

Domain-structure analysis of recombinant rat hormone-sensitive lipase

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Hormone-sensitive lipase (HSL) plays a key role in lipid metabolism and overall energy homeostasis, by controlling the release of fatty acids from stored triglycerides in adipose tissue. Lipases and esterases form a protein superfamily with a common structural fold, called the α/β -hydrolase fold, and a catalytic triad of serine, aspartic or glutamic acid and histidine. Previous alignments between HSL and lipase 2 of *Moraxella* TA144 have been extended to cover a much larger part of the HSL sequence. From these extended alignments, possible sites for the catalytic triad and α/β -hydrolase fold are suggested. Furthermore, it is proposed that HSL contains a structural domain with catalytic capacity and a regulatory module attached, as well as a structural N-terminal domain unique to this enzyme. In order to test the proposed domain structure, rat HSL was overexpressed and

purified to homogeneity using a baculovirus/insect-cell expression system. The purification, resulting in > 99% purity, involved detergent solubilization followed by anion-exchange chromatography and hydrophobic-interaction chromatography. The purified recombinant enzyme was identical to rat adipose-tissue HSL with regard to specific activity, substrate specificity and ability to serve as a substrate for cAMP-dependent protein kinase. The recombinant HSL was subjected to denaturation by guanidine hydrochloride and limited proteolysis. These treatments resulted in more extensive loss of activity against phospholipid-stabilized lipid substrates than against water-soluble substrates, suggesting that the hydrolytic activity can be separated from recognition of lipid substrates. These data support the concept that HSL has at least two major domains.

INTRODUCTION

Hormone-sensitive lipase (HSL) is a critical enzyme in lipid metabolism and overall energy homeostasis in mammals. It catalyses the rate-limiting step in the mobilization of fatty acids from adipose tissue, i.e. the hydrolysis of triacylglycerols to diacylglycerols [1,2]. Like glycogen phosphorylase, the corresponding enzyme in carbohydrate metabolism, the activity of HSL is under strict hormonal and neural control by a mechanism involving phosphorylation by cAMP-dependent protein kinase (cAMP-PK) [3,4]. The site phosphorylated by cAMP-PK *in vitro* has been identified as Ser-563 in rat HSL [5,6]. However, in contrast to glycogen phosphorylase, the mechanism whereby phosphorylation causes activation is unknown. A second phosphorylation site (Ser-565 in rat HSL), the basal site, is phosphorylated in unstimulated adipocytes, presumably by 5'-AMP-activated protein kinase, without any concomitant change in enzyme activity [7,8].

HSL has been purified from adipose tissue of several species, and the limited amounts of pure protein obtained have been used for partial characterization [9–12]. HSL cDNAs have been isolated from rat, mouse and human adipose tissue, which, together with the biochemical characterization, have provided information about enzyme structure of this 84 kDa protein [5,13–15]. Using site-directed mutagenesis and transient expression in COS cells [16], the active-site serine, of an expected catalytic triad [17], has been identified as Ser-423 in rat HSL [18].

HSL is not closely related to any other mammalian lipases, but is presumably a member of an esterase subfamily of a newly described lipase/esterase superfamily [19]. This relationship is based on the discovery that a stretch of 88 amino acids (Ile-345–Ala-433) within HSL shows sequence similarity to the lipase

2 of *Moraxella* TA144 [14,20], as well as to other lipases and esterases [19,21].

From further sequence alignment, a model structure of HSL containing two major domains is proposed. The first domain, largely encoded by exons 1–4, shows no significant sequence similarity to other known protein sequences. The second domain is largely encoded by exons 5–9 and harbours the presumed catalytic triad. The regulatory module is inserted in the catalytic domain and is largely encoded by exon 8. In order to verify this domain structure, large amounts of pure HSL are cardinal. Thus, a baculovirus/insect-cell system was used for expression of recombinant rat HSL. The recombinant protein was purified to > 99% homogeneity and was comparable with native rat HSL purified from adipose tissue [9,10] with respect to several biochemical features, including phosphorylation and activation by cAMP-PK. We report here an initial HSL structure determined through limited proteolysis and denaturation studies of the recombinant protein.

MATERIALS AND METHODS

Construction of recombinant transfer vector and isolation of recombinant virus

A full-length rat HSL cDNA, lacking all but 14 of the 5'-untranslated nucleotides [5,13] was inserted into the *Sma*I site of the plasmid pVL1393 (Invitrogen). The resulting 12.4 kb construct was purified using Qiagen maxipreps (Qiagen). Recombinant baculovirus expressing β -galactosidase and wild-type baculovirus were from Clontech.

