Pregastric esterase (PGE) (EC 3.1.1.3) was purified to homogeneity from calf pharyngeal tissue. The enzyme had an apparent molecular mass of 50 kDa, as determined by SDS/PAGE. The serine-binding reagent diethyl p-nitrophenyl phosphate was a potent inhibitor of PGE. This is in accordance with the claim that a functional serine residue is necessary for the lipolytic activity of lipases. PGE was not inhibited by the thiol reagents 5,5′-dithiobis(2-nitrobenzoic acid) or 4,4′-dithiopyridine. A partial inhibition with dodecylthio-5-(2-nitrobenzoic acid) was observed, but the same degree of inhibition was caused by the non-esterified fatty acid C₁₂:0. PGE shows a great sequence similarity to gastric lipases. Gastric lipases have three cysteine residues, and two of these form a disulphide bridge. Blocking the remaining free cysteine with thiol reagents inactivates the gastric lipases. The fact that PGE is not inhibited by thiol reagents indicates that PGE has no functional free thiol group. The PGE cdna codes only for two cysteines, and their involvement in the formation of a disulphide bridge was demonstrated.

INTRODUCTION

Pregastric esterase (PGE) is a major fat-digesting enzyme in the calf. PGE is secreted by the von Ebner’s glands on the dorsal posterior region of the tongue and also by glands located in the pharynx wall. Although the digestion of fat is almost exclusively attributed to the activity of pancreatic lipase, previous reports showed the importance of preduodenal lipases in the initial digestion of dietary triacylglycerols [1]. This preduodenal fat digestion is a precondition for efficient intestinal lipolysis. It certainly becomes important in cases of pancreatic insufficiency, and in the newborn, where pancreatic function is still not fully developed.

The existence of preduodenal lipases has been described in several mammals [2] and the genes coding for rat lingual lipase (RLL), for calf PGE and for gastric lipases of dog (DGL), rabbit (RGL) and human (HGL) have been cloned [3–6]. These preduodenal lipases show a great similarity in both their nucleotide and amino acid sequences. The amino acid sequences, deduced from the cDNA sequences, are up to 85% identical.

Pregastric esterase (PGE) was observed. But the same degree of inhibition was caused by the non-esterified fatty acid C₁₂:0. PGE shows a great sequence similarity to gastric lipases. Gastric lipases have three cysteine residues, and two of these form a disulphide bridge. Blocking the remaining free cysteine with thiol reagents inactivates the gastric lipases. The fact that PGE is not inhibited by thiol reagents indicates that PGE has no functional free thiol group. The PGE cDNA codes only for two cysteines, and their involvement in the formation of a disulphide bridge was demonstrated.

Experimental

Reagents

DTNB, 4-PDS, E600, lauric acid (C₁₂:0), p-nitrophenyl butyrate (PNPB), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) were purchased from Sigma (St. Louis, MO, U.S.A.). C₁₂:0-TNB was generously given by Professor R. Verger (CNRS, Marseilles, France). Tributyrin was from Fluka (Buchs, Switzerland). Low-molecular-mass protein markers were obtained from Pharmacia LKB (Uppsala, Sweden).

BCA protein assay reagent was from Pierce (Rockford, IL, U.S.A.).

Determination of PGE activity

pH-stat method

For quantitative purposes lipase activity was measured titrimetrically at room temperature in a pH-stat (Metrohm) at pH 5.5, with a tributyrin emulsion as the substrate. This emulsion was prepared as follows: 0.125 ml of tributyrin was added to 2.5 ml of 5% gum arabic, 0.9% NaCl and 2 μM BSA and mixed.

Abbreviations used: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; C₁₂:0, lauric acid; C₁₂:0-TNB, dodecylthio-5-(2-nitrobenzoic acid); DGL, dog gastric lipase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); E600, diethyl p-nitrophenyl phosphate; HGL, human gastric lipase; NBT, Nitro Blue Tetrazolium; 4-PDS, 4,4′-dithiopyridine; PGE, pregastric esterase; PNPB, p-nitrophenyl butyrate; RGL, rabbit gastric lipase; RLL, rat lingual lipase.

