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Short Communication

Separation and characterization of the precursor and activated forms of porcine and human pancreatic colipase by reversed-phase liquid chromatography

Nathalie Rugani, Christine Dezan and Laurence De La Fournière

Laboratoire de Biochimie, Case 65, Faculté des Sciences St-Charles, 3 Place Victor Hugo, 13331 Marseille Cedex 3 (France)

Patrick J. Cozzone

C.R.M.B.M., Faculté de Médecine la Timone, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 5 (France)

Bernard Bellon and Louis Sarda

Laboratoire de Biochimie, Case 65, Faculté des Sciences St-Charles, 3 Place Victor Hugo, 13331 Marseille Cedex (France)

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ABSTRACT

Reversed-phase liquid chromatography was used as an alternative method for the characterization of the precursor and activated forms of porcine and human pancreatic colipase. Using a Beckman Ultrasphere column with an increasing acetonitrile gradient in 0.1% trifluoroacetic acid, it was possible to obtain well-resolved separation of the precursor form of colipase (procolipase) from its trypsin-activated derivative. This protocol was used (1) to study the activation of porcine procolipase by trypsin or thrombin *in vitro*, (2) to assess the homogeneity of porcine colipase preparations used in tridimensional structure studies and in combination with immunoaffinity chromatography, (3) to identify the form of colipase present in samples of human pancreatic juice.

INTRODUCTION

Colipase is a non-enzymic protein secreted by pancreas. Its biological function is to act as a co-factor of pancreatic lipase during intestinal fat digestion. It specifically enables pancreatic lipase to bind to lipid–water interfaces in the presence of bile salt. Colipase is a polypeptide of 95 amino acid residues with a central core, tightly held by five disulphide bridges, and free amino- and carboxy-terminal segments of sixteen and eight amino acid residues, respectively. Comparison of the primary structures of colipases from vertebrates reveals a high degree of homology among proteins [1].

Colipase is secreted in all species as procoli-

Correspondence to: Louis Sarda, Laboratoire de Biochimie, Case 65, Faculté des Sciences St-Charles, 3 Place Victor Hugo, 13331 Marseille Cedex 3, France.

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pase. Procolipase reverses the inhibition of pancreatic lipase by bile salts in lipolysis systems containing pure triacylclycerol. Conversely, procolipase is weakly active in presence of phospholipid. *In vitro* studies have shown that procolipase is converted by trypsin into a short form that specifically cleaves the arginine–glycine peptide bond at positions 5 and 6 [2]. Limited proteolysis of procolipase by trypsin is considered as an activation process of physiological significance, since removal of the N-terminal pentapeptide enhances the capacity of the cofactor to bind to interfaces in phospholipid-containing lipolysis systems [2,3]. There is also evidence that the free N-terminal pentapeptide plays a regulatory function as a satiety signal for fat intake [4,5].

In addition to the well-characterized procolipase and trypsin-activated product, several active forms of the cofactor were isolated from homogenate of porcine pancreas and characterized [3,6]. It has been shown, in particular, that carboxypeptidase A removes the last two residues of the polypeptide, thus converting the 95-residue procolipase, identified as procolipase A, into the 93-residue procolipase B [7]. Forms A and B of porcine procolipase are equally active and are converted into activated product by trypsin.

Procolipase, which is obtained from porcine pancreas in much larger amounts than from any other species, is currently used in structure–function studies. Purification of the protein is generally achieved by conventional techniques, including salt and solvent precipitation and ion-exchange chromatography [8,9]. The major form of the cofactor obtained from tissue extract is procolipase A. However, depending on the conditions used for collecting pancreatic glands and addition of protease inhibitors to the crude extracts, procolipase B and trypsin-activated colipases (forms A and B) are also found in variable amounts. Separation of partially degraded proteins from procolipase A is achieved at the final stage of purification. Homogeneity of porcine procolipase preparations is generally assessed by using polyacrylamide gel electrophoresis (PAGE) and end-group (amino) analysis. These techniques, however, do not allow a precise quantitation of minor amounts of protein contaminants. Indeed, it has appeared that limited proteolysis of procolipase by endogenous proteases active in pancreas homogenate might represent the major source of microheterogeneity accounting for the difficulties encountered, so far, to obtain crystals of the protein suitable for X-ray structure studies [10].

