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Purification and Characterization of Lamb Pregastric Lipase

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ABSTRACT

Lamb pregastric lipase was purified from a commercial source using delipidation, solubilization with KSCN, acid-precipitation, pepsin-digestion, affinity chromatography with agarose-Cibacron Blue F3GA, gel filtration, and elution from a native 10% (w/v) polyacrylamide gel. The enzyme had a single subunit of 68,000 Da with maximum esterase activity when measured at pH 6.0 and 30°C. The enzyme preferentially hydrolyzed short- and medium-chain (C4, C6, and C8) synthetic esters and short-chain (C4 and C6) monoacid triglycerides. The NH_2-terminal sequence demonstrated high homology with gastric and lingual lipases.

Index Entries: Lamb pregastric lipase; lipase; preduodenal.

INTRODUCTION

Pregastric lipases (PGL) are lipolytic enzyme constituents of the oral secretions of young ruminants (*1*). These preduodenal enzymes, like the monogastric lingual lipases, play an important part in fat digestion in newborn animals (*2–4*). Pregastric lipases from calf, kid-goat, and lamb sources are used in the manufacture of cheese and other foods to enhance flavor (*5–7*). Lamb PGL produces a sharp, lingering flavor in Romano, Provolone, Mozzarella, and Feta cheeses (*1,8–11*) by the liberation of mixtures of short- and medium-chain (C2–C10) free fatty acids from milk-fat (*12*). Pregastric lipases have alsi been used as therapeutic agents for the

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treatment of scours and diarrhea in calves (*13,14*) and malabsorption syndrome in children (*15*).

Recent studies on preduodenal lipases have resulted in the purification and characterization of calf pregastric lipases (*16,17*), rat lingual lipases (*18–20*), rabbit gastric lipase (*21*), and human gastric lipase (*22*). The extent of structural similarity between preduodenal lipases of monogastric and polygastric origin remains unclear, however, since only very limited structural sequence data for members of the latter group is available. This paper describes the first purification and characterization of lamb pregastric lipase.

MATERIALS AND METHODS

Materials

Capalase L®, a commercial lamb pregastric lipase powder, is a product of Dairyland Food Labs, Inc., Waukesha, WI. α-Naphthyl esters, monoacid triglycerides, pepsin, gum arabic, bovine serum albumin (BSA), bromophenol blue, low molecular weight markers, and silver staining kit were purchased from Sigma Chemical Co. (St. Louis, MO). The protein assay kit and the Econo-Pac Blue cartridge were from Bio-Rad Laboratories (Richmond, CA). Amicon PM-10 and Amicon XM-50 filters were from Amicon Division (W.R. Grace & Co.-Conn., Danvers, MA). High molecular weight markers were supplied as a kit from BRL (Life Technologies, Inc., Gaithersburg, MD). The immune-screening kit was from Promega Biotec (Madison, WI). All other chemicals were research grade.

Esterase Activity

Esterase activities utilized α-naphthyl ester substrates and were measured at 30°C by a modification of the method of Van Asperen (*23*). A reaction mixture of 2.5 mL of 0.1 *M* α-naphthyl ester as the substrate in 0.02 *M* potassium phosphate buffer (pH 6.0) and 0.5 mL of enzyme solution was incubated at 30°C for 30 min, then 0.5 mL of a solution of 5 parts 1% (w/v) Fast Blue B (sodium salt) and 2 parts 5% (w/v) SDS, both in 0.02 *M* phosphate buffer (pH 6.0), were added and the absorbance at 600 nm measured. Unless stated otherwise, all esterase assays were performed using α-naphthyl butyrate as substrate. A standard curve of α-naphthol was used to convert absorbance units into enzyme activity units. One unit (U) of esterase activity is defined as the amount of enzyme producing 1 μmol of α-naphthol/min.

Lipase Activity

The lipase assay was a modification of a turbidometric technique (*24*). A volume of 1 mL of substrate (3 m*M* concentration) containing 200 μL 5% (w/v) gum arabic and 100 μL 0.2 *M* potassium phosphate buffer (pH

6.0) was emulsified at high power with an ultrasonicator probe (Cole-Parmer Instruments, Chicago, IL) for 30 s. After enzyme addition, the decrease (per min) in absorbance at 340 nm was measured. Control experiments utilized an equivalent amount of buffer replacing the enzyme. One unit of lipase activity is defined as the amount of enzyme that leads to a decrease of 0.001 absorbance units (at 340 nm) per minute under the conditions described above.

Protein Assay

Protein was assayed by the method of Bradford (*25*) using the Bio-Rad protein assay kit. Bovine serum albumin was used as the standard for protein determination.

