HIGH AND LOW MOLECULAR WEIGHT FORMS OF PANCREATIC CHOLESTEROL ESTERASE

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SUMMARY

Cholesterol esterase has been isolated from pig pancreas powder as a complex of molecular weight > 800,000. By lipid extraction, the complex was converted into enzyme sub-units of 15,000-20,000 molecular weight, which were still capable of hydrolysing and synthesising cholesterol esters. The active sub-units could be partially re-aggregated by incubation with a lipid emulsion. All the enzyme preparations showed similar cholesterol ester hydrolytic : cholesterol esterifying activity ratios, suggesting that the same enzyme catalyses both reactions.

Several recent reports on the purification of lipolytic enzymes from different tissue sources have revealed certain similarities in the chromatographic properties of these enzymes. Lipolytic protein of molecular weight > 800,000 has been separated from pig pancreas homogenates. Subsequent delipidation of the enzyme preparation produced an active peak of much lower molecular weight (1,2). Lyophilised extracts of pig pancreas were shown to contain lipolytic protein of high molecular weight. However, using extracts of fresh pancreas, lipolytic activity was isolated only in a low molecular weight form (3).

Lipases from tissues other than the pancreas have shown similar properties (4-6).

From these reports it appears that lipolytic enzymes in general have a tendency to form lipoprotein complexes of high molecular weight. Until the pre-

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sent report however, there has been no evidence for the involvement of lipid with cholesterol esterase in such a way as to facilitate the existence of the enzyme in different molecular weight forms, although rat pancreatic cholesterol esterase has been shown to complex with crystalline cholesterol (7) and bile salts (9).

The present paper is concerned primarily with the molecular weight properties of pig pancreatic cholesterol esterase. The enzyme will be shown to exist as a high molecular weight complex with lipid and a low molecular weight delipidised form, thus exhibiting similar behaviour to other lipolytic enzymes.

EXPERIMENTAL

Materials

The material used for enzyme extraction and purification was an acetone-extracted hog pancreas powder produced, under the commercial name of Viokase, by the Viobin Corporation, Monticello, Illinois.

Bile salts were obtained from the Maybridge Chemical Company, Launceston, Cornwall, UK, and their purity confirmed by thin-layer chromatography (9).

Lipomul (15% cottonseed oil) was provided by AB Astra, Sweden.

