Volume 327, number 1, 79–84 FEBS 12737 July 1993

One-step purification and characterization of human pancreatic lipase expressed in insect cells

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Received 22 April 1993; revised version received 8 June 1993

A cDNA clone encoding the sequence of human pancreatic lipase (HPL) was subcloned into the baculovirus transfer vector pVL1392 and used in co-transfection of *Spodoptera frugiperda* (Sf9) insect cells with wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA. A single recombinant protein (50 kDa) secreted by Sf9 cells was detectable in the culture medium 24 h post-infection using both anti-HPL polyclonal antibodies and potentiometric measurements of lipolytic activity. The expression level reached 40 mg/l of enzyme at 6 days. A single cation-exchange chromatography was sufficient to obtain a highly pure recombinant HPL as demonstrated by N-terminal sequencing, amino acid composition and carbohydrate analysis, as well as by mass spectrometry. These analyses revealed the production of mature protein with the correct processing of signal peptide and an homogenous glycosylation pattern. The kinetic properties of recombinant and native HPL were compared. Both enzymes showed similar profiles of interfacial activation, inhibition by bile salts and re-activation by colipase.

Lipase; Baculovirus expression system; Cation-exchange chromatography; Lipolytic activity; *N*-Glycosylation; Mass spectrometry

1. INTRODUCTION

Human pancreatic lipase (HPL; triacylglycerol hydrolase; EC 3.1.1.3) is the main lipolytic enzyme involved in the digestion of dietary fat and represents 3% of the total proteins secreted by the exocrine pancreas [1,2]. Contrary to most of the pancreatic enzymes which are secreted as proenzymes and further activated by proteolytic cleavage in the small intestines, HPL is directly secreted as an active enzyme. HPL is a 50 kDa glycoprotein [3] consisting of a 449 amino acid polypeptide [4,5], including a high mannose or complex-type glycan chain N-linked to Asn^{166} as found also in porcine pancreatic lipase [6,7]. The resolution of the HPL 3D structure revealed the presence of a catalytic triad (Ser^{152}–Asp^{176}–His^{263}) similar to that found in other serine hydrolases, Ser^{152} being part of the G-X-S-X-G consensus sequence [5]. This structure furthermore confirmed the existence of two distinct domains in HPL, as previously suggested [8].

A large N-terminal domain contains the active site covered by a 'lid' domain, as seen in the crystal structure. In its closed conformation, the lid prevents access of substrate to the active site. In the presence of a lipid-water interface, however, the lid is displaced to one side, thus exposing both the active site and a larger hydrophobic surface as also demonstrated in the case of *Rhizomucor miehei* lipase [9–11]. These studies have suggested a structural basis for 'interfacial activation', a significant property of pancreatic lipases, as described by Sarda et al. [12]. Whereas the rate of breakdown of a dilute solution of short-chain triglycerides by pancreatic lipase is very slow, there is a sharp increase in the enzymatic activity once the substrate solubility is exceeded. Thus, pancreatic lipases were defined as a particular class of esterases, active on an unsoluble triglyceride substrate.

The smaller C-terminal domain in HPL is required for colipase binding, as shown by the recent 3D structure of HPL–porcine procolipase complex [13]. In addition to interfacial activation, pancreatic lipases are also characterized by their behavior in the presence of bile salts. They are inactive on an emulsified triglyceride substrate in the presence of a micellar concentration of bile salts. The bile salts coating of triglyceride globules present a negatively charged surface to the surrounding medium, thus inhibiting pancreatic lipase adsorption and activation; however, a specific lipase-anchoring protein present in the exocrine secretion of pancreas, colipase, counteracts this effect [14,15] through the formation of a specific 1:1 complex with lipase that facilitates adsorption at bile salts-covered lipid–water interfaces.

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Abbreviations: FPLC, fast protein liquid chromatography; Fuc, fucose; GlcNAc, *N*-acetyl glucosamine; HPLC, high pressure liquid chromotography; IEF, isoelectric focusing; Man, mannose; MES, 2-[*N*-morpholino] ethane-sulphonic acid; NaTDC, sodium taurodeoxycholate.

