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Molecular cloning and nucleotide sequence of rat lingual lipase cDNA

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ABSTRACT

Purified rat lingual lipase (EC3113), a glycoprotein of approximate molecular weight 52,000, was used to generate polyclonal antibodies which were able to recognise the denatured and deglycosylated enzyme. These immunoglobulins were used to screen a cDNA library prepared from mRNA isolated from the serous glands of rat tongue cloned in E. coli expression vectors. An almost full length cDNA clone was isolated and the nucleotide and predicted amino acid sequence obtained. Comparison with the N-terminal amino acid sequence of the purified enzyme confirmed the identity of the cDNA and indicated that there was a hydrophobic signal sequence of 18 residues. The amino acid sequence of mature rat lingual lipase consists of 377 residues and shares little homology with porcine pancreatic lipase apart from a short region containing a serine residue at an analogous position to the ser 152 of the porcine enzyme.

INTRODUCTION

The serous (von Ebner's) glands at the back of the rat tongue secrete an acid stable lipolytic activity which hydrolyses triglycerides to diglycerides, monoglycerides and free fatty acids in the pH range 2-6.5 (1) and which aids emulsification and digestion of dietary fat in the stomach. Similar activities have been found in homogenates of human lingual serous glands and in gastric aspirates of humans and rats (2-5). Purified rat lingual lipase (rat LL) has an approximate mol. wt. of 52,000 (6), which is similar to that of porcine pancreatic lipase (PPL) (7).

Several conditions in humans, including cystic fibrosis, pancreatitis, premature birth and alcoholism, are associated with pancreatic lipase insufficiency and in the former case enzyme replacement therapy with LL has been suggested as a method of treatment (8). Expression of a cloned cDNA gene encoding human LL in a microorganism may provide a source of acid stable lipase for use as a dietary aid in the above conditions.

Molecular cloning techniques have been used to obtain the amino acid sequences of a large number of proteins. Frequently, clones have been

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isolated from libraries using oligonucleotide probes with sequences predicted from partial amino acid sequence data (9). An alternative strategy, useful in the absence of any protein sequence data, is to construct cDNA libraries in vectors which ensure that at least a proportion of inserted cDNA will be transcribed into mRNA and translated into protein, usually as a fusion with a bacterial protein. These libraries are then screened with an antibody against the protein of interest. Both plasmid (10-13) and λ phage vectors (14, 15) have been developed for this purpose.

In this paper we report the molecular cloning and nucleotide sequence of a cDNA for rat LL mRNA. A cDNA library from rat serous glands was made in the pUR series of vectors. These lac promoter driven β-galactosidase gene fusion vectors permit the fusion of cDNA onto the 3' end of the β-galactosidase gene in all three reading frames. As a result peptides encoded by cDNA expressed in these vectors are fused to almost full length β-galactosidase and can be detected with specific antibodies (12). The library was probed with a rabbit antiserum to rat LL and a full length cDNA clone was identified.

MATERIALS AND METHODS

Purification of Rat LL

Acetone-dried powder of homogenised and delipidated serous glands from Wistar rats was fractionated by the butanol-ammonium sulphate procedure of Verger *et al* (16). The resulting lipase rich cream was extracted with 2% Triton X100 and the enzyme was purified by sequential chromatography on carboxymethyl Sepharose CL6B and Sephadex G100 in 50mM sodium acetate pH5.0.

Preparation of Antiserum

Rabbits were immunised with Sephadex-purified enzyme (100μg each) in Freund's complete adjuvant. The inoculation was repeated after 14 days using incomplete adjuvant and a test-bleed taken on day 28. Rabbits were further boosted after 3 months and bled out one week later. Antibody specificity and titre were determined by Ouchterlony radial immunodiffusion and by precipitation of ^{125}I-labelled rat LL.