Wild-type baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA (Invitrogen) and transfer vector, containing the full-length HSL cDNA, were co-transfected into

Abbreviations used: AcNPV, *Autographa californica* nuclear polyhedrosis virus; C₁₃E₁₂, an alkylpolyoxyethylene ether-type detergent, Berol 058; CO, cholesteryl oleate; cAMP-PK, cAMP-dependent protein kinase; GdnCl, guanidinium chloride; GL, gastric lipase; HL, hepatic lipase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; MOI, multiplicity of infection; MOME, mono-oleoyl-2-O-mono-oleylglycerol; PL, pancreatic lipase; PNPB, *p*-nitrophenyl butyrate; PP2A, protein phosphatase 2A; TO, trioleoylglycerol; Sf9 cells, *Spodoptera frugiperda* cells.

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Spodoptera frugiperda (Sf9) cells by calcium phosphate coprecipitation [22]. Recombinant viruses were detected in a plaque assay [22] by visual screening for occlusion-negative plaques. The identities of recombinant viruses were confirmed by dot-blot hybridization [22], and subsequently by Northern-blot analyses, SDS/PAGE, Western blots and HSL-activity assays (see below). For these tests, monolayer Sf9 cells ($\sim 4 \times 10^6$) in 60 mm-diam. plates were inoculated with virus stock for 1 h in Ex-cell 401 with L-glutamine (JRH Biosciences) supplemented with 10% foetal bovine serum and 100 units/ml streptomycin/penicillin (Gibco/BRL). After infection, the medium was replaced with 5 ml of fresh medium and the cells were incubated at 27 °C. At various times after infection, cells were either processed for RNA extraction and Northern-blot analysis or rinsed once with 3 ml of PBS and then homogenized in 0.7 ml of 0.25 M sucrose/1 mM EDTA/1 mM dithioerythritol/20 μ g/ml leupeptin/2 μ g/ml anti-pain/1 μ g/ml pepstatin (homogenization buffer) using a small glass homogenizer. These homogenates were used for HSL-activity measurements, SDS/PAGE and Western-blot analysis.

Total RNA from 4×10^6 Sf9 cells was prepared by the method of Chomczynski and Sacchi [23]. RNA samples were electrophoresed in 1% agarose/2.2 M formaldehyde gels, transferred to nylon membrane, and hybridized using washing conditions as previously described [16].

SDS/PAGE analysis and Western-blot analysis

SDS/PAGE analyses were performed using either glycine [24,25] or tricine [26] buffer. Protein bands were stained with either Kenacid Blue (BDH), Coomassie Brilliant Blue G-250 (Serva) or silver nitrate as described [27]. Western-blot analyses were carried out using a polyclonal rabbit antibody directed against rat adipose-tissue HSL [28,29], anti-rabbit IgG, conjugated to alkaline phosphatase, and a chromogenic substrate (ProtoBlot II AP Systems; Promega). Quantitative analysis of SDS/polyacrylamide gels and Western blots was done using a videodensitometric system developed by Lars Kopp, Makab, Göteborg, Sweden. The amount of HSL used for these analyses was maintained in the linear range of staining to allow quantitative estimations of the proteins (C. Holm and E. Degerman, unpublished work). The 110000 g supernatant of rat adipose tissue [9] and the partially purified rat adipose-tissue HSL [10] were used as reference materials.

HSL activity assays and protein determinations

Recombinant HSL and rat adipose-tissue HSL [9,10] were assayed against tri- and di-acylglycerol and cholesteryl ester substrates. These assays are based on measurements of release of [3 H]oleic acid from tri[3 H]oleoylglycerol (TO), 1(3)-mono[3 H]oleoyl-2-*O*-mono-oleylglycerol (MOME; a diacylglycerol analogue not hydrolysable at position 2) and cholesteryl [3 H]oleate (CO) respectively [9,30,31]. All substrates were emulsified with phospholipids by sonication, and BSA was used as fatty acid acceptor. For each 4 ml substrate, 50×10^6 c.p.m. [3 H]TO, 16×10^6 c.p.m. [3 H]MOME or 20×10^6 c.p.m. [3 H]CO was used at a final substrate concentration of 1.67 mM TO, 5 mM MOME or 0.45 mM CO. The TO and MOME substrates were emulsified with 0.6 mg and the CO substrate with 1.4 mg of phospholipid (phosphatidylcholine/phosphatidylinositol, 3:1, w/w). Briefly, unlabelled and labelled lipids plus phospholipids (in heptane, toluene and chloroform respectively) were mixed in a 4 ml reaction vial, and the solvent was evaporated using N_2 . Potassium phosphate (2 ml, 0.1 M), pH 7.0, was added, and the substrate was sonicated for 2×1 min (with a 1 min interval), using a

