Purification, cloning and regulation of a novel acid-lipase-like protein of hamster expressed in lacrimal glands and tears during lactation☆

Anupam Paliwal, Prabir K. De*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500007, Andhra Pradesh, India

Received 11 June 2006; received in revised form 9 October 2006; accepted 23 October 2006

Available online 28 October 2006

Abstract

We report a novel 48-kDa tear acid-lipase-like protein (TALLP), which is markedly induced in lacrimal glands (LG) and secreted in tears of hamster dams during lactation. TALLP is undetectable in LG and tears of normal hamsters, but is also induced after gonadectomy in both sexes and this is prevented by androgen, estrogen or thyroid hormone treatment. These observations and the obliteration of TALLP upon cessation of lactation suggest that endogenous estrogens (in females) and androgens (in males) completely repress TALLP expression. Purified TALLP is monomeric, contains ∼18% N-glycosylation and several pI isoforms. TALLP expression was tissue-specific and immunolocalized in LG acinar cells. The cDNA deduced amino-acid sequence of TALLP precursor (398 residue, containing a 19 residues signal-peptide) showed only 43–48% identity with all known mammalian acid-lipases, including even those of other rodents, suggesting that TALLP is a prototype of a new category, within the acid-lipase family. Surprisingly, although the catalytic triad residues and other sequence features important for lipolytic activity are conserved in TALLP, it has no detectable lipase activity. However, TALLP binds the polarity sensitive hydrophobic probe, 1-aminoanthracene (Kd = 12 μM). TALLP might have a unique substrate-specificity or a lipid-binding/carrier function in tears of hamster dams. This is the first report of an acid-lipase-like protein secreted in tears of any species. Since TALLP lacks the usual lipase activity, it can be an excellent model to understand better what other structural features in acid-lipases influence their catalytic activity.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Acid lipase; Sex hormone; Thyroid hormone; Lactation; Tear film lipid; Lacrimal gland; Syrian hamster; Lipocalin; Harderian gland

1. Introduction

The preocular tear film maintains ocular surface integrity, preserves visual acuity and defends against microbial challenge [1–4]. The film contains an inner protein-rich aqueous layer secreted by lacrimal gland (LG) and an outer lipid layer secreted by accessory lacrimal glands [1–4]. The lipid layer prevents evaporation of the underlying aqueous layer, provides lubrication, antimicrobial activity and may be a site for production of pheromones from lipid precursors [2–6]. Abundant lipid-binding proteins e.g. lipocalins, present in the aqueous layer modify the physicochemical properties of the tear film by binding tear lipids and possibly also serve as scavengers for harmful lipophilic compounds and/or as transport proteins for pheromonal ligands [2–4,7–9]. Lipases, if present in secretions of LG, could also modify and/or bind tear lipids and alter properties of the tear lipid layer, affecting tear film’s integrity and ocular health [10–12].

A phospholipase A2 secreted by LG is known, and is believed to have an antimicrobial function in tears [12,13]. PCR amplified short cDNA fragments of pancreatic-, lipoprotein- and endothelial cell-derived lipases from mouse, rat and rabbit lacrimal and accessory glands, and also transcript of a phosphatidylserine phospholipase A1 in rabbit LG, have been detected [11]. Additionally, in female mouse LG, transcripts for pancreatic lipase-related protein-1 has also been reported [10]. However, in all these cases, neither expression of the corresponding lipase proteins nor their lipase activity was demonstrated.

Acid-lipases [14–18] hydrolyze triacylglycerols and cholesterol esters (substrates, which are also present in tear lipid layer [2–4,10,11]). Mammalian acid-lipases consist of the closely
similar preduodenal lipases (secreted by glands in tongue, pharynx or stomach) that hydrolyze only dietary triglycerides [14–17], and the related intracellular lysosomal lipases, which are active additionally on cholesteryl esters [14,18,19]. Among mammalian lipases, enzymes of the acid-lipase family are stable to acidic conditions, have low pH optimum, require no protein co-factor for activity, are highly N-glycosylated, have MM of ~50 kDa and show no sequence homology with any other known lipase family [14,15,20]. However, they contain an α/β hydrolase fold and a decapetide with a loose consensus sequence, [LIV]-X-[LIVFY]-[LIVMST]-X-G-[GSTAC] (PROSITE pattern PS00120) conserved in almost all known lipases. The completely conserved serine in this decapetide is part of a catalytic triad, S/H/(D or E), which is also conserved in all lipases [14,21,22]. Like many lipases, acid-lipases undergo a conformational change, from an inactive (closed) to an active (open) form upon interfacial substrate binding, which involves the displacement of a lid domain that otherwise blocks access to the active site [21,23,24].

Here we report a tear acid-lipase like protein (TALLP), which is massively expressed in LG of hamsters only during lactation and after gonadectomy. TALLP has an unusual hormonal regulation in hamster LG, since both androgens and estrogens, and also thyroid hormone markedly repress its expression. Surprisingly, no lipase activity could be demonstrated for TALLP. Sequence analysis showed that TALLP was a prototype of a new category of lipase-like proteins within the acid-lipase family. Possible physiological function of TALLP, naturally expressed only in lactating hamsters is discussed.

2. Materials and methods

2.1. Animal experimentation for regulation studies

Syrian hamsters were bred and maintained in our Institute’s animal house. Our Institutional animal experimentation ethics committee approved all animal experiments. Experiments were done on groups of hamsters. Representative results are reported. Hamsters of both sexes were gonadectomized at 2 months age. Estradiol-17β (5 μg), dihydrotestosterone (50 μg), progesterone (75 μg), dexamethasone (75 μg), tri-iodothyronine (20 μg) (all from Sigma) were daily administered for 15 days by subcutaneous injections to 15-day gonadectomized hamsters. Lactating hamsters were treated for 15 days from 1-day post-partum. Hamsters were sacrificed on the day after last injection along with appropriate controls. Sham surgery or treatments with vehicle alone had no significant effect. Timed-pregnant and lactating hamster damms were sacrificed at different days of gestation and post-partum respectively. The day pups were born was considered as 0-day parturition and the following day was considered as 1-day post-partum (1-day lactation). In experiments involving lactating hamster dams, only those with litter size of 6 pups were born. Normal weaning of pups (separation from dam) was done at 20-day post-partum. Immediately after sacrifice, a pair of exorbital lacrimal gland (LG) and other tissues, if required, from each hamster were excised out, cleaned of any extraneous tissue, weighed and stored at 2–5 °C. Ocular surface washings (diluted tears) of individual hamsters were collected following anesthesia, as described previously [9].

