Preparation of Porcine Pancreatic Lipase Free of Co-lipase Activity

JAKOB DONNÉR

Department of Physiological Chemistry, University of Lund,
P.O.B. 750, S-220 07 Lund 7, Sweden

Porcine pancreatic lipase L_B was purified and released from co-lipase activity by reduction with β -mercaptoethanol in the presence of guanidine chloride. The amino acid and carbohydrate composition of the enzyme is presented. The following physical constants were measured or calculated: Molecular weight 52 000, sedimentation coefficient ($s_{20,w}^0$) 4.0 S, diffusion coefficient ($D_{20,w}^0$) 6.7×10^{-7} cm² s⁻¹, Stokes' radius (r) 30.3 Å, partial specific volume ($\bar{v}$) 0.72 cm³ g⁻¹, frictional ratio (f/f_0) 1.23 and isoelectric point (pI) 5.18.

Pancreatic lipase (EC 3.1.1.3) is inhibited by conjugated bile salts in concentrations above their CMC (critical micellar concentration).¹⁻³ Addition to such systems of co-lipase, a polypeptide co-factor for lipase,^{4,5} restores the activity of lipase.^{1,2} A method for preparing a pure lipase – as judged by disc electrophoresis – from porcine pancreas was described by Verger *et al.*⁶ In our hands lipase prepared according to that method still exhibits co-lipase activity³ to an extent of 20–30 % of the lipase activity. Similar values have also been reported by others.⁷

The mechanism of the interaction between lipase, co-lipase and conjugated bile salts is being investigated at present in this laboratory. It was thus of interest to see whether the residual co-lipase activity was an inherent property of lipase or a property due to contamination. A method is presented in this paper by which the co-lipase activity was lowered considerably. Besides, the resulting lipase was characterised further with respect to a few physico-chemical properties and for

comparative purposes the carbohydrate and amino acid composition is also presented.

EXPERIMENTAL

Determination of lipase and co-lipase activity. Determination of lipase activity was done potentiometrically at pH 8.0 as described by Borgström *et al.*² using a Mettler titrator. Co-lipase activity is defined as the hydrolysis of tributyrin at pH 7.0 in the assay system for lipase modified such that the incubation mixture is 0.004 M with respect to sodium taurodeoxycholate and contains 50 lipase units of rat pancreatic lipase.¹ A lipase or a co-lipase unit of activity expresses the number of micromol of butyric acid released per min in the assay system used at 25 °C.

Determination of protein. Protein concentration was determined by measuring the absorbance at 280 nm. When the molecular weight of lipase was determined on a Sepharose 6B column a fluorescence method was used.⁸

Preparation of porcine pancreatic lipase free of co-lipase activity. Lipase was prepared from fresh porcine pancreas by the method of Verger *et al.*⁶ and the homogeneity of the preparation was verified by disc electrophoresis. According to the method mentioned a delipidated water extract is chromatographed on DEAE-cellulose (pH 8.0) using a salt gradient, on Sephadex G-100 and finally on CM-cellulose (pH 5.0) whereby two lipases, lipase L_A and L_B , respectively, are separated when eluted from the CM-cellulose column with a pH-gradient.

A sample of lipase L_B was taken (specific activity 11 300, E (280 nm, 1 %) = 13.3) containing 240 000 lipase units and 54 000 co-lipase units (0.225 co-lipase units per unit of lipase) in 0.020 M Tris-HCl pH 7.0 and 0.02 % NaN₃ in a total volume of 20 ml. To the sample was added guanidine chloride, β -mercaptoethanol and EDTA to final concentrations of 0.7 M, 0.4 M, and 0.002 M, respectively. It was then

kept at 4 °C for 4 h under stirring. After centrifugation at 35 000 *g* for 20 min the sample was put on a Sephadex G-100 column (100 × 5 cm) equilibrated with 0.020 M Tris-HCl pH 7.0, 0.4 M β -mercaptoethanol, 0.002 M EDTA and 0.02 % NaN_3 . After elution with the same buffer (flow rate 55 ml/h, fraction time 20 min) fractions containing more than 100 lipase units per ml were pooled. This pool of 200 ml was dialyzed against 5 l of 0.050 M Tris-HCl pH 7.0 and 0.02 % NaN_3 for 8 h twice.