The recent 3D structures of HPL have not only contributed considerably to the structure–function characterization of pancreatic lipases but also to that of hepatic and lipoprotein lipases which belong to the same gene family [16–18]. Based on the HPL structure and using site-directed mutagenesis, the role of the lid domain in lipoprotein lipase substrate specificity has been investigated [19,20]; also chimeric hepatic/lipoprotein lipases were used to characterize the respective roles of the N- and C-terminal domains in these two lipases [21]. Similarly, expression of recombinant HPL and mutants thereof is of particular interest in order to investigate the role of the lid domain in interfacial activation and lipase–colipase interactions.

In this report, we describe the high-level expression of HPL in Baculovirus-infected insect cells. The recombinant enzyme (rHPL) obtained after a one-step purification procedure was characterized and compared to native HPL (nHPL).

2. MATERIALS AND METHODS

2.1. *Materials*

The baculovirus transfer vector, pVL1392, *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA, *Spodoptera frugiperda* (Sf9) cells, Baculovirus agarose, Grace media + supplements and Cationic liposome solution were from Invitrogen Corp (San Diego, CA). The serum-free insect cell culture medium SF-900 II was from GIBCO BRL/Life Technologies (Copenhagen, Denmark) Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs/FINNZYMES (Espoo, Finland). Mono S cation-exchange column was from Pharmacia LKB (Copenhagen, Denmark) Colipase was from Boehringer-Manheim (Manheim, Germany) Sodium taurodeoxycholate was from Sigma (St. Louis, MO). Purum tributyrin was from FLUKA (Buchs, Switzerland). Native HPL and anti-HPL polyclonal antibodies were a generous gift from Alain De Caro and Robert Verger (Laboratoire de lipolyse enzymatique, CNRS Marseille, France)

2.2. *Production of recombinant virus*

A cDNA clone for the HPL was obtained from human placenta mRNA by PCR technology based upon the complete sequence of HPL, including signal peptide [4]. A 1,410 bp *Bam*HI fragment, containing the entire coding region of HPL, was subcloned into the pVL1392 transfer vector. The plasmid was purified by CsCl-gradient ultracentrifugation and then used for co-transfection with AcMNPV DNA into Sf9 cells.

Transfection was performed as described in the Baculovirus Expression System Manual, from Invitrogen Corp. (version 1.5.5). 1 μg of linear AcMNPV DNA and 3 μg of recombinant pVL1392 were transfected into Sf9 cells (2×10^6 cells in 60 mm dishes). The resulting culture supernatant was collected after 5 days. Fresh monolayers of Sf9 cells in 100 mm plates were infected with virus supernatant at various dilutions and then overlaid with 1.5% agarose containing complete TNM-FH medium [22]. After 6 days recombinant plaques were identified as occlusion-negative (seen in a light microscope) The presumed recombinant plaques were used to infect 6-well plates containing Sf9 cells and after 5 days the corresponding virus DNA was purified and subjected to a PCR reaction with reverse and forward primer as previously described [23,24]. The PCR fragments from recombinant plaques were also analysed by restriction enzyme digestion. The recombinant viruses were subjected to another round of plaque purification to ensure that the recombinant virus stock was free of wild-type virus.

2.3. *Expression of rHPL in Sf9 cells*

The Sf9 cells were grown at 27°C to a concentration of 8×10^5 cells/ml in 250 ml SF-900 II medium using 1,000 ml screw-capped Erlenmeyer flask in an orbital shaker set at 80 rpm. The recombinant baculovirus containing the HPL cDNA was added to the cells at a multiplicity of infection close to 1. Sampling was performed each day for 7 days in order to check cell viability and measure lipase production. After centrifugation to remove cells and debris, lipolytic activity was assayed using the standard measurement of pancreatic lipases [25] and an aliquot was stored at −20°C for SDS-PAGE and immunoblotting.

2.4. *Purification of rHPL*

Cultures of recombinant baculovirus-infected Sf9 cells were harvested after 3 days and cells were pelleted by centrifugation at 10,000 rpm for 10 min. The supernatant was lyophilyzed during 24 h and the dry material was dissolved in a few ml of distilled water and dialyzed overnight against 10 mM MES buffer, pH 6.5. Prior to chromatography, the solution was passed through a 0.8 μm Millipore filter. Using FPLC (Pharmacia), cation-exchange chromatography was performed on a Mono S HR 5/5 column equilibrated in 50 mM NaCl, 10 mM MES buffer, pH 6.5 After sample injection, a linear NaCl concentration gradient was applied, increasing over 60 min from 50 mM to 175 mM NaCl in 10 mM MES buffer pH 6.5. The flow rate was adjusted to 1 ml/min and the pressure was maintained between 20 and 25 bar. The protein elution profile was recorded spectrophotometrically at 280 nm and the lipase activity was measured potentiometrically in all fractions collected (see analytical assays).