2.2. Preparation of tissue extracts for detection of TALLP expression

Lacrimal glands from each hamster or other tissues (if required) from hamster and rat (e.g. von Ebner’s lingual glands) were homogenized (2.5% w/v) in chilled 20 mM Tris–HCl (pH 7.5) and centrifuged at 30,000 × g (30 min at 4 °C). For LG tissue extracts and tear samples to be compared, equal volumes of supernatants were loaded in SDS-PAGE (10.5%) and gels were either stained with Coomassie R-250 (Sigma) to detect presence of the 48-kDa TALLP band or used for Western blots probed with TALLP antiserum.

2.3. Purification and partial characterization of TALLP

2.3.1. Purification

Purification fractions were monitored for the presence of the major 48-kDa LG protein (TALLP) by SDS-PAGE followed by protein staining. Absorbance at 280 nm and a protein estimation kit (Bio-Rad), were used to monitor protein concentration. Pooled exorbital lacrimal glands (~4 g tissue from 35, one-month ovariecetomized hamsters) were homogenized (5% w/v) in chilled 20 mM Tris–HCl, pH 7.5 (buffer-A) and centrifuged at 105,000 × g for 1 h and supernatant subjected to (NH4)2SO4 fractionation. The proteins precipitated between 35 and 95% (NH4)2SO4 saturation, containing most of TALLP, were dissolved in buffer-A/0.5 M NaCl and slowly passed through a Concanavalin-A Sepharose (Amersham) column (1.6 × 10 cm), in the cold room and column washed extensively to remove unbound proteins. Specific elution with buffer-B containing 0.5 M NaCl and 0.5 M α-methyl mannoside, released the Con-A-bound TALLP from the column. Pooled eluate was dialyzed extensively against 10 mM potassium phosphate buffer (pH 7.5) (buffer-B) and then loaded on a BioGe-HTP (Bio-Rad) column (2.5 × 3.5 cm). The flow through containing TALLP was collected and directly applied on a phenyl Sepharose (Sigma) column (1.6 × 5 cm). After extensive washing with buffer-B, elution was carried out with a linear gradient of 0–70% ethylene glycol. Fractions containing pure TALLP, eluting between 30 and 40% ethylene glycol, were pooled and dialyzed against buffer-B. After reconfirming purity of the final dialyzed 48-kDa TALLP preparation by silver staining, it was aliquoted and stored at ~20 °C.

2.3.2. MM and pl estimation, deglycosylation and glycan differentiation

Purified TALLP was incubated for 16 h with either N-glycosidase F or O-glycosidase (Boehreinger) as described previously [25] and analyzed by SDS-PAGE. In SDS-PAGE, MM estimation was done using molecular mass markers run alongside. For determination of native MM, an aliquot of purified TALLP was run on a precalibrated column of Sephadex G-75 (Amersham). For two-dimensional electrophoresis, of purified TALLP immobilized pH gradient strips from Bio-Rad (17 cm; pH 4–11) were used for isoelectric focusing (IEF). IEF was carried out in a Protean IEF cell (Bio-Rad), according to the protocol recommended by the manufacturer, followed by SDS-PAGE in second dimension. Gels were silver stained. The pl values of isoforms of pure TALLP were estimated using IEF marker proteins (Bio-Rad). Binding of TALLP to different lectins, GNA (Galanthus nivalis agglutinin), SNA (Sambucus nigra agglutinin), MAA (Maackia amurensis agglutinin), PNA (peanut agglutinin) and DSA (Datura stramonium agglutinin) were checked using a glycan differentiation kit (Boehringer), according to the protocol provided with the kit.

2.3.3. N-terminal sequencing and determination of internal amino acid sequence tags

For N-terminal sequencing, purified TALLP was resolved in SDS-PAGE and transferred onto PVDF membrane for sequencing in a 473A Applied Biosystem automated protein sequencer. For determination of internal sequence tags, MS/MS fragmentation spectra of tryptic digest of TALLP was obtained using QSTAR Pulsar (ESI-Q-TOF) from PE SCIEX with nano spray source. TOF-MS (Time of Flight-Mass Spectrometry) was obtained at 1000 V spraying voltage. Multiple charged species obtained were subjected to MS/MS with collision energy ranging from 30 to 50 eV. These MS/MS data were analyzed using Bio-Analyzer from Applied Biosystems to get peptide sequences.

2.4. Antibody production, Western blots and immunohistochemistry

Polyclonal antiserum against pure TALLP was raised in Balb/c mice using standard immunization procedures. Western blots of different samples, after resolution in SDS-PAGE, were done essentially as described previously [9,25]. Blots were probed with TALLP antiserum and crossreactions with antibody was detected using peroxidase or alkaline phosphatase conjugated anti-mouse IgG (Boehringer). For immunohistochemical localization, 5 μm sections of formalin fixed exorbital LG from male, gonadectomized and lactating hamsters were probed with TALLP antiserum and final detection was done using peroxidase.
2.5. Lipase activity assays

Lipase activity was assayed at 37 °C at different pH, as described earlier by Gargouri et al. [26] using tributyrin as substrate. Briefly, the assay was carried out in 15 ml containing 10% gum arabic, 50 mM NaCl and 0.01% BSA. In some assays, 5.0 ml of 30% emulsion of olive oil in 10% gum arabic-water [26] or a total lipid extract of Harderian gland or pooled ocular surface washings of hamsters [5,6,9] (emulsified with gum arabic or sodium taurodeoxycholate) was used as substrate instead of tributyrin. In other lipase assays, substrates like triolein, triocanoin, intralipid, diacylglycerols, monoacylglycerols and egg-phosphatidylcholine were also used. Liberated fatty acids were titrimetrically measured, with one unit corresponding to the release of 1 μmol of fatty acid per min at 37 °C [26]. Esterase activity was checked colorimetrically using paranitrophenyl-butyrate, -palmitate or -oleate as chromogenic substrates using the method of Timmermans et al. [27].

