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AN IMPROVED LARGE SCALE PROCEDURE FOR THE PURIFICATION OF PORCINE PANCREATIC LIPASE

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Summary

A modified procedure for purifying porcine pancreatic lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) is described. In comparison to the previous procedure reported by Verger, R., de Haas, G.H., Sarda, L. and Desnuelle, P. (1969) Biochim. Biophys. Acta 188, 272—282) it is more rapid, more reproducible and results in a purer enzyme preparation. No colipase could be detected in the mixture of isoenzymes and, naturally, in the different separated lipases. In this process, butanol treatment is omitted. After pancreas powder extraction, a batch procedure was used for adsorption on DEAE-cellulose. Sephadex filtration (pH 8.0) was made in a larger size column. Finally the isoenzymes were separated on CM-cellulose as in the Verger procedure, but under slightly modified conditions. Lipase L_B was fully homogeneous as judged by end group determination, gel electrophoresis (in the presence or absence of sodium dodecyl sulfate) and sedimentation equilibrium.

Introduction

The procedure mostly used for the purification of lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) from porcine pancreas is that described in 1969 by Verger et al. [1]. It essentially includes an aqueous extraction of a defatted pancreas powder, the partial delipidation of the extracts by *n*-butanol and two chromatography steps on DEAE-cellulose (at pH 8.0) and Sephadex G-100. Two isoenzymes, lipases L_A and L_B, later shown to differ by the composition of their sugar moiety [2] were separated by chromatography on CM-cellulose.

However, when this procedure was followed by the present authors, lower yields were obtained in the first steps of the preparation. Difficulties were encountered with the DEAE-cellulose chromatography. The column often became clogged by small amounts of lipids remaining in the lipase solution.

Moreover, the preparations were reported to be contaminated with varying proportions of colipase. A complete or partial removal of the cofactor required an additional chromatography step on hydroxyapatite or concanavalin A-Sepharose [3] or guanidine-mercaptoethanol treatment followed by Sephadex G-100 filtration [4].

In earlier studies on the primary structure of porcine lipase [5], the original procedure of Verger et al. [1] was progressively modified to reduce the purification time to a minimum, improve the reproducibility of the technique and release the colipase contaminant.

Materials and Methods

Materials. The use of commercial delipidated pancreas powder as starting material for the purification of lipase was first recommended by Dr. R. Verger. The powder employed in the present work was from Choay-Chimie (Paris, France). DEAE-cellulose (DE-11) and CM-cellulose (CM-32) were purchased from Whatman Biochemicals Ltd. (Maidstone, U.K.). Aquacide III, from Calbiochem (San Diego, U.S.A.), was employed in some preparations for the concentration of enzyme solution in dialysis bags.

Activity determination. The lipase activity was measured titrimetrically against Methocel-stabilized long-chain triglyceride emulsions [6] or emulsions of purified tributyrin [7]. With both substrates, one lipase unit was defined as the amount of enzyme hydrolysing one ester bond per min. The specific activity was taken as the number of units per mg protein, taking the absorbance value of $E^{1\%}_{1cm} = 13.3$ at 280 nm for the pure enzyme [8]. The specific activity of lipase towards emulsified tributyrin is known to be about twice as high as that towards long-chain triglycerides [6].

N-terminal residues. 20 nmol lipase were oxidized by performic acid, lyophilized, dansylated in 8 M urea and precipitated by 10% trichloroacetic acid [9]. After hydrolysing for 4 h in boiling 5.6 M HCl, the liberated dansyl amino acids were determined according to Charles et al. [10]. Protein oxidation, prior to dansylation, removed any remaining traces of proteolytic activity, and favored reagent accessibility. Dansylation analysis (without the use of urea and trichloroacetic acid precipitation) was also made for testing low molecular weight contaminants [10].

Sialic acid determination. Glycoprotein samples containing 5—10 μg sialic acid were incubated in 100 μl 0.05 M H_2SO_4 at 80°C for 1 h and the liberated sialic acid was determined according to Warren [11], with half the proportions of reagents recommended by the authors. The standard was sheep thyroglobulin known to contain 1.4% sialic acid (w/w).

Results

Purification procedure

All assays were carried out at 0—4°C.

100 g pancreas powder were suspended in 1 l water/1 mM benzamidine/1 mM phenylmethylsulfonyl fluoride. The mixture (adjusted to pH 9.0) was mechanically stirred for 2 h and centrifuged.