Branson Sonifier 250 at a setting of 1–2. An additional 1 ml (1.6 ml for MOME) of 0.1 M potassium phosphate, pH 7.0, was added, and sonication was continued for 4×30 s (with 30 s intervals) on ice. The CO substrate was heated to 37 °C before sonication. After the second sonication, 1 ml (0.4 ml for MOME) of 20% BSA in 0.1 M potassium phosphate, pH 7.0, was added. For each assay, 100 μ l of substrate was mixed with 100 μ l of HSL sample (0.5–2 m-units for MOME and CO and 5–20 m-units for TO) in 20 mM potassium phosphate (pH 7.0)/1 mM EDTA/1 mM dithioerythritol/0.02% BSA. The mixture was incubated at 37 °C for 30 min. Hydrolysis was stopped by the addition of 3.25 ml of methanol/chloroform/heptane (10:9:7) followed by 1.05 ml of 0.1 M potassium carbonate/0.1 M boric acid, pH 10.5. The mix was shaken vigorously for 10 s and centrifuged at 800 g for 20 min. The upper phase (1 ml), containing the released fatty acids [32], was used for liquid scintillation counting. For further details on preparation of the substrate emulsions see [33]. One unit of enzyme activity is equivalent to 1 μ mol of fatty acids released/min at 37 °C, using MOME as substrate. Specific activity is expressed as units/mg of protein. Unless otherwise stated, MOME was used as substrate.

In addition to the lipid substrates above, two substrates were used at concentrations where they were fully soluble in water. First, *p*-nitrophenyl butyrate (PNPB; Sigma) was used for determinations of substrate specificity and for K_m determinations [34]. For assays with this substrate, 10 μ l of PNPB (in acetonitrile) was added to 990 μ l of 0.1 M sodium phosphate/0.9% NaCl/1 mM dithioerythritol, pH 7.25, containing 5–10 m-units of HSL. The final concentration of PNPB ranged from 25 μ M to 10 mM for K_m determinations and was 2 mM (V_{max} is reached at 1.5 mM) for measurement of specific activity. Incubation was for 10 min at 37 °C. Hydrolysis was stopped by addition of 3.25 ml of methanol/chloroform/heptane (10:9:7), followed by vigorous shaking and centrifugation as above and incubation for 3 min at 42 °C. Activity was measured as absorbance at 400 nm of the supernatant containing *p*-nitrophenol. Secondly, a tributyrin (TB) assay was used as described by Shirai et al. [35]. Briefly, to HSL (5–10 m-units), in 490 μ l of the same buffer as used for PNPB assays, containing 2% BSA, was added 10 μ l of [3 H]TB (Fluka) in acetonitrile to a final concentration of 400 μ M.

For estimation of specific activity, purified recombinant HSL protein mass was determined by absorbance at 280 nm using an absorption coefficient of $50490 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [36]. Amino acid composition analysis was performed at the Central Amino Acid Laboratory, Institute of Biochemistry, University of Uppsala, after precipitation with chloroform/methanol [37]. Protein was routinely determined as described by Bradford [38], using BSA as a standard.

Infection of Sf9 suspension cultures, HSL overexpression and purification

For large-scale production of recombinant HSL, Sf9 cells in suspension cultures (2×10^6 /ml) were infected with recombinant virus stock and incubated at 27 °C with shaking. Cells were harvested after 60 h by centrifugation at 1200 g for 10 min at 4 °C and then homogenized in 3 vol. of homogenization buffer (see above). This homogenate was the starting material for purification of HSL. The homogenate was solubilized with 1% $C_{13}E_{12}$, a detergent from the alkyl polyoxyethylene group (Berol 058; Berol Kemi AB, Stenungsund, Sweden) and 10 mM NaCl using sonication with a Branson Sonifier 250 at setting 1–2 (3–4 min in 30 s pulses; cooling with ice). Insoluble material was removed by centrifugation at 10000 g for 10 min at 4 °C. The clear supernatant was dialysed overnight at 4 °C against 20 mM

Tris/acetate (pH 7.5)/20% glycerol/1 mM dithioerythritol/0.2% $C_{13}E_{12}$ /2 μ g/ml leupeptin and then subjected to anion-exchange chromatography on Q-Sepharose FF (Pharmacia), essentially as reported for purification of rat adipose-tissue HSL on Mono Q [10]. Briefly, the Q-Sepharose column was equilibrated with buffer A [50 mM Tris/acetate (pH 7.5)/20% glycerol/1 mM dithioerythritol/0.2% $C_{13}E_{12}$]. After loading the sample (typically 15 g of total protein on a 300 ml column), the column was washed with 10 vol. of buffer A, after which a gradient from 0 to 0.15 M sodium acetate in buffer A, pH 7.0, was applied, followed by a plateau at 0.1 M sodium acetate in buffer A, pH 7.0. A second gradient from 0.15 to 0.3 M sodium acetate in buffer A, pH 7.0, was used to elute the HSL. After adding salt (0.8 M NaCl final concn.) the eluted protein was subjected to hydrophobic-interaction chromatography on phenyl-Sepharose CL-4B (Pharmacia), essentially as described for the purification of bovine adipose-tissue HSL on phenyl-silica [11]: The pooled material was applied to a phenyl-Sepharose column (100 ml) that had been equilibrated with 20 vol. of 5 mM potassium phosphate (pH 7.4)/2 M NaCl/2% $C_{13}E_{12}$ /1 mM dithioerythritol/10% glycerol, followed by 10 vol. of buffer B [0.1 M sodium phosphate (pH 7.4)/0.8 M NaCl/1 mM dithioerythritol/0.005% $C_{13}E_{12}$ /10% glycerol]. After loading and washing, HSL was eluted with a four-component gradient in buffer B; sodium phosphate from 0.1 M, pH 7.4, to 5 mM, pH 7.0, NaCl from 0.8 M to 0 M, $C_{13}E_{12}$ from 0.005 to 0.2% and glycerol from 10 to 50%.