This paper describes a technique for the separation and characterization of porcine pancreatic procolipase and its trypsin-activated product, based on reversed-phase high-performance liquid chromatography (RP-HPLC). This method, which allows the identification and precise quantification of the major active forms of the porcine cofactor found in pancreatic extracts, is useful to assess the homogeneity of procolipase preparations, to follow the *in vitro* conversion of porcine procolipase into its short form by trypsin or thrombin, and to characterize colipase separated from human pancreatic juice in one step by immunoaffinity chromatography.

EXPERIMENTAL

Chemicals

Acetonitrile was from Carlo Erba (Milan, Italy) and trifluoroacetic acid (TFA) was from Pierce (Beijerland, Netherlands). All the chemicals used in PAGE were from Sigma (St. Louis, MO, USA). All other chemicals, including proteolytic enzymes (trypsin or thrombin) and enzyme inhibitors, were from Sigma, except α-NAPAP (N-α-(2-naphthylsulphonylglycyl) 4-amidinophenylalanine piperide), a specific inhibitor of thrombin, which was purchased from Interchim (Montluçon, France).

Analytical reversed-phase liquid chromatography

All chromatography was performed on a Beckman (System Gold) liquid chromatography system. The eluted proteins were detected at 230 or 280 nm by a UV-166 variable-wavelength detector, and the chromatograms were obtained with either integration graphical or integration tabular options. The identity of the peaks eluted from the column was checked by measuring colipase

activity after peak collection. Actually, more than 95% of cofactor activity placed onto the column was recovered, indicating that porcine colipase was stable in acetonitrile–TFA under the conditions used for chromatography. A Beckman Ultrasphere column (Octyl 5 μm, 250 × 4.6 mm I.D.) was used. Deionized water containing 0.1% TFA, ammonium formate (pH 2.75, 12 mS), or ammonium acetate (pH 3.75, 12 mS) and acetonitrile were used as gradient system. Details of the experiments are given in the legends to the figures. Samples of proteins were prepared in deionized water and concentrations were adjusted to 1 mg/ml. Samples were loaded onto the column via a fixed-volume loop injector.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out according to the method of Laemmli [11] on polyacrylamide slab gel 0.75 mm thick with 5 mm wide sample wells. In the absence of sodium dodecyl sulphate (SDS), the running gel contained 0.025 *M* Tris–HCl–0.2 *M* glycine (pH 8.8) and 7.5% acrylamide. In SDS gel electrophoresis, the running gel contained 0.1% SDS in the same buffer and 15% acrylamide. The protein samples (1 mg/ml) were prepared in the running buffer and delivered to the gel slots in 20-μl amounts. Gels were stained with R-250 Coomassie Blue.

Colipase samples

Porcine procolipase was purified from pancreatic tissue according to a procedure already described [9]. Porcine pancreas were collected at the slaughterhouse immediately after the death of animals and kept on ice for *ca.* 60 min before starting the purification. Proforms A and B of the porcine cofactor were separated by ion-exchange chromatography on DEAE-cellulose [9]. Procolipase was converted into its trypsin-activated product by treatment with trypsin *in vitro*, under conditions previously reported [2,3]. Treatment of procolipase by thrombin was as follows: 0.3 mg of protein in 0.3 ml of $5 \cdot 10^{-2}$ *M* ammonium bicarbonate (pH 8.4) was mixed with 0.1 ml of the same buffer containing 30 μg (60 NIH units) of thrombin and kept at 37°C for 6 h. In a control experiment, thrombin was mixed with 130 μg of α-NAPAP, a specific inhibitor of the enzyme, prior to incubation with procolipase. Native and trypsin- or thrombin-treated porcine procolipase were reduced with dithiothreitol (DTT) at the final concentration of 40 m*M* for 5 min at 96°C. Porcine pancreatic juice (lyophilized powder) was kindly provided by Dr. T. Corring (INRA, Jouy en Josas, France). Human pancreatic juice was a gift from Dr. J. C. Dagorn (INSERM, Marseille, France). Human pancreatic juice was collected on ice and immediately mixed with phenyl propionate 5 m*M*, benzoyl arginine 1 m*M*, phenylmethylsulphonyl fluoride 0.6 m*M* and benzamidine 50 m*M*. Colipase represented 1–2% of the total proteins of the juice. Isolation of human colipase from pancreatic juice in one step by immunoaffinity chromatography on immobilized monoclonal antibody (mAb 72.11) has already been reported [12].