2.6. Fluorescence assays for 1-amoanthracene binding

Assays for the binding to TALLP of the polarity sensitive fluorescent probe, 1-amoanthracene (1-AMA) was performed as described earlier [24,28,29]. Spectra were recorded at 25 °C with a spectrofluorimeter (Hitachi F-4500) using a 1 nm slit width for emission recordings. Excitation was fixed at 295 nm and gradually increasing concentrations of added 1-AMA (5 mM stock dissolved in acetate buffer (pH 5.0), containing 0.1 M NaCl), were taken in presence of 0.25 μM of purified TALLP (2 μM) in 10 mM sodium acetate buffer (pH 5.0), containing 0.1 M NaCl, which were in presence of 150 mM NaCl and 0.01% BSA. In some assays, 5.0 ml of a 30% emulsion of other ocular tissues (not shown), which included the infraorbital lacrimal gland, Harderian gland (a tear lipid secreting gland) [5,6,9], eye-globe and eye-lids from normal, gonadectomized male and female hamsters (see Materials and methods for details). Soluble extract of LG of gonadectomized males and females (lanes 8 and 9) and lactating hamsters (lane 10) but was undetectable in normal males and females (lanes 6 and 7). Fig. 1 also indicates that expression pattern of the 48-kDa protein in LG and tears is similar to the major 20-kDa lipocalin, except that significant levels of the lipocalin is expressed in normal females. Inspection of other ocular tissues (not shown), which included the infraorbital lacrimal gland, Harderian gland (a tear lipid secreting gland) [5,6,9], eye-globe and eye-lids from normal, gonadectomized and lactating hamsters revealed that, the infraorbital lacrimal gland also contains a major 48-kDa protein with a similar pattern of expression as that seen for exorbital lacrimal gland. This suggested that both exorbital lacrimal gland and the much smaller infraorbital lacrimal gland, which are indistinguishable histologically, biochemically and in protein profiles [9,30,31] further inspection of protein profiles of LG extracts from hamsters in different hormonal states (Fig. 1) revealed presence of a 48-kDa protein in lactating female (lane 5), gonadectomized female (lane 4) and gonadectomized male (lane 3) hamsters, which was however trace or absent in normal females (lane 2) and males (lane 1). A similar 48-kDa major protein band was also present in ocular surface washings (tears) of gonadectomized males and females (lanes 8 and 9) and lactating hamsters (lane 10) but was undetectable in normal males and females (lanes 6 and 7). Fig. 1 also indicates that expression pattern of the 48-kDa protein in LG and tears is similar to the major 20-kDa lipocalin, except that significant levels of the lipocalin is expressed in normal females. Inspection of other ocular tissues (not shown), which included the infraorbital lacrimal gland, Harderian gland (a tear lipid secreting gland) [5,6,9], eye-globe and eye-lids from normal, gonadectomized and lactating hamsters revealed that, the infraorbital lacrimal gland also contains a major 48-kDa protein with a similar pattern of expression as that seen for exorbital lacrimal gland. This suggested that both exorbital lacrimal gland and the much smaller infraorbital lacrimal gland, which are indistinguishable histologically, biochemically and in protein profiles [9], secrete the major 48-kDa protein into tears. All further investigations were done only on the exorbital lacrimal glands (LG).

3. Results

3.1. Identification and expression pattern of a novel 48-kDa protein in lacrimal gland and tears of hamster

We had earlier identified by comparing protein profiles of hamster exorbital lacrimal glands, a female-specifically expressed abundant 20-kDa protein (a lipocalin), which displays marked changes in its level of expression in different sex hormonal states [9,30,31]. Further inspection of protein profiles of LG extracts from hamsters in different hormonal states (Fig. 1) revealed presence of a 48-kDa protein in lactating female (lane 5), gonadectomized female (lane 4) and gonadectomized male (lane 3) hamsters, which was however trace or absent in normal females (lane 2) and males (lane 1). A similar 48-kDa major protein band was also present in ocular surface washings (tears) of gonadectomized males and females (lanes 8 and 9) and lactating hamsters (lane 10) but was undetectable in normal males and females (lanes 6 and 7). Fig. 1 also indicates that expression pattern of the 48-kDa protein in LG and tears is similar to the major 20-kDa lipocalin, except that significant levels of the lipocalin is expressed in normal females. Inspection of other ocular tissues (not shown), which included the infraorbital lacrimal gland, Harderian gland (a tear lipid secreting gland) [5,6,9], eye-globe and eye-lids from normal, gonadectomized and lactating hamsters revealed that, the infraorbital lacrimal gland also contains a major 48-kDa protein with a similar pattern of expression as that seen for exorbital lacrimal gland. This suggested that both exorbital lacrimal gland and the much smaller infraorbital lacrimal gland, which are indistinguishable histologically, biochemically and in protein profiles [9], secrete the major 48-kDa protein into tears. All further investigations were done only on the exorbital lacrimal glands (LG).

3.2. Purification, partial characterization and cellular localization of the 48-kDa LG/tear protein

Fig. 2A shows purification of the 48-kDa protein from LG of gonadectomized female hamsters (see Materials and methods for details). Soluble extract of LG (lane 1), upon (NH4)2SO4 fractionation precipitated most of the 48-kDa protein at 35–95% saturation. The dissolved precipitate (lane 2) was passed through a Con-A Sepharose column. Specific eluate of the Con-A-bound proteins (containing the 48-kDa protein) (lane 3) was applied on a column of hydroxylapatite (Bio-Gel-HTP)}
wherein, the 48-kDa protein eluted unbound along with minor contaminants (lane 4). The pooled unbound fraction was loaded on a phenyl Sepharose column, which bound the 48-kDa protein. Ethylene glycol gradient elution released the pure 48-kDa protein (lane 5) at a 30–40% concentration. Silver staining (lane 6) confirmed that the purified protein was homogeneous. 

∼3 mg of purified protein was obtained from 4 g of LG and our estimation showed that the 48-kDa protein comprises ∼2% of the total soluble proteins in LG of gonadectomized and lactating hamsters. Gel filtration of the purified protein on a precalibrated Sephadex G-75 column gave a single peak (not shown) of estimated MM ~53.7 kDa, which was close to the MM of 48-kDa estimated in SDS-PAGE, indicating that the purified protein was a monomer. Incubation of the purified protein with N-glycosidase F (Fig. 2B, lane 2) shortened it to a ∼39 kDa species in SDS-PAGE, indicating presence of considerable (~18%) N-glycosylation. Similar incubation with O-glycosidase had no effect on mobility (lane 3). After 2D electrophoresis, purified 48-kDa protein showed presence of at least four isoforms (two major and two minor), which focused within the pl range of 5.8–6.2 (Fig. 2C). This heterogeneity might be due to presence of differential N-glycosylation [20,32,33]. The purified protein showed strong binding with lectins like GNA, DSA and Con-A, but not with PNA, MAA or SNA (not shown). This, and results of N-glycosidase F treatments, indicated presence of terminally linked mannose with complex and hybrid type N-glycans in the 48-kDa protein. N-terminal sequencing and sequencing of tryptic fragments of the purified 48-kDa protein gave the following sequences: LFETTTNPEAYMKVSIV for N-terminal and three internal sequence tags: (i) IPTAMWSSGK, (ii) TADFSELDFTVKG and (iii) NAYVSNIDLK. Interestingly, BLAST search of the N-terminal and the first two internal sequence tags showed best matches with acid-lipases of mammalian origin.