Phosphorylation and activation of recombinant HSL by cAMP-PK

To measure phosphorylation and activation of the recombinant HSL, equal amounts (0.7 units/ml; 0.039 μ M) from various purification steps, and rat-adipose tissue HSL [10], were phosphorylated by incubation with 180 units (as defined by Sigma)/ml cAMP-PK (catalytic subunit from bovine heart; Sigma) in 3.33 mM imidazole/HCl (pH 7.0)/5 mM $MgCl_2$ /2 mM dithioerythritol/0.1% $C_{13}E_{12}$ /0.2 mM ATP at 37 °C for 30 min. An excess of ice-cold 10 mM EDTA and 2 mM dithioerythritol was added to stop the reaction, and HSL was assayed with a 0.5 mM TO substrate, as described above except that the substrate was prepared in 10 mM Tris/HCl (pH 8.3)/5 mM NaCl/0.5 mM EDTA. For time-course activation, performed with the purified HSL, aliquots were removed at different time points for analysis with 0.5 mM TO substrate. In parallel incubations, using [γ - ^{32}P]ATP (1600 c.p.m./pmol), aliquots were removed and subjected to SDS/PAGE. ^{32}P -labelled HSL was revealed by digital imaging of ^{32}P , using a Fujix BAS 2000 PhosphorImager (Fuji). To determine the stoichiometry of HSL phosphorylation, maximally phosphorylated enzyme was subjected to SDS/PAGE and [^{32}P]phosphate incorporation was quantified by Čerenkov counting of polyacrylamide-gel slices. The amount of phosphate was calculated from the specific radioactivity of [^{32}P]ATP in the incubations and the amount of HSL from the specific activity of the enzyme (see above).

Treatment of HSL with protein phosphatase 2A

To monitor the degree of phosphorylation of the recombinant purified HSL, it was treated with protein phosphatase 2A (PP2A) prior to phosphorylation by cAMP-PK (see above). A 200–300 m-unit portion of HSL (2–3 μ l) was diluted to 40 μ l in 5 mM Tris/HCl (pH 7)/0.1% β -mercaptoethanol/0.1 mM EDTA/5% glycerol was treated with 10 nM PP2A (UBI) for 1 h at room temperature. The reaction was stopped by addition of 20 nM okadaic acid (Boehringer-Mannheim). Half of the PP2A-treated HSL was then phosphorylated as described above. In

parallel incubations, HSL was phosphorylated in the same buffer without prior PP2A treatment. HSL activity was measured against 0.5 mM TO as described above.

To ensure that PP2A could act effectively on phosphorylated HSL, HSL was phosphorylated with [^{32}P]ATP prior to dephosphorylation. Both phosphorylated and dephosphorylated HSL was electrophoresed on PhastGels (Pharmacia) 8–25% SDS/PAGE gels as recommended by the manufacturer, followed by analysis of the gels by digital imaging. The PP2A treatment of labelled HSL showed more than 80% reduction in labelling.

Limited proteolysis

Recombinant HSL was subjected to limited proteolysis, using trypsin, chymotrypsin or endoproteinase Glu-C. HSL (110 units/ml) in 200 μ l of 5 mM sodium phosphate (pH 7.0)/1 mM dithioerythritol/0.2% $C_{13}E_{12}$ /50% glycerol was mixed with 50 μ l of 1.1 units/ml trypsin, 0.9 units/ml chymotrypsin or 2 units/ml endoproteinase Glu-C (all Boehringer-Mannheim) in 100 mM potassium phosphate, pH 7. At different time points, aliquots were taken for electrophoresis and, in the case of trypsin and chymotrypsin digests, HSL activity measurements. The aliquots (20 μ l) for SDS/PAGE were directly added to tubes containing 20 μ l of 2 \times SDS/PAGE loading buffer [26] and subjected to Tricine/SDS/PAGE with 8% polyacrylamide in the separation gel and 4% in the stacking gel. For activity measurements, 5 μ l aliquots of the digests were added to 1 μ l of 9 mg/ml trypsin/chymotrypsin inhibitor (Sigma) and monitored for hydrolysis of TO, CO, MOME, TB and PNPB as described above.