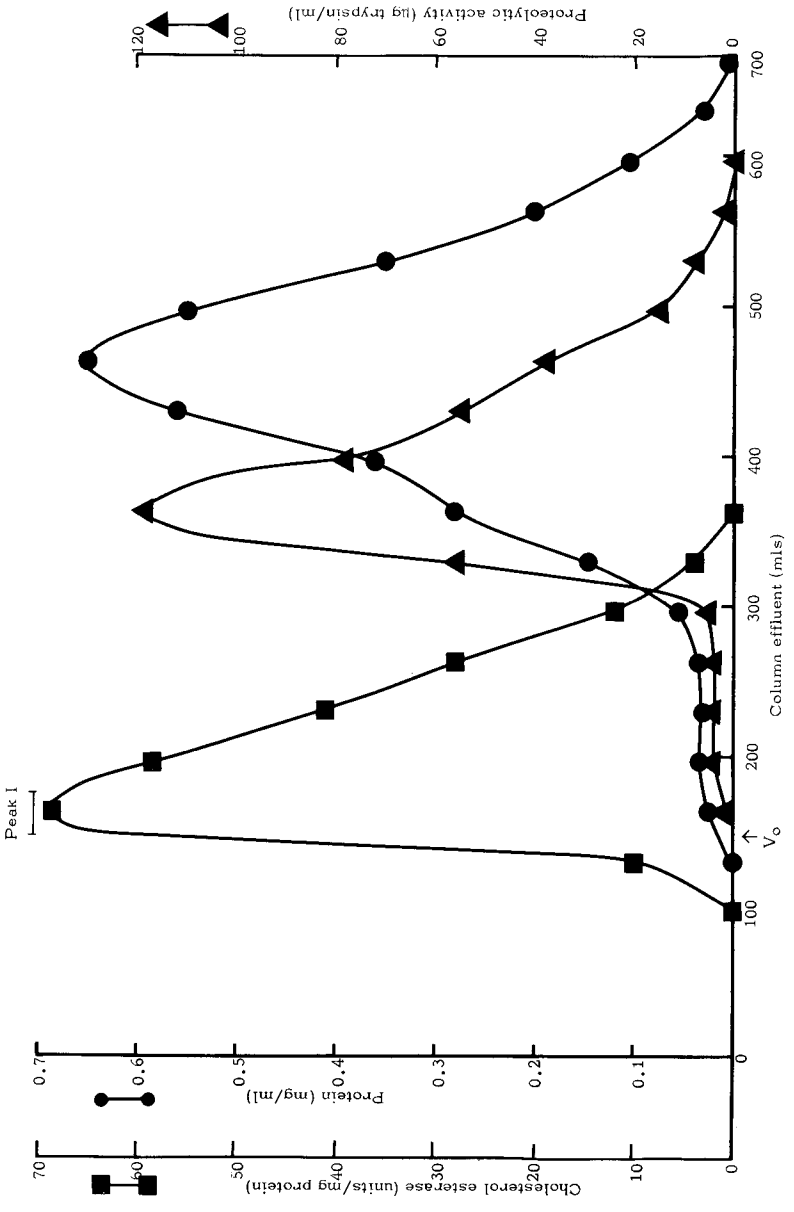
Enzyme assay

Cholesterol ester hydrolytic activity was assayed by incubation of 0.75 ml of 0.25 M tris-HCl buffer, pH 7.4, with 0.75 ml of column effluent and 1.50 ml of heat-treated human plasma (10,11). The latter contained 4 mM cholesterol esters. Taurocholate and taurodeoxycholate were added to concentrations of 4 mM and 12 mM respectively (12,13).

Cholesterol esterifying activity was assayed by incubation of 2.5 ml of 0.03 M Veronal-acetate buffer, pH 5.2, with 1.0 ml of a 95 mM solution of taurocholate, 1.0 ml of column effluent and 0.5 ml of a substrate solution of 20 mM cholesterol and 40 mM oleic acid in ethanol (14).

Assay mixtures were pre-incubated for 30 minutes before addition of substrate and the complete systems incubated for 2 hours at 37°C.

After incubation 0.5 ml of assay system was extracted according to the



1 The separation of pancreas powder extract on Sephadex G-200

method of Folch, Lees and Sloane-Stanley (15). Cholesterol ester and free cholesterol in Folch extracts were separated on Florisil columns according to Carroll (16). The solvent of each fraction was removed by evaporation under nitrogen and the cholesterol (both free and esterified) estimated by the method of Webster (17).

One unit of activity is defined as the amount of enzyme catalysing the esterification or production of 1 μmole of free cholesterol per hour.

Proteolytic activity was assayed by the method described by Kunitz (18), using casein as substrate. Activities were compared with that of a standard solution of pure trypsin and results expressed in μg of trypsin.

Protein estimations of the column effluent were carried out using the Technicon Autoanalyster (Technicon Methodology Sheet, Protein 1C). Bovine serum albumin was used as reference protein.

Enzyme separation

One gram of Viokase powder was extracted with 15 ml distilled water for 30 minutes at 4°C. The debris was removed by centrifugation at 3,000 rpm for 15 minutes. Five mls of supernatant (containing approximately 100 mg protein) were mixed with 75 mg sodium taurocholate and incubated for 10 minutes at 37°C. This mixture was then applied to a preparative Sephadex G-200 column (60 cm x 3.25 cm diam.) of bed volume 500 ml and void volume 145 ml. The column was eluted with a 40 ml/hr upward flow of 0.05 M formate buffer, pH 7.4. As the cholesterol esterase was eluted at the void volume, this will be referred to as Peak I (Figure 1).

Thirty mls of Peak I solution containing approximately 2 mg protein and 150 units of cholesterol esterase, were concentrated to a volume of 6 mls by overnight dialysis against a 20% solution of polyvinyl pyrrolidone (PVP). A 60% loss in enzyme activity occurred during this treatment.