2.5. *Analytical assays*

Lipase activity in insect cell culture supernatant and in fractions collected during the purification procedure was measured potentiometrically at pH 8.0 and 37°C using a pH-stat (TTT 80 Radiometer, Copenhagen). The standard assay conditions consisted of a mechanically stirred emulsion of 0.5 ml tributyrin in a thermostated reaction vessel containing 14.5 ml of 0.28 mM Tris, 150 mM NaCl, 1.4 mM $CaCl_2$, 4 mM NaTDC [25]. Colipase was added at a molar excess of 2. The specific activity of nHPL was 8,000 U/mg under these standard assay conditions. 1 unit (U) represents one μmol of butyric acid released per min.

Electrophoresis was performed on 12% SDS-polyacrylamide gels. Two gels were run in parellel One gel was stained with Coomassie blue and the second gel was transfered onto nitrocellulose membrane [26] and immunoblotting was performed using rabbit polyclonal antibodies raised against nHPL purified from human pancreatic juice. The revelation of the bound antibodies was done with anti-rabbit IgG peroxidase conjugate and 4-chloro 1-naphtol as substrate.

The isoelectric points of rHPL and nHPL were determined on IEF 3–9 PhastGel using the Pharmacia Phastsystem.

Prior to N-terminal sequencing, a sample of the purified rHPL (250 μl containing 125 μg rHPL) from the Mono S column was run through an HPLC column. The sample was injected onto a Vydac 214TD54 reverse-phase C4 HPLC column (0.46 × 25 cm) equilibrated at 30°C at a flow rate of 1.5 ml/min with 0.1% TFA in 20% (v/v) acetonitrile. The concentration of acetonitrile in the eluting solvent was raised to 70% (v/v) over 25 min. Absorbance was measured at 280 nm. The purified rHPL eluted at a retention time of 16.2 min, corresponding to 48.4% of acetonitrile. The material eluting in the main peak was collected and concentrated to 60 μl by vacuum centrifugation. Amino acid sequence analysis on this sample was carried out by automated Edman analysis using an Applied Biosystem Model 470A gas-phase sequencer [27].

Amino acid composition analysis was carried out by hydrolysis of 50 μg rHPL with 6 M HCl for 24 h at 110°C as previously described [28].

Carbohydrate composition analysis was carried out by hydrolysis of 50 μg rHPL with 2 M HCl for 1, 2 and 4 h at 100°C, and monosaccharides were separated on a CarboPac PA1 (Dionex, Sunyvale, CA) column (4 × 250 mm) eluted with 14 mM NaOH. The monosac-

charides were detected by pulsed amperometric detection (Dionex, PAD detector). The amount of monosaccharides was corrected to time zero of hydrolysis and calculated as nmol of monosaccharide per nmol protein.

Electrospray mass spectrum was recorded on API III LC/MS/MS system (Perkin-Elmer Sciex instrument, Thornhill Canada). The triple quadrupole instrument has a mass-to-charge (m/z) range of 2,400 and is fitted with an articulated, pneumatically assisted electrospray (also referred to as ion-spray) interface and an atmospheric pressure ionization source. Sample introduction was done by a syringe infusion pump (Sage instruments, Cambridge, MA) through a fused capillary (75 μm i.d.) with a liquid flow-rate set at 0.5–1 μl/min. The instrument m/z scale was calibrated with selected (covering m/z 50–2,400) ammonium adduct ions of poly(propylene glycols) (PPG's) under unit resolution. The accuracy of mass measurements is generally better than 0 02%.

3. RESULTS AND DISCUSSION

3.1. *Expression of purification of rHPL*

A time–course experiment of rHPL expression in insect cells was carried out (Fig. 1), demonstrating that rHPL activity is present in the culture medium after 24 h following infection with recombinant baculovirus. The activity accumulated in the medium reached a maximal level of 40 μg/ml after 6 days, similar to yields previously described with the expression of pancreatic cholesterol esterase using the same expression system [29]. The activity was directly measured from the culture medium using tributyrin as substrate (see section 2) and the amount of active enzyme was deduced from its specific activity.

