HETEROGENEITY OF SALT-RESISTANT LIPASE FROM HUMAN POSTHEPARIN PLASMA

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1. Introduction

Catabolism of plasma triglyceride-rich lipoproteins is thought to be mediated by a specific triglyceride hydrolase, lipoprotein lipase in the capillary bed of extrahepatic tissues [1]. Lipoprotein lipase is released into plasma after heparin administration [2], and it has been assumed that all triglyceride lipase activity in postheparin plasma is due to this enzyme. Recent studies have shown, however, that a second triglyceride lipase activity is present in postheparin plasma of man and experimental animals [3–6]. This activity differs from lipoprotein lipase in its resistance to protamine and 1 M NaCl and in its lack of activation by serum cofactors. The 'salt-resistant' or 'protamine-insensitive' lipase is present in heparin perfusate of liver [7,8], but not in postheparin plasma of hepatectomized animals [5,9], and has been assumed to originate from liver.

In the present study we describe a refined purification procedure for human postheparin triglyceride lipases. By this method the saltresistant triglyceride hydrolase is separated into three subfractions. Further purification of these fractions and of lipoprotein lipase is attained by calcium gel chromatography. After this treatment the enzyme activities are no more retained by heparin-Sepharose.

2. Material and methods

2.1. *Enzyme assay*

Triglyceride hydrolase activity was assayed in duplicate with a synthetic [^{14}C]triolein substrate emulsified in the presence of 5% gum arabic [10].

2.2. *Sources of enzyme*

Postheparin plasma was obtained by plasmapheresis from healthy young males after injection of heparin (100 IU/kg body weight, Heparin, Medica, Finland). Plasma was stored frozen at –20°C until assayed.

2.3. *Enzyme purification*

In a typical experiment 400 ml of postheparin plasma from a single donor was mixed with 100 ml of 20% triglyceride emulsion (Intralipid®, Vitrum, Stockholm, Sweden). The mixture was incubated at 37°C for 15 min. After centrifugation at 76 000 *g* for 60 min, the fat layer at the top of the centrifuge tube was collected. The clear infranatant was again mixed with Intralipid® and the incubation and centrifugation were repeated. The fat layers were combined and added to 250 ml centrifuge tubes containing 150 ml of cold acetone. After stirring the tubes were centrifuged at 20 000 *g* for 10 min. The precipitate was then washed with 150 ml of acetone and twice with 70 ml of ether and finally dried under nitrogen. The dried powder was dissolved in 5 mM sodium barbital buffer, pH 7.4, containing 20% of glycerol. The solution was centrifuged at 27 000 *g* for 15 min. The pellet contained no triglyceride lipase activity.

The clear supernatant was applied on a Sepharose 4B column (2.5 × 17.5 cm) containing covalently bound heparin [11]. The column was equilibrated with 0.15 M NaCl in 5 mM sodium barbital buffer, pH 7.4, containing 20% glycerol. After washing with 0.4 M NaCl the enzyme was eluted with a linear NaCl gradient (600 ml; 0.4 ml/min) from 0.4 to 1.5 M NaCl

in 5 mM sodium barbital buffer, pH 7.4, containing 20% glycerol.

2.4. *Calcium phosphate gel adsorption*

Calcium phosphate gel adsorption was carried out as described by Fielding [12]. Pooled fractions from the heparin-Sepharose chromatography were mixed with calcium phosphate gel (Sigma Chemical Co. 7 mg of dry gel/ml of eluate) and the suspension was stirred at 4°C overnight. After centrifugation at 9000 *g* for 10 min, the supernatant was removed and the gel was washed with 20 ml of 50 mM NH_4OH–NH_4Cl buffer, pH 8.3, containing 0.5 mM potassium oleate, 10 mM desoxycholate and 0.1 M potassium oxalate and finally with 50 mM NH_4OH–NH_4Cl buffer containing 0.1 M potassium oxalate. The enzymes were eluted twice with 4 ml of 50 mM sodium citrate–50 mM NH_4OH–NH_4Cl buffer, pH 8.3, containing 0.5 mM potassium oleate.