It was observed that during this process a white precipitate was formed in the dialysis sac. The precipitate was subsequently shown to contain all the cholesterol esterase activity and was solubilised by the addition of sodium

chloride and calcium chloride to final concentrations of 0.75 M and 0.025 M respectively. The salt additions were found to be necessary to ensure complete removal of lipid in the subsequent extraction. The resultant solution was then agitated continuously at 4°C with 5 times its own volume of ether. On separation of the aqueous and ether layers, the lower aqueous layer, containing the delipidised cholesterol esterase, was freed of residual ether by a stream of nitrogen bubbled through the solution.

The insoluble complex formed during concentration of a Peak I preparation was removed by centrifugation and Folch extracted. Qualitative examination of the extracted lipid by thin-layer chromatography revealed that the bulk of the lipid was made up of the more polar classes of lipid, namely free fatty acids and phospholipids, although small amounts of both free and esterified cholesterol and a trace of triglycerides were also detected.

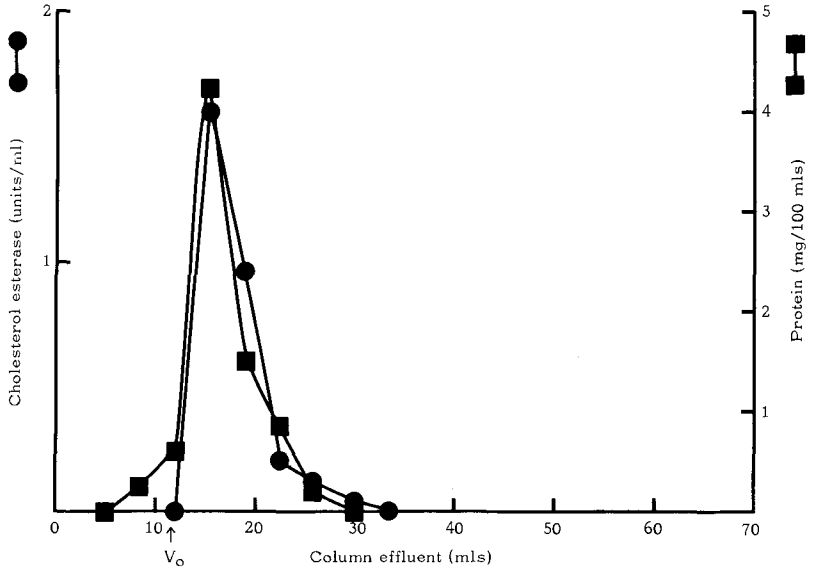
A 2 ml aliquot of the delipidised Peak I solution containing 20 units of cholesterol esterase activity was chromatographed on a Sephadex G-200 column (28 cm x 1.50 cm diam.) of bed volume 50 ml and void volume 11 ml. The column was eluted by a 15 ml/hr downward flow of buffer. The column was calibrated with proteins of known molecular weight, in a similar manner to that described by Andrews (19,20) (Table 1).

TABLE 1

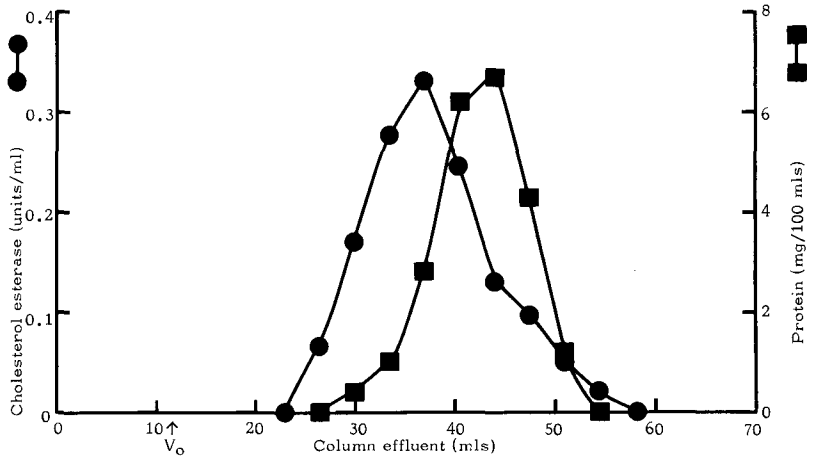
K_{av} values of standard proteins, calculated from their elution volumes from Sephadex G-200