SDS-PAGE

Samples obtained at each stage of purification were run on discontinuous SDS-PAGE (4% w/v stacking gel, 10% w/v running gel) with bromophenol blue as the tracking dye, using gel and buffer formulations described previously (*26*). Silver stained gels were calibrated with standard proteins between 14,000 and 200,000 Da.

Delipidation and Solubilization of Pregastric Lipase

Delipidation of the pregastric lipase utilized a method described earlier (*16*). 100 g of Capalase L® were added to 500 mL cold acetone (−20°C) for 15 min. The mixture was centrifuged at 15,000×*g* for 10 min in the cold (−20°C). The supernatant was discarded and the pellet resuspended in 500 mL *n*-butanol at −20°C. The mixture was stirred for 15 min, then centrifuged as before. The pellet was treated three more times with cold acetone, centrifuged, dried under vacuum for 2 h, and stored desiccated at −70°C until used. 5 g of the dried powder was mixed in 50 mL of 20 m*M* Tris-HCl (pH 7.0) containing 0.25 *M* KSCN. The suspension was stirred for 15 min at room temperature and then centrifuged (5,000×*g*; ambient temperature). The supernatant was recovered and the pellet re-extracted with a further 50 mL of buffer and KSCN as before. The supernatants were combined, clarified by centrifugation (50,000×*g*; 20°C) and filtered successively through 0.45 μm and 0.22 μm cellulose nitrate filters. The filtrate was labeled as crude PGL.

Acid-Precipitation on Non-PGL Proteins

Crude PGL (90 mL) was dialyzed against 2 L of sodium acetate buffer (50 m*M*, pH 4.8) at 4°C for 12 h with a fresh buffer change at 6 h. The dialyzed sample was centrifuged for 1 h (50,000×*g*, 4°C) and the supernatant retained.

Pepsin Digestion

Calf pregastric lipase has been shown to be resistant to pepsin digestion (*27*), providing an opportunity for removal of nonlipase proteins. To test this approach for lamb PGL, pepsin was added to 0.5 mL of the acid-treated preparation in 50 m*M* sodium acetate (pH 4.8) at concentrations ranging from 50–400 μg pepsin/mg protein. After incubating at 25°C for various times, aliquots (20 μL) were withdrawn and mixed with 20 μL Tris-HCl (40 m*M*, pH 8.0) and stored on ice. The pepsin-digested protein mixture was concentrated with buffer change (new buffer: 20 m*M* Na_2HPO_4 buffer, pH 7.1) using an Amicon XM-50 filter in an Amicon Ultrafiltration Cell (25 cm diameter, 50 mL capacity) at 20 lbs/in^2 pressure.

Affinity Chromatography

The Econo-Pac blue cartridge contains a beaded, crosslinked agarose with covalently attached Cibacron Blue F3GA dye, which has a affinity for albumin (*28*). The cartridge was equilibrated in application buffer (20 m*M* Na_2HPO_4 buffer, pH 7.1) and the concentrated enzyme sample from the previous purification step applied to the cartridge using a 5 mL plastic syringe. The cartridge was then washed with 10 mL of the application buffer (flow-rate of 1 mL/min) and the effluent collected. The effluent was concentrated to 2 mL and the buffer changed to 100 m*M* Tris-HCl, pH 8.0 using the Amicon XM-50 filter unit as before.

Gel Filtration

200 μL (≈2.2 mg) of the Econo-Pac blue cartridge effluent was applied to a Superose 12 column (HR 10/30, Pharmacia FPLC system), pre-equilibrated with 100 m*M* Tris-HCl, pH 8.0. The enzyme was eluted at a flow-rate of 0.5 mL/min, the fraction size being 0.5 mL, and monitored with a Pharmacia Single Path UV-1 absorption detector at 280 nm. Selected fractions with esterase activity were pooled and concentrated using an Amicon PM-10 filter (10,000 Da cutoff).

Native (non-SDS) Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed on 1.5 mm slab gels (4% stacking gel, 10% running gel) using Tris-glycine buffer, pH 8.3 at 100 V at 4°C. One section containing a sample lane and BSA as marker was silver stained. A duplicate sample lane was equilibrated in 20 m*M* phosphate buffer, pH 6.0 for 30 min at room temperature and then transferred to a 50 mL solution containing 20 m*M* phosphate buffer (pH 6.0), 100 mg *o*-dianisidine (Fast Blue B), and 10 m*M* α-naphthyl butyrate and incubated at 30°C until esterase products were visible. The corresponding regions were excised from a third sample lane. The gel slices in dialysis

tubing (Spectropor, 12,000 Da cutoff, Spectrum Medical Industries, Los Angeles, CA) containing 20 m*M* Tris-HCl, pH 7.5, were immersed in the same buffer contained in a horizontal slab gel electrophoresis unit and subjected to 100 V for 1 h at 4°C. The current was then reversed (100 V for 2 min) and the eluted protein solution in the dialysis tubing removed, pooled, concentrated, and re-dialyzed in Spectropor tubing against 10 m*M* Tris-HCl, pH 7.5 at 4°C with four buffer changes over a period of 48 h. The dialyzed proteins were concentrated once again using Centricon-10 concentrators (10,000 Da cutoff, Amicon) and assayed for esterase and lipase activities.