Immunoaffinity Chromatography

Immunoglobulins precipitated from high titre serum by precipitation with sodium sulphate (18% w/v) were redissolved in 0.1M sodium bicarbonate pH9 and dialysed extensively against the same buffer. The immunoglobulins were coupled to CNBr activated Sepharose (Pharmacia) using the manufacturers' instructions at 1.3mg IgG/ml settled gel. The affinity matrix was blocked with 1M ethanolamine (pH8) for 2h at room temperature and washed with 0.1M sodium

bicarbonate pH9 and 0.1M sodium acetate pH5 buffers before use.

Proteins solubilised from the 'lipase rich cream' were applied to a column of the affinity matrix and washed with 1M KCl in phosphate buffered saline. Tightly bound protein was eluted in 0.1M glycine-HCl pH2.3, dialysed against 50mM NH_4 acetate pH5, freeze dried, and stored at -20°C. From 5 or 6 rats, approximately 1mg of purified enzyme could be obtained by this procedure.

Assay of Enzyme Activity

The lipolytic activity of the enzyme was assayed by the measurement of hydrolysis of ^3H-triolein essentially as described by Abrams et al (8), with the exception that free fatty acids were extracted into 0.1M glycine pH12.5.

Polyacrylamide Gel Electrophoresis and Western Blotting

SDS polyacrylamide gels were run as described by Laemmli (17). For Western blotting (18), proteins were electroblotted (Bio-Rad) onto nitrocellulose paper (Schleicher & Schuell). Filters were blocked with 5% w/v casein in buffer and incubated with 1ml of an appropriate dilution of rabbit antiserum. After a high salt buffer wash the filter was reacted with 2μCi ^{125}I protein A (Amersham) in a volume of 1ml.

Endoglycosidase H Treatment

Endoglycosidase H (EndoH, Miles) digests were carried out at 37°C in 50mM sodium acetate pH5.5 containing 1mM phenylmethylsulphonylfluoride, 10μM Pepstatin A, 10U/ml EndoH and 100μg/ml rat LL for up to 21h. Control experiments were carried out using ovalbumin to confirm Endo H activity and lack of protease activity.

N-terminal Sequence Analysis

N-terminal sequence analysis of rat LL, purified on the immunoadsorbent column, was determined by Dr. B. Dunbar, Univ. of Aberdeen, Scotland, using a Beckman spinning cup sequenator.

mRNA Preparation and in vitro Translation

Lingual serous glands of newly sacrificed mature female Sprague Dawley rats were dissected from the back of the tongue and frozen in liquid nitrogen. The glands were crushed under liquid N_2 and total RNA isolated from the powder by guanidinium thiocyanate-phenol extraction as described by Maniatis et al (19). Polyadenylated mRNA was selected by oligo dT-cellulose (Collaborative Research) column chromatography (20) and integrity checked by agarose gel electrophoresis and in vitro translation in a rabbit reticulocyte lysate in the presence of ^{35}S methionine (21). Immunoprecipitation of the in vitro translation products was undertaken as described (22) using protein A sepharose to precipitate the complexes. Polypeptides were analysed on 10%

SDS polyacrylamide gels (17).

Cloning and Screening Procedures

Standard procedures were used to synthesise cDNA from serous gland mRNA (23, 24). Half of the cDNA was treated with an excess of Sau3A while the rest was treated with S_1 nuclease (Boehringer) before addition of BamH1 linkers. Excess linkers were removed by Sephadex G75 chromatography (19). Sau3A digested or BamH1 linked cDNA was ligated into BamH1 cleaved and alkaline phosphatase (CIP, Boehringer) treated pUR290, 291 and 292 vector DNA under standard conditions (19) and the mixtures were then used to transform E. coli DH1 (25).

