 291.).

and was claimed to be more than 95% pure. Although our preparation had a higher specific activity than that of Fielding, it may still be inhomogenous since the specific activity was not constant over the peak of enzyme activity eluted from the heparin-Sepharose column.

The recovery of LPL activity from the column was about 50%. The enzyme had the characteristics of a lipoprotein lipase both in the dialyzed skim milk and after purification (table 2) (1,2,5,16). Thus, it was active only in the presence of serum, it was stimulated by heparin, and it was inhibited by protamine and by high salt concentrations.

In conclusion, a strong and reversible binding of LPL to heparin has been demonstrated. This finding confirms previous evidence (see introduction) for an interaction between the two macromolecules.

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