Molecular Weight Estimation of Native PGL

Purified lipase was applied to a Superose 12 column (HR 10/30, Pharmacia FPLC system) and equilibrated in 100 m*M* Tris-HCl, pH 7.5. Fractions of 0.5 mL size were eluted at the rate of 0.3 mL/min. The elution was monitored as before at 280 nm. The column was calibrated with standard proteins (12,400 Da–200,000 Da) obtained from Sigma Chemical Company.

Amino Acid Analysis

The amino acid composition of the purified pregastric lipase was determined, after 24 h hydrolysis in HCl, using a Waters amino acid analyzer (Waters Assoc., Milford, MA) at the Macromolecular Structure Facility (Department of Biochemistry, Michigan State University). The NH_2-terminal amino acid sequence of purified enzyme was determined using an Applied Model 477A Sequencer with on-line (model 120) PTH-AA analyzer (Applied Biosystems, Inc., Foster City, CA).

Immunological Characterization

A western blot (nitrocellulose filter) of the purified enzyme electrophoresed on SDS-polyacrylamide gel (10% w/v) was used to test the immunoreaction with rabbit anti-serum prepared toward purified enzyme. Immune reactions between antigen and antibody were detected using anti-rabbit IgG-alkaline phosphatase conjugate and alkaline phosphatase color development reagents (Nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate), obtained as a kit from Promega Biotec (Waukesha, WI), referred to here as the BCIP-NBT substrate-dye system.

Temperature Stability Studies

Enzyme stability was measured between 30°C and 65°C. The enzyme was pre-incubated at the selected temperatures and aliquots of 1 μL (1 μg) were removed at intervals of 15 min to 1 h and assayed for esterase activity at 30°C using the standard esterase assay.

Effect of Carbon Chain Length on Esterase and Lipase Activities

Esterase and lipase activities were measured using 0.1 m*M* α-naphthyl ester substrates and 3.0 m*M* mono-acid triglycerides, respectively. The α-naphthyl esters used were α-naphthyl-acetate (C2), -propionate (C3), -butyrate (C4), -caproate (C6), -caprylate (C8), -caprate (C10), -laurate (C12), and -myristate (C14). The monoacid triglycerides used were tributyrin (C3), tricaproin (C6), tricaprylin (C8), tricaprin (C10), trilaurin (C12), trimyristin (C14), and tristearin (C18). 20 μL (20 μg) of electrophoretically pure enzyme was used for each determination.

RESULTS

Purification of Pregastric Lipases

The initial steps of purification included acid precipitation, pepsin digestion, and Econo-Pac blue affinity chromatography. The crude lamb pregastric lipase demonstrated resistance to pepsin, as seen in Fig. 1. A pepsin concentration of 100 μg/mg protein, and an incubation time of 30 min, was selected for use in the purification process, as higher concentrations of pepsin resulted in a greater loss of enzyme activity (Fig. 1) without significant improvement in specific activity. The Econo-Pac blue affinity step was introduced to remove an albumin-like contaminant that, unless removed, copurified with the pregastric lipase in subsequent steps.

Following initial purification steps, the pregastric lipases eluted as a broad activity peak from the Superose 12 column (Fig. 2). Analysis of the pooled fractions from the Econo-Pac blue cartridge effluent (Fig. 3A) and the Superose 12 column (Fig. 3B) by activity staining of native (non-SDS) gels showed the presence of four activity bands (Fig. 3) designated I, II, III, and IV, and IV being the major activity band. Enzymes I and IV were successfully eluted whereas enzymes II and III could not be eluted in detectable amounts. SDS-PAGE of enzyme I (labeled PGL1) revealed a major band of 55,500 Da with an occasional faint band at 28,000 Da (Fig. 4A), whereas enzyme IV (labeled PGL2, Fig. 4B) gave a single band of molecular weight 68,000 Da. Approximate molecular weights of 240,000 Da and 70,000 Da for PGL1 and PGL2, respectively, were estimated from elution patterns using gel filtration (Superose 12) column chromatography with standard protein markers (12,400–200,000 Da). Although PGL1 is of interest, having a lower molecular weight in SDS-PAGE than PGL2 and in crossreacting serologically (Fig. 5), the enzyme rapidly lost activity in the highly purified state and further enzymatic characterization was not possible. The overall purification scheme is presented in Table 1, in which PGL2 was purified 61-fold, with a yield of 0.2%.