In Western blots, antisera raised in mice against the purified 48-kDa protein showed a single crossreaction with the pure protein (not shown). Moreover, as shown in Fig. 3A, a single 48-kDa crossreaction was detected in Western blots of crude LG extracts and tears of gonadectomized males (lanes 3 and 8), gonadectomized females (lanes 4 and 9) and lactating female hamsters (lanes 5 and 10) whereas LG and tears of normal males (lanes 1 and 6) or females (lanes 2 and 7) showed almost no crossreaction. These results confirmed the expression pattern seen earlier in protein profile studies (Fig. 1). It needs mention, that in Western blots of LGs of a large number of normal male and female hamsters, all males and majority of the females showed no crossreaction. However, a small percentage of female LGs displayed a faint crossreaction at 48 kDa, which was at a much lower level than gonadectomized or lactating hamsters (e.g. see lane 2, Fig. 3A and lane 1, Fig. 5). In immunohistochemical localization studies, antisera against the 48-kDa protein showed strong immunoreactivity in cytoplasm of acinar cells of LG of gonadectomized males (Fig. 3B, lower panel) as well as gonadectomized females (not shown), but no immunoreactivity was detectable in male LG (Fig. 3B, upper panel). Western blots of other hamster tissues (not shown), including liver, brain, kidney, spleen, stomach, eyelids, ovary, testis, prostate, Harderian, salivary, lactating mammary and lingual von Ebner’s glands, showed no crossreaction.

Fig. 2. Purification and partial characterization of the LG 48-kDa protein (TALLP). (A) Protein-stained SDS-PAGE profiles of major purification fractions (see text for details): lane 1, crude soluble extract of LG from gonadectomized female hamsters; lane 2, 35–95% (NH₄)₂SO₄ pellet; lane 3, pool of Con A-bound proteins eluted with α-methyl mannoside; lane 4, unbound pool of BioGel HTP column; lane 5, pool of purified TALLP eluted from phenyl Sepharose column; lane 6, silver stain of purified TALLP. (B) Effect of glycosidases on purified TALLP: lane 1, purified TALLP after control incubation without glycosidase; lane 2, TALLP after incubation with N-glycosidase F; lane 3, TALLP after incubation with O-glycosidase. After incubation, samples were run in SDS-PAGE (10.5%). (C) 2-D electrophoresis profile of purified 48-kDa TALLP. Four pl isoforms (two major and two minor spots) are indicated by arrowheads.

Fig. 3. Western blots of TALLP in hamster LG and tear extracts and its immunohistochemical localization in LG. (A) Western blots of hamster exorbital LG (lanes 1–5) and tears (lanes 6–10) showing presence or absence of a single 48-kDa crossreaction (TALLP) in different states: intact males (lanes 1 and 6); intact females (lanes 2 and 7); gonadectomized males (lanes 3 and 8); gonadectomized females (lanes 4 and 9); lactating females (lanes 5 and 10). Blots were probed with mouse antiserum raised against purified TALLP. (B) LG sections of male (top) and gonadectomized male (bottom) were probed with TALLP antiserum. Intense cytoplasmic immunoreactivity as evidenced by golden brown diaminobenzidine reaction product, is present in almost all acinar cells in LG of gonadectomized females but is absent in males. Sections were 5 µm thick; magnification, 200×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3. Regulation of expression of 48-kDa LG/tear protein

As shown in Figs. 1 and 3, massive expression of 48-kDa protein is seen only in LG and tears of lactating and gonadectomized hamsters. We had earlier found that high expression of 20-kDa lipocalin in LG of gonadectomized hamsters was markedly repressed upon treatment with androgen, estrogen and thyroid hormone [9,30,31]. Fig. 4 shows protein-stained profiles and Western blots of LG and tears of gonadectomized hamsters after different hormonal treatments. The high levels of 48-kDa protein expressed in LG and present in tears of gonadectomized males (lane 1, panels A to D) and gonadectomized females (lane 5), is completely obliterated after 15 days treatment with dihydrotestosterone (DHT; an androgen) (lanes 2 and 6), or estradiol-17β (E2; an estrogen) (lanes 3 and 7), or tri-iodothyronine (T3; a thyroid hormone) (lanes 4 and 8). However, similar 15 days treatment of gonadectomized females with progesterone (a progestin) (lane 9) or dexamethasone (a synthetic glucocorticoid) (lane 10), and similar treatments of gonadectomized males (not shown) had no effect on expression of the 48-kDa protein in LG and tears. Above results show a marked repressive effect of androgen, estrogen and thyroid hormone on expression of the 48-kDa protein.

Since LG of normal virgin females usually contain undetectable levels of the 48-kDa protein, it was investigated whether the high expression seen during lactation was due to an induction, which occurred pre-partum (i.e. during pregnancy) or post-partum. Moreover, since lactation is a temporary state, it was also investigated whether the high expression continues after cessation of lactation following normal pup-weaning or early pup-deprivation. Fig. 5 shows that LG of virgin female (lane 1), displays a faint crossreaction in Western blot indicating low expression of the 48-kDa protein, whereas no expression is detectable in late-pregnant dams (13-day gestation) (lane 2) and also in 1-day post-partum dams (lane 3). However, a marked post-partum induction of the 48-kDa protein is clearly apparent in the high level of crossreaction detected in 9-day post-partum lactating dams (lane 4), which persists even in 20-day post-partum dams (i.e. on normal pup-weaning day) (lane 6). This expression is completely obliterated 20 days after normal weaning (removal) of pups (lane 8) and also is undetectable in dams, which were maintained with their pups for 20 days beyond the normal weaning day (not shown). Interestingly, dams that were early deprived of their pups, either immediately following parturition (lane 5) or at 9-day post-partum (lane 7) showed no expression of the 48-kDa protein, when checked on 9- and 20-day post-partum respectively. Finally, daily post-partum treatment of nursing hamster dams with E2, DHT or T3 prevented expression of the 48-kDa protein in their LG (not shown).

