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PURIFICATION AND FURTHER CHARACTERIZATION OF CO-LIPASE FROM PORCINE PANCREAS

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SUMMARY

Co-lipase has been purified from porcine pancreas by a method involving the following steps: acid extraction of pancreas homogenate, heat treatment, $(NH_4)_2SO_4$ fractionation, treatment with ethanol, chromatography on SP-Sephadex, chromatography on DEAE-cellulose and filtration through Sephadex G-75. The method can be applied on a relatively large preparative scale. Starting from 500 g of pancreatic tissue a yield of 30 mg of co-lipase is obtained after a 300-fold purification.

Co-lipase was characterized by physical and chemical methods. Co-lipase is a single-chain polypeptide containing about 95 residues per mole with isoleucine as the N-terminal amino acid. It has an excess of acidic residues (21) over basic residues (10) and an isoelectric point of 5.0. It contains five disulfide bridges. The molecular weight of co-lipase determined with several techniques is in the order of 10 000 with no significant difference between the value based on amino acid analysis and the values based on other methods indicating that the molecule is essentially free of non-protein components.

INTRODUCTION

Recently we reported about the physiological importance of pancreatic co-lipase as a co-factor for lipase[1]. In order to further study the mode of action of this co-factor a large-scale purification scheme has been worked out for the isolation of co-lipase starting from porcine pancreas. The method reported here takes advantage of the remarkable stability of co-lipase against heat and against acid and appears to be a more rapid and convenient method for the purification of pancreatic co-lipase on a large scale than that earlier described by Maylié *et al.*[2].

MATERIALS AND METHODS

Porcine pancreas was obtained from the slaughterhouse shortly after the death of the animal and kept on ice until processed. DEAE-cellulose (microgranular DE 52)

was purchased from Whatman, SP-Sephadex, Sephadex G-25, Sephadex G-75 and Sephadex G-100 were obtained from Pharmacia, Uppsala and Bio-Gel P-30 from Bio-Rad Laboratories. Ampholine carrier ampholytes (LKB 8141) in the pH range of 3–10 were products from LKB-produkter, AB, Stockholm. Bovine serum albumin and bovine pancreatic ribonuclease were purchased from BDH, egg albumin from Serva, bovine α-chymotrypsinogen and pepsin from Sigma, human serum albumin from Mann Research Lab. Bile salts were prepared as described by Hofmann[4]. Tributyrine was obtained from BDH and subjected to fractional destillation before use.

Lipase, free of co-lipase, used for co-lipase determinations was prepared in two chromatographic steps starting from rat pancreatic juice. 100 ml of rat pancreatic juice, containing 2 g of protein, was first desalted by filtration through a Sephadex G-25 column (5 cm × 100 cm) equilibrated in 0.01 M phosphate buffer pH 6.2. The desalted protein was immediately applied on a DEAE-cellulose column (2.5 cm × 40 cm) equilibrated in the same buffer. The cationic fraction obtained after elution with 0.01 M phosphate buffer, pH 6.2, contained lipase activity which was completely free of co-lipase activity. Further purification of lipase was not performed as highly purified lipase, prepared according to Gidez[5], proved to be labile, while the partially purified lipase, described above, was quite stable. The lipase was kept in sealed ampoules at −30 °C, each ampoule containing about 1000 units/ml (using 0.5 ml of tributyrine in 15 ml of 2 mM Tris–HCl–0.15 M NaCl, pH 8, at 25 °C for assay of lipase as described earlier[6]).

Assay of co-lipase

The assay of co-lipase was based on the fact that bile salt inhibits lipase at pH 6.5 and that co-lipase releases the inhibition at this pH[1]. The assays were performed by potentiometric titration with 0.2 M NaOH using a Metrohm pH-meter 300 B, Impulsomat E 473 and Dosimat with a 1-ml burette. The substrate was prepared in a vial by adding 0.5 ml of tributyrine to 15.0 ml of 2 mM Tris–HCl buffer, pH 6.5, 4.0 mM in sodium taurodeoxycholate, 1 mM in $CaCl_2$ and 0.15 M in NaCl. The incubation was performed at 25 °C. Stirring was maintained with a magnetic rod under standardized conditions. An excess of lipase (an aliquot of 50 μl from the purified lipase ampoules described above containing about 50 lipase units) was first added and the rate of reaction, which should be less than 0.5 μmole/min, recorded for a few minutes. On addition of co-lipase, the activity of lipase was restored and the released butyric acids were automatically titrated. Under the assay conditions described it was found that the reaction followed essentially zero-order kinetics with a linear relationship between the rate of hydrolysis and the amount of co-lipase added up to rate of 15 μmoles fatty acid released per min.

Determination of protein

Protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry *et al.*[7] using crystalline bovine serum albumin as standard.

Electrophoreis

Electrophoresis was carried out by the disc technique on polyacrylamide using a Tris–glycine buffer at pH 9.3 and a 7.5% polyacrylamide gel[8,9]. The gel was prepared by adding 7.5 g acrylamide and 0.200 g *N,N'*-methylenebisacrylamide into a 100-ml

measuring cylinder and adding Tris–HCl buffer (4.55 g Tris *plus* 6.0 ml 1 M HCl *plus* water to 100 ml; pH 8.9) with stirring to make 99 ml. After addition of 60 μl *N,N,N′,N′*-tetramethylethylenediamine, the solution was filtered. Polymerization was initiated by adding 1.0 ml of freshly dissolved ammonium persulfate solution (70 mg/ml). The upper buffer of the tank buffer solution was prepared by dissolving 5.16 g Tris and 3.48 g glycine in 1000 ml of water. The lower buffer contained 14.5 g Tris and 60 ml 1 M HCl diluted to 1000 ml of water.

The salt-free protein sample solutions, made more dense by adding glycerol, were applied on the gels in 10 μl. Electrophoresis was run at a constant current of 4 mA per gel rod for 60 min. After fixation of the gels for 1 h in 10% trichloroacetic acid the gels were stained with 0.04% coomassie blue in 10% trichloroacetic acid for 2–3 h and finally destained in 10% trichloroacetic acid.

Isoelectric focusing

Isoelectric focusing was carried out in the LKB 8101 Electrofocusing column at 4 °C during 48 h as described by Svensson[10]. The pH gradient was made with ampholine carrier ampholytes in the pH range 3–10. After completed focusing fractions of 2–3 ml were collected and assayed for co-lipase activity.

Ultracentrifugation

Sedimentation equilibrium analysis. Sedimentation equilibrium analysis for calculation of the molecular weight was performed using a modified technique of Yphantis[11] as described by Chervenka[12]. The protein was dissolved in 0.1 M Tris–HCl buffer, pH 7.4, and centrifuged at 44 000 rev./min for 18 h at 20 °C in a Spinco Model E ultracentrifuge*. Recordings were made with interference optics.

Calculation of the apparent molecular weight was computed from the following equation:

$$M = 2RT\,(\mathrm{d}\,Mc/\mathrm{d}r^2)/(1-\bar{v}\varrho)w^2$$

The partial specific volume of the protein was calculated from the amino acid composition by using the amino acid specific volume values as described by Cohn and Edsall[13].

Sedimentation velocity analysis. Sedimentation velocity analysis for determination of the sedimentation coefficient was performed as described by Schachman[14,**]. The protein was dissolved at four different concentrations in 0.05 M phosphate buffer, pH 7, containing 0.5 M NaCl and centrifuged at 59 870 rev./min at 20 °C in a Spinco Model Ultracentrifuge. The sedimentation coefficient was calculated by extrapolation to infinite dilution[15]. Partial specific volume of the protein was estimated from the amino acid analysis[13].

Amino acid analysis

Amino acid analyses were performed in a Jeol amino acid analyser[15,***]. The protein (1.00 mg for each analysis) was hydrolysed in 6 M HCl at 110 °C *in vacuo* for

* The determination was kindly performed by T. Laurent at the Institute of Medical Chemistry, University of Uppsala.

** The analysis was kindly performed by Dr Ulla-Britt Hansson, Chemical Center, Lund.

*** The analysis was kindly performed by Dr J. O. Jeppson at the Department of Clinical Chemistry, Malmö.

24 and 72 h. The individual values obtained for each residue were taken from the 24-h value except for valine and isoleucine for which the concentration determined after 72 h was used and of serine and threonine for which linear extrapolations to zero time were made. Norleucine was incorporated as internal standard. Cystine was estimated as cysteic acid after performic acid oxidation. This analysis gave a second evaluation of methionine as methionine sulfone. Tryptophan was determined by spectrophotometry as described by Edelhoch[17]. For calculation of the molecular weight of the protein based on the amino acid analysis the method as described by DeLaage[18] was used. According to this procedure a set of molecular weights to be tested is chosen. For each molecular weight of the protein the number of residues of each amino acid is calculated. The difference between the calculated numbers and the nearest integers for each amino acid is taken as a measure of the fitness of the molecular weight.

Analytical gel chromatography

Stokes' molecular radius was determined by analytical gel chromatography on a Sephadex G-100 column (100 cm × 1.5 cm) equilibrated with 0.02 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl[19]. The experiments were carried out at +4 °C. The void volume, V_0, was estimated by chromatographing Blue Dextran ($M_r > 1.4 \cdot 10^6$). The void volume *plus* the inner volume was estimated from the elution position of 0.1 μCi ^3H_2O in admixture with the chromatographed samples. Calculation of the volume of the gel matrix or the polymer volume, V_p, was based on a bed volume of 30 ml/g dry Sephadex G-100 and a partial specific volume of 0.6 ml/g Sephadex. The total volume, V_t, was taken as the sum of the void volume, inner volume and polymer volume. The elution volume, V_e, of a given solute zone was taken as the concentration maximum of the zone.

Stokes' molecular radius was computed from the equation $K_{av} = e^{-\Pi \cdot L (r_r + r_s)^2}$ given by Laurent and Killander[19], where K_{av} is the volume available for the analysed solute in the gel, r_s is Stokes' molecular radius and L and r_r are constants characteristic of each gel. K_{av} was calculated from the formula

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

r_r was assumed to be 6.5 Å[19,20] and L was determined by chromatographing human albumin ($r_s = 35.5$ Å).

Calculation of diffusion coefficient, molecular weight and frictional ratio

The free diffusion coefficient ($D_{20,w}$) was calculated from Stokes' radius by use of the Stokes–Einstein equation as described by Pedersen[21]

$$D = \frac{\boldsymbol{k} \cdot T}{6 \Pi \eta r}$$

where $\boldsymbol{k}$ is the Boltzman constant, T the absolute temperature, η is the system viscosity and r is the Stokes' radius of the protein. The molecular weight was calculated from the free diffusion coefficient, sedimentation coefficient and partial specific volume by use of the Svedberg equation:

$$M_r = \frac{s}{D} \cdot \frac{RT}{1 - \bar{v}\varrho}$$

where R is the gas constant and ϱ is the solvent density. The frictional ratio f/f_0 was calculated from the expression:

$$f/f_0 = \frac{r}{\frac{(3\bar{v}M_r)^{1/3}}{4\Pi N}}$$

where N is Avogadros' number.

The molecular weight was also estimated by the gel filtration method according to Andrews[22] with use of reference proteins with known molecular weights. The following proteins were used: bovine serum albumin (M_r 69 000), egg albumin (M_r 45 000), pepsin (M_r 35 000), bovine pancreatic chymotrypsinogen (M_r 26 000), bovine pancreatic ribonuclease (M_r 13 600). For molecular weight calibration curve the K_{av} value for each protein was calculated as described above and plotted *versus* the logarithm of its molecular weight.

End-group analysis

Analysis for N-terminal amino acid was done with dansyl chloride as described by Gray[23]. Dansyl amino acids were identified by polyamide thin-layer chromatography as described by Woods and Wang[24].

Determination of free SH groups

The content of free SH groups was determined by titration with 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Ellman[25,26]. Solutions of 1 ml containing about 1 mg of colipase were prepared and added with 0.1 ml of the reagent. Titrations were performed in buffer at pH 8.0, and in buffers containing 8 M urea or 6 M guanidine–HCl[27].

Determination of sulfur

Sulfur analysis were performed by oxygen flask combustion followed by titration according to Fritz *et al.*[28] *.

RESULTS

Purification procedure

Acid extraction of pancreas. 500 g of defatted porcine pancreas were homogenised for 30 min in an Ultraturax (Type Janke-Winkel T 45) with 2 l of 0.1 M H_2SO_4. The turbid extract was then filtered through gauze to remove crude material. The filtrate was subsequently heated to 80 °C and after reaching this temperature left to cool at 4 °C. After 12 h the homogenate was filtered through Hyflo at 4 °C by suction. Through this procedure floating fat as well as insoluble material was removed.

Precipitation with $(NH_4)_2SO_4$. To the filtrate which still had a slight turbidity was added 300 g of solid $(NH_4)_2SO_4$ per l (about 50% saturation). After 1–3 h with stirring at 4 °C the precipitate, which contained the co-lipase activity was collected by filtration and suspended in distilled water to a volume of 125 ml.

Treatment with ethanol. To the preceeding solution was added 125 ml of 95%

* The analysis was kindly performed by Dr E. Bladh, Division of analytical chemistry, Chemical Center, Lund.

ethanol. The mixture was left at room temperature for 1 h after which the supernatant which contained the co-lipase activity was filtered through Hyflo to remove precipitate. Co-lipase was then precipitated by increasing the ethanol to a concentration of 90% by adding 2120 ml of 95% ethanol. The precipitate which contained co-lipase activity was dissolved in distilled water and filtered through Hyflo. The final filtrate in 90 ml, which was waterclear had a conductivity of about $20 \cdot 10^{-3}\ \Omega^{-1} \cdot cm^{-1}$ (corresponding to an ionic strength of 0.2 M NaCl solution) and a pH of about 5.

Chromatographic procedure

SP-Sephadex chromatography. The solution obtained after the ethanol treatment described above, was adjusted to pH 4 by addition of acetic acid and subsequently diluted with distilled water to a conductivity of about $5 \cdot 10^{-3}\ \Omega^{-1} \cdot cm^{-1}$. It was then applied on a SP-Sephadex column (2.5 cm × 40 cm) equilibrated with 0.01 M sodium acetate buffer, pH 4.0. After application of the sample, the column was eluted with a further 500 ml of acetate buffer, pH 4.0, and then with the same buffer containing 0.1 M NaCl. The remaining proteins were then eluted with a linear concentration gradient of NaCl (0.1–0.6 M) in 0.01 M acetate buffer, pH 4.0 (Fig. 1). Two different fractions of co-lipase activity were obtained; one was eluted with the initial buffer (Fraction I:

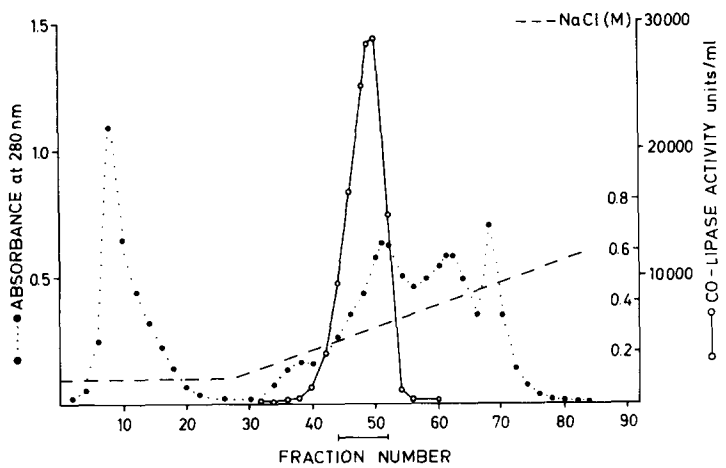

Fig. 1. Chromatography on SP-Sephadex. The SP-Sephadex column (2.5 cm × 40 cm) was equilibrated in 0.01 M acetate buffer, pH 4.0. After application of the protein, the column was eluted with 0.01 M acetate buffer pH 4.0 containing 0.1 M NaCl and then with a linear concentration gradient of NaCl (0.1–0.6 M). ●···●, absorbance at 280 nm; ○—○, co-lipase units/ml; ———, molarity of NaCl.

total activity $1.0 \cdot 10^6$ units; spec. act. 6600) and the other with the gradient at a molarity of NaCl of about 0.30 M (Fraction II: total activity $2.4 \cdot 10^6$ units; spec. act. 45 000). Fraction II being the main peak of co-lipase activity was lyophilized and further processed.

DEAE-cellulose chromatography. The lyophilized powder was dissolved in 20 ml of distilled water and freed of NaCl by passage through a Sephadex G-25 column (2.5 cm × 40 cm) equilibrated in 5 mM $NaHCO_3$ buffer (pH 8.0), containing 5 mM NaCl. The resulting solution was then applied to a DEAE-cellulose column (2.5 cm ×

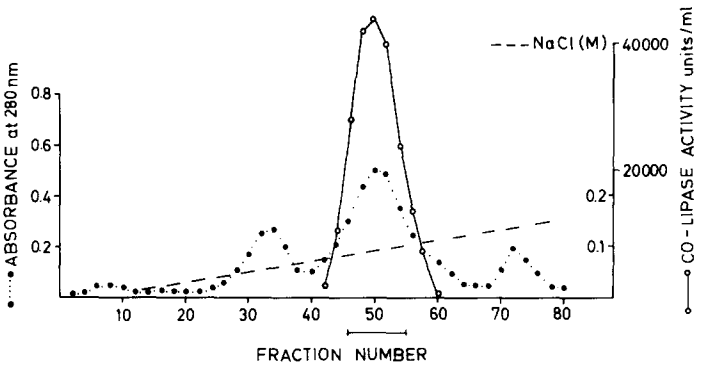

Fig. 2. Chromatography on DEAE-cellulose. The DEAE-cellulose column (2.5 cm × 40 cm) was equilibrated in 5 mM $NaHCO_3$ buffer, pH 8.0, containing 5 mM NaCl and eluted with a linear concentration gradient of NaCl (5–150 mM). ●···●, absorbance at 280 nm; ○—○, co-lipase units/ml; — — —, molarity of NaCl.

40 cm) equilibrated in the same buffer (Fig. 2). The column was washed with 300 ml of the buffer and then eluted by a slow linear concentration gradient in NaCl (5–150 mM). This chromatographic step was similar to that described by Maylié *et al.*[2]. The co-lipase-containing fractions, eluted at an ionic strength of 0.09 M NaCl were combined and lyophilized.

Sephadex G-75 chromatography. The lyophilized powder was dissolved in 5.0 ml of distilled water and applied on a Sephadex G-75 column (2.5 cm × 100 cm) equilibrated in distilled water. Elution was performed with the same solution (Fig. 3). The co-lipase-containing fractions which possessed a constant specific activity were combined and lyophilized. The purified protein was dried *in vacuo* over P_2O_5 and kept in sealed ampoules at −30 °C.

The yield at each step in the purification procedure and the degree of purification obtained have been summarized in Table I. In the chromatographic steps (7–8) only the peak fractions containing about 60% of the recovered co-lipase activity were used for further chromatography.

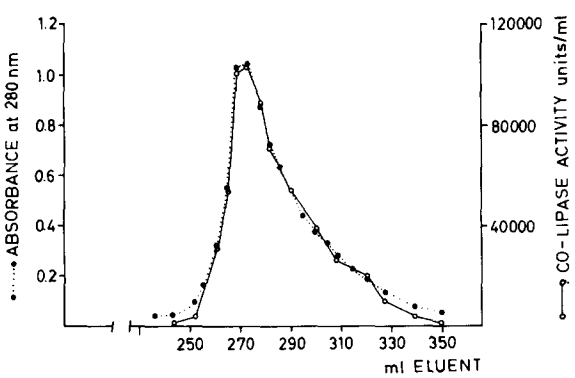

Fig. 3. Filtration through Sephadex G-75. The Sephadex G-75 column (2.5 cm × 100 cm) was equilibrated and eluted with distilled water. Void volume 170 ml; total volume 480 ml. ●···●, absorbance at 280 nm; ○—○, co-lipase units/ml.

TABLE I

PURIFICATION OF CO-LIPASE

Step	*Total activity (units × 10^{-6})*	*Spec. act.*	*Protein (g)*	*Yield (%)*
1. Acid extract	10.4	140*	74	100
2. Heat treatment	10.4	140*	74	100
3. Filtration through Hyflo	5.6	180*	31.0	54
4. 50% $(NH_4)_2SO_4$	4.7	740**	6.35	45
5. 50% ethanol solution	4.7	8 200**	0.570	45
6. 90% ethanol precipitate	4.3	16 000**	0.270	41
7. SP-Sephadex chromatography	2.4	45 000**	0.053	23***
8. DEAE-cellulose chromatography	1.5	90 000**	0.017	14***
9. Sephadex G-75 filtration	1.2	100 000**	0.012****	12

* The protein has been determined with the technique of Lowry *et al.*[7].
** The protein has been determined by measuring the absorbance at 280 nm.
*** Only the peak fractions were pooled and further chromatographed.
**** This figure is based on the absorbance of the protein solution at 280 nm. The $E_{1\,cm}^{1\%}$ of the pure protein is, however, 4.0, why this figure corresponds to 30 mg of protein by weight.

Comments on the purification procedure

Filtration and centrifugation. Various procedures were tried to remove the insoluble material and the floating fat layer present after heat treatment of the acid pancreatic extract. Centrifugation (using an MSE 18 centrifuge) of the acid extract was not efficient alone but must be followed by filtration through Hyflo to remove the floating fat. Similar results were obtained by isoelectric precipitation at pH 5 followed by centrifugation. In neither procedure the recovery of co-lipase in this step was better than 50%.

Precipitation with $(NH_4)_2SO_4$. In spite of its low molecular weight co-lipase was precipitated to 80–90% already at 40% saturated $(NH_4)_2SO_4$. Increasing the $(NH_4)_2$ SO_4 to 50% saturation gave an almost quantitative precipitation without appreciably lowering the specific activity.

Treatment with ethanol. The subsequent treatment with 50% ethanol resulted in severalfold purification with a quantitative recovery. Initially the ethanol was removed by evaporation on a rotatory evaporator at 25 °C, and the remaining solution lyophilized. Upon redissolving the lyophilized material a turbid and jelly-like solution was formed which was difficult to further chromatograph. If the co-lipase was instead precipitated by increasing the ethanol to 90% as described above the redissolved precipitate upon filtration formed a water-clear solution which was suitable for further chromatography.

Chromatographic steps. For the isolation of substantial amounts of co-lipase it was found convenient to start the chromatographic procedure with a solution of about $15 \cdot 10^6$ co-lipase units (spec. act. 10 000–15 000). By the SP-Sephadex chromatography co-lipase activity was obtained in two different fractions (Fraction I and Fraction II). The major part of the recovered co-lipase activity (about 70%) was in Fraction II. This was eluted from the SP-Sephadex column at least 3-fold purified and was the one chosen to be further purified.

When this fraction was subjected to chromatography on the DEAE-cellulose on additional 2-fold purification of the protein was obtained, but it still contained some

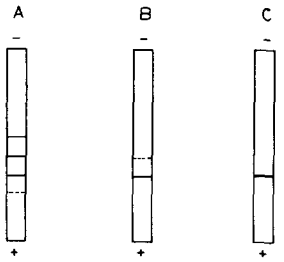

Fig. 4. Disc electrophoresis of co-lipase fractions at various stages of the purification. (A) After chromatography on SP-Sephadex at pH 4.0, Fraction II. (B) After chromatography no DEAE-cellulose at pH 8.0. (C) After Sephadex G-75 filtration.

impurity as detected by disc electrophoresis (Fig. 4). In the final Sephadex G-75 filtration the peak absorbance at 280 nm showed a complete correspondance with the co-lipase activity. The elution volume of the peak of co-lipase suggested a M_r of the protein of around 12 000. The peak was not symmetrical probably due to adsorption of the protein to the gel matrix as the column was run in distilled water.

After each step in the purification procedure 1 ml of the respective fraction was filtered through a Sephadex G-100 column (100 cm × 1.5 cm) equilibrated in 0.02 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl. These chromatographies revealed that co-lipase did not change its molecular weight during the whole purification procedure but was always eluted at a position corresponding to a molecular weight of around 12 000.

Dialysis. Co-lipase could not be efficiently dialysed without leakage of the protein (about 10–15% of total activity) through cellophan bags or by ultrafiltration using Diaflo membranes. Desalting by filtration through Sephadex G-25 gave a quantitative yield when buffers of low ionic strength were used. Elution with distilled water gave 70–80% recovery.

Physical and chemical properties of co-lipase

Criterion for homogeneity. Purified co-lipase revealed only one band on polyacrylamide-disc electrophoresis (Fig. 4). Furthermore, co-lipase sedimented as a single symmetrical component when subjected to high speed centrifugation. In the sedimentation equilibrium analysis performed for determination of the molecular weight a straight line was obtained when log c was plotted against r^2.

Determination of isoelectric point. The isoelectric point was found to be about 5.0.

Specific activity. The specific activity of the purified co-lipase was $\simeq$ 40 000 units/mg when the protein was weighed out as a dry powder. When the protein was determined from the absorbance in solution at 280 nm assuming an $E^{1\%}_{1\,cm}$ of 10.0 for co-lipase, as was done for Steps 4–9 in the purification procedure the specific activity was found to be about 100 000 per mg. The difference between these figures is due to the fact that the molecular extinction coefficient of pure co-lipase; $E^{1\%}_{1\,cm}$ at 280 nm is only 4.0 a figure also reported by Maylié *et al.*[2].

The method of Lowry was used in Steps 1–3 as these solutions were turbid. 1 mg of the purified co-lipase was found to correspond to 0.9–1.0 mg crystalline bovine serum albumin in the method of Lowry.

Determination of molecular weight. The molecular weight of co-lipase was calculated from (a) sedimentation equilibrium analysis, (b) sedimentation velocity analysis combined with analytic gel filtration for calculation of Stokes' radius and the diffusion coefficient, (c) filtration through Sephadex G-100 and Bio-Gel P-30 by use of reference proteins, (d) the amino acid composition, (e) the content of sulfur.

(a) The molecular weight determined by sedimentation equilibrium ultracentrifugation was found to be 11 300 based on a partial specific volume of the protein of 0.714 $cm^3 \cdot g^{-1}$.

(b) The molecular weight calculated from the sedimentation coefficient, diffusion coefficient and Stokes' radius was found to be 9700. The sedimentation coefficient ($s_{20,w}$) was calculated to 1.46 $\pm$ 0.14 S and the diffusion coefficient $12.8 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ based on a Stokes' radius of 16.7 Å.

(c) The molecular weight calculated from the elution position of co-lipase on a Sephadex G-100 by use of reference proteins indicated a molecular weight of 11 000–12 000 (Fig. 5) (K_{av} = 0.61). The same value for the molecular weight was also obtained by filtration through Bio-Gel P-30, where co-lipase had an elution volume of $1.55 \cdot V_0$.

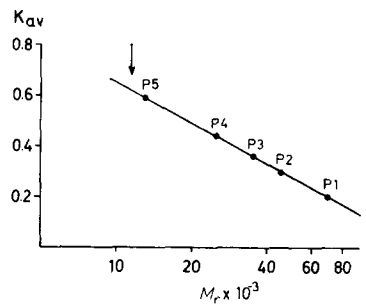

Fig. 5. Molecular weight determination of co-lipase by the Sephadex filtration technique. In this experiment, the Sephadex G-100 column (1.5 cm × 100 cm) was equilibrated with and eluted by a 0.02 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl. The reference proteins bovine serum albumin (P_1, M_r 69 000), egg albumin (P_2, M_r 45 000), pepsin (P_3, M_r 35 000), bovine chymotrypsinogen (P_4, M_r 26 000) and bovine ribonuclease (P_5, M_r 13 600) were dissolved in the equilibration buffer at a 1% concentration. The figure reproduces a semi-logarithmic plot of the molecular weight of the protein *versus* the K_{av} value. The arrow indicates the K_{av} value (0.61) observed for co-lipase.

(d) The molecular weight of co-lipase calculated from the amino acid composition was 10 300 (Table III).

(e) The molecular weight of co-lipase calculated from the content of sulfur was 9640 $\pm$ 150 (see below).

The frictional ratio f/f_0 was found to be 1.19 based on a molecular weight of 9700 of co-lipase. The physical properties of co-lipase have been summarized in Table II.

Amino acid composition. The amino acid composition is given in Table III. A most striking feature is the high amount of acidic residues (aspartic + glutamic acid, 21 residues) which accounts for the acidic isoelectric point of the co-lipase (pI = 5.0). Moreover, co-lipase contains ten half-cystine residues. Titration with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) at pH 8.0 even in the presence of 8 M urea or 6 M guanidine–HCl, showed the absence of any free sulfhydryl groups. Co-lipase there-

TABLE II

PHYSICAL PROPERTIES OF PORCINE PANCREATIC CO-LIPASE

Stokes' radius	16.7 Å
Diffusion coefficient $D_{20,w}$	$12.8 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1}
Sedimentation coefficient $s_{20,w}$	$1.46 \cdot 10^{-13} \pm 0.14 \cdot 10^{-13}$ s
Partial specific volume	0.714 cm$^3 \cdot$g^{-1}
Frictional ratio	1.19
Molecular weight	
Method of Chervenka[12]	11 300 ± 1130
From $D_{20,w}$, $s_{20,w}$ and $\bar{v}$ data	9 650 ± 1000
From amino acid analysis	10 300
From sulfur analysis	9 640 ± 160
Gel filtration	12 000

fore contains five disulfide bridges, which readily account for the thermal stability of the molecule as pointed out by Maylié *et al.*[2]. The total absence of methionine and tryptophan is in agreement with the report of Maylié *et al.*[2] and is an additional criterium for the purity of the co-lipase. The low content of aromatic residues (tryptophan 0; tyrosine 3 residues) was consistent with the low extinction coefficient at 280 nm (see above). In addition, the amino acid analysis demonstrated the total absence of hexosamines in co-lipase. The total number of residues was calculated to be 95 and the

TABLE III

AMINO ACID COMPOSITION OF CO-LIPASE

Residues	*Number of residues*		*Corresponding weight in 1 mole protein*
	*Experimental**	*Nearest integral number*	
Alanine	5.21	5	355
Arginine	5.05	5	780
Aspartic acid	12.29	12	1380
Cysteine**	10.31	10	1021
Glutamic acid	9.13	9	1152
Glycine	7.74	8	456
Histidine	2.21	2	274
Isoleucine***	6.03	6	678
Leucine	9.34	9	1017
Lysine	4.71	5	640
Methionine	0.00	0	—
Phenylalanine	2.08	2	294
Proline	0.97	1	97
Serine†	10.00	10	870
Threonine†	5.84	6	606
Tryptophan††	0.00	0	—
Tyrosine	2.66	3	489
Valine***	2.05	2	198
Total number of residues		95	
Total weight of residues			10325

* Except where noted the figures are taken from the 24-h hydrolysis value.
** Half-cystine was determined as cysteic acid after performic acid oxidation.
*** 72-h hydrolysis value.
† Values obtained by extrapolation to zero time of hydrolysis.
†† Determined spectrophotometrically.

corresponding molecular weight 10 300. Similar results have later been found for preparations in which co-lipase was initially extracted from the gland with ice-cold 70% aqueous ethanol at pH 2.5. (These preparations were kindly supplied by Novo Terapeutisk Laboratorium AS, Copenhagen, Denmark.)

Terminal amino acid. After dansylation of the protein, co-lipase was found to possess only one amino-terminal amino acid, isoleucine.

Analysis for sulfur. Co-lipase was found to contain 3.32 ± 0.05% by weight of sulfur. Assuming a content of ten half-cystine residues per mole of protein the molecular weight of co-lipase was calculated to be 9640 ± 150 (see above).

DISCUSSION

Co-lipase has been purified from porcine pancreas and characterized by physical and chemical methods. The results indicate that co-lipase is a single-chain polypeptide with a molecular weight of around 10 000. The molecular weight was determined by several techniques (Table II). The values obtained are in fair agreement and no significant difference can be seen within the range of experimental error. A fairly accurate value appears to be the value based on the content of sulfur (3.32%) assuming the presence of ten half-cystine residues per mole of co-lipase. This value was found to be 9640 ± 150. The molecular weight obtained from amino acid analysis (10 300) and the value based on sedimentation coefficient, diffusion coefficient and Stokes' radius (9700) are in close agreement. The molecular weight determined with sedimentation equilibrium analysis is somewhat larger (11 300 ± 1100) although the lower value within the range of experimental error (10 200) well suits the other values above. This would indicate that co-lipase does not contain any non-protein part as was suggested by Maylié *et al.*[2]. These authors found the molecular weight of co-lipase to be around 8100 based on amino acid analysis and between 9400 and 9900 determined by ultracentrifugation and therefore assumed co-lipase to contain a non-protein component in the order of 15%.

On gel filtration the molecular weight estimated by the method of Andrews was found to be around 12 000. This somewhat larger value obtained by gel filtration compared to the other methods may be explained by the asymmetry of the molecule.

The amino acid composition obtained in our preparation is in principal similar to that obtained by Maylié *et al.*[2]. Thus no methionine or tryptophan was found. Also the presence of five disulfide bridges in co-lipase is in agreement. Our analysis showed a somewhat higher content of acidic and hydroxylated amino acid residues. The high content of acidic residues (21 residues) is in agreement with the acidic isoelectric point of co-lipase (pI = 5.0). Both types of residues are known to favour hydrogen bonding: their high content together with the existence of five disulfide bridges in co-lipase indicate a rather compact tertiary structure which is also verified by the low frictional ratio ($f/f_0 = 1.19$) found for co-lipase and undoubtedly contributes to the very high stability of the protein against heat. The low content of aromatic residues agrees with the low absorbance value at 280 nm.

The elution of co-lipase in two different fractions on ion-exchange chromatography indicates that co-lipase could exist in at least two different forms with the same molecular weight but with different charge. Studies are continued to investigate this polymorphism of co-lipase.