Disc electrophoresis. To check the purity of the preparations disc electrophoresis was performed. A 7.5 % polyacrylamide gel was used with a pH 8.9 system⁹ and the gels were stained with Coomassie Brilliant Blue G 250.¹⁰

Amino acid analysis. Amino acid analysis was done in a Jeol amino acid analyser* after 25 and 72 h hydrolysis in 6 M HCl at 110 °C *in vacuo*. Only samples that had been subjected to performic acid oxidation were taken after 18 h hydrolysis. 1.7 mg of protein were used for the analysis. After lyophilisation the samples were dried over phosphorus pentoxide in a drying gun with boiling toluene to constant weight as judged by measurements on a Cahn microbalance. The individual values obtained for each residue were averaged except in the cases of isoleucine, leucine, proline, and valine for which the highest concentration determined after 72 h was used, and of serine and threonine for which extrapolations to zero time were done. Norleucine was incorporated as internal standard. The 18 h values were used for cystine measured as cysteic acid and for methionine measured as methionine sulfone after performic acid oxidation. The cysteic acid value was corrected as described by Moore.¹¹ Tryptophan was determined by spectrophotometry.¹²

Carbohydrate analysis. The lyophilized samples were dried over phosphorus pentoxide as described above. Neutral sugars were determined with the aid of gas chromatography according to a method by Lindahl¹³ which had been modified by Carlstedt.** 2.9 mg of lipase L_B was used for the analysis. Hexosamine was determined on a Biocal BC 200 amino acid analyzer.¹⁴*** 2.4 mg of lipase L_B was used for the analysis.

Molecular weight determinations. a. *Sedimentation equilibrium analysis.* Sedimentation equilibrium analysis for calculation of the molecular weight was performed according to the technique of Yphantis.¹⁵ The protein was dissolved in 0.1 M sodium chloride to a final concentration of 0.5 mg/ml and centri-

fuged at 72 000 *g* for 23 h at 20 °C in a Spinco Model E ultracentrifuge.* An interferometric optical system was used. The partial specific volume of the protein was calculated from the amino acid and carbohydrate composition using the amino acid specific volume values given by Schachman¹⁶ and carbohydrate specific volume values given by Gibbons.¹⁷ The weight of each amino acid and carbohydrate species per unit weight of protein was multiplied by its specific volume value. The sum of the obtained volume values is the volume of the protein per unit weight.

b. *Sedimentation velocity analysis.* Sedimentation velocity analysis for determination of the sedimentation coefficient was done as described by Schachman.¹⁶ The protein was dissolved at three different concentrations in 0.1 M NaCl and 0.003 M potassium phosphate pH 8.0 and centrifuged at 52 000 rpm at 20 °C in a Spinco Model E ultracentrifuge with a schlieren optical system. The sedimentation coefficient *s* was combined with the free diffusion coefficient *D* and the partial specific volume $\bar{v}$ in the Svedberg equation

$$M = (s/D)[RT/(1 - \bar{v}\rho)]$$

(where *R* is the gas constant, ρ solvent density and *T* the absolute temperature) to yield the molecular weight. The free diffusion coefficient was obtained from the Stokes' radius by use of the Stokes-Einstein equation¹⁸

$$D = kT/6\pi\eta r$$

where *k* is the Boltzmann constant and η the viscosity of the medium. The Stokes' radius was calculated from gel filtration data according to the method of Laurent and Killander.¹⁹ Sephadex G-100 was used and 7×10^{-8} cm was chosen as the value of r_s .¹⁹ The concentration of the dextran chains, $\bar{L}$, was calculated by determining the K_{av} for bovine serum albumin and soybean trypsin inhibitor on the gel when chromatographed at 22 °C in a phosphate buffer, 0.10 M pH 6.0 and 0.05 M pH 7.0, respectively. The Stokes' radius for the two proteins was calculated from the Stokes-Einstein equation quoted above.¹⁸ For soybean trypsin inhibitor *D* was calculated from *s* and molecular weight data given by Sheppard *et al.*²⁰ using the Svedberg equation and *D* for human serum albumin has been measured by Ehrenberg.²¹ Lipase was chromatographed at 22 °C in 0.10 M sodium chloride and 0.003 M potassium phosphate pH 8.0.

c. *Amino acid composition.* Calculations of the molecular weight were also done using the

* The analysis was kindly done by Miss Inger-Marie Karlsson and Dr. J. O. Jeppson at the Department of Clinical Chemistry, Malmö.