3.4. Cloning and analysis of cDNA of hamster 48-kDa LG/tear protein

Employing RT-PCR and RACE techniques and using different degenerate and specific primers (see Materials and methods for details), full-length cDNA of the 48-kDa protein was cloned from LG of gonadectomized female hamsters. The 1765 bp cDNA (GenBank,DQ144735) has a 47 bp 5′-UTR preceding the translation start codon (ATG) and a 521 bp 3′-UTR containing a typical polyadenylation site located 497 bp downstream of the stop codon. The cDNA encodes a 398 amino acid long precursor protein, having a 19-residue signal peptide. The 379 amino acid long mature protein has the same N-terminal sequence as that obtained experimentally and also contained all the three internal sequence tags obtained by MS/MS, confirming that the cDNA encodes the hamster 48-kDa LG/tear protein. The mature protein has a calculated pl of 8.42 and calculated mass of 42.5 kDa, which is close to the MM of 39-kDa found for the deglycosylated 48-kDa LG protein (Fig. 2B). The lower pl (5.8–6.2) isoforms found for the purified protein (Fig. 2C), could be an effect of N-glycosylation present in this protein. Consistent with this, three potential N-glycosylation sites were found in the deduced sequence of the
mature protein (see Fig. 6). However, no predicted potential for O-glycosylation was found.

Interestingly, GenBank search using this hamster LG cDNA revealed homology with cDNAs of known and characterized mammalian acid-lipases [18,34–38]. Surprisingly, higher identity scores were found with several cDNA entries (mostly of mouse, rat and human) encoding predicted (hypothetical) acid-lipases, with some having expressed sequence tag (EST) evidence. Maximum identity was with a predicted cDNA of mouse, XM_285300 (EST evidence reported in NEIBank’s mouse lacrimal gland EST library; http://neibank.nei.nih.gov/cgi-bin/showDataTable.cgi?lib=NbLib00081). While the coding DNA sequence (CDS) of hamster cDNA has 80% identity with that of the above mentioned mouse cDNA, it has only 58–61% identity with CDS of all known and characterized mammalian acid-lipases (i.e. acid-lipases of human, dog, cow, rabbit, rat and mouse [18,34–39]). Notably, the hamster cDNA has a single EcoR1 site within its CDS, which is conserved in almost all homologous acid-lipase cDNAs (see also Fig. 6). Since, cDNA homology search strongly suggested that the hamster 48-kDa tear/LG protein is an acid-lipase-like protein, it is henceforth referred to as TALLP (tear acid-lipase-like protein).

3.5. Absence of any detectable lipase activity in TALLP

Since sequence homology strongly indicated that TALLP is an acid-lipase-like protein, presence of lipase activity in TALLP was investigated. Using tributyrin or olive oil (commonly used acid-lipase substrates) and titrimetric assay protocol of Gargouri et al. [26], we could not detect any lipase activity using purified TALLP. Moreover, no activity could be detected even after using different amounts of TALLP (up to 300 μg) or varying the assay pH (4.0 to 8.0) and/or concentration of substrates, emulsifiers (sodium taurodeoxycholate/gum arabic) or additives (NaCl and BSA). Additionally, no activity was also found for emulsifiers (sodium taurodeoxycholate/gum arabic) or additives using different amounts of TALLP (up to 300 μg). Moreover, no activity could be detected even after overnight incubation of rat lingual lipases (lysosomal lipases of human and rat, and lingual/gastric lipases of mouse, rabbit and dog; not shown in alignment). However, TALLP had conspicuously higher identity (67%) with XM_285300 (Fig. 6). Alternately, 99 residues in mature TALLP (shown unbolded) have no similarities with any of the four aligned active acid-lipases. Notably, in the alignment of mature proteins, a total of four gaps are required for TALLP, three for XM_285300, while bovine lipase was the only active acid-lipase, which required a gap (Fig. 6). The alignment also shows that out of the three potential N-glycosylation sites in TALLP, N70, N268 and N316 (all present in XM_285300), N70 is not present in any active acid-lipase, while N268 is conserved in all and N316 is conserved only in mouse lysosomal lipase and also in lysosomal lipases of rat and human [33,38]. N-268 is likely to be glycosylated in TALLP, since N-glycosylation at this site is confirmed for several acid-lipases [24,32,33]. Interestingly, TALLP lacks a N-glycosylation site (at K32), which is conserved in all known active acid-lipases of mammalian origin and also in XM_285300. Free thiol of a cysteine has been shown to be important for lipase activity of human and rabbit gastric lipases and substrate specificity of human lysosomal lipase [19,40–42]. Out of the four cysteines in mature TALLP (C80, C193, C243, C252), the first two are uniquely positioned, while the latter two are very likely disulfide-linked since they are completely conserved and believed to be disulfide-linked in all active acid-lipases [24,27,39–41]. However, TALLP, XM_285300 and also bovine pregastric lipase [27], lack a cysteine (substituted by threonine; T260 in TALLP), which is however conserved in all other known mammalian acid-lipases [39]. Additionally, Fig. 6 shows that, the EcoR1 site present in cDNA of TALLP and other mammalian acid-lipases, segments all the translated proteins at an identical position (see also Discussion).
Importantly, TALLP has the catalytic triad residues (S170, H340, D369) and two oxyanion hole residues (Q171, L84), which comprise the essential catalytic machinery, conserved in all lipolytically active lipases [21,22,39]. Additionally, a decapeptide known to contain the catalytic serine residue and which comprises a conserved nucleophilic elbow in almost all lipases [21,39,41] is also conserved in TALLP (I164 to A173). Above features are also conserved in XM_285300 Fig. 6.