Denaturation of HSL

Recombinant HSL was denatured using guanidinium chloride (GdnCl). HSL activity was measured against TO, CO, MOME, TB and PNPB, as described above, with varying concentrations of GdnCl (UBI) in the assay buffers. HSL was incubated with the GdnCl containing assay buffer for 2 h on ice, before the assays were started. In the lipid emulsion assays (TO, CO, MOME) additional GdnCl was added together with the substrate to compensate for the dilution.

RESULTS AND DISCUSSION

Sequence analysis of HSL

Initially, rat HSL showed no strong sequence similarity to other mammalian lipases such as pancreatic lipase (PL), lipoprotein lipase (LPL) or hepatic lipase (HL). A subsequently discovered lipase of *Moraxella* TA144 has shown similarity to a stretch of 88 amino acids in HSL [14, 20], and recently this similarity has been extended to include several other bacterial hydrolases [21]. Further analysis has shown that lipases and esterases constitute a large superfamily of hydrolytic enzymes with two distantly related subfamilies [19]. From biochemical and crystallographic analyses it is known that, in both subfamilies, hydrolysis is performed by a catalytic triad of serine, aspartic acid (or in a few cases glutamic acid) and histidine, analogous to what is found in proteinases [17,39]. The secondary structure of lipases and esterases shows alternating β -strands and α -helices (with some variations) [17,39]. The β -strands form a β -sheet surrounded by the α -helices, defined as the α/β -hydrolase fold [39]. The active-site serine residue and the acidic residue are found in tight turns between β -strands and α -helices [17].

From the exon/intron organization of HSL genes [14,15] it has been suggested that HSL is a mosaic protein, with functional motifs encoded by different exons. The sequence exhibiting high similarity to the *Moraxella* TA144 lipase 2 is encoded by most of