Determination of colipase activity and immunoinactivation studies

Cofactor activity was determined at pH 9 and 25°C with the pH-stat method, using triolein emulsified in gum arabic as substrate, in the presence of 8 m*M* sodium deoxycholate and purified pancreatic lipase, free of colipase, in excess to cofactor (40 lipase units). Activity was derived from the zero-order kinetic of lipolysis and expressed in units [13]. One colipase unit corresponded to the liberation of one micro-equivalent fatty acid per minute under standard conditions. In the assay system used, procolipase (forms A and B) and trypsin-activated products showed very similar activity. Immunoinactivation experiments were performed on human pancreatic juice and purified human colipase with monoclonal antibody mAb 72.11 obtained at the laboratory, as described previously [12,14].

Protein determination

The protein concentration of the purified porcine or human pancreatic colipase was determined spectrophotometrically using an absorption coefficient, $A_{1\,cm}^{1\%}$ at 280 nm of 3.6 [15], and with the colorimetric method of Lowry *et al.* using serum albumin as standard [16].

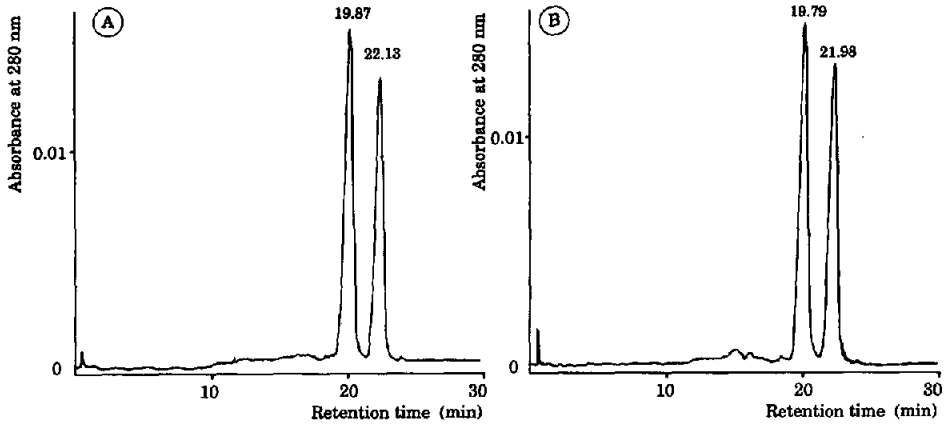

Fig. 1. RP-HPLC of purified porcine pancreatic procolipase and its trypsin-activated product. Mixtures of equal amounts (30 μg each) of procolipase (form A or B) and the product formed by limited proteolysis by trypsin were chromatographed at room temperature with a Beckman apparatus (System Gold) equipped with a Beckman Ultrasphere column (octyl 5 μm, 250 × 4.6 mm I.D.). Elution was achieved with a linear gradient from 30 to 60% acetonitrile in 0.1% TFA in water at a flow-rate of 1.0 ml/min. The detection wavelength was 280 nm. (A) Mixture of procolipase A (t_R = 19.87 min) and trypsin-activated colipase A (t_R = 22.13 min). (B) Mixture of procolipase B (t_R = 19.79 min) and trypsin-activated colipase B (t_R = 21.98 min). Similar elutions diagrams are obtained by using the same acetonitrile gradient in ammonium formate or ammonium acetate at a concentration corresponding to conductivity of 12 mS and pH 2.75 and 3.75, respectively.