3.7. Sequence analysis shows that TALLP is distinct from preduodenal and lysosomal acid-lipases

Two categories of acid-lipases (preduodenal and lysosomal) comprise the mammalian acid-lipases [14,15,21,43]. If TALLP is the hamster counterpart of either preduodenal or lysosomal lipase, it should have distinctly higher identities with members of any one of the four active acid-lipases. When a residue in TALLP and all its aligned residues in the four active acid-lipases are identical/conserved, all five aligned residues are dark shaded. Within the first five sequences (i.e. excluding XM_285300), wherever any four aligned residues are identical/conserved, they are light shaded. The bottom sequence (XM_285300) is not included in any shading, but its residues, which are identical/conserved with TALLP, are bolded. Four cysteines in mature TALLP are indicated by dots. A dashed line joins two cysteines, which are conserved and disulfide-linked in all known active acid-lipases. The C to T substitution in TALLP, BPGE and XM_285300 is indicated ($). Putative N-glycosylation sites (three in TALLP) are underlined in all sequences. The conserved catalytic triad residues (S, D and H) are indicated by an asterisk (*). The conserved oxyanion hole residues (L and Q) are indicated by #. Gaps in alignment are indicated by a dash (−). Arrow pointing down corresponds to the single EcoRI site in TALLP cDNA, conserved in all other sequences. Percent amino acid sequence identities with mature TALLP are indicated at the end of each sequence. RLL, rat lingual lipase; HGL, human gastric lipase; BPGE, bovine pregastric esterase; MLYL, mouse lysosomal lipase; XM_285300, PREDICTED: Mus musculus similar to Gastric triacylglycerol lipase precursor. (The novel cDNA sequence of TALLP precursor has been submitted to GenBank with accession numberDQ144735).

Fig. 6. Multiple alignment of TALLP with four lipolytically active mammalian acid-lipases and a hypothetical acid-lipase-like protein of mouse. Arrowhead pointing down indicates cleavage point for predicted signal peptide of all sequences. A bolded residue in mature TALLP indicates presence of either an identical or conserved residue (which is also bolded) in at least any one of the four active acid-lipases. When a residue in TALLP and all its aligned residues in the four active acid-lipases are identical/conserved, all five aligned residues are dark shaded. Within the first five sequences (i.e. excluding XM_285300), wherever any four aligned residues are identical/conserved, they are light shaded. The bottom sequence (XM_285300) is not included in any shading, but its residues, which are identical/conserved with TALLP, are bolded. Four cysteines in mature TALLP are indicated by dots. A dashed line joins two cysteines, which are conserved and disulfide-linked in all known active acid-lipases. The C to T substitution in TALLP, BPGE and XM_285300 is indicated ($). Putative N-glycosylation sites (three in TALLP) are underlined in all sequences. The conserved catalytic triad residues (S, D and H) are indicated by an asterisk (*). The conserved oxyanion hole residues (L and Q) are indicated by #. Gaps in alignment are indicated by a dash (−). Arrow pointing down corresponds to the single EcoRI site in TALLP cDNA, conserved in all other sequences. Percent amino acid sequence identities with mature TALLP are indicated at the end of each sequence. RLL, rat lingual lipase; HGL, human gastric lipase; BPGE, bovine pregastric esterase; MLYL, mouse lysosomal lipase; XM_285300, PREDICTED: Mus musculus similar to Gastric triacylglycerol lipase precursor. (The novel cDNA sequence of TALLP precursor has been submitted to GenBank with accession numberDQ144735).

Importantly, TALLP has the catalytic triad residues (S170, H340, D369) and two oxyanion hole residues (Q171, L84), which comprise the essential catalytic machinery, conserved in all lipolytically active lipases [21,22,39]. Additionally, a decapeptide known to contain the catalytic serine residue and which comprises a conserved nucleophilic elbow in almost all lipases [21,39,41] is also conserved in TALLP (I164 to A173). Above features are also conserved in XM_285300 Fig. 6.

3.7. Sequence analysis shows that TALLP is distinct from preduodenal and lysosomal acid-lipases

Two categories of acid-lipases (preduodenal and lysosomal) comprise the mammalian acid-lipases [14,15,21,43]. If TALLP is the hamster counterpart of either preduodenal or lysosomal lipase, it should have distinctly higher identities with members of any one of these two categories, which is however not seen. Thus, although pairwise protein sequence identities among lysosomal lipases of rat, mouse and even the more distant species human are in the range of 84–73% and those among preduodenal lipases of these species are 88–68%, TALLP’s identities with different members of either of these two categories (even those from mouse and rat, which are closely related to hamster) vary within a similar and low range of 48–43%. On the other hand, TALLP had considerably higher identities (60–67%) with several predicted (hypothetical) acid-lipases annotated in the available genome sequences, which includes the best match XM_285300 of mouse. Moreover, like TALLP, XM_285300 also had only low level of identities (44–50%) with all acid-lipases (even with the preduodenal and lysosomal lipases of mouse). All above findings strongly suggest that although TALLP belongs to the acid-lipase gene family, it is not the hamster counterpart of preduodenal or lysosomal lipase. Fig. 7 shows a dendrogram obtained by analyzing the mature protein sequences of selected acid-lipases of mammalian origin. Results show that TALLP (along with XM_285300) constitutes a new category of acid-lipase-like proteins, which is distinct from other mammalian acid-lipases. This conclusion is also supported by the identities seen between the sequences of their signal peptides (Fig. 6).
3.8. Interaction of TALLP with the polarity sensitive hydrophobic fluorescent probe 1-AMA

Although TALLP does not have lipase activity on any of the commonly used lipid substrates, there exists a possibility that it might have the property of binding to hydrophobic lipid molecules. 1-aminoanthracene (1-AMA), an excellent probe for hydrophobic binding studies [29], has been previously found to bind the substrate-binding pocket (active site) of the open (inhibited/inactive) forms of dog gastric [24], and human pancreatic [28] lipases. In an aqueous buffer AMA showed weak fluorescence emission with maxima at 563 nm (not shown) when excited at 297 nm. Fig. 8 shows that when native TALLP was incubated with increasing concentrations of AMA, both an increase in AMA fluorescence intensity and a blue shift in its emission maxima were observed, indicating accommodation of AMA in a hydrophobic environment, likely to be due to its binding to TALLP. This binding of AMA to TALLP followed a hyperbolic one-site binding curve with an estimated $K_d$ of 12.0 μM. Thus, unlike dog gastric and human pancreatic lipases, which bind AMA only in their open (covalently modified and inhibited) configuration [24,28], unmodified native TALLP has a property of binding a hydrophobic ligand, AMA, with high affinity.

4. Discussion

Our results show that a novel 48-kDa tear acid-lipase-like protein (TALLP) lacking any detectable enzymatic activity is abundantly expressed in LG of lactating and gonadectomized hamsters. TALLP displays an unusual regulation in being markedly repressed by physiological levels of androgens (in males) and estrogens (in females). Thus, TALLP is undetectable in male and female hamsters, but is massively induced in LG (and secreted in tears) of both sexes after gonadectomy (an artificial low sex-hormonal state), and exogenous androgens or estrogens obliterate this gonadectomy-induced expression. TALLP expression in gonadectomized hamsters is also obliterated by thyroid hormone treatment. However, since no significant decrease in endogenous levels of thyroid hormones is known to occur after gonadectomy in hamsters [31,44], the repressive effect of exogenous thyroid hormone treatment on TALLP expression might be an effect of supraphysiological levels of this hormone. This needs further investigation.