Protein	Elution Volume (Ve)	K_{av}	Molecular Weight	Amount Applied	Monitor
Trypsin	36 ml	0.65	24000	2 mg	Enzyme assay
Pepsin	33 ml	0.57	36000	25 mg	UV
Bovine serum albumin	28 ml	0.44	67000	25 mg	UV

Molecular weight values were obtained from Documenta Geigy (1962)

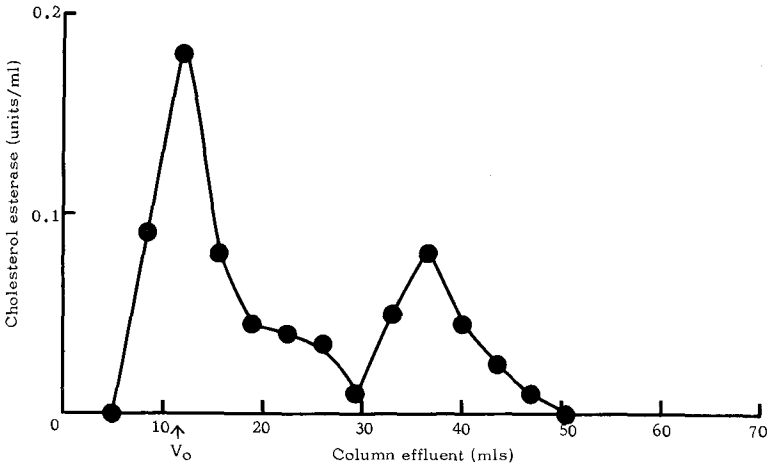


2 The separation of concentrated Peak I on Sephadex G-200



3 The separation of delipidised concentrated Peak I on Sephadex G-200

A single protein peak (Peak II) containing a total of 6 units of cholesterol esterase activity was eluted from the column (Figure 3) much later than a non-delipidised but concentrated Peak I preparation (Figure 2). Calculation of the average K_{av} of the cholesterol esterase protein contained in Peak II indicated a molecular weight of between 15,000 and 20,000 for this protein.



4 The separation of Lipomul-treated delipidised concentrated Peak I on Sephadex G-200

Two mls of delipidised Peak I solution containing 20 units of cholesterol esterase activity were incubated for 30 minutes at 37°C after the addition of 0.06 ml Lipomul. The mixture was chromatographed on the smaller Sephadex G-200 column (Figure 4). Fifty-five per cent of the recovered cholesterol esterase activity was eluted at the void volume, forty-five per cent remaining as Peak II.

Throughout the chromatographic studies the different enzyme preparations showed the same activity ratio for hydrolytic and esterifying reactions (Table II).