The supernatant was mixed with 2 l water and 2.2 l DEAE-cellulose (previously equilibrated with a 5 mM Tris · HCl buffer (pH 8.0)/3.3 mM $CaCl_2$/18.2 mM NaCl/1 mM benzamidine (buffer I)) and stirred for 2 h. After decanting, the thick DEAE-cellulose suspension was poured into a 9 cm diameter column. The column was washed with 1 l buffer I and then eluted by a linear NaCl gradient (18.2—200 mM) in the same buffer (Fig. 1).

After $(NH_4)_2SO_4$ precipitation of the lipase fractions (50 g $(NH_4)_2SO_4$ per 100 ml), the precipitate was taken up in 50 ml 5 mM Tris · HCl buffer (pH 8.0)/3.3 mM $CaCl_2$/0.4 M NaCl/0.77 mM NaN_3/1 mM benzamidine (buffer II). The resulting suspension (to which 50 μmol phenylmethylsulfonyl fluoride was added) was dialyzed for 20 h against buffer II. The clear solution was passed through a Sephadex G-100 column (6 × 220 cm) eluted by buffer I. The chromatography profile was comparable to the one given in Fig. 5 of the Verger publication [1]. Lipase was found under the first peak (fraction 3240—3720 ml) with high specific activity (Table I). The second, inactive peak (fraction 3720—4200 ml) was larger than in the Verger procedure. Colipase was present in a later fraction (4728—5040 ml). The fractions containing lipase were stored at −10°C.

Two preparations purified up to Sephadex elution were concentrated by Aquacide III, dialyzed for 24 h against 50 mM sodium acetate buffer (pH 5.0) and applied to a CM-cellulose column equilibrated with the same buffer and then eluted by a pH gradient (5.0—5.7). The elution pattern (Fig. 2) shows the separation of a small, inactive and strongly anionic peak and of four active peaks. The largest peak which emerged last, corresponded to the weakly-anionic lipase B. characterized by Verger et al. [1]. The location of the next two peaks suggested that they were formed by lipase L_A which had split into two components designated L_{A1} and L_{A2}. The existence of an additional enzyme form designated L_C was not previously reported. At the end of the procedure, 142, 31, 19 and 23 mg of lipases L_B, L_{A1}, L_{A2} and L_C, respectively, were obtained. The purification procedure and activities are given in Table I.

Properties of purified lipase

Lipase L_C, L_{A1}, L_{A2} contained, respectively, 0.4, 0.4 and 0.1 mol sialic acid

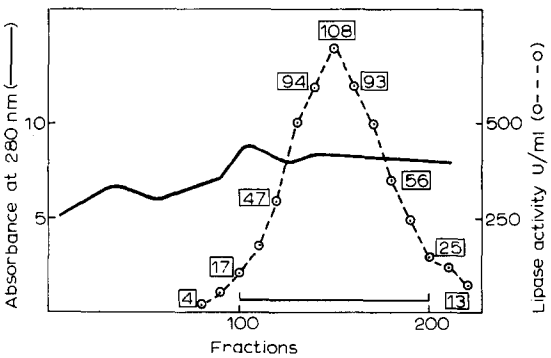

Fig. 1. Elution profile from DEAE-cellulose. The column (1 × 28 cm) was eluted by a linear NaCl concentration gradient. (18.2—200 mM) in buffer I. Chamber volume, 4 l. Flow rate, 300 ml/h. 27.5-ml fractions. The figures along the lipase peak indicate the specific enzyme activity in the fractions.

TABLE I

FLOW SHEET OF THE PURIFICATION PROCEDURE

Step	Protein (mg)	Lipase units * (10^{-3})	Specific activity *	Yield
Aqueous extract	— **	1900	— **	100
DEAE-cellulose	—	1200	—	63
$(NH_4)_2SO_4$ precipitation dialysis	2160	916	423	48
Sephadex filtration	290	780	2700	41
Aquacide treatment dialysis	460 ***	1060 ***	2300	28
CM-cellulose				
Lipase L_B	142	485	3400	13
Lipase L_{A1}	31	96	3140	2.5
Lipase L_{A2}	19	55	2800	1.5
Lipase L_C	22	44	2020	1.1
Total	214	680	—	18

* In these assays, the substrate was Methocel-stabilized emulsions of long-chain triglycerides.

** Protein and specific activity values are not given before $(NH_4)_2SO_4$ precipitation which removes most of the nucleic acids strongly absorbing at 280 nm.