| | | |
|---------|---|-----|
| Rat HSL | MDLRTMTQSLVLAEDNMAFFSSQGPGETARRLSNVFAGVREQALGLEPTLGQLLGVVAHFDLDTETPANGYRSL | 75 |
| Mur HSL | MDLRTMTQSLVTLAEDNMAFFSSQGPGETARRLSNVFAGVREQALGLEPTLGQLLGVVAHFDLDTETPANGYRSL | 75 |
| Hum HSL | MDLRTMTQSLVTLAEDNIAFFSSQGPGETARRLSGVFAGVREQALGLEPALGRLGVAHFDLDDPETPANGYRSL | 75 |
| | VHTARCCLAHLLHKSRYVASNRKSIFFRASHNLAELEAYLAALTQLRALAYYAQRLLTINRPGVLFEGDEGLISA | 150 |
| | VHTARCCLAHLLHKSRYVASNRKSIFFRASHNLAELEAYLAALTQLRAMAYYAQRLLTINRPGVLFEGDEGLITA | 150 |
| | VHTARCCLAHLLHKSRYVASNRKSIFFRTSHNLAELEAYLAALTQLRALVYYAQRLLVTINRPGVLFEGDEGLTA | 150 |
| | DFLQDYVTLHKGCFYGRCLGFQFTPAIRPFLQTLISIGLVSFGEHYKRNETGLSVTASSLFTGGRFAIDPELRGAE | 225 |
| | DFLQDYVTLHKGCFYGRCLGFQFTPAIRPFLQTLISIGLVSFGEHYKRNETGLSVTASSLFTGGRFAIDPELRGAE | 225 |
| | DFLREYVTLHKGCFYGRCLGFQFTPAIRPFLQTLISIGLVSFGEHYKRNETGLSVTASSLFTSGRFAIDPELRGAE | 225 |
| | FERITQNLVDVHFWKAFWNITETEVLSLANMASTTVRVSRLSLPPEAFEMPLTSDPKLTVTISPLAHTGPGPV | 300 |
| | FERITQNLVDVHFWKAFWNITETEVLSLANMASTTVRVSRLSLPPEAFEMPLTSDPRLTVTISPLAHTGPAVPV | 300 |
| | FERITQNLVDVHFWKAFWNITETEVLSLANMASATVRVSRLSLPPEAFEMPLTADPTLTVTISPLAHTGPGPV | 300 |
| | 4 5 | |
| Rat HSL | LARLISYDLREGQDSKMLNSTAKSEGPRLELRPRPQOAPRSRALVVIHGGGFVAQTSKSHPEYLYKNWAQELGV | 374 |
| Mur HSL | LARLISYDLREGQDSKVLNSTAKSEGPRLDVPRPQOAPRSRALVVIHGGGFVAQTSKSHPEYLYKNWAQELGV | 374 |
| Hum HSL | LVRLISYDLREGQDSEELSSLIKSNQORSLELWPRPQOAPRSRSLIVHFGGGFVAQTSRSHEPYLKSWAQELGA | 375 |
| Mor Li2 | SERKSTDEAAMLFF HGGGF CIGDIDT H HEFCHTVCQAT GW | 190 |
| | S | |
| | PIISIDYSLAPEAFPRALEECFFAYCWAVKHCDLLGSTGERICLAGDSAGGNLCITVSLRAAAYGVRVPDGIMA | 449 |
| | PIFISIDYSLAPEAFPRALEECFFAYCWAVKHCDLLGSTGERICLAGDSAGGNLCITVSLRAAAYGVRVPDGIMA | 449 |
| | PIISIDYSLAPEAFPRALEECFFAYCWAIKHCDLLGSTGERICLAGDSAGGNLCFTVALRAAAYGVRVPDGIMA | 450 |
| | AVVSVDYRMAYEPAPITALKDCLAYAWLAELHSQSLGASPSRITLS GDSAGC LALMAQQVIT-KPIDALWQDNN | 264 |
| | D | |
| | AYPMTTLOSSASPSRLLSLMDPLLPLSVLSK-CVSAYSSTETEDHFSDQKALGVMGLVQRDTSLEFLRDLRLGAS | 523 |
| | AYPMTTLOSSASPSRLLSLMDPLLPLSVLSK-CVSAYSSTEAEDHFSDQKALGVMGLVQRDTSLEFLRDLRLGAS | 523 |
| | AYPATMLQPAASPSRLLSLMDPLLPLSVLSK-CVSAYAGAKTEDHNSDQKALGVMGLVRRDTSLEFLRDLRLGAS | 524 |
| | QAPAAD-KKVNDTFK-N SLAD LPRPLAQLPLYPMTDYEAEPYPSWELYGEGLLLDHNDAAEVFN SAYTQHS- GLFOS | 336 |
| | S S | |
| | SWLNSFLELSGRKPKITPLPATEITLRPTDSGRLETSMRRSVSEAAALQPEGLLGTDSLKKLTIKDLSEFKGNSEF- | 597 |
| | SWLNSFLELSGRKPKITTSPTAE-----SVRPTESMRRSVSEAAALQPEGLLGTDSLKKLTIKDLSEFKGNSEF- | 588 |
| | SWLNSFLELSGRKSKMSEPIAE-----FMRRSVSEAAALQPEGLLGTDSLKKNLTLRDLSEFKGNSEF- | 587 |
| | HPLISVM----- | 343 |
| | SDSPEMSQSMETLGPSTPSDVNFFLRSGNSQEEAETRDDISPMDGIPRVRAAFPDGFHPRSSQGVLMPLYTSSP | 672 |
| | SDSPEMSQSMETLGPSTPSDVNFFLRPGNSQEEAAKDEVPRMDGVPRVRAAFPEGFHPRSSQGVLMPLYTSSP | 663 |
| | SDTPMSLSAETLGPSTPSDVNFFLRPPEDAGEEAAKNELSPMDRGLGVRAAFPEGFHPRSSQGVLMPLYTSSP | 662 |
| | D DD H | |
| | IVKNPFMSPLLAPDVMKTLPPVHIVACALDPMLDDSVMFARRLKD LGQPVTLLKVVEDLPHGFTSLAALCRETRQ | 747 |
| | IVKNPFMSPLLAPDSMLKTLPPVHIVACALDPMLDDSVMFARRLKD LGQPVTLLKVVEDLPHGFTSLAALCRETRQ | 738 |
| | IVKNPFMSPLLAPDSMLKSLPPVHIVACALDPMLDDSVMLARRLRLNGQPVTLLKVVEDLPHGFTSLAALCRETRQ | 737 |
| | -----HGDNTQLCPYSYIVVAELDILRDEGLAYAEELLOKEGVQVQTYTTLGAP PHGF INLMSVHQ---- | 401 |
| | AAELCVQRIRLLTTPPAAP-LT | 768 |
| | AEEFCVQRIRLLTTPPAAP-LN | 759 |
| | AAELCVERIRLLVLTTPPAGEGPSGETGAAGVDGGCGGRH | 775 |
| | GLGNQTTYTINEFACLVQNLLTSE-GDKPNLRA | 433 |

Figure 1 Alignment of HSL sequences and *Moraxella* TA144 lipase 2

The amino acid sequence of rat, murine and human HSL were aligned to the last 283 amino acids of *Moraxella* TA144 lipase 2. Conserved amino acids among the three HSL sequences are framed. Those residues also conserved in the bacterial lipase are in **bold** letters. The region of HSL amino acid sequence corresponding to the boundary between exon 4 to 5 is marked '4|5'. Suggested amino acids of the catalytic triad are marked with capital letters above the lines, and the phosphorylation sites by closed circles. The catalytic aspartic acid residue is unknown, so all four conserved candidates are marked.