RESULTS

Separation of the precursor and trypsin-activated forms of porcine colipase: criteria of purity

RP-HPLC separations were performed on porcine procolipase A and B, purified from pancreas, mixed with an equal amount of the product obtained by treatment of the proform with trypsin *in vitro*. The elution profiles in Fig. 1 show that the resolution between procolipase and its trypsin-activated derivative was excellent. As indicated by Fig. 2, contamination of porcine procolipase or trypsin-activated colipase by either one of two forms of the cofactor can be detected at a very low level (less than 1.25%). The retention times (t_R) of procolipase A and procolipase B, which differ by two residues missing at the C-terminus of form B, are almost identical (19.87 and 19.79 min, respectively). Similarly, the retention times of the trypsin-activated derivatives show no significant difference (22.13 and 21.98 min, respectively). This method, therefore, fails to distinguish between forms A and B of the cofactor. However, as shown by data presented in Fig. 3, forms A and B of the precursor and activated forms of porcine colipase, which differ by one negative charge (aspartic acid at position 94), can be separated and identified unambigously by PAGE carried out under non-denaturating conditions.

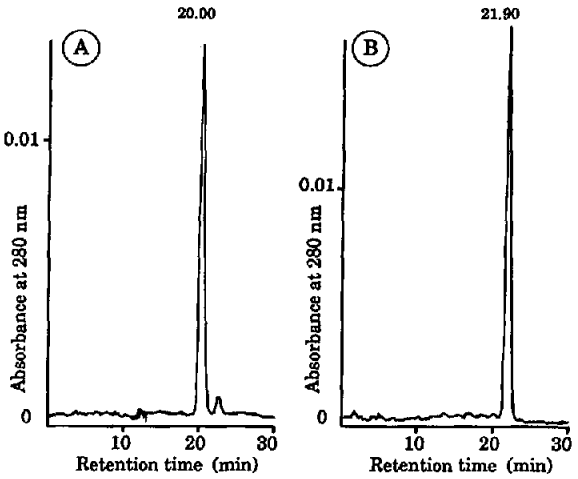

Fig. 2. Purity check of porcine pancreatic colipase by RP-HPLC. Samples of porcine procolipase A (A) and trypsin-activated product (B) were chromatographed under the same conditions as described in Fig. 1.

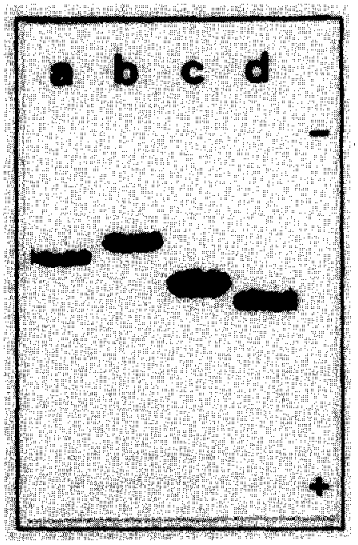

Fig. 3. PAGE of purified preparations of porcine procolipase and trypsin-activated products (forms A and B). Lanes: a = trypsin-activated colipase B; b = procolipase B; c = procolipase A; d = trypsin-activated colipase A.

Study of the activation of porcine procolipase by thrombin

In vitro treatment of procolipase by thrombin caused no loss in activity. The product resulting from thrombin action was first characterized with the PAGE and RP-HPLC techniques and by the dansylation method. Thrombin-treated procolipase showed electrophoretic and chromatographic behaviour identical with that of trypsin-activated colipase, and glycine was the only amino-terminal residue. Experiments performed under the same conditions, but with thrombin previously mixed with α-NAPAP, yielded a product that could not be distinguished from procolipase. These results indicated that thrombin, like trypsin, had cleaved the arginine–glycine bond at positions 5 and 6. Since porcine procolipase contains a second arginine–glycine peptide bond at positions 65 and 66, it was necessary to establish wether this bond located in the central region of the polypeptide was accessible to thrombin in the native conformation of the cofactor. This was done by running SDS electrophoresis on the thrombin-treated protein after reduction by DTT. Only one protein component, of molecular mass *ca.* 10 000 and identical with reduced colipase, was identified indicating that thrombin had no effect on the Arg_{65}–Gly_{66} bond of native colipase.