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for 20 s at full speed with a Sorvall mixer. One unit of activity is defined as the amount of enzyme that liberates 1 µmol of butyric acid/min.

Colorimetric method
A stock solution of 50 mM PNPB was prepared by dissolving 10.45 mg of PNPB in 1 ml of acetonitrile. The reaction mixture contained 0.75 mM PNPB and 10 µl of the sample in a final volume of 200 µl of PBS. The hydrolysis of PNPB was monitored for 10 min by measuring the colour development at 405 nm. This method was used as a fast qualitative test to determine the enzyme activity in the column fractions.

Purification of PGE

Tissue extraction
Pharyngeal tissue of 24-week-old calves was obtained from a local slaughterhouse. The tissue was cut in 1 cm pieces after removal of most of the muscle. PGE extracts were prepared by soaking 40 g of pharyngeal tissue in 100 ml of phosphate buffer (28 mM KH₂PO₄, 39 mM Na₂HPO₄, 15 mM NaCl and 1 mM benzamidine, pH 7) at 4 °C for at least 1 h. After centrifugation for 15 min at 12000 g, the tissue pellet was successively extracted three more times with the same buffer. The supernatants were pooled and used as starting material for subsequent purification steps.

Anion-exchange chromatography
Anion-exchange chromatography was performed on a 50 ml Q₅₀ FASTFLOW Sepharose column (26 mm × 20 cm; Pharmacia) equilibrated with phosphate buffer, pH 7. The tissue extract was loaded at a flow rate of 4 ml/min. The column was washed with 2 column vol. of phosphate buffer and the bound material was eluted with a linear gradient of 0–0.5 M NaCl. Fractions of volume 12 ml were collected, lipolytic activity was measured by colorimetry with the PNPB method, and the active fractions were pooled.

(NH₄)₂SO₄ precipitation
The pooled fractions were concentrated by precipitation at 50 % saturation of (NH₄)₂SO₄. An equal volume of saturated (NH₄)₂SO₄ solution was slowly added at 4 °C with continuous stirring. The mixture was left for 30 min at 4 °C and centrifuged at 12000 g for 15 min. The precipitate was resuspended in 10 ml of PBS and centrifuged again. The clear supernatant was used for further purification by gel filtration.

Gel-filtration chromatography
For this purpose a Sephacryl S-200 HR column (26 mm × 85 cm; Pharmacia) equilibrated with PBS was used. A 10 ml sample was loaded on the column. Elution was carried out with PBS at a flow rate of 2 ml/min, and 8 ml fractions were collected. Lipolytic activity was measured with PNPB as substrate and the active fractions were pooled. The column was calibrated with BSA, ovalbumin and carbonic anhydrase as molecular-mass markers.

Determination of protein concentration
Protein concentrations were determined by the BCA protein assay with BSA as the standard. Specific activities are expressed as units/mg of protein.

Gel electrophoresis
Discontinuous SDS/PAGE was performed by the standard procedure of Laemmli [17]. Samples were subjected to gel electrophoresis under either non-reducing (without β-mercaptoethanol) or reducing conditions. Proteins were stained by silver staining [18]. Phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa) were used as the molecular-mass standards.

Antibody production and immunoblot analysis
Polyclonal antibodies against PGE were raised in rabbits by subcutaneous injection of 160 µg of purified PGE diluted 1:2 (v/v) in Freund’s complete adjuvant. Monthly booster injections were given with the same amount of PGE in incomplete adjuvant. After the second booster, the animals were bled every 2 weeks after immunization and the serum was recovered. These antibodies were able to precipitate PGE activity (results not shown).

For immunoblot experiments, proteins were transferred to a poly(vinylidene difluoride) membrane (Millipore) by semi-dry blotting. Electroblotting was performed as described by Bartles and Hubbard [19]. To block the remaining unoccupied sites, the membrane was incubated overnight in PBS containing 10 % non-fat dry milk. Thereafter the membrane was incubated for 1 h with anti-PGE antibodies diluted 1/1000 in PBS containing 0.2 % Tween 20 and 3 % non-fat dry milk. The membrane was rinsed three times with PBS/Tween 20 and further incubated for 1 h with a 1/1000 dilution of alkaline phosphatase-conjugated swine anti-rabbit antibodies (Dako D306). After washing the membrane three times with PBS/Tween 20 and twice with the alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl₂, pH 9.5), the immunoreaction was revealed with 0.016 % BCIP and 0.03 % NBT in alkaline phosphatase buffer.