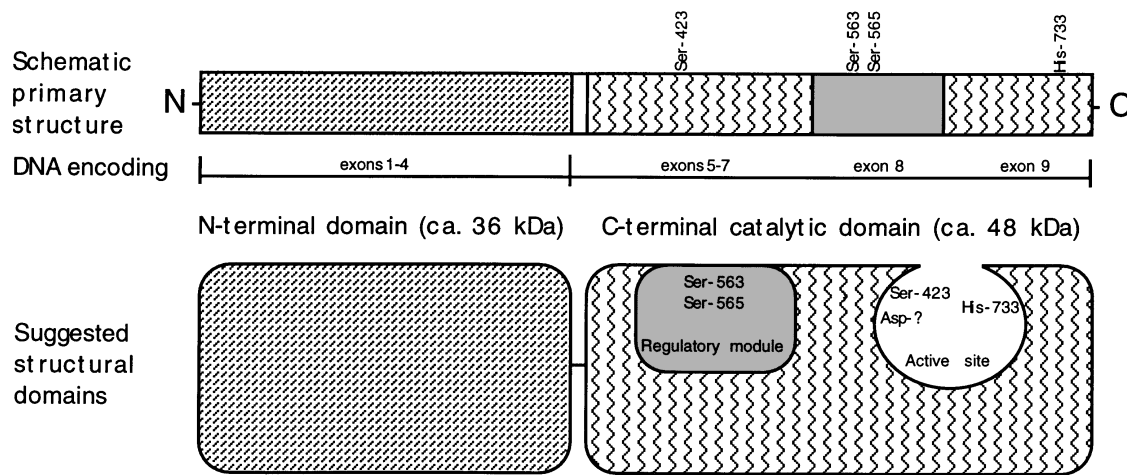


Figure 2 Proposed domain structure for HSL

Top: linear representation of HSL amino acid sequence. Middle: corresponding HSL coding sequence showing location of exons. Bottom: hypothesized domain structure for HSL. The N-terminal domain (■) encoded by exons 1–4 may contain sites for HSL-specific functions such as intracellular lipid droplet recognition. The C-terminal domain is mainly formed by an α/β -hydrolase fold (▨) and harbours the active site with the catalytic triad (Ser-423, Asp-? and His-733). The C-terminal domain also contains a regulatory module (▩) with phosphorylation sites (Ser-563 and Ser-565).

exon 5 and the beginning of exon 6, where the active-site serine residue is found (see below). The phosphorylation sites are encoded by exon 8 and a putative lipid binding site by exon 9 [14]. The consensus motif around the active site serine is Gly-Xaa-Ser-Xaa-Gly [17], and this motif is found around Ser-423 in rat HSL. The importance of this serine residue has been confirmed by site-directed-mutagenesis experiments [18]. In order to identify the aspartic acid/glutamic acid and histidine residues of the HSL catalytic triad and to predict more about HSL domain structure, the three known HSL sequences were compared in more detail. In Figure 1 the known HSL protein sequences for rat [5,18], mouse [15] and human ([14]; see GenBank accession no. L11706 for an update of DNA and protein sequences) were aligned with the last 283 amino acids of *Moraxella* TA144 lipase 2. This extended alignment between the bacterial lipase and HSL begins in a region close to the boundary between parts of HSL encoded by exons 4 and 5 respectively (Figure 1). Interestingly, this region is much less conserved between the different HSL proteins than the surrounding sequences, possibly suggesting a hinge region between domains. The first 313 amino acids are encoded by exons 1–4 and share no significant similarity to any known proteins in the protein databases, but the identity between the three species in this region is 91% (Figure 1).

A putative HSL regulatory domain was proposed to be formed by amino acids 521–669 (numbering is from rat HSL), based on a gap in the alignments to various bacterial lipase sequences [19]. This region of HSL includes 26 amino acids encoded by exon 7 and all but the last 31 amino acids encoded by exon 8. These previous alignments, furthermore, suggested His-733 as part of the catalytic triad [19]. A similar sized gap occurs in the alignment to the *Moraxella* lipase presented here, suggesting a regulatory module of the same size (from amino acid 531 to 685 by rat sequence numbering), which contains the two phosphorylation sites (Ser-563 and Ser-565 in the rat sequence).