For immunological screening, colonies were transferred onto nitrocellulose filters and lysed on Whatman 3MM paper wetted with 9M urea in 10mM Tris-HCl pH7.6, 1mM EDTA in a $CHCl_3$-saturated atmosphere. Cell debris and DNA were removed by washing extensively in 160mM NaCl, 10mM Tris-HCl pH7.6 (Buffer A), first containing 0.01% w/v SDS, and then 10mM $MgCl_2$ 5µg/ml pancreatic DNase. Non specific antibody binding sites were then blocked by incubating with Buffer A, 0.5% w/v casein. The filters were next incubated colony side down in Buffer A, 0.1% w/v Triton, 0.1% w/v SDS 1mM EDTA containing 2µg/ml IgG (purified as described above and adsorbed with a urea extract of E. coli DH1). Unbound antibody was removed by vigorous washing in the Triton-SDS buffer and the filters then incubated with 1µCi/ml ^{125}I protein A. The filters were finally washed extensively with the Triton-SDS buffer and subjected to autoradiography.

Further Analysis of Clones

Plasmids were prepared from positive colonies by the small scale method of Ish-Horowicz & Burke (26) and DNA digested with various restriction enzymes (Amersham) under recommended conditions. For nucleotide sequencing, fragments of cDNA were subcloned into M13 mp8 or mp9 and transformed into JM101 (27) for sequence determination by the dideoxy procedure (28). Southern and Northern blots were carried out essentially as described in Maniatis et al (19), using 1% w/v agarose or 1.2% v/v formaldehyde-agarose gels respectively. Hybrid selection was undertaken as described (29).

Primer extension reverse sequencing was done by dideoxy incorporation (30) using a primer complementary to nucleotides 142-159 of the pLL10 DNA sequence (5'CATCTGACTAATATTCAT3' synthesised by automated solid phase phosphotriester chemistry [31]).

RESULTS

Characterisation of Rat LL Antisera

Rabbits immunised with the Sephadex purified rat LL, produced antibodies to the enzyme which reached a high level titre (>1 in 25,000) after four months, as detected by precipitation of ^{125}I-labelled LL, inhibition of enzyme activity and immunodiffusion tests. Antisera were tested further for specificity and ability to recognise denatured and deglycosylated protein by Western blotting. The antibody reacted with a protein of mol. wt. 52,000, the size expected for lingual lipase, suggesting specificity and recognition of denatured polypeptide under conditions similar to those to be used for clone detection (Fig. 1, lane 1).

After Endonuclease H (Endo H) treatment three bands of lower mol. wt. were detected with the antibody (Fig. 1, lane 2) presumably representing polypeptides containing different numbers of residual N-linked oligosaccharide chains. Treatment of LL with neuraminidase or heat denaturation in SDS before Endo H did not alter the pattern of deglycosylation or the size of the band with the lowest mol. wt. These results provide evidence that the antiserum reacts with denatured rat LL and several partially deglycosylated species and that the molecular weight of the core polypeptide is approximately 41,000.

Immunoaffinity Chromatography

Immunoaffinity chromatography was able to provide sufficient quantities of pure protein for amino acid sequencing. Immunoglobulin purified from high titre antisera was coupled to CNBr-activated Sepharose and used to select proteins from a crude extract of serous glands. The affinity purified protein ran as a single band of mol. wt. 52,000 (Fig. 1, lane 3). Enzyme assays confirmed that the activity was selectively bound to the matrix, not dissociated by a high salt wash and eluted by acid pH with a recovery of 80%. Immunoaffinity purification afforded the isolation of milligram quantities of pure protein for sequencing which had not been possible by conventional purification techniques.

In vitro Translation of mRNA from Rat Serous Glands

Total mRNA was isolated from von Ebner's glands and as a control from fragments of front tongue. The mRNAs were translated *in vitro* in a reticulocyte lysate in the presence of ^{35}S methionine and the translation products analysed by polyacrylamide gel electrophoresis. Fig. 1, lanes 5 and 6 show that a different spectrum of polypeptides are synthesised by the mRNA isolated from either serous gland or from front tongue tissue. Furthermore, a prominent protein with a mol. wt. of about 40,000 in the products from serous gland mRNA