Importantly, natural expression of TALLP is seen temporarily, only during lactation, a physiological state during which hamster dams are anestrus (acyclic) with little or no circulating estrogens [25,31,45]. Thus, the gonadectomy-like low-estrogen state prevailing during lactation must be the reason for TALLP induction in LG of hamster dams. It is well known that normal weaning or early pup-deprivation leads to a resumption of estrus cycles, resulting in a rise of estrogen levels [25,31,45,46]. This possibly explains the lack of TALLP expression in hamster dams after such treatments.

Interestingly, hormonal regulation of TALLP is similar to that of hamster 20 kDa tear lipocalin, which has been shown to be transcriptionally repressed by androgen, estrogen and thyroid hormone [9,25,31]. Given the way these hormones are known to act [47], their repressions of TALLP are likely to be also at the transcriptional level and mediated by their respective receptors expressed in LG [48]. TALLP is however, the first instance of a lipase-like protein displaying an unusual multi-hormonal regulation (repression).

Lipolytically inactive, lipase-like proteins are known [49,50]. Abundant insect yolk proteins (distantly related to mammalian lipases) are lipolytically inactive due to altered residues in their catalytic triad and they also lack an alpha helical flap, essential for interaction with lipid substrates [49,51]. *Drosophila* yolk proteins, related to pancreatic lipases, have been shown to be storage molecules for the hormone (and secreted in tears) of both sexes after gonadectomy (an artificial low sex-hormonal state), and exogenous androgens or estrogens obliterate this gonadectomy-induced expression. TALLP expression in gonadectomized hamsters is also obliterated by thyroid hormone treatment. However, since no significant decrease in endogenous levels of thyroid hormones is known to occur after gonadectomy in hamsters [31,44], the repressive effect of exogenous thyroid hormone treatment on TALLP expression might be an effect of supraphysiological levels of this hormone. This needs further investigation.

Importantly, natural expression of TALLP is seen temporarily, only during lactation, a physiological state during which hamster dams are anestrus (acyclic) with little or no circulating estrogens [25,31,45]. Thus, the gonadectomy-like low-estrogen state prevailing during lactation must be the reason for TALLP induction in LG of hamster dams. It is well known that normal weaning or early pup-deprivation leads to a resumption of estrus cycles, resulting in a rise of estrogen levels [25,31,45,46]. This possibly explains the lack of TALLP expression in hamster dams after such treatments.

Interestingly, hormonal regulation of TALLP is similar to that of hamster 20 kDa tear lipocalin, which has been shown to be transcriptionally repressed by androgen, estrogen and thyroid hormone [9,25,31]. Given the way these hormones are known to act [47], their repressions of TALLP are likely to be also at the transcriptional level and mediated by their respective receptors expressed in LG [48]. TALLP is however, the first instance of a lipase-like protein displaying an unusual multi-hormonal regulation (repression).

Lipolytically inactive, lipase-like proteins are known [49,50]. Abundant insect yolk proteins (distantly related to mammalian lipases) are lipolytically inactive due to altered residues in their catalytic triad and they also lack an alpha helical flap, essential for interaction with lipid substrates [49,51]. *Drosophila* yolk proteins, related to pancreatic lipases, have been shown to be storage molecules for the hormone (and secreted in tears) of both sexes after gonadectomy (an artificial low sex-hormonal state), and exogenous androgens or estrogens obliterate this gonadectomy-induced expression. TALLP expression in gonadectomized hamsters is also obliterated by thyroid hormone treatment. However, since no significant decrease in endogenous levels of thyroid hormones is known to occur after gonadectomy in hamsters [31,44], the repressive effect of exogenous thyroid hormone treatment on TALLP expression might be an effect of supraphysiological levels of this hormone. This needs further investigation.

Importantly, natural expression of TALLP is seen temporarily, only during lactation, a physiological state during which hamster dams are anestrus (acyclic) with little or no circulating estrogens [25,31,45]. Thus, the gonadectomy-like low-estrogen state prevailing during lactation must be the reason for TALLP induction in LG of hamster dams. It is well known that normal weaning or early pup-deprivation leads to a resumption of estrus cycles, resulting in a rise of estrogen levels [25,31,45,46]. This possibly explains the lack of TALLP expression in hamster dams after such treatments.

Interestingly, hormonal regulation of TALLP is similar to that of hamster 20 kDa tear lipocalin, which has been shown to be transcriptionally repressed by androgen, estrogen and thyroid hormone [9,25,31]. Given the way these hormones are known to act [47], their repressions of TALLP are likely to be also at the transcriptional level and mediated by their respective receptors expressed in LG [48]. TALLP is however, the first instance of a lipase-like protein displaying an unusual multi-hormonal regulation (repression).

Lipolytically inactive, lipase-like proteins are known [49,50]. Abundant insect yolk proteins (distantly related to mammalian lipases) are lipolytically inactive due to altered residues in their catalytic triad and they also lack an alpha helical flap, essential for interaction with lipid substrates [49,51]. *Drosophila* yolk proteins, related to pancreatic lipases, have been shown to be storage molecules for the hormone (and secreted in tears) of both sexes after gonadectomy (an artificial low sex-hormonal state), and exogenous androgens or estrogens obliterate this gonadectomy-induced expression. TALLP expression in gonadectomized hamsters is also obliterated by thyroid hormone treatment. However, since no significant decrease in endogenous levels of thyroid hormones is known to occur after gonadectomy in hamsters [31,44], the repressive effect of exogenous thyroid hormone treatment on TALLP expression might be an effect of supraphysiological levels of this hormone. This needs further investigation.