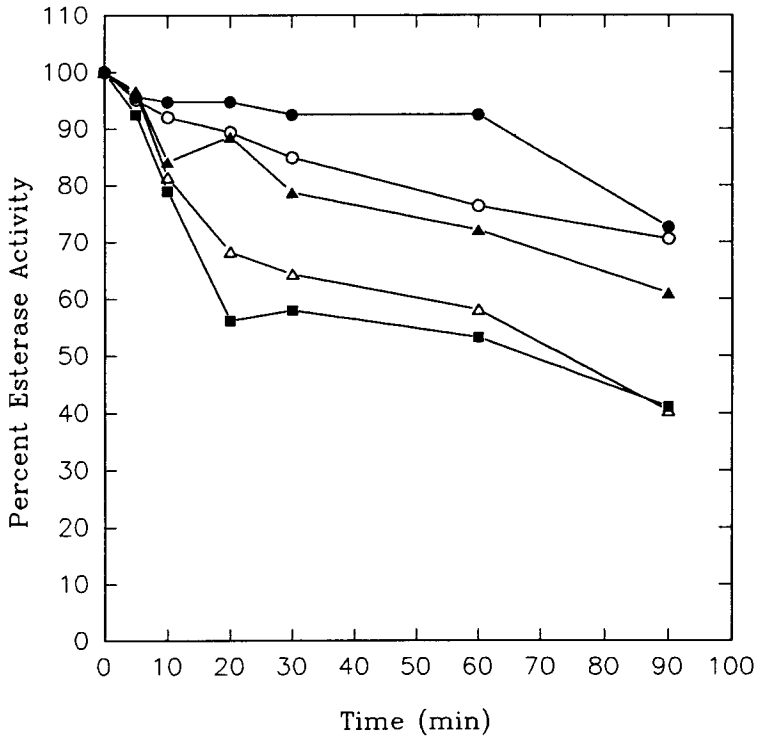

Fig. 1. Effect of pepsin on the activity of lamb pregastric lipase. Partially purified enzyme (supernatant of acid-treated step) was incubated with various concentrations of pepsin (μg/mg protein) including 50 (○); 100 (▲); 200 (△); or 400 (■) with no pepsin (●) as control. After incubation at various times, aliquots were removed and assayed using α-naphthyl butyrate as substrate. Activity is expressed as a percentage of the activity at zero time and data presented are the means of three readings.

Amino-Acid Composition and NH_2-terminal Sequence Analysis

The amino acid analysis of PGL2 is presented in Table 2. The ratio of 1.8:1 for acidic (Glx+Asx) to basic (Arg+His+Lys) amino acids indicates that PGL2 is highly acidic. Comparable ratios of 2.2:1 for rabbit gastric (*21*), 1.9:1 for calf pharyngeal (*17*) and rat lingual lipases (*29*), and 1.8:1 for human gastric lipase (*30*) were reported, the latter two ratios being deduced from cDNA sequences.

The NH_2-terminal amino acid sequence of purified PGL2 was determined to be Ser-His-Pro-Thr-(Asp/Lys)-Pro-Glu. Comparison of this sequence with reported preduodenal lipase NH_2-terminal amino acid sequences (*17,21,29,30*), after alignment, reveals close homology between PGL2 and the preduodenal lipases, especially rabbit gastric lipase (Table 3).