The viability of the Sf9 cells in the culture medium was also followed by light microscopy each day. A sharp decrease in cell viability was observed between day 3 and 4 (data not shown).

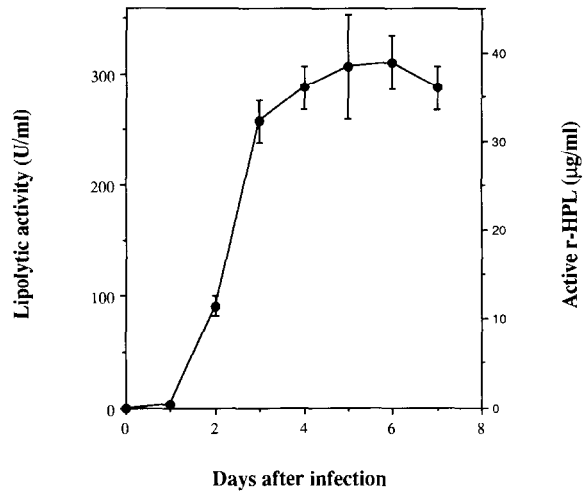

Fig 1. Time-dependent expression of rHPL in Sf9 cells (mean of three identical experiments ± S.D.). The Sf9 cells were infected with recombinant baculovirus containing the HPL cDNA. Each day after the initial infection, an aliquot of the culture medium was removed and tested for rHPL expression and cell viability. Lipolytic activity was determined using the pH-stat technique and tributyrin as substrate (see section 2).

In parallel with the activity measurements, the rHPL secreted by the infected Sf9 cells was analysed by SDS-PAGE and immunoblotting (Fig. 2A and B). We demonstrated that the patterns on the SDS gel and immunoblot follow the activity measurements as rHPL expression increases. The rHPL was clearly the major protein observed at 50 kDa. At day 4 the background level of other proteins increased, in accordance with increasing cell lysis. As shown in Fig. 2B, anti-HPL