*** Pool of two preparations.

per mol protein. Lipase L_B was devoid of this compound. The results can be compared to those of Plummer and Sarda [2] who found 0.3 mol sialic acid per mol lipase L_A and no trace of this compound in L_B.

After Sephadex filtration, lipase preparation yielded a single band by electrophoresis at pH 8.5 on a 1% sodium dodecyl sulfate (SDS)/5.6% polyacrylamide gel. As shown in Fig. 3 (tube 3) a single band was also obtained with lipase L_B in the absence of SDS. Lipase L_{A1} and L_{A2} each yielded two bands (tubes 1 and 2) and their mixture with lipase L_B gave a total of three bands under the same conditions. Gel electrophoresis assays performed as in tube 4 but with

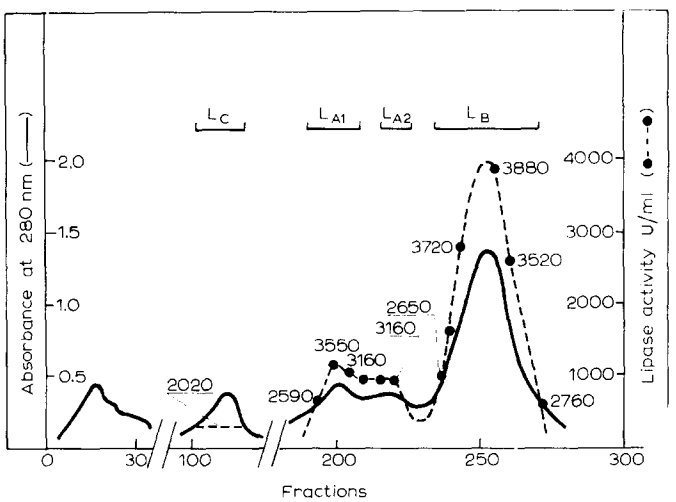

Fig. 2. Chromatography on CM-cellulose. The column (2.5 × 40 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) was charged with two preparations after Sephadex filtration and it was eluted by a pH gradient (5.0—5.7). Chamber volume, 1.2 l. 6-ml fractions. Flow rate, 35 ml/h.

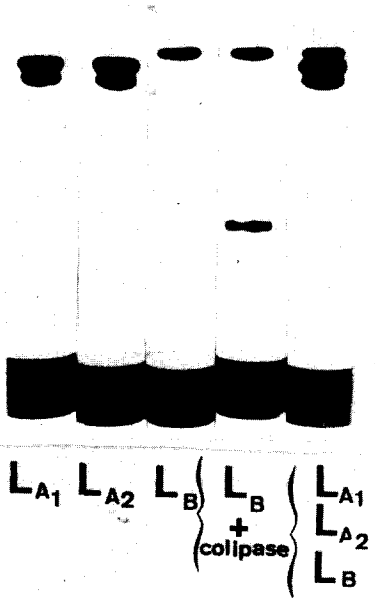

Fig. 3. Gel electrophoresis at pH 8.5 (350 V for 2 h, in the absence of SDS) of the lipases separated by CM-cellulose. 7.5% polyacrylamide gel. Tube size 0.6 × 14 cm. 1, lipase L_{A1}; 2, lipase L_{A2}; 3, lipase L_B; 4, lipase L_B (30 μg) + colipase (137 μg); 5, $L_{A1} + L_{A2} + L_B$.

decreasing amounts of added colipase, indicated that the limit of detection of colipase in lipase preparation was less than 1% (w/w).

No traces of N-terminal residues other than serine, already known to be N-terminal in porcine lipase [5], could be detected in the separated lipases L_{A1}, L_{A2}, L_B, and L_C and also in their unfractionated mixture. The two possible N-terminal residue derivatives of colipase are valine or glycine [12,13]. Taking into account the relative stability of the above derivatives in boiling HCl compared to that of dansyl serine, this result suggests that lipase preparation was not contaminated by colipase impurity.

Lipase L_B was found to be homogeneous by sedimentation equilibrium studies. The partial specific volume ($\bar{v}$) of the enzyme was measured by an Alton Parr digital microdensimeter and found to be 0.73 at 20°C. The corresponding molecular weight value, similar to that already reported by Verger et al. [1], was 50 000 ± 1000.