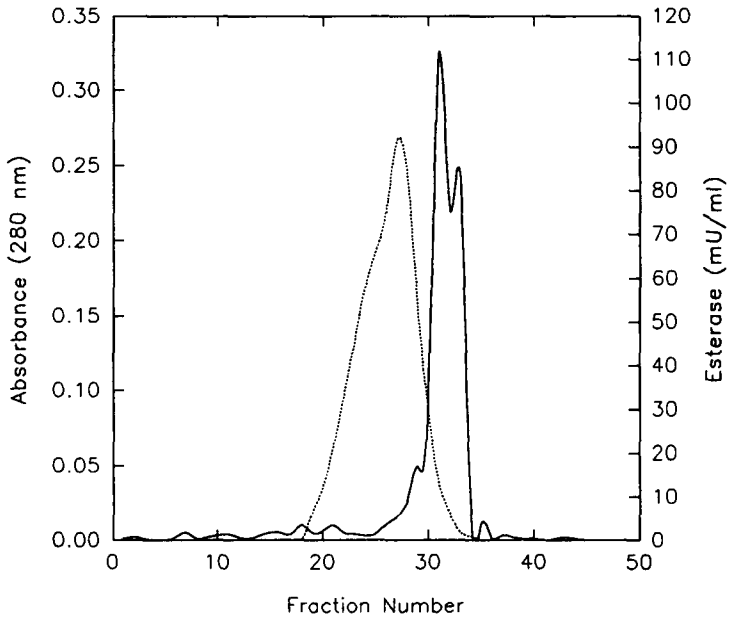

Fig. 2. Gel filtration on a Superose 12 column (HR 10/30, Pharmacia FPLC system) at room temperature. 200 μL (≈ 2.2 mg) of the concentrated EconoPac blue cartridge effluent was loaded on the column pre-equilibrated with 100 m*M* Tris-HCl, pH 8.0. The elution rate was 0.5 mL/min and fractions of 0.5 mL were collected. Esterase activity of the fractions was determined using α-naphthyl butyrate as substrate (dotted line), and protein was measured as absorbance at 280 nm (solid line).

There is no homology between the NH_2-terminal sequence of PGL2 and that reported for calf pregastric lipase (*17*), both calf and lamb being ruminants belonging to polygastric species. In the limited study on calf pregastric lipase (*17*), only four terminal amino acids were determined, however, and experimental details of the terminal sequence analysis were not given.

Characterization of PGL2

Effect of pH

The esterase activities of PGL2 was measured at pH values ranging from 3.0–7.0. PGL2 had maximum activity at pH 6.0 (Fig. 6). The esterase assay system could not be used at pH values above 7.0 because of instability in the assay reaction.

Temperature Stability

PGL2 was stable at temperatures up to 30°C, but it was rapidly inactivated at temperatures higher than 30°C (Fig. 7). Results are expressed as a percentage of the initial activity at 30°C.

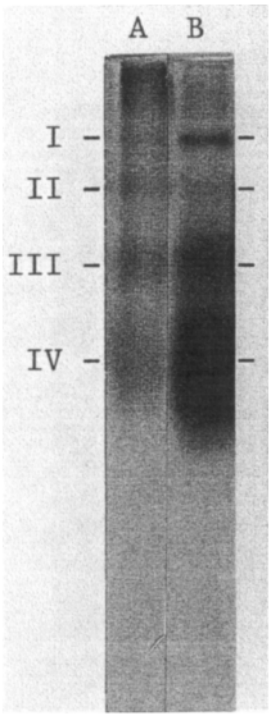

Fig. 3. Esterase activity staining of native (non-SDS) polyacrylamide (10% w/v) gel electrophoresed lamb pregastric lipase. [A] EconoPac blue cartridge effluent (10 μg); [B] Pooled Superose 12 fractions (10 μg). The gel was stained for activity as described in the Materials and Methods section.

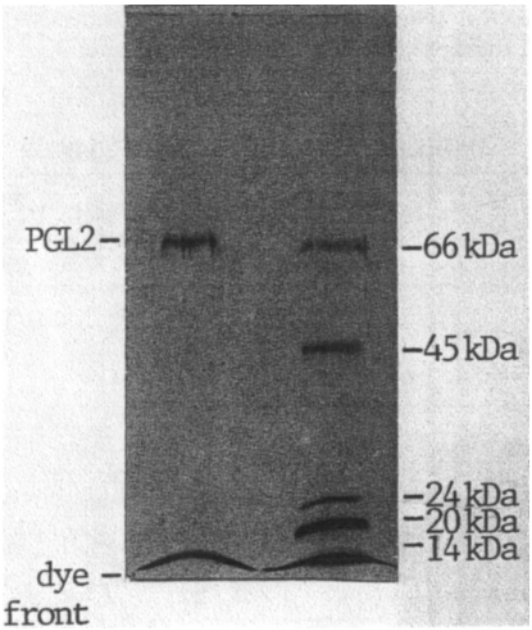

Fig. 4. SDS-polyacrylamide (10% w/v) gel electrophoresis of purified PGL2. Approximately 3 μg of the enzyme was applied to the gel. The protein standards used were: bovine serum albumin (66 kDa); ovalbumin (45 kDa); trypsinogen (24 kDa); trypsin inhibitor (20 kDa); α-lactalbumin (14 kDa).