2.5. *Antisera*

Antiserum against salt-resistant lipase has been described earlier [13]. The antiserum against lipoprotein lipase from bovine milk [14] was a gift from Drs. Hernell, Egelrud and Olivecrona, Umeå, Sweden.

3. Results

Optimal results were obtained in the formation of the enzyme–substrate complex by adding 1 part of 20% Intralipid® to 4 parts of postheparin plasma. Under these conditions more than 30% of the total lipolytic activity in postheparin plasma was recovered after delipidation of the enzyme–substrate complex. Immunological studies indicated that 40–45% of the salt-resistant lipase and 5–10% of the serum stimulated lipase activity remained in the infranatant after two treatments with Intralipid®. Thus, both lipases bind to Intralipid® although the affinity of the salt-resistant lipase is distinctly lower.

When the delipidated preparation was subjected to affinity chromatography on heparin-Sepharose, several peaks of triglyceride lipase activity were constantly seen (fig.1). Plasma obtained from two donors yielded three separate peaks (II, III and IV, fig.1), while an additional peak (I) eluting in front of the other fractions was discernible in the preparation from a third donor. The four peaks of activity were eluted at 0.5 (I), 0.7 (II), 0.85 (III) and at 1.05 M NaCl (IV), respectively. The effect of serum and NaCl on the activity of lipase in fractions II-IV is shown in fig.2. Peak I (not shown), II and III were not

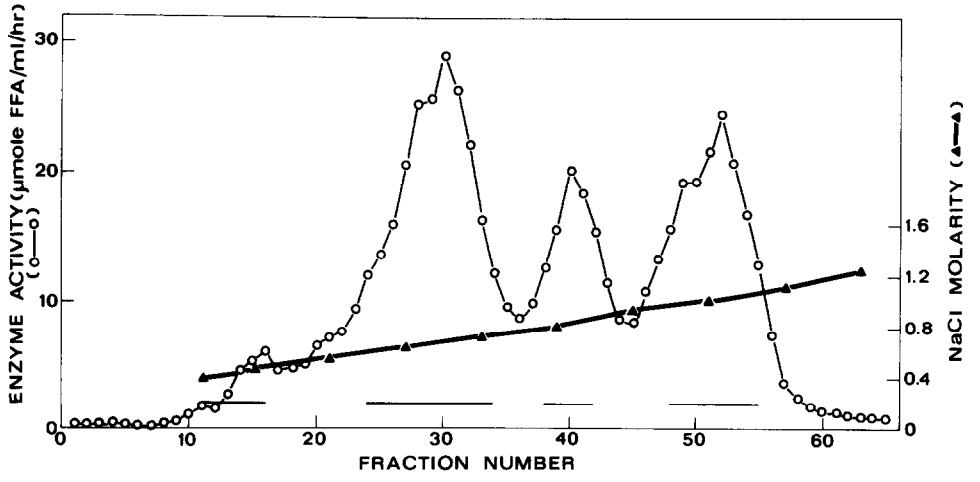

Fig. 1. Heparin-Sepharose chromatography of delipidated triglyceride lipase preparation obtained after the enzyme-substrate complex step (for details see Material and methods). Triglyceride lipase activity (o——o) and NaCl concentration (▲——▲) are as indicated. Peaks I, II, III and IV were pooled as shown by the black bars in the panel.