Tryptic cleavage
A 1 ml portion of purified PGE (200 µg) was cleaved with 35 units of tosylphenylalanylcitoromethane-treated trypsin attached to beaded agarose (Sigma) for 25 min at room temperature.

N-terminal sequence analysis
To determine N-terminal sequences, protein bands from SDS gels were transferred to a Problott membrane (Applied Biosystems). Transfer was performed as described by Madsudaira [20]. Automated Edman protein degradation was performed with a protein sequencer (Applied Biosystems; model 471 A).

Reactions of PGE with DTNB, 4-PDS, C₁₂:0-TNB and C₁₂:0
A 1 ml (200 µg) portion of purified PGE was preincubated with DTNB, 4-PDS, C₁₂:0-TNB or C₁₂:0 at final concentrations of 0.45 mM, 0.4 mM, 0.4 mM and 0.5 mM respectively (at least a 100 molar excess). After different incubation times, 50 µl samples were taken for determination of the residual lipase activity by using the pH-stat with tributylin as substrate.
mercaptoethanol, and the residual enzyme activity was measured in 50 µl samples at different times, by the pH-stat method.

**Reaction of PGE with E600**

E600 is a well-known serine protease inhibitor. E600 was dissolved at a concentration of 40 mM in acetonitrile. A final concentration of 0.48 mM was added to a 1 ml sample of purified PGE (200 µg), which corresponds to a molar ratio of 120:1. The residual lipase activity in 50 µl samples was measured at different times with tributyrin as substrate.

**RESULTS**

**Purification of PGE**

The elution pattern obtained by anion-exchange chromatography of the pooled extracts of pharyngeal tissue on a Q\textsuperscript{FASTFLOW} column is shown in Figure 1. The fractions eluted between 175 mM and 250 mM NaCl were pooled. Their combined activity accounted for 30% of the PGE activity initially loaded on the column.

The precipitate obtained at 50% saturation with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} of the pooled fractions was resuspended in PBS. The supernatant obtained after centrifugation of this suspension contained 26% of the lipase activity in the original extract.

**Figure 1** Ion-exchange chromatography of a pharyngeal extract on a Q\textsuperscript{FASTFLOW} column

Protein (continuous curve) was determined by monitoring the A\textsubscript{280} of the effluent. Elution was performed with 600 ml of a linear NaCl gradient (0 to 0.5 M), followed by a final washing with 150 ml of 1 M NaCl (straight line). PGE activity (D; A\textsubscript{405}) was measured with PNPB.

**Figure 2** Elution profile of PGE activity from a Sephacryl S-200 column

Pooled Q\textsuperscript{FASTFLOW} fractions were loaded on the column after precipitation with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and resuspension in PBS. Measurement of the A\textsubscript{280} was used for monitoring the protein content (continuous curve) in the effluent. PGE activity (O; A\textsubscript{405}) was measured with PNPB.

Chromatography of this supernatant on a Sephacryl S-200 gel-filtration column gave the elution profile shown in Figure 2. This step resulted in fractions containing essentially pure PGE, as revealed by SDS/PAGE and silver staining of the gel (Figure 3).

Table 1 shows the complete purification flow-sheet. A 13-fold purification was achieved, with an overall recovery of 14% of the enzyme activity present in the original tissue extract. About 6.3 mg of purified PGE was obtained starting from 40 g of fresh calf pharyngeal tissue.

Purified PGE appeared as a single band on SDS/PAGE with a molecular mass of 50 kDa. The molecular mass as determined by gel filtration was about 46 kDa.

The activity of purified PGE was to a large extent dependent on the presence of BSA in the reaction medium. When purified PGE was assayed on a tributyrin emulsion without BSA, a rapid loss of activity occurred. By contrast, optimal activity was reached and maintained for a considerable length of time when the medium was supplemented with 2 µM BSA before adding the enzyme. Addition of BSA to an emulsion in which the enzyme was already inactivated did not restore the action of PGE (results not shown). Obviously PGE is subject to irreversible interfacial denaturation, a phenomenon that also occurs with the gastric lipases [12–14]. In crude PGE extracts this surface inactivation was not observed, as it was probably prevented by the presence of other proteins, mainly BSA.