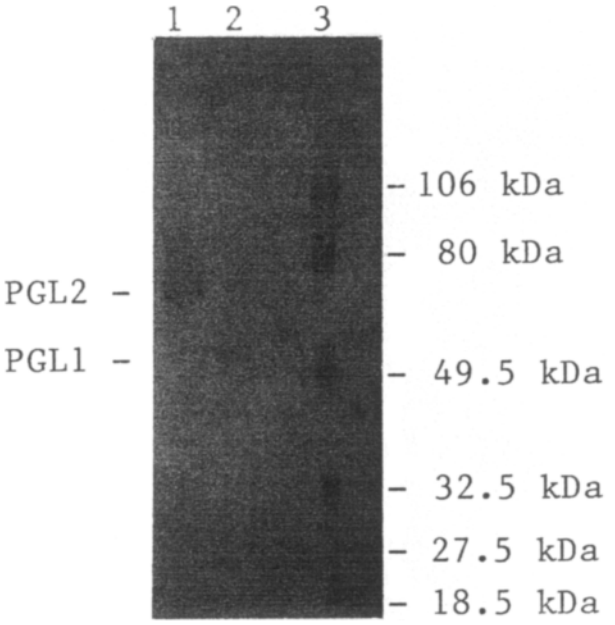

Fig. 5. Immuno-staining of a western blot of SDS-polyacrylamide (10% w/v) gel electrophoresed PGL1 and PGL2. Approximately 3 μg of each enzyme was applied to each lane (PGL2, lane 1; PGL1, lane 2). The western blot was treated with purified rabbit antiserum toward purified PGL2. Antirabbit alkaline phosphatase conjugate was used as the secondary antibody, and the immune-reaction was detected using the BCIP-NBT substrate-dye system. Prestained molecular weight markers (Bio-Rad) were run in lane 3.

Table 1
Purification of Lamb Pregastric Lipase

Purification steps	Total activity, mU	Total protein, mg	Specific activity, mU/mg	Yield, %
Crude enzyme	33335	1576.00	21	100.0
Acid precipitation (supernatant)	15335	240.00	64	46.0
Pepsin digestion (50,000 Da cutoff ultrafilter retentate)	11335	82.20	138	34.0
Econo-Pac blue cartridge effluent	8335	22.00	379	25.0
Superose 12 column fraction (pooled)	2170	3.30	658	6.5
PGL2	65	0.05	1292	0.2

Table 2
Amino Acid Composition of Purified PGL2

Amino acid	Composition,[a] mol%	Number of residues
Aspartic acid/asparagine	9.1	54
Threonine	9.2	55
Serine	8.1	49
Glutamic acid/glutamine	11.2	67
Proline	7.3	44
Glycine	7.3	44
Alanine	9.5	57
Cysteine	0.7	4
Valine	6.9	41
Methionine	n.d.	n.d.
Isoleucine	3.3	20
Leucine	8.9	53
Tyrosine	2.7	16
Tryptophan	n.d.	n.d.
Phenylalanine	4.1	25
Lysine	6.2	37
Histidine	1.9	11
Arginine	3.6	21
Total number of residues		598
Average molecular weight range (Da)		64,000

n.d.: not determined.
[a]Normalization was based on Alanine.

Table 3
Comparison of the NH_2-terminal Amino Acid Sequences of PGL2 and Other Preduodenal Lipases

Lipase	Sequence
Lamb pregastric (PGL2)[a]	Ser-His-Pro-Thr-($^{Asp}_{Leu}$)-Pro-Glu
Rabbit gastric[a] (*21*)	Lys-Ser-Ala-Pro-Thr-Asn-Pro-Glu
Human gastric[b] (*31*)	Leu-Phe-Gly-Lys-Leu-His-Pro-Gly-Ser-Pro-Glu
Rat lingual[b] (*30*)	Leu-Phe-Gly-Lys-Leu-Gly-Pro-Gly-Asn-Pro-Glu
Calf pregastric[c] (*17*)	Phe-Leu/(Ile)-Gly-

[a]sequence aligned for maximum homology
[b]derived from cDNA sequence
[c]sequence not aligned