Figure 1 shows the conserved Ser-423, His-733 and four aspartic acid residues that are candidates for the catalytic triad (marked in capital letters). Exactly which of the four marked aspartic acid residues that participates in catalysis is unknown. On the basis of the presented alignment, we suggest that HSL is composed of two major structural domains (Figure 2). The part

of HSL that aligns with the *Moraxella* lipase (basically encoded by exons 5–7 and 9) probably forms the α/β -hydrolase fold and active site. This C-terminal catalytic domain also contains a regulatory module (largely encoded by exon 8) with phosphorylation sites (Ser-563 and Ser-565). Exons 1–4 are suggested to encode a separate N-terminal structural domain. Both the N-terminal domain and the regulatory module appear unique to HSL by sequence comparison with other lipases. We speculate that the N-terminal domain carries out functions specific to HSL, for example recognition of intracellular lipid droplets.

Large-scale production and purification of HSL in insect cells

In order to test the proposed structural model (Figure 2), large quantities of purified rat HSL were needed. Thus HSL was expressed in Sf9 cells by a baculovirus vector under control of the strong polyhedrin promoter. Northern analysis of RNA extracted from Sf9 cells infected with recombinant baculovirus (not shown) demonstrated that these cells expressed a major transcript consistent in size with the expected mRNA. This transcript, however, was not present in cells infected with wild-type virus. SDS/PAGE analysis showed that the major protein expressed in infected Sf9 cells is of 84 kDa (Figure 3), consistent with the size of rat adipose-tissue HSL [9,10]. This protein accounted for approx. 5% of total cellular protein as determined by densitometric scanning of the Kenacid-stained gel. The corresponding expression in rat adipose tissue is 0.02% [9]. All expressed HSL activity in Sf9 cells was found intracellularly, as expected.

Large-scale production of HSL was performed in suspension cultures. Sf9 cells, infected with recombinant baculovirus, and harvested at time points between 48 and 96 h, showed an optimal HSL activity expression at 60 h (results not shown). Varying the multiplicity of infection (MOI) between 0.5 and 10 demonstrated that the level of expression reached a plateau at a MOI of about 2. On the basis of these results, all subsequent large-scale productions were performed at an MOI greater than 2, usually 8–10, and cells were harvested after 60 h. Under these conditions it was found that approx. 40 mg of HSL protein was produced per litre of culture (2×10^9 cells). By comparison, it was demon-

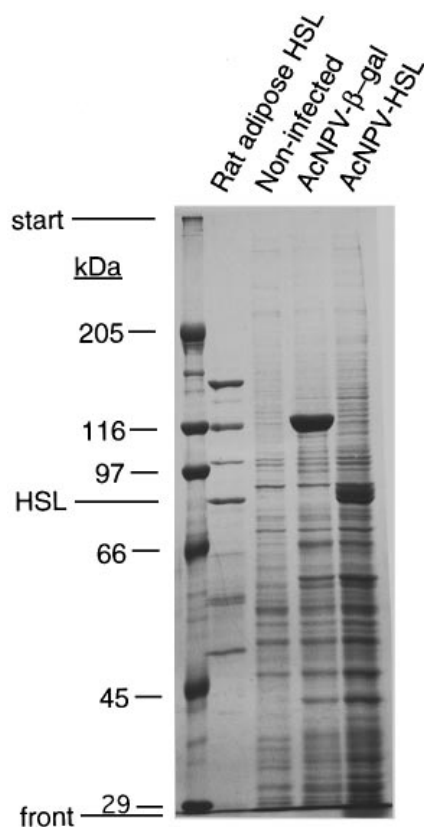


Figure 3 SDS/PAGE analysis of infected Sf9 cells

A 20 μg portion of total protein from uninfected cells and cells infected with recombinant virus for either HSL (AcNPV-HSL) or β -galactosidase (AcNPV- β -gal) were subjected to SDS/PAGE analysis and stained with Kenacid Blue. For comparison, 2 μg of partially purified rat adipose tissue HSL was loaded. Mobilities of molecular-mass markers and HSL are indicated on the left.

Table 1 Purification of recombinant HSL

Recombinant HSL was purified to homogeneity as described in the Materials and methods section. Shown are the results from one purification, which is representative of four.

| Purification step | Specific activity (units/mg of protein)* | Purification (fold) | Recovery (%) |
|--|--|---------------------|--------------|
| I. Cell homogenate | 6.0 | 1 | 100 |
| II. Detergent solubilization + 10 000 g centrifugation | 5.3 | 0.9 | 92 |
| III. Q-Sepharose | 37 | 6.2 | 80 |
| IV. Phenyl-Sepharose | 217† | 36 | 45 |

* Enzyme activity determined using the monoether diacylglycerol analogue and protein determined as described by Bradford [38], unless otherwise indicated.

† Protein determined using UV absorption at 280 nm and the calculated absorption coefficient [36] for rat HSL ($50\,490\text{ M}^{-1}\cdot\text{cm}^{-1}$).

strated that the fat-pads of 200 rats contain approx. 800 μg of HSL protein [9].

Since the recombinant enzyme is intended for studies requiring a high degree of purity of the protein, such as crystallization studies, a purification scheme resulting in large amounts of homogenous enzyme was established. A modification of previous