** This was kindly done by Dr. I. Carlstedt, Department of Medical Chemistry 2, Lund.

*** This was kindly done by Dr. D. Heinegård, Department of Medical Chemistry 2, Lund.

* The determination was kindly done by Doc. H. Pertoft at the Institute of Medical Chemistry, University of Uppsala.

amino acid composition as described by De-laage.^{22*}

d. *Gel chromatography of reduced protein in guanidinium chloride.* The molecular weight of lipase was determined by means of gel chromatography in a 120 × 0.9 cm glass column with Sepharose 6B (Pharmacia Lot. No. 5686) as described by Mann and Fish.²³ The proteins were dissolved in 0.5 ml of the eluent, 6 M guanidinium chloride and 0.05 M potassium phosphate pH 7.0 for the reduction. The solution was made 0.01 M with respect to dithiothreitol and when carboxymethylation was to be started the solution was made 0.025 M with respect to iodoacetic acid. The following proteins were used for calibrating the column; bovine serum albumin (M 65 400) (Ref. 24), the heavy chain of human γ -globulin (M 53 000) (Ref. 25), ovalbumin (M 40 000) (Ref. 24).

Frictional ratio. The frictional ratio was calculated from the expression $f/f_0 = r/(3\eta M/4\pi N)^{1/3}$ where N is Avogadro's number.

Isoelectric focusing. The isoelectric focusing was performed as described by Vesterberg and Svensson²⁶ in a column of 110 ml volume purchased from LKB-Produkter. An Ampholine (LKB-Produkter) pH gradient between pH 4 and 6 was used and 0.1 mg of lipase was introduced into the column. The water circulating through the mantle was kept at +3 °C in a thermostated bath. The electrolysis was interrupted after 72 h and fractions of 1 ml volume were taken from the column and the lipase activity as well as the pH of the fractions were measured. The pH measurements were done at +3 °C in a Radiometer (Copenhagen) pHM 71 Mk 2.

RESULTS

Pancreatic lipase free of co-lipase activity. The dialyzed pool contained 125 500 lipase units and 618 co-lipase units, i.e. 0.005 co-lipase units per unit of lipase. The specific activity of lipase was unchanged by the procedure and the lipase was homogenous by disc electrophoresis. When co-lipase activity was looked for in the eluate from the G-100 column a peak was found with a K_D -value of 0.64. This result could not be reproduced if the sample of lipase was simply rechromatographed on G-100 in the presence of 0.4 M NaCl as in the original method⁶ without preceding treatment with β -mercaptoethanol and guanidine chloride.

Amino acid composition. The amino acid composition of the lipase prepared in the above mentioned manner is given in Table 1.

* These calculations were kindly done by Dr. Jan Sternby.

Table 1. Amino acid and carbohydrate contents of lipase L_B free of co-lipase activity. Number of residues per mol of lipase L_B free of co-lipase activity using the mean value (52 000) of the molecular weight determinations given in this paper.

	Exp. values	Next integers	
Ala	22.7	23	
Arg	19.9	20	
Asx	58.5	59	
Cys	5.8	6	
Glx	40.2	40	
Gly	44.0	44	
His	9.6	10	
Ile	27.3	27	
Leu	30.9	31	
Lys	22.0	22	
Met	2.8	3	
Phe	25.2	25	
Pro	27.1	27	
Ser	33.1	33	
Thr	24.9	25	
Trp	9.2	9	
Tyr	15.0	15	
Val	35.2	35	
Fucose	0.7	1	1
Galactose	0.5	1	— ^a
Galactosamine	1.4	1	2 ^a
Glucose	0.2	—	— ^a
Glucosamine	2.4	2	3 ^a
Mannose	6.1	6	6

^a In case galactose and glucose result from the deamination of galactosamine and glucosamine.