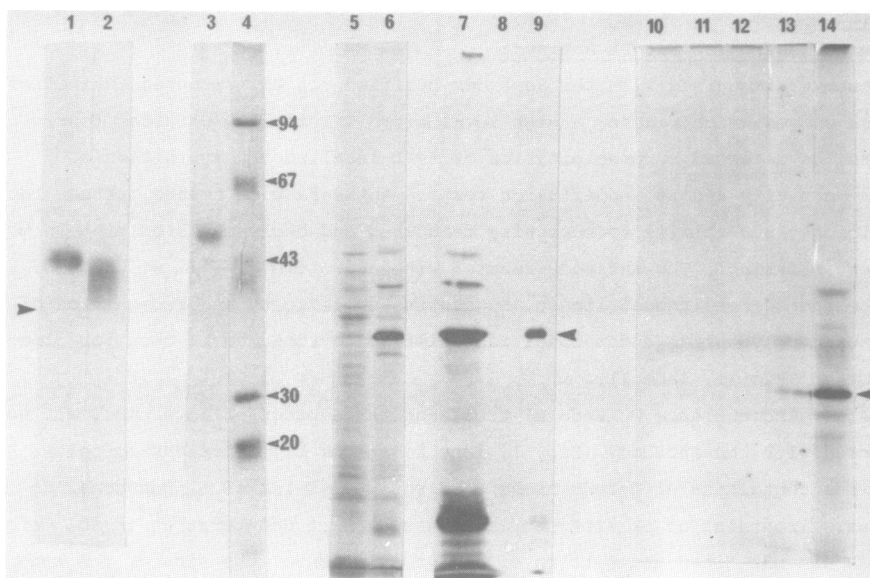

Figure 1, lanes 1 and 2

Western blot of lingual lipase after incubation with endoglycosidase H at 37°C for 0 and 40 min respectively. The arrow shows the position of a calf prochymosin marker (40,600).

Lanes 3 and 4

SDS-polyacrylamide gel (8.75%) showing Coomassie blue stained affinity purified lingual lipase (lane 3) and marker proteins (lane 4).

Lanes 5-9

Autoradiograph of polyacrylamide gels of ^{35}S methionine labelled polypeptides synthesised *in vitro* in reticulocyte lysates in response to: lane 5, mRNA isolated from rat front tongue; lane 6, mRNA isolated from rat serous glands. Lanes 7-9 show polypeptides immunoprecipitated from reticulocytes lysates primed with serous gland mRNA: lane 7, not precipitated; lane 8, precipitated with preimmune serum; lane 9, precipitated with LL antiserum. The arrow indicates the position of LL.

Lanes 10-14

Autoradiograph of a polyacrylamide gel of ^{35}S methionine labelled polypeptides synthesised in reticulocyte lysates in response to hybrid selected mRNA. Lane 10, no RNA in lysate; lane 11, no DNA on filter; lane 12, vector DNA on filter; lane 13, plasmid DNA from positive clone on filter; lane 14, serous gland mRNA in lysate (no selection). The arrow indicates the position of LL.

is specifically immunoprecipitated by LL antiserum and not by preimmune serum (Fig. 1, lanes 7-9). The size of this major polypeptide is similar to the smallest form of deglycosylated LL (Fig. 1, lane 2) which is consistent with it being the unmodified native form of the polypeptide. The fact that this polypeptide is not a major constituent of the translation products of front

tongue mRNA, but is abundant in the products from serous gland mRNA, suggests that rat LL mRNA is relatively abundant in serous glands.

Cloning of Lingual Lipase cDNA

The pUR vectors were chosen for this study because during preliminary experiments they gave stronger and more frequent signals than other expression vectors such as pUC8 and 9 (32) which give rise to relatively short N-terminal fusions. Following transformation into DH1 about 28,000 colonies with the Sau3A digested cDNA and 7,500 colonies with the BamH1 linked cDNA were obtained with each of the pUR vectors at a density of about 3,000 per 13.5 cm petri dish. After transfer to nitrocellulose the colonies were lysed with 9M urea prior to exposure to chloroform vapour because in our hands this treatment increased the frequency and strength of the signals obtained. After screening with IgG purified from rabbit anti rat LL serum followed by ^{125}I Protein A sixty colonies were found to produce positive signals. These were streaked out for single colonies and rescreened. Forty of the original 60 colonies again gave clear signals. Positive clones were identified arising from all three pUR vectors with both BamH1 linked and Sau3A digested cDNA. A similar detection frequency of about 5.10^{-4} was recorded for each vector-cDNA combination.