Using tributyrin as substrate [7] purified lipase L_B had a specific activity amounting to 7500 units/mg. As expected from previous investigations on the activating effect exerted by colipase in the absence of bile salts [6], this activity rose to 9500 units/mg on addition of 2 mol cofactor per mol enzyme. By contrast, the activity was 250 units/mg in the presence of 2—4 mM taurodeoxycholate; the activity was 8000 units/mg in the presence of both taurodeoxycholate and colipase (2 mol per mol lipase).

Discussion

The above reported procedure is a modification of that worked out by Verger et al. [1]. The principal difference is that a batch procedure was used for

adsorption on DEAE-cellulose and directly performed on pancreatic powder extracts. The elution on column which followed, was therefore rapid. On account of the large volume of solution and the great amount of DEAE-cellulose used, the batch-step was not affected by small quantities of remaining lipids. The latter impurities were released either in the supernatant or adsorbed without disadvantage in the DEAE-cellulose bulk. Therefore, butanol treatment (used in Verger procedure for the removal of an acidic phospholipid) was omitted, thus improving the yield without affecting the purity of the final product. Likewise one can surmise that almost all the defatted pancreas powders supplied by various firms and laboratories could be suitable for use in the modified procedure.

It was observed by the present authors that when $(NH_4)_2SO_4$ precipitation was carried out prior to the DEAE-cellulose step it induced severe lipase losses. A further difference was that the Sephadex column was buffered at pH 8.0 to increase lipase stability. In this manner it was possible to prepare 300 mg highly purified lipase in about a week with a reproducible yield of about 40%. Moreover it has been recently shown that the same Sephadex column can be loaded with twice the quantity indicated. The technique could be scaled up with appropriate equipment.

A further chromatography of the Sephadex eluate on CM-cellulose separated a small amount of an inactive impurity and four isoenzymes L_{A1}, L_{A2}, L_B, L_C (instead of two in the Verger procedure). It was confirmed that the less anionic lipase L_B was by far the major form existing in porcine pancreas (about 70% of the total activity).

No colipase impurity could be detected by various tests in the unfractionated isoenzymes after Sephadex filtration or in the different separated lipases. Colipase-lipase complex dissociation was favored by the large dilution of lipase solution in batch step and in Sephadex filtration.

Serine is the N-terminal residue of the different forms of lipase. On gel electrophoresis, lipases L_{A1} and L_{A2} further split into two bands suggesting the existence of other isoenzymes. It was not possible to confirm the great difference in the isoleucine content of lipases L_A and L_B reported by Verger et al. [1]. The amino acid compositions were found very similar. Therefore a likely assumption is that the isoenzymes differ only in the sugar moieties.

In conclusion, the lipase preparations after Sephadex filtration could probably be used for a number of investigations including sequence determination. Lipase L_B is somewhat purer and chemically better defined but its isolation is associated with a marked decrease in yield from 40 to 13%.

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References

1 Verger, R., de Haas, G.H., Sarda, L. and Desnuelle, P. (1969) Biochim. Biophys. Acta 188, 272—282
2 Plummer, T.H. and Sarda, L. (1973) J. Biol. Chem. 248, 7865—7869

3 Verger, R., Rietsch, J., van Dam-Mieras, M.C.E. and de Haas, G.H. (1976) J. Biol. Chem. 251, 3128—3133
4 Donnér, J. (1976) Acta Chem. Scand. 30, 430—434
5 Rovery, M., Bianchetta, J. and Guidoni, A. (1973) Biochim. Biophys. Acta 328, 391—395
6 Maylié, M.F., Charles, M., Astier, M. and Desnuelle, P. (1973) Biochem. Biophys. Res. Commun. 52, 291—297
7 Sémériva, M., Dufour, C. and Desnuelle, P. (1971) Biochemistry 10, 2143—2149
8 Sémériva, M., Chapus, C., Bovier-Lapierre, C. and Desnuelle, P. (1974) Biochem. Biophys. Res. Commun. 58, 808—813
9 Gros, C. and Labouesse, B. (1969) Eur. J. Biochem. 7, 463—470
10 Charles, M., Erlanson, C., Bianchetta, J., Joffre, J., Guidoni, A. and Rovery, M. (1974) Biochim. Biophys. Acta 359, 186—197
11 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
12 Maylié, M.F., Charles, M., Gache, C. and Desnuelle, P. (1971) Biochim. Biophys. Acta 229, 286—289
13 Erlanson, C., Fernlund, P. and Borgström, B. (1973) Biochim. Biophys. Acta 310, 437—445