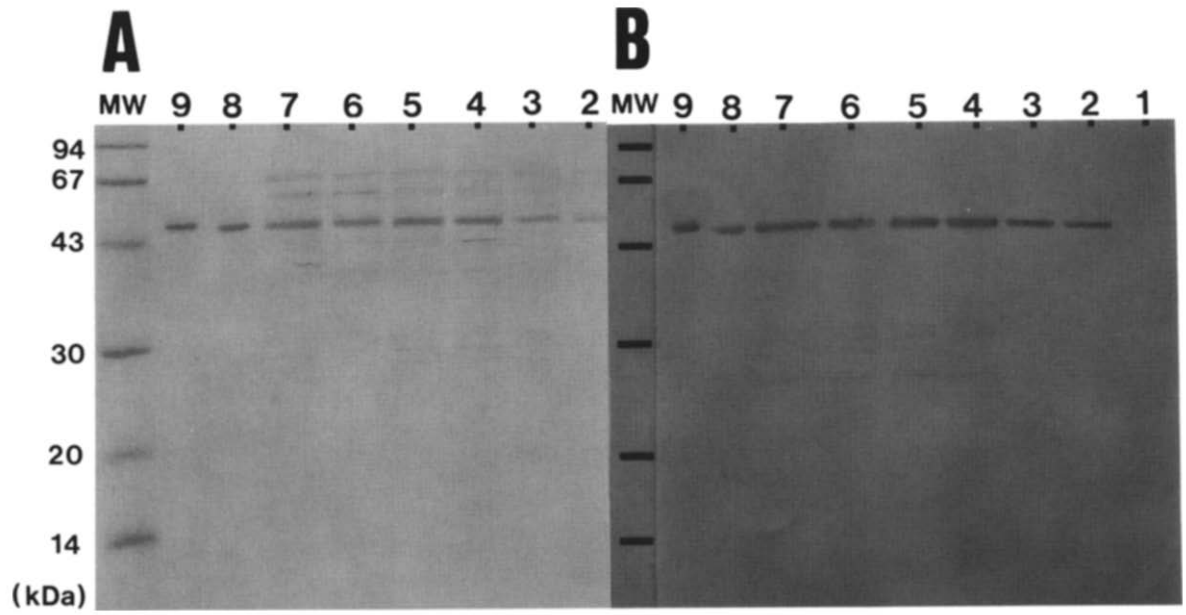

Fig. 2. SDS-PAGE (A) and immunoblotting (B) of rHPL expressed in Sf9 cells. Each day after the initial infection of Sf9 cells an aliquot of the culture medium was removed and subjected to electrophoresis and immunoblot analysis. In panels A and B, lanes 1–7 correspond to day 1–7 after infection. Lane 8 is the purified rHPL obtained after cation-exchange chromotography on a Mono S column. Lane 9 is nHPL purified from human pancreatic juice. Molecular weight markers were loaded on lane 10.

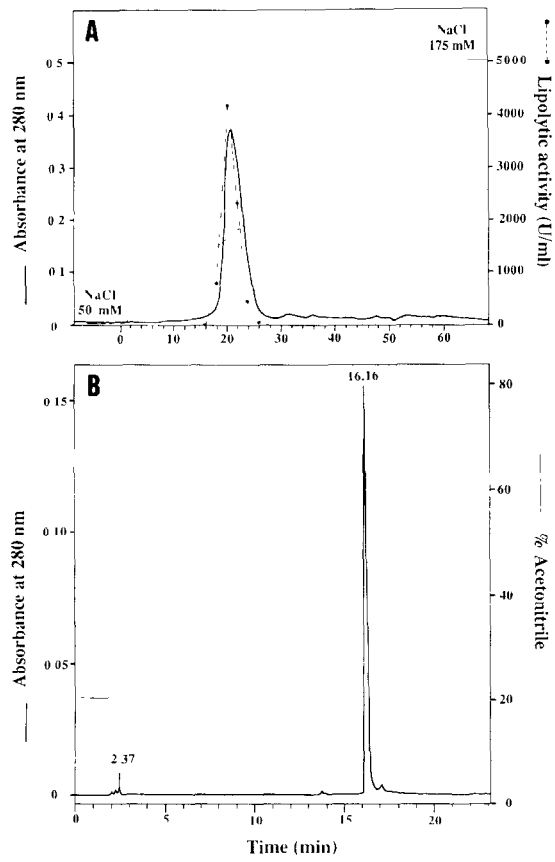

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Fig. 3. Purification of rHPL expressed in baculovirus-infected insect cells. Pure rHPL was obtained using FPLC and a single purification step on a Mono S HR 5/5 column (A). Protein elution was recorded by measuring absorbance at 280 nm, and lipolytic activity was assayed in all the collected fractions using the pH-stat technique and tributyrin as substrate (see section 2). The fractions containing rHPL activity were pooled and further subjected to HPLC analysis (B) on a reverse-phase column (see section 2)

antibodies also reacted with lower molecular weight compounds appearing in the medium from day 4. These minor compounds might be proteolytic fragments of rHPL generated by proteases released from lysed cells.

Accordingly, culture supernatants were harvested after 3 days and rHPL was purified as described (see section 2). As shown in Fig. 3A, a single step procedure using a cation-exchange column was highly efficient for isolating the rHPL. The solution containing rHPL was loaded onto a Mono S HR 5/5 column and lipolytic activity was assayed in the eluant in order to check for the absence of non-bound lipase. A linear salt gradient was then applied and rHPL was eluted at about 80–90 mM NaCl. An average recovery of about 90–95% rHPL lipolytic activity was obtained in the fractions corresponding to the single peak observed in Fig. 3A. Lipolytic activity and absorbance at 280 nm were clearly correlated.

The purified rHPL was analyzed by SDS-PAGE. The results showed a single protein of 50 kDa that co-migrated with native HPL (Fig. 2A and B, lanes 8 and 9).

Purified rHPL was then run through an HPLC column as described (section 2). As shown in Fig. 3B, rHPL recovered from the Mono S column is 98% pure.

The isoelectric point of rHPL was found to be 7.5, which is identical to the pI of the major nHPL isoform (data not shown) as previously reported [30–32] and significantly different from the value found by De Caro et al. [3].