The plasmids contained in positive colonies were examined by restriction enzyme digestion and Southern blotting using the longest cDNA insert as a probe. The insert DNAs were found to be related and one of the plasmids (pLL10) isolated from the pUR292/BamHI linked library, had a 1350 base pair insert, sufficient to encode rat LL. One of the clones was also used to hybrid select a serous gland mRNA that translated *in vitro* to give a polypeptide of the same size as that immunoprecipitated by anti rat LL serum (Fig. 1, lanes 10-14).

The efficiency of the antibody screening was examined by probing the BamHI library with the cDNA from pLL10. This should detect LL encoding inserts which are out of frame or in the wrong orientation with respect to the β-galactosidase gene. A probe covering the entire coding region of LL (see below) should also detect any colonies with inserts which encode portions of LL not reacting with the antibody. Approximately 6-fold more positive colonies from each library were detected with the DNA probe. It was assumed that the lower frequency of detection with the antibody was because only one in six recombinants have an insert in the correct orientation and reading frame with respect to the β-galactosidase coding sequence rather than inability of the antibody to recognise all portions of LL produced in *E. coli*. This conclusion

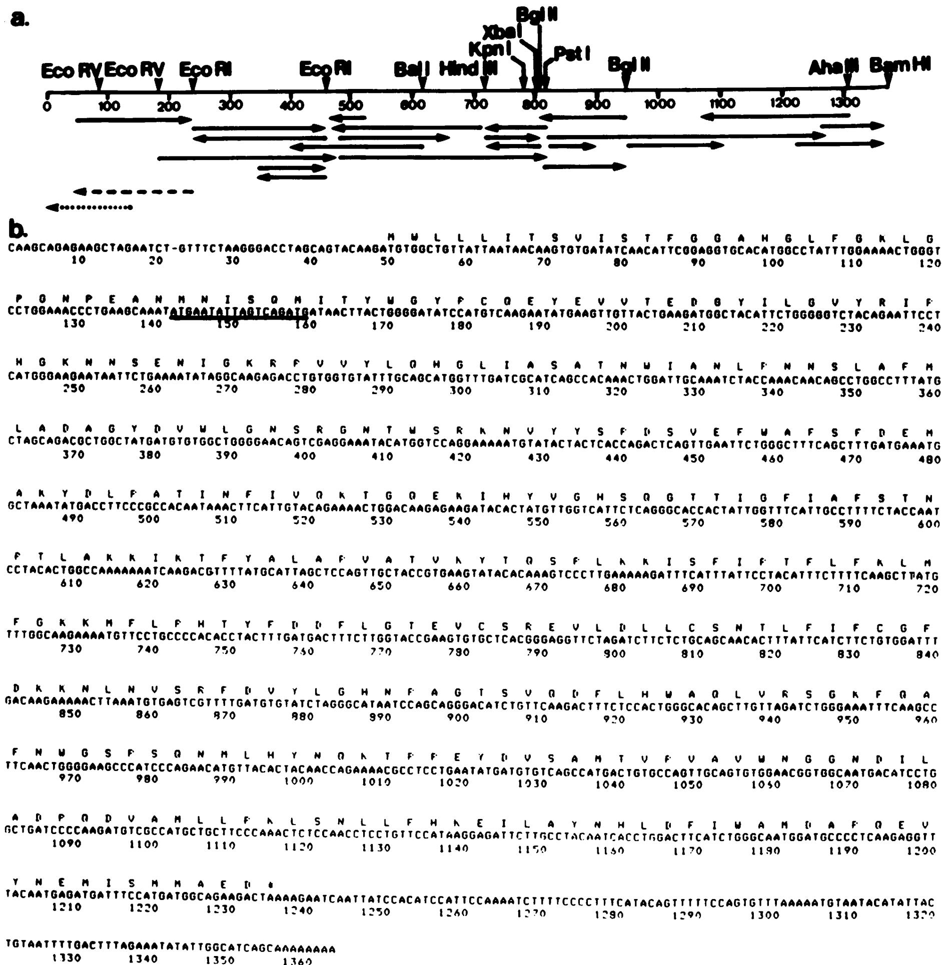

Figure 2(a)