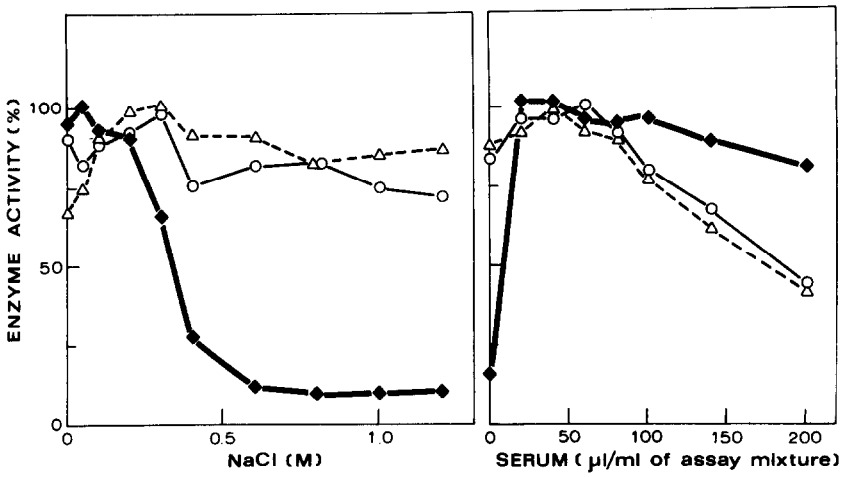

Fig. 2. Effect of NaCl and serum on the activity of triglyceride lipase in fractions II (△--△), III (○——○) and IV (■——■). The assays were performed under standard conditions varying the concentrations of serum and NaCl in the assay medium.

inhibited by 1 M NaCl and did not require the presence of serum for full activity. In contrast to these fractions, triglyceride lipase in peak IV was activated 6-fold by serum and was strongly inhibited by high salt concentrations.

The lipase fractions were further purified by adsorption on calcium phosphate gel. All four fractions were retained by the gel and could be eluted with a citrate-containing buffer. This step resulted in 1.5–2.5-fold additional purification of all four fractions with the recovery of lipase activity varying between 10 and 40%. The specific activities of the final preparations were 107, 1103, 801 and 1789 µmol FFA released/mg protein/hr for the fractions I, II, III and IV, respectively.

When the enzyme preparations were subjected to chromatography on heparin-Sepharose after the calcium phosphate gel step, none of the activities were retained by the column. The recoveries of the enzyme activities in this step were constantly more than 100% ranging from 140 to 290% in different batches of enzymes. The reason for this activation is not known. Antiserum prepared against the salt-resistant lipase abolished the activity in fractions I, II and III while the activity in fractions IV was not changed (fig.4). An antiserum against the lipoprotein lipase from bovine milk precipitated the activity in fraction IV but did not affect the enzymes in the three other preparations.

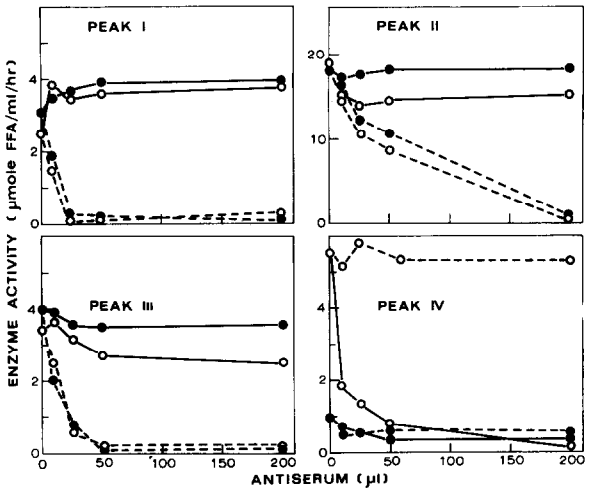

Fig. 3. Effect of antiserum against salt-resistant lipase from human postheparin plasma (– – – –) and antiserum against lipoprotein lipase from bovine milk (——) on the activity of triglyceride lipase in fractions I, II, III and IV (see text). Lipase fractions were preincubated at 4°C for 2 hr with the indicated amounts of antisera (diluted to a constant volume with normal rabbit serum). Open symbols (○) indicate the assays performed at 0.1 M NaCl and solid symbols (●) those performed at 1.0 M NaCl concentration.