**Influence of thiol reagents and β-mercaptoethanol on PGE activity**

With DTNB or 4-PDS, PGE inhibition was less than 5% after more than 3 h of incubation. C\textsubscript{14}0-TNB partially inhibited PGE
Table 1 Flow-sheet of the purification of PGE

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Activity recovery (%)</th>
<th>Purification factor</th>
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<td>11 933</td>
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<td>28</td>
<td>111</td>
<td>26</td>
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<tr>
<td>Sephacryl S-200 gel filtration</td>
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<td>6.5</td>
<td>260</td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>

Influence of E600 on PGE activity

E600 is a well-known serine-enzyme inhibitor, which binds covalently to serine residues. A rapid and complete inhibition of PGE was observed after 45 min incubation with 0.48 mM E600, the inhibition half-time being 5 min.

Characterization of PGE fragments after trypsin digestion

Mild trypsin cleavage of PGE under reducing conditions resulted in the appearance of three bands on immunoblotting (P1, P2 and P3; see Figure 6). Under non-reducing conditions only P1 was present. Identical N-terminal sequences for the fragments P1 and P2 were found: Ile-Ala-Lys-Asn-Pro-Glu-Ala-Ser-Met-Asn-Val-Ser-Gln-Met-Ile-Ser-Tyr, indicating a first trypsin cleavage site at Lys. The N-terminal sequence of P3 (Glu-Thr-Leu-Asp-Val-Leu) points to a second trypsin cleavage site at Arg. The N-terminal amino acid sequences of the fragments of PGE, determined by Edman degradation, are identical with those deduced from the cDNA sequence and clearly show that the purified enzyme corresponds to the protein encoded by the cDNA sequence of PGE [4]. N-terminal sequence analysis of the native purified enzyme was impossible, due to a blocked N-terminus.

DISCUSSION

PGE was purified about 13-fold by the combination of QFASTFLOW anion-exchange chromatography, (NH₄)₂SO₄ precipitation and Sephacryl S-200 gel filtration of extracts of calf pharyngeal tissue. The final preparation gave a single band of 50 kDa on SDS/PAGE and an overall recovery of 14%. Two previously published purification procedures [15,16] reported a similar molecular mass and resulted in a yield of 15% and 26%, respectively. Neither of these two methods gave a satisfactory result in our laboratory. In the method of Sweet et al. [15] a lipid extraction was crucial to convert PGE–lipoprotein complexes into single low-molecular-mass units. Bernbaek et al. [16] encountered the formation of catalytically inactive PGE aggregates during the purification. Furthermore, the final preparations showed a strong tendency to form non-active complexes upon freezing and thawing. Although our method has a lower yield, it has the major advantage that no lipoprotein complexes are formed. Consequently no lipid-extraction step was required to dissociate the aggregates. Furthermore, our method is less cumbersome and time-consuming.

The purified enzyme was used to investigate whether a serine residue and a free thiol group are involved in its activity. PGE activity is completely inhibited by E600, a serine inhibitor. This
is in agreement with observations made with other lipases and is a confirmation of the fact that a serine residue is important for lipolytic activity in lipases in general [9]. The preduodenal lipases show no sequence identity with other lipases, except for a conserved Gly-Xaa-Ser-Xaa-Gly sequence which is found in all lipases sequenced so far. X-ray-diffraction analysis of crystalline lipase from Mucor miehei [8] and human pancreas lipase [7] predicted a Ser/His/Asp triad, with a central role for the serine residue of the pentapeptide in the catalytic site. When the enzyme is not attached to an oil/water interface, this putative hydrolytic site is covered by a surface loop and is therefore inaccessible. Crystal structure analysis on Mucor miehei lipase inhibited by n-hexylphosphonate [21] and E600 [22] showed a conformational change by which the conserved serine was exposed. The lipase-inhibitor complex was proposed to be equivalent to the activated state generated when the enzyme binds to an oil/water interface. HGL and RGL, when inactivated by E600, lose their activity on both water-soluble and emulsified tributyrin, but are still able to bind to lipid/water interfaces [9]. This provides strong evidence that the essential serine residue is involved in catalysis and not in lipid binding.