Restriction map of pLL10 showing restriction sites used for M13 cloning. The arrows denote the directions of dideoxy sequencing. The broken arrow indicates a confirmatory sequence obtained by the Maxam-Gilbert procedure and the dotted arrow indicates sequence obtained by primer extension using an oligonucleotide complementary to the sequence underlined at nucleotides 142-159.

Figure 2(b)

Nucleotide sequence encoding rat LL and the predicted amino acid sequence. The nucleotide at position 22 was not identified. The numbers refer to the sequence of the mRNA.

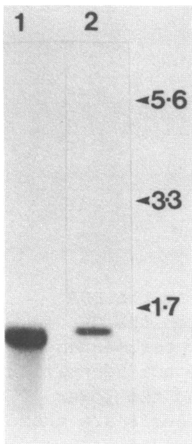

Figure 3
Northern blot of serous gland mRNA probed with the cDNA insert of pLL10 (lane 1). The band in lane 2 is a marker mRNA of 1.45kb obtained by probing RNA from recombinant yeast cells with a restriction fragment of pMA91 (40) containing part of the phosphoglycerate kinase gene. The other markers (1.7, 3.3 and 5.6 kb) were obtained from the same gel and represent other specific yeast mRNAs (J. Mellor personal communication).

is supported by the finding that all three LL Sau3A fragments predicted from the DNA sequence (see below) were picked up in the Sau3A library by the antibody.

Nucleotide Sequence of Rat LL cDNA and Predicted Amino Acid Sequence of the Protein

The restriction map and nucleotide sequence of pLL10 cDNA is shown in Fig. 2. The sequence is notable for being AT rich (57% A+T) and this is reflected in a lack of restriction sites particularly towards the 3' end of the sequence. The poly A at the 3' end of the cDNA indicates that the 3' end of the mRNA is represented. It is interesting that an AATAAA polyadenylation signal is apparently not present although the sequence AAATATA occurs 11 bases from the poly A. Northern blots of serous gland mRNA probed with pLL10 DNA gave a single band with a size of about 1.4kb (Fig. 3). The 1350bp cDNA insert in pLL10 was therefore an almost full length cDNA and so primer extension sequencing with an oligonucleotide complementary to nucleotides 142-159 was used to determine the sequence missing from the 5' end of the clone. This confirmed the sequence of the terminal 100 nucleotides in pLL10 and derived a sequence for the 42 nucleotides missing from the 5' end (Fig. 2).

The complete LL cDNA contains an open reading frame of 1203 nucleotides (nucleotides 31-1233 in Fig. 2) with the first in-frame methionine being encoded by nucleotides 49-51. There is therefore a coding capacity for 395 amino acids. This methionine is followed by a sequence of twelve hydrophobic amino acids, consistent with it being the start of translation, and the hydrophobic region being the core of a signal sequence for secretion (33). From the sequence we were unable to determine precisely where cleavage occurs to

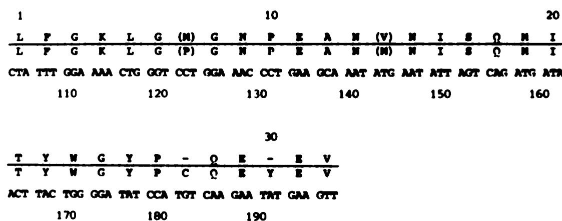

Figure 4

The N-terminal sequence of rat lingual lipase predicted from the DNA sequence of pLL10 (lower sequence) compared to that obtained from the N-terminus of the affinity purified enzyme by protein sequence (upper sequence). In the latter case, the amino acids at positions 27 and 30 were not identified. The upper numbers refer to amino acids in the mature protein and the lower numbers to the sequence of the mRNA. The amino acids in parenthesis are those at which the sequences do not correspond.

liberate the mature protein. To resolve this question LL was purified on the immunoadsorbent column (Fig. 1, lane 3) and the sequence of the first 32 amino acids determined. From this sequence (Fig. 4) it was concluded that LL is synthesised with an 18 amino acid signal sequence and that the cDNA selected by the immunoscreening is derived from LL mRNA.