N-Terminal sequence, KEVCYERLGCFSDDS, is identical to the N-terminal sequence of mature HPL

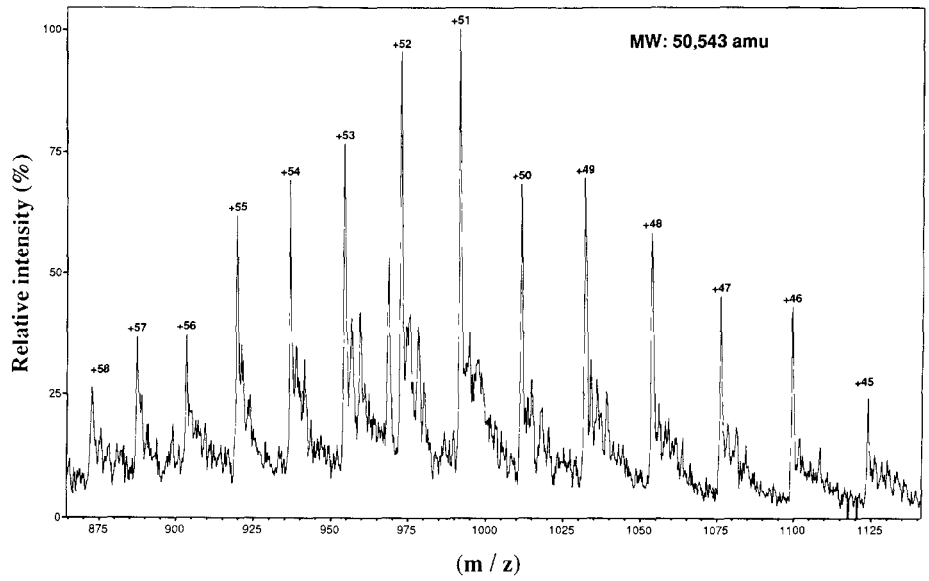

Fig. 4. ESMS spectrum of 20 pmol purified rHPL. The mass spectrum displays a series of multiply protonated molecular ions. The molecular weight of rHPL is determined to 50,543 amu or Da.

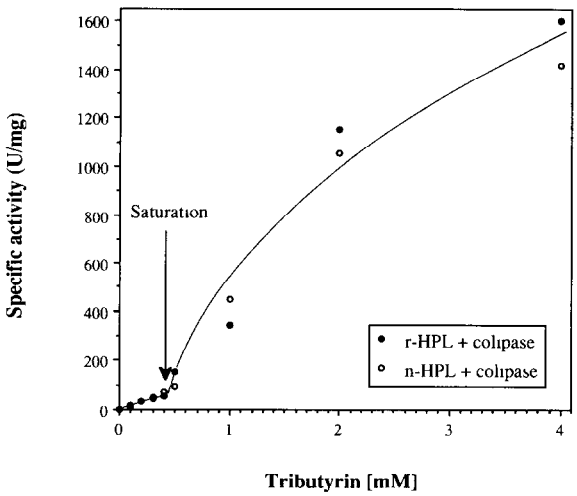

Fig. 5. Interfacial activation. The influence of tributyrin concentration on both rHPL and nHPL was measured by the pH-stat technique. As indicated by the arrow, tributyrin saturation under the present experimental conditions is found at 0.4 mM. The assay (37°C, pH 8.0) contained 1 μg and 50 μg enzyme above and below tributyrin saturation, respectively, in 15 ml NaCl solution (150 mM) and various concentrations of tributyrin in the presence of a molar excess of colipase of 2.

[4,5], confirming that rHPL is correctly N-terminally processed and no degradation products were observed either by HPLC analysis (Fig. 3B) or by N-terminal sequencing.

The amino acid composition confirmed that rHPL consist of 449 amino acid residues corresponding to a molecular weight of 49,505 Da. Carbohydrate analysis revealed the presence of 2.59 nmol Man, 1.61 nmol GlcNAc and 0.69 nmol Fuc per nmol of rHPL. These results suggest the N-linked glycosylation pattern,

$$-\mathrm{Asn}^{166}-\mathrm{GlcNAc}-\mathrm{GlcNAc}-\mathrm{Man}\begin{cases}\mathrm{Man}\\ \mathrm{Fuc}\end{cases}\begin{matrix}\diagup\\ \diagdown\end{matrix}\mathrm{Man}$$

where the glycan chain is only the core of the classical N-linked polysaccharides.