It should be noticed that PGE, just like the other preduodenal lipases, accommodates two of these conserved sequences, specifically Gly-Asn-Ser$_{99}$-Arg-Gly and Gly-His-Ser$_{102}$-Gln-Gly. There is at present no indication which of these two serine residues is implicated in the formation of the catalytic triad. No information is available on the three-dimensional structure of preduodenal lipases, and, because of the limited sequence similarity to other lipases, it is not evident that preduodenal lipases employ the same catalytic mechanism.

In contrast with the gastric lipases, PGE is not inhibited by the thiol reagents DTNB and 4-PDS. In the gastric lipases three cysteine residues are conserved (Cys$_{237}$, Cys$_{256}$ and Cys$_{241}$). PGE has only two of these conserved cysteine residues (Cys$_{236}$ and Cys$_{235}$). Sequence alignment of PGE with the gastric lipases shows the presence of one additional amino acid for the gastric lipases after position 4, which explains the shift in amino acid numbering. For HGL, RGL and DGL inhibited by thiol reagents, only one out of the three cysteines was titrated per enzyme molecule [12–14]. The thiol-modified gastric lipases could still penetrate into lipid monolayers, but they were unable to hydrolyse the substrate [23]. From the inhibition of the gastric lipases by DTNB, 4-PDS and C$_{12,0}$-TNB, it was assumed that these enzymes possessed a thiol group that is essential for their catalytic activity. Therefore they were called ‘thiol enzymes’. The thiol group of gastric lipases is assumed to be either directly or indirectly involved in the catalytic mechanism. It is proposed that the modification of this thiol group prevents the access of the substrate to the catalytic site. This led to the conclusion that gastric lipases have one free thiol group and one disulphide bridge. In the gastric lipases Cys$_{237}$ and Cys$_{241}$ are separated by an Arg$_{239}$. For RGL and HGL the fragments generated after trypsin digestion at Arg$_{239}$ were separated under both reducing and non-reducing conditions. This indicates that the essential free cysteine is Cys$_{241}$ and that Cys$_{237}$ and Cys$_{236}$ are involved in a disulphide bridge [24]. The fact that PGE, which contains only two of these conserved cysteines, is not inhibited by DTNB or 4-PDS indicates that no free thiol group is involved in its activity. The inhibition of PGE by β-mercaptoethanol assumes the presence of a disulphide bridge, which is important for the lipolytic activity. The presence of this disulphide bridge in PGE was proved by the fact that fragments P2 and P3, generated by tryptic cleavage at Arg$_{238}$, were not separated in the absence of a reducing agent. This is in contrast with what was found for RGL and HGL, and clearly indicates that in PGE there is a disulphide bridge between Cys$_{236}$ and Cys$_{235}$, linking P2 and P3.

PGE is only partially inhibited by C$_{12,0}$-TNB, a hydrophobic analogue of DTNB. This inhibition is due to the C$_{12,0}$ moiety of this thiol reagent, as shown by the inhibition with the non-esterified fatty acid C$_{12,0}$. From the present study it is clear that, although all preduodenal lipases share a great sequence identity, their behaviour in the presence of thiol reagents is quite different. Our results indicate that no thiol group is involved in the catalytic mechanism of PGE, making the enzyme distinct from the gastric lipases. Accordingly, the term ‘thiol enzyme’ does not apply to PGE. In this respect it is noteworthy that RLL has four cysteine residues [5] which might be involved in the formation of two disulphide bridges. Should this be the case, RLL, similarly to PGE, may not have a free thiol group. Preduodenal lipases could then be classified in two separate categories: first, the pregastric lipases, which are not produced by the stomach and do not possess a free thiol group; secondly, the gastric lipases, originating from the stomach. These gastric enzymes all have one functional free thiol.
group that plays an important role in their catalytic activity. Unfortunately, inhibition experiments, as described in this study on PGE, are not yet available for RLL.

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