Separation of the precursor and activated forms of human colipase

mAb 72.11, an antiporcine procolipase monoclonal antibody, reacts specifically with the precursor forms of porcine, equine and human colipase. It inhibits colipase-dependent pancreatic lipase activity by preventing the binding of the cofactor to triacylglycerol in a bile salt containing lipolysis system. The amount of procolipase in pancreatic juice can be estimated by measuring the decrease in cofactor-dependent lipolytic activity following incubation of an aliquot of the juice with mAb 72.11. Also, mAb 72.11 coupled to Sepharose 4B can be used to separate procolipase from proteins of pancreatic extract or juice in one step.

Human pancreatic juice containing more than 90% of its colipase activity in the precursor form was passed through a column of immobilized mAb 72.11. All colipase activity sensitive to immunoinactivation by mAb 72.11 (procolipase) was retained on the column. Adsorbed procolipase was eluted with a glycine–HCl buffer at pH 3.4 or pH 2.8. The eluted protein fraction, which contained all cofactor activity separated from the juice, was identified by the dansylation method and by immunoinactivation with mAb 72.11. It appeared that protein eluted at pH 3.4 was identical with the proform of the cofactor, as shown by N-terminal alanine and sensitivity to inactivation by mAb 72.11. In contrast, elution at pH 2.8 yielded human colipase in the activated form (N-terminal glycine). These observations were confirmed by chromatographic analysis. Chromatograms of the human colipase fractions eluted from the immunoadsorbent column at pH 3.4 and pH 2.8 are presented in Fig. 4. These show two well-resolved peaks, corresponding to human procolipase and its activated derivative. The activated form of the human cofactor had a retention time higher by *ca.* 1 min than that of the proform, as already observed with the porcine

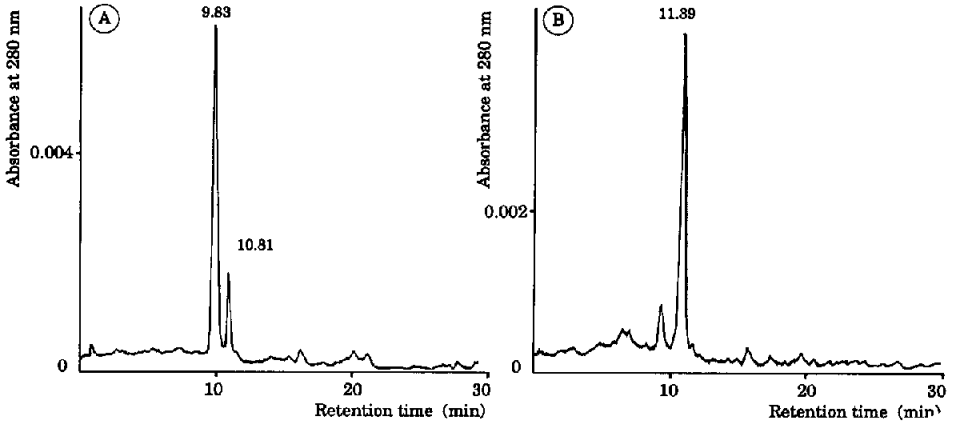

Fig. 4. RP-HPLC separation and characterization of the precursor and activated forms of human colipase. Procolipase was separated from human pancreatic juice by immunoaffinity chromatography on immobilized mAb 72.11. The adsorbed cofactor was recovered by eluting with 0.1 *M* glycine–HCl buffer pH 3.4 (A) or pH 2.8 (B). RP-HPLC analyses were performed with 10 μg of protein.

cofactor (Fig. 1). These chromatograms show that elution at pH 3.4 prevents partial conversion of the procolipase into activated colipase, in contrast to what was observed at more acidic pH (pH 2.8).