The Electrospray (ES) mass spectrum of purified rHPL is shown in Fig. 4. It displays a series of ions corresponding to multiply protonated molecular ions (45+ to 58+). The determined molecular mass (50,543 Da) is consistent with the theoretical value of the polypeptide (49,505 Da) corrected for the presence of the proposed N-linked glycan (+1,038 Da).

3.2. *Kinetic characterisation of rHPL and comparison with nHPL*

Interfacial activation of HPL is shown in Fig. 5, demonstrating a sharp increase in activity when the tributyrin reaches its saturation point (at 400 μM under the present experimental conditions). The measurements were done in the absence of bile salts and in the presence of colipase in order to avoid irreversible inactivation of HPL at the interface (see below).

As shown in Fig. 6, rHPL and nHPL also display similar properties with respect to the bile salts inhibition above their critical micellar concentration, and to the restoration of activity by colipase. Without bile salts (NaTDC) and without colipase, rHPL and nHPL are irreversibly inactivated at the interface. These results are distinct from earlier observations with porcine pancreatic lipase, which is active and stable under these conditions [33].

Using tributyrin as substrate, micellar concentrations of bile salts (2 mM NaTDC) and colipase in excess, we have measured optimal specific activity of 12,000 U/mg for both rHPL and nHPL in the pH range 7.0–7.5. Under these conditions it is possible to increase by 50% the specific acticity of HPL, as compared to the activity (8,000 U/mg) obtained under standard assay conditions (pH 8.0, 4 mM NaTDC, [25]).

4. CONCLUSION

We have shown that the baculovirus system can be used to produce a recombinant pancreatic lipase in substantial yield for basic research. The enzyme is easy to purify in a one-step procedure. The highly purified ma-

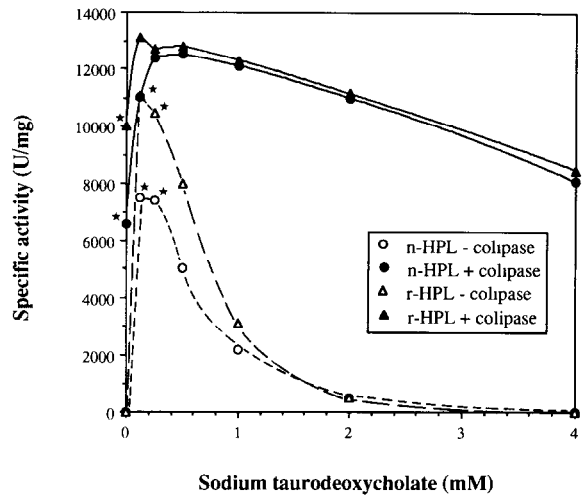

Fig. 6. Sensitivity to bile salts and colipase effect. In the absence of colipase (*open symbols*), both rHPL and nHPL are inhibited by bile salts concentrations above the critical micellar concentration (around 1 mM NaTDC). In the presence of colipase (*closed symbols*), the inhibition by bile salts of both rHPL and nHPL is supressed. The lipase specific activity was measured with the pH-stat technique at pH 8.0 and at 37°C. The final assay volume consisted of 15 ml, containing 0.5 ml tributyrin, 14.5 ml of 0.28 mM Tris, 150 mM NaCl, 1.4 mM $CaCl_2$ and various concentrations of NaTDC. The assay contained between 1 μg and 5 μg enzyme and a molar excess of colipase of about 2. The asterisks (*) indicate lipolytic activities based on initial velocities when the enzyme is inactivated at the interface during titration. For all the other points the kinetics of fatty acid titration were found to be linear, except when, in the total absence of bile salts and colipase, neither rHPL or nHPL displayed activity and were irreversibly inactivated.

terial obtained and its biochemical characterization similar to that of the native enzyme show that the baculovirus expression system is promising for the expression of pancreatic lipases, and mutants thereof, for further structure–function studies.

Acknowledgements Our thanks are due to Esper Boel, Sannie Rasmussen, Mogens Christensen, Dorte M. Gundesen and Anni Demandt for fruitful discussions and technical assistance. This research was carried out with financial support of the BRIDGE-T-lipase programme of the European Communities under contract No. BIOT-CT90–0181 (SMA).

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