Carbohydrate composition. The carbohydrate composition is presented in Table 1 together with the amino acid composition using a value of 52 000 for the molecular weight of lipase L_B free of co-lipase activity.

Molecular weight. a. The molecular weight determined by sedimentation equilibrium ultracentrifugation was found to be $52\,000 \pm 5\%$. The value of 0.72 for the partial specific volume was used as derived from the amino acid and carbohydrate composition. When $\log C$ is plotted against r^2 a straight line is obtained. This is an indication of homogenous molecular weight distribution in the sample.¹⁵

b. The sedimentation coefficient obtained by extrapolation to zero concentration ($s_{20,w}^0$) was found to be 4.0×10^{-13} s using $s_{20,w}$ for solutions with a lipase concentration of 1.25 mg/ml, 2.5 mg/ml and 5.0 mg/ml, respectively.

Fig. 1. The function $Y = \sum_i (n_i/N_i - 1)^2$ (see text) plotted against a computer program test factor. The minima indicated by numbers correspond to molecular weight as follows: I = 36 754, II = 40 258, III = 53 245, IV = 57 746, V = 71 070.

The peaks were symmetrical. When gel filtration data were analysed according to the method of Laurent and Killander¹⁹ the Stokes' radius was found to be 30.3 Å and the diffusion coefficient ($D_{20,w}$) 7.08×10^{-7} cm²/s. These values correspond to a molecular weight of 49 500 when combined in the Svedberg equation.

c. Amino acid composition. When molecular weight was calculated according to the method of Delaage²² a value of 53 245 was obtained. The function $Y = \sum_i (n_i/N_i - 1)^2$ is represented in Fig. 1 as a function of a test factor in the computer program. n_i is the number of mol of the amino acid i per mol of protein (this value is varied by assuming different molecular weights of the protein) in the analysed material and N_i is the nearest integral. The result was the same whether methionine or cystine or both were omitted from the calculations. These amino acids occur least often (Table 1) in the protein and should be expected to influence the positions of the minima of the curve corresponding to different molecular weight values if the amino acid analysis was far from correct with respect to these amino acids. To the molecular weight value of 53 245 should be added the weight of the carbohydrate molecules in the lipase. That sum gives a molecular weight value of 55 200.

d. Gel chromatography of reduced protein in guanidinium chloride. The K_D values were

Fig. 2. Chromatography of reduced and carboxymethylated bovine serum albumin (BSA), lipase L_B free of co-lipase activity (indicated by the arrow), heavy chain of human γ -globulin and ovalbumin on Sepharose 6B in 6 M guanidine chloride.

analyzed as described by Porath.²⁷ According to that method $K_D^{1/3}$ is plotted against $M^{0.555}$. The interpolated value for lipase (see Fig. 2) corresponds to a molecular weight of 50 800.

Frictional ratio. Using the average value 52 000 of the molecular weight determinations the frictional ratio was found to be 1.23.

Isoelectric focusing. The pI value of lipase L_B free of co-lipase activity was found to be 5.18.

The physicochemical constants of lipase L_B free of co-lipase activity are summarized in Table 2 using the mean value of 52 000 for the molecular weight.

Table 2. Physicochemical properties of lipase L_B free of co-lipase activity.

Stoke's radius, r	30.3 Å
Sedimentation coefficient, $s_{20,w}^0$	4.0×10^{-13} s
Diffusion coefficient, ^a $D_{20,w}^0$	6.7×10^{-7} cm ² s ⁻¹
Partial specific volume, ^b $\bar{v}$	0.72 cm ³ g ⁻¹
Frictional ratio, f/f_0	1.23
Isoelectric point, pI	5.18

^a Derived from the Svedberg equation using a value of 52 000 for the molecular weight. ^b Derived from carbohydrate and amino acid analyses.

DISCUSSION

Co-lipase activity is found in the eluate from the G-100 column in a peak with a K_D value of 0.64 which is separate from the lipase peak. This K_D value is the same as for pure co-lipase when reduced and chromatographed under the same conditions as described for lipase L_B in this paper. It is thus probable that the contaminating amount of co-lipase is bound to lipase by a disulfide bridge.