DISCUSSION

E. coli clones synthesising rat LL have been identified with a polyclonal antibody that reacted with the denatured and unglycosylated protein. The antibody detected both Sau3A fragments and longer BamHI linked inserts expressed as C-terminal fusions to β-galactosidase. The rat LL coding region contains 3 Sau3A fragments, each of which start in a different reading frame. All three were found to have fused correctly to the appropriate vector. Furthermore, the frequency of detection with the antibody was similar whether BamHI linked or Sau3A digested insert DNA was used. This indicates that the combination of vector system, antiserum and screening procedure was sufficient for frequent detection of cDNA clones encoding rat LL. In the latter case, the urea lysis step probably helped by dissolving insoluble β-galactosidase fusions with the consequence that they were made more available for antibody detection. Over-production of β-galactosidase is known to give rise to insoluble inclusion bodies (34, 35) and DH1 cells harbouring pLL10 were found to contain such bodies. These cells were shown to be making 5-10% of their total cell protein as fusion protein by Western blotting (data not shown). Although such levels of insoluble protein may not be lethal there were marked differences in the growth rate of cells harbouring these plasmids and in the

absence of selection plasmid loss was observed. This problem may be overcome by growing the plasmids in a strain of _E. coli_ with the _lac_ I^q mutation (36). β-galactosidase can then be induced with IPTG as required prior to screening.

The cDNA identified using the antibody to rat LL has been confirmed to be from the cognate mRNA by comparison with the N-terminal sequence of the LL protein. The amino acid sequence predicted for rat LL indicates that the mature lipase consists of 377 amino acids. The predicted mol. wt. of this protein is 42,564, in close agreement with the mol. wt. of the deglycosylated protein (Fig. 1, lane 2) and of the core polypeptide synthesised _in vitro_ (Fig. 1, lanes 5-9)obtained by SDS gel electrophoresis. Mature lingual lipase contains five potential sites for N linked glycosylation (Asn X Ser or Thr); judging by the deglycosylation pattern with Endo H digestion (Fig. 1, lane 2), it seems likely that at least three of these sites have oligosaccharide side chains in the native enzyme.

The sequence of LL has also been compared to that of porcine pancreatic lipase (PPL). Rat LL is 72 amino acids shorter than PPL (7) and is striking in that it bears little amino acid sequence homology. However, there is some similarity in the region of the essential serine 152 in PPL. This serine reacts with micellar diethyl-p nitrophenyl phosphate and may participate in the fixation of the enzyme to lipids (37-39). It is present in the sequence Gly His Ser$_{152}$ Leu Gly in PPL and in an equivalent position in rat LL (Gly His Ser$_{153}$ Gln Gly). Another point of similarity is the single glycosylation site in PPL (Asn$_{166}$ Gly Thr) and a potential site in rat LL (Asn$_{166}$ Pro Thr).

It is interesting that the N-terminal sequence predicted from the cDNA does not exactly correspond to the amino acid sequence obtained for the purified protein. The nucleotide sequence of the mRNA has been confirmed by primer extension. It is unlikely therefore that the difference in sequence is due to a cloning artefact or to the isolation of a clone for a minority mRNA species. As different strains of rats were used for the enzyme purification and mRNA isolation it seems more likely that the differences represent true allelic variants.

Finally, the cDNA for rat LL should allow the isolation of genomic clones in order to determine the structure of the rat gene. In addition, it can be used to isolate cDNA clones specific for the analogous human enzyme.

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