Importantly, natural expression of TALLP is seen temporarily, only during lactation, a physiological state during which hamster dams are anestrus (acyclic) with little or no circulating estrogens [25,31,45]. Thus, the gonadectomy-like low-estrogen state prevailing during lactation must be the reason for TALLP induction in LG of hamster dams. It is well known that normal weaning or early pup-deprivation leads to a resumption of estrus cycles, resulting in a rise of estrogen levels [25,31,45,46]. This possibly explains the lack of TALLP expression in hamster dams after such treatments.
ecdysone involved in insect embryogenesis [49,51]. Lipase activity is also undetectable in pancreatic lipase-related protein-1 (PLRP-1), despite its having a conserved catalytic triad and ~68% identity with the classical pancreatic lipase [50,52]. Interestingly, site-directed mutagenesis of two residues, predicted to cause steric hindrance at the active site of dog PLRP1, resulted in a highly active lipase [50,52]. Since, TALLP has conserved catalytic machinery, its lack of activity could be due to some unidentified sequence features resulting either in steric hindrance, a misfolded protein or an inability to penetrate and bind the interface (critical for catalytic activity for all lipases [43]). Circular dichroism analysis (not shown) of purified TALLP was in accordance with a folded protein, comparable to that of human gastric lipase (Canaan, S., personal communication). Inspection of modeled 3D structure of TALLP (not shown) and its comparison with crystal structures of human and dog gastric lipases [24,39] revealed no obvious reason for lack of activity in TALLP. Moreover, an inhibitor of gastric lipases (the C11Y4 phosphonate inhibitor [24]) corresponding to a triglyceride substrate, could be positioned within the ‘active site’ cleft of modeled TALLP in a similar manner (not shown), as observed in the three dimensional structure of dog gastric lipase-C11-phosphonate inhibitor complex [24]. Nevertheless, solving the crystal structure of TALLP may provide useful clues to its lack of activity, as it was possible for PLRP-1 [50,52] but this is unlikely to reveal the physiological role of TALLP, the role of PLRP-1 being still unknown [53].

Our sequence comparisons suggest that a reason for lack of activity in TALLP may possibly reside among the 99 residues, which have no match whatsoever in known mammalian acid-lipases, and among them, more suspect should be the 48 residues (Fig. 6) whose counterparts are conserved in all such lipases. However, neither the absence of a conserved free cysteine (at T260) nor the absence of a conserved N-glycosylation site at K32 in TALLP, are likely to be reason for its lack of activity, since bovine pregastric lipase is active without this cysteine [27], and deglycosylation or mutagenesis of N-glycosylation sites in gastric and lysosomal acid-lipases did not result in inactive lipases [20,32,33]. Nevertheless, it cannot be completely ruled out whether such changes, in the context of TALLP’s sequence, may result in its lack of lipase activity. Alternately, it is possible that presence of a unique N-glycosylation site at N-70, two uniquely located cysteines (C80, C193) or deletions in TALLP (indicated by gaps in alignment) may be responsible for its lack of lipase activity or imparts it unusual and yet unidentified substrate specificity. In this context, it needs mention that no activity of TALLP on vinyl butyrate, dicaprin monolayers, radiolabeled triglycerides (Canaan, S., personal communication) or radiolabeled cholesterol oleate (Tiwari, R., personal communication) could be detected.

Interestingly, presence of the conserved EcoRI site in cDNAs of TALLP and other active acid-lipases could be conveniently utilized for preparing chimeric overexpression constructs. Checking lipase activity in chimeric proteins might reveal, whether the reason for lack of activity resides in the N- or C-terminal segments of TALLP flanking the EcoRI site. TALLP is thus an interesting model of an acid-lipase-like protein, whose lack of activity could be investigated by preparing chimeras with active acid-lipases and 3D crystal structure analysis, to provide new insights regarding the sequence and structural features, which affect activity and/or substrate-specificity of acid-lipases.

Mammals express two related but distinct categories of acid-lipase genes, encoding lysosomal and preduodenal acid-lipases [14,15,21,43]. Although hamster preduodenal and lysosomal lipases (or their cDNAs) are not yet isolated, our analysis of sequence identities suggests that TALLP is a prototype of a new category within the acid-lipase gene family, distinct from mammalian preduodenal and lysosomal lipases. Notably, several additional acid-lipase-like genes have been predicted and annotated in the available genome sequences, but unlike TALLP, no evidence of expression of their proteins is yet available.

TALLP is the first acid-lipase-like protein detected in LG and tears of any species. The temporary massive induction of TALLP in hamster dams is intriguing, and this should be an important clue to its function. Secretions of lacrimial and accessory lacrimal glands are believed to be sources of pheromones [5,6,9,54,55]. Hamster tears contain the unusual lipid 1-alkyl-2,3-diacylglycerol in which compositions of alkyl and acyl groups differ markedly between males and females [5,6], and such lipids in lactating dams, were proposed to have a role in mother-pup pheromonal communication [6]. TALLP might function in the modification of such specific lipid substrates by a lipase activity (which may have remained undetected). Interestingly, in rats, dodecyl propionate (a potential lipase substrate) has been identified as a pheromone important for mother-pup communication [56]. Alternately, since tears are voided externally, a lipolytically inactive TALLP might still perform a significant function by binding and transport of pheromonal tear lipids to the exterior for their dissemination. Our result showing that purified TALLP binds the hydrophobic ligand 1-AMA suggests an open conformation exposing a hydrophobic surface, which may bind lipid. An open conformation in purified TALLP might also result due to presence of bound lipids. Although uninvestigated, the possibility of any bound lipids in purified TALLP seems unlikely, considering the different steps and extensive dialysis employed during its purification. A lipid binding/storage function has also been proposed for the lipolytically inactive PLRP-1 [50,52,53] and insect yolk proteins [49,51]. Additionally, like tear lipocalin [2–4,7–9,12], if TALLP interacts with tear lipids, it can potentially alter tear film’s physiochemical characteristics. Thus, it would be interesting to know whether the exaggerated expression of TALLP and lipocalins in tears of lactating hamster is beneficial, deleterious or has no net effect on their tear film and ocular surface. Finally, since tear film disorders and dry eye diseases in humans show a marked gender difference (ten times higher in females) and are associated with hormonally altered states e.g. menopause, lactation, etc. [3,4], presence of any hormonally regulated lipase-like protein in human tears needs investigation.
Acknowledgements

A.P. thanks CSIR, India for a research fellowship. Authors thank Mr. K. Ram for technical assistance, Drs V. M. Dhople and M. V. Jagannadham for help in internal and N-terminal sequencing, Dr. B. Sesikeran (NIN, India) for suggestions on immunohistochemistry and Prof. R. Schneider and Ms. R. Tiwari (University of Fribourg, Switzerland) for cholesterol esterase assays. Authors also gratefully acknowledge the gift of reagents, helpful suggestions and critical comments on the manuscript kindly provided by Dr. S. Canaan, of EIPL, France.

References