The residual co-lipase activity exhibited by lipase L_B after the treatment described in this article could be explained by incomplete inhibition by the sodium taurodeoxycholate. Co-lipase is rich in half-cystines⁵ containing 10 mol of half-cystine per mol. It is noteworthy that the half-cystine value reported in this paper for lipase L_B free of co-lipase activity is less than half the value given by Verger *et al.*⁶ for lipase L_B .

As can be seen from the results presented compared to those given by Verger *et al.*⁶ no drastic changes in composition or in physico-chemical constants have been introduced by the treatment to which the lipase has been subjected in this work. A method, which does not result in any major modification of protein structure, is thus available for the preparation of a lipase that can be used in experiments designed to elucidate the activating effect of co-lipase on lipase activity.

Acknowledgements. I wish to thank Mr. S. Svensson for his expert technical assistance. I am also grateful to Dr. L. Fagerlind at Försvarets Forskningsanstalt, Stockholm for his generous gifts of DFP. This research was supported by Dr. P. Håkansson's Stiftelse.

REFERENCES

1. Borgström, B. and Erlanson, C. *Biochim. Biophys. Acta* 242 (1971) 509.
2. Borgström, B. and Erlanson, C. *Eur. J. Biochem.* 37 (1973) 60.
3. Maylié, M. F., Charles, M., Astier, M. and Desnuelle, P. *Biochem. Biophys. Res. Commun.* 52 (1973) 291.
4. Maylié, M. F., Charles, M., Gache, C. and Desnuelle, P. *Biochim. Biophys. Acta* 229 (1971) 286.
5. Erlanson, C. and Borgström, B. *Biochim. Biophys. Acta* 271 (1972) 400.
6. Verger, R., de Haas, G. H., Sarda, L. and Desnuelle, P. *Biochim. Biophys. Acta* 188 (1969) 272.
7. Esposito, S., Sémériva, M. and Desnuelle, P. *Biochim. Biophys. Acta* 302 (1973) 293.
8. Weidekamm, E., Wallach, D. F. H. and Flückiger, R. *Anal. Biochem.* 54 (1973) 102.
9. Maurer, H. R. *Disk-Elektrophorese*, Walter de Gruyter, Berlin 1968, p. 42.
10. Diezel, W., Kopperschläger, G. and Hofmann, E. *Anal. Biochem.* 48 (1972) 617.
11. Moore, S. J. *Biol. Chem.* 238 (1963) 235.
12. Edelhoch, H. *Biochemistry* 6 (1967) 1948.
13. Lindahl, U. *Biochem. J.* 116 (1970) 27.
14. Antonopoulos, C. A., Axelsson, I., Heinegård, D. and Gardell, S. *Biochim. Biophys. Acta* 338 (1974) 108.
15. Yphantis, D. A. *Biochemistry* 3 (1964) 297.
16. Schachman, H. K. *Methods Enzymol.* 3 (1957) 32.
17. Gibbons, R. A. In Gottschalk, A., Ed., *Glycoproteins*, BBA Library, Elsevier, Amsterdam 1966, Vol. 5, pp. 29–111.
18. Pedersen, P. L. *J. Biol. Chem.* 243 (1968) 4305.
19. Laurent, T. C. and Killander, J. *J. Chromatogr.* 14 (1964) 317.
20. Sheppard, E. and McLaren, A. D. *J. Am. Chem. Soc.* 75 (1953) 2587.
21. Ehrenberg, A. *Acta Chem. Scand.* 11 (1957) 1257.
22. Delaage, M. *Biochim. Biophys. Acta* 168 (1968) 573.
23. Mann, K. G. and Fisch, W. W. *Methods Enzymol.* 26 (1972) 28.
24. Edsall, J. T. In Neurath, H. and Bailey, K., Eds., *The Proteins*, Academic, New York 1953, Vol. 1B, p. 634.
25. Dorrington, K. J. and Tanford, C. *Adv. Immunol.* 12 (1970) 333.
26. Vesterberg, O. and Svensson, H. *Acta Chem. Scand.* 20 (1966) 820.
27. Porath, J. *Pure Appl. Chem.* 6 (1963) 233.

Received October 1, 1975.