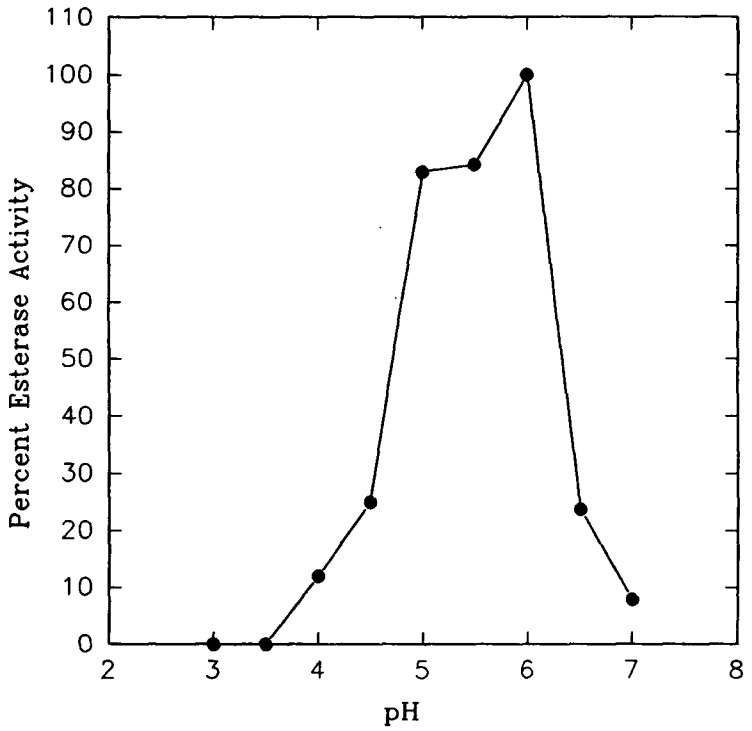

Fig. 6. Effect of pH on esterase activity of PGL2. The activity of PGL2 was tested in the pH range 3.0–7.0 using α-naphthyl butyrate as substrate. The buffers used were 20 m*M* citrate (pH 3.0–5.5), 20 m*M* phosphate (pH 6.0–7.0). The data presented are the mean of three readings. The assay system was unstable above pH 7.0.

Carbon Chain Specificity

PGL2 was found to preferentially hydrolyze C4, C6, and C8 α-naphthyl esters and C4 and C6 mono-acid triglycerides (Fig. 8).

Stability of PGL2

Crude enzyme lost 45% at room temperature, 5% at 4°C, and 2% at −20°C of its esterase activity in 30 d. The electrophoretically pure PGL2 was found to be inactive after 7 d at room temperature, and lost 30% at 4°C, and 9% at −20°C of its activity after 30 d.

Effect of Various Compounds on Esterase Activity

BSA, Triton X-100, and bile salts had no effect on the esterase activities of PGL2. EDTA and SDS inhibited PGL2 activity (Table 4).

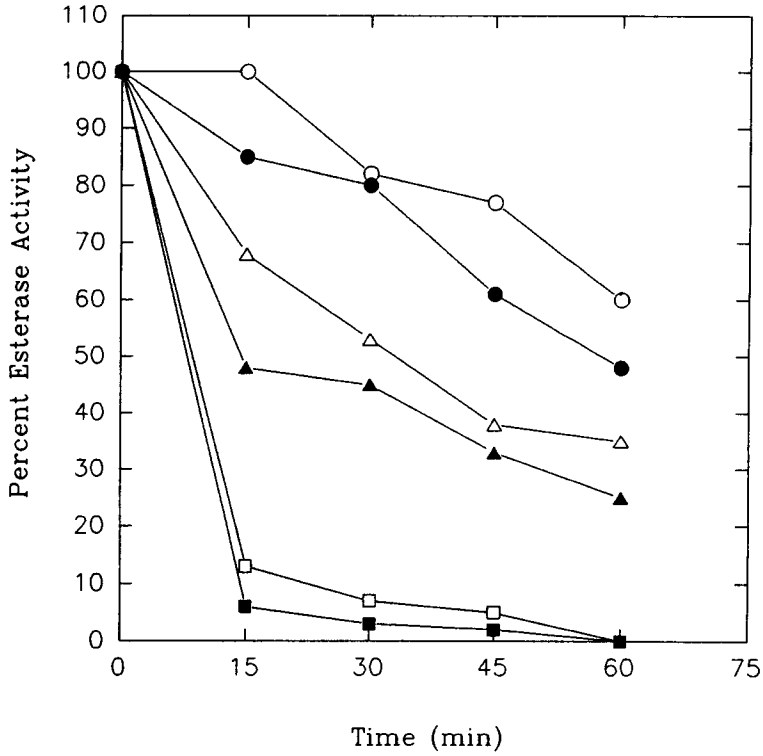

Fig. 7. Temperature stability of pregastric lipase PGL2. The enzyme was incubated at each of the various temperatures and aliquots removed at different times (15–60 min), cooled to room temperature, and the esterase activity determined. The data presented are the means of three readings and are expressed as a percentage of the activity at zero time. The temperatures used were: 30°C (○); 37°C (●); 45°C (△); 50°C (▲); 55°C (□); 65°C (■).

DISCUSSION

Earlier steps in this purification scheme were focused on the removal of nonlipase proteins from the crude enzyme starting material, and included acid-precipitation of non-PGL proteins, pepsin-digestion of non-PGL proteins, and affinity chromatography using the EconoPac Blue cartridge to remove an albumin-like protein.

The molecular weight of PGL2 was 70,000 Da by gel filtration, and 68,000 Da by SDS-PAGE, indicating a single subunit. Calf pregastric lipase has been reported as having molecular weights of 49,000 Da (*16*) and 55,000 Da (*17*). It is not known whether carbohydrate, if present, may be reflected in this difference.