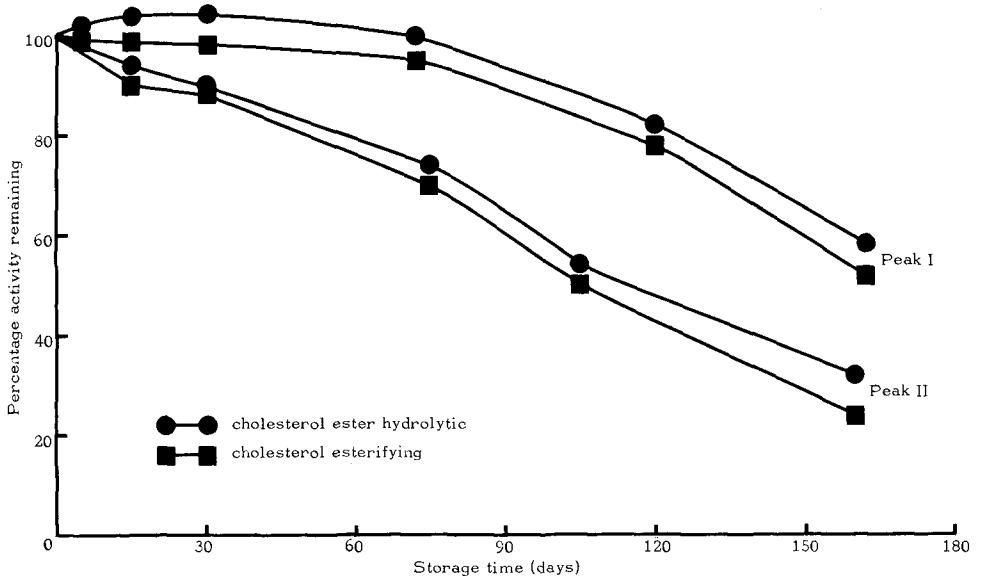
TABLE II

Comparison of the ratios of cholesterol ester hydrolytic and cholesterol esterifying activities of the enzyme preparations obtained during column chromatographic separations

Enzyme Preparation	Hydrolytic Activity (units/ml)	Esterifying Activity (units/ml)	Ratio
I	5.15	1.32	3.9:1
CI	8.40	2.20	3.8:1
DCI	8.15	2.00	4.1:1
II	0.49	0.12	4.1:1

I : Peak I preparation
II : Peak II preparation
CI : Concentrated Peak I solution
DCI : Delipidised concentrated Peak I solution

Peak I and Peak II preparations were assayed for both hydrolytic and esterifying activities over a period of 160 days storage at 4°C. Figure 5 shows the activity losses with time. The curves have two obvious features. The Peak I preparation was more stable than the Peak II preparation,



5 The stability of cholesterol ester hydrolytic and cholesterol esterifying activities during storage

retaining 50-60% of its original activity after 6 months. Also the ratio of the two activities in both preparations remained approximately the same throughout storage.

DISCUSSION

Cholesterol esterase, when extracted from an acetone-extracted pancreas powder, has been shown in the present work to resemble other lipases (1-6) in its capacity to form a soluble high molecular weight complex with lipid. The formation of this complex does not appear to be simply the collection of enzyme sub-units at a lipid-water interface, which occurs during normal lipolytic action (21). The complex appears to be lipoprotein in nature since during concentration of Peak I solution, as well as water being removed from the solution, some PVP may have migrated into the solution, allowing the

formation of an insoluble complex between the enzymic lipoprotein and PVP. The lipoprotein nature of Peak I enzyme is thus indicated since plasma lipoproteins are known to produce insoluble complexes with PVP, which are resolubilised in dilute salt solutions (22).

The molecular weight value of 15,000 to 20,000 obtained for the Peak II enzyme preparation is lower than those reported earlier for other lipolytic enzymes. The majority of these reports describe the production of a low molecular weight lipase (or esterase) having a molecular weight of between 35,000 and 40,000. Morgan et al, however, reported lipolytic activity in a preparation of about 20,000 molecular weight, which was described as possibly being the monomeric form of the 40,000 molecular weight enzyme (23).

The cholesterol esterase sub-units were re-aggregated into an apparently high molecular weight complex on incubation with Lipomul, although the lipid emulsion used for this purpose was excluded from Sephadex G-200 and it is possible that the low molecular weight enzyme units were simply adsorbed in their low molecular weight form onto this emulsion. Therefore it cannot be confirmed irrefutably that a lipoprotein complex, similar to that originally isolated from Viokase, is being re-formed during this treatment.

Although cholesterol esterase has been separated into sub-units of comparatively low molecular weight, no evidence has been produced as to its molecular state during enzyme assay. Since it has been suggested that the active form of cholesterol esterase is a polymer with bile salts (9), it is possible that polymerisation with either bile salts or substrate lipid could occur in the assay systems used here.

The observation of similar values for the ratios of cholesterol ester hydrolytic to cholesterol esterifying activities shown by the different enzyme preparations is consistent with reports (24,25) postulating that the same enzyme is involved in both reactions. Murthy and Ganguly (14) have claimed to be able to separate hydrolytic and esterifying activities by the use of calcium phosphate gel, but this has yet to be confirmed.

The results of the storage experiments indicate that both forms of the enzyme are reasonably stable, but that the high molecular weight lipoprotein form is the more stable. These findings show the same pattern of protection as that reported by Schoor and Melius for pancreatic lipase (26). These workers showed that the presence of lipid, when associated with the enzymic protein, afforded good protection from denaturation.

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