4. Discussion

The purification procedure described in this paper involves two steps specific for triglyceride lipases. The use of enzyme–substrate complex formation gives excellent purification of triglyceride lipases and simultaneously circumvents the laborious acetone–ether treatment of plasma employed in earlier studies [5,6]. Both lipoprotein lipase and salt-resistant lipase were complexed to Intralipid® although the affinity of the latter activity to triolein emulsion was distinctly lower. Chromatography of the delipidated preparation on heparin-Sepharose resulted in four separate fractions, three of which had the characteristics of salt-resistant lipase. The fourth fraction clearly differed from the others and was identical with lipoprotein lipase in its behaviour towards various activators and inhibitors.

Further purification of the four lipase preparations by calcium phosphate gel chromatography yielded fractions which were no more retained by heparin-Sepharose. This observation is in agreement with the results of Fielding [12] who has shown that rat post-heparin plasma triglyceride lipase is not retained by heparin-Sepharose after calcium phosphate gel treatment. On the other hand, it is obvious from our results that complete separation of lipoprotein lipase and salt-resistant lipase can be achieved only by heparin-Sepharose chromatography performed *before* calcium phosphate gel treatment.

The presence of two immunologically different triglyceride lipases in postheparin plasma has earlier been inferred from studies using antisera against salt-resistant lipase [13] and bovine milk lipoprotein lipase [14]. The present study provides conclusive evidence for this suggestion by demonstrating that three of the fractions separated on heparin-Sepharose were precipitated by antiserum against salt-resistant lipase while the fourth was immunologically similar to bovine milk lipoprotein lipase. The molecular basis for the chromatographic heterogeneity of salt-resistant triglyceride lipase is currently not known. Further studies are needed to establish whether these fractions represent activities with different origins and/or physiological functions or whether they are formed from a single enzyme species during the purification.

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References

[1] Robinson, D. S. (1970) in: Comprehensive Biochemistry (Florkin, M. and Stotz, E. M., eds.), Vol. 18, pp. 51–116, Elsevier, Amsterdam.
[2] Korn, E. D. (1959) Methods Biochem. Anal. 7, 145.
[3] Krauss, R. M., Windmueller, H. G., Levy, R. I. and Fredrickson, D. S. (1973) J. Lipid Res. 14, 286–295.
[4] Greten, H., Walter, B. and Brown, W. V. (1972) FEBS Lett. 27, 306–310.
[5] Ehnholm, C., Bensadoun, A. and Brown, W. V. (1973) J. Clin. Invest. 52, 26a.
[6] Ehnholm, C., Shaw, W., Greten, H., Lengfelder, W., Brown, W. V. (1974) in: Atherosclerosis (Schettler, G. and Weizel, A., eds.), pp. 557–560, Springer-Verlag, Berlin.
[7] Hamilton, R. L., Jr. (1964) in: Postheparin plasma lipase from hapetic circulation. University Microfilms, Ann Arbor, Michigan.
[8] Assman, G., Krauss, R. M., Fredrickson, D. S. and Levy, R. I. (1973) J. Biol. Chem. 248, 1992–1999.
[9] Greten, H., Sniderman, A. D., Chandler, J. G., Steinberg, D. and Brown, W. V. (1974) FEBS Lett. 42, 157–160.
[10] Bensadoun, A., Ehnholm, C., Steinberg, D. and Brown, W. V. (1974) J. Biol. Chem. 249, 2220–2227.
[11] Iverius, P.-H. (1971) Biochem. J. 124, 677–683.
[12] Fielding, P. E., Shore, V. G. and Fielding, C. J. (1974) Biochemistry 13, 4318–4323.
[13] Huttunen, J. K., Ehnholm, C., Nikkilä, E. A. and Ohta, M. (1975) Eur. J. Clin. Invest., in press.
[14] Hernell, O., Egelrud, T. and Olivecrona, T. (1975) Biochim. Biophys. Acta, in press.