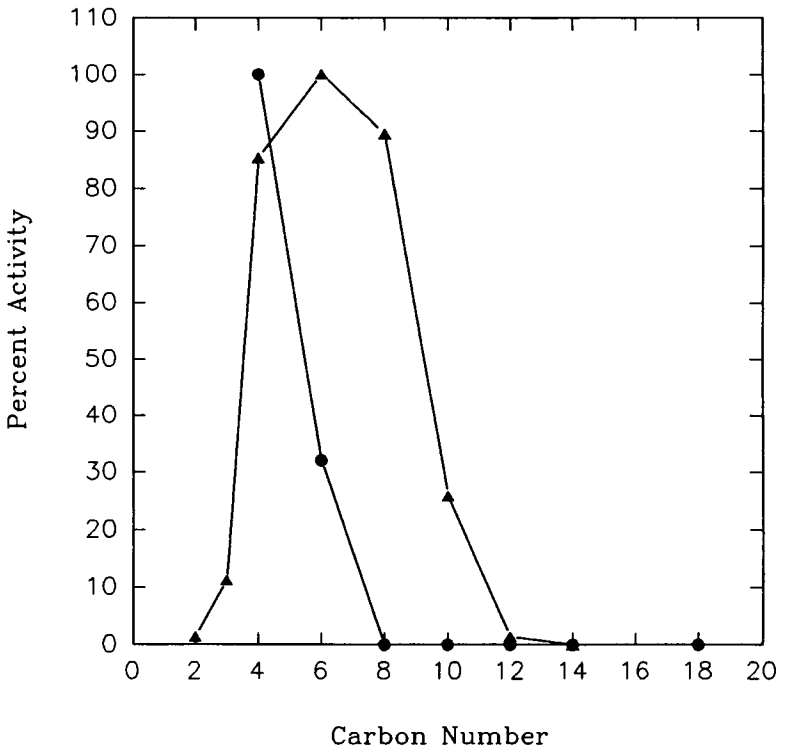

Fig. 8. Activity of PGL2 toward α-naphthyl esters and monoacid triglycerides. The various substrates used are described in the text. 100 m*M* α-naphthyl esters (▲), and 3 m*M* monoacid triglycerides (●) were used as substrates. For α-naphthyl esters, the maximum activity was toward α-naphthyl caproate (C6), and for monoacid triglycerides, maximum activity was toward tributyrin (C4). These activities were taken as 100%, and the activities toward the other substrates are a percentage of the maximum activities. Results are means of three readings.

Table 4
Effect of Various Inhibitors/Activators on Activity of PGL2

Inhibitor/Activator	Concentration	Residual activity, %
Sodium dodecyl sulfate	0.1%	0
Na_2EDTA	0.1 m*M*	62
	1.0 m*M*	0
Triton X-100	0.1%	100
	1.0%	100
Sodium deoxycholate	20 m*M*	100
Sodium taurocholate	20 m*M*	100

Values are means of 3 determinations. The standard esterase assay was used with α-naphthyl butyrate as substrate (see Materials and Methods). Residual activity is expressed as percentage of control (without the addition of inhibitor/activator).

Optimal pH values for activity for PGL2 were found to be between pH 5.5 and 6.0 with maximum activity at pH 6.0, similar to reports on calf pregastric lipase (*16,17,31*).

PGL2 was found to be specific for short- and medium-chain carbon substrates. This result agrees with previous reports that pregastric lipases from ruminants are specific for these substrates (*1,10*), an important property in their biotechnological applications. Maximum activities were observed with C6 (α-naphthyl caproate) when α-naphthyl esters were used as substrates, and with C4 (tributyrin) when monoacid triglycerides were used as substrates. This variation may reflect differences in enzyme activity on single and two-phase substrates.

In conclusion, this is the first report on the purification and characterization of lamb pregastric lipase. The possible presence of a second, minor, less stable lipase (PGL1), antigenically related but with a lower subunit molecular weight, requires further investigation. The purified pregastric lipase PGL2 is similar to calf pregastric lipase with respect to optimum pH, substrate specificity, amino-acid composition, but differs in molecular weight. The NH_2-terminal sequence analysis reveals a close homology between PGL2 and rabbit gastric lipase. This finding suggests a significant similarity between lipases of a ruminant (lamb, polygastric species) and a nonruminant (rabbit, monogastric species). Both PGL2 and rabbit gastric lipase are preduodenal lipases, leading one to speculate that all preduodenal lipases may be highly related. Sequencing of the lamb pregastric lipase cDNA, which is underway, should provide further details on this relationship.

ACKNOWLEDGMENTS

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