DISCUSSION

Several active forms of colipase, the protein cofactor of pancreatic lipase, have been isolated from the pancreas or pancreatic juice of mammals. Unlike the two equine isoproteins, which differ by four amino acid substitutions [17,18], the various forms of the porcine and human proteins characterized so far were found to result from limited proteolysis of procolipase, the secreted form of the cofactor, at both ends of the polypeptide. Trypsin cleaves the Arg_5–Gly_6 bond, which is present in all species, yielding a short form considered as the activated form of the cofactor acting *in vivo* [2,3]. Carboxypeptidase A removes the last two residues at the C-terminus of porcine procolipase with no change in activity [3,7]. The presence of partially degraded forms of colipase in pancreatic extracts depends on the conditions of collection of pancreatic glands or juice and/or of specific protease inhibitors during purification. The homogeneity of purified procolipase is generally assessed by using end-group analysis and PAGE. In this study we used RP-HPLC to characterize porcine and human procolipase and their products formed by limited proteolysis by enzymes active in pancreatic extracts, in order to improve the detection of minor amounts of protein contaminants in samples prepared for X-ray and NMR structural studies.

Porcine and human procolipase are well separated from their trypsin-activated products on a reversed-phase column. Removal of the N-terminal pentapeptide increases the retention time of the cofactor by 1.5–2 min. This agrees with the early suggestion by Borgstrom *et al.* [2] that the new N-terminal sequence (Gly_6–Ile–Ile–Ile_9) resulting from limited trypsinolysis enhances the capacity of the protein cofactor to bind to hydrophobic surfaces. Of interest is the observation that, after complete reduction of the disulphide bridges with DTT, the retention times of inactive procolipase and its trypsin-treated derivative are identical and decreased by 10 min with respect to the native proteins (data not shown). This finding agrees well with the idea that, in their native conformation, procolipase and trypsin-activated colipase possess a structually well-defined surface lipid recognition site, hydrophobic in nature, and that this domain is disrupted after reduction of the disulphide bridges.

As thrombin, a protease possibly active in pancreatic tissue homogenate, is known to cleave certain arginine–glycine bonds in proteins and resembles trypsin in many enzymic properties including the ability to convert chymotrypsinogen into chymotrypsin [19,20], we investigated its effect on porcine procolipase by using RP-HPLC in combination with PAGE and end-group analysis. Porcine procolipase contains two arginine–glycine bonds. The first bond, at positions 5 and 6, is readily cleaved by thrombin yielding the activated form of the cofactor. The second bond, located at positions 65 and 66 in the central region of the polypeptide, close to half-cystine residues 61, 63 and 69, is not accessible to thrombin, in the native configuration of colipase. It appears, then, that the activated form of colipase present in pancreatic extract might originate from limited proteolysis not only by trypsin but also by thrombin.

Finally, we used RP-HPLC to characterize the precursor and activated form of the cofactor isolated from human pancreatic juice by immunoaffinity chromatography [12]. We have confirmed the previous observations by different groups [12–24] that, in human procolipase, the Arg_5–Gly_6 bond is far more labile to proteolysis than in the porcine protein, especially at acidic pH. However, it is not known whether this is correlated with the two amino acid substitutions occurring at positions 1 (Val → Ala) and 3 (Asp → Gly) in the human protein.

CONCLUSION

RP-HPLC is a reliable technique for identifying the various forms of porcine and human colipases found in pancreatic extracts and assessing the homogeneity of protein samples prepared for structural studies. It is useful to follow the activation of the proform of the cofactor (procolipase) *in vitro* by trypsin or thrombin. RP-HPLC, in combination with immunoinactivation, provides a straightforward method for the specific identification and quantitation of procolipase isolated from samples of human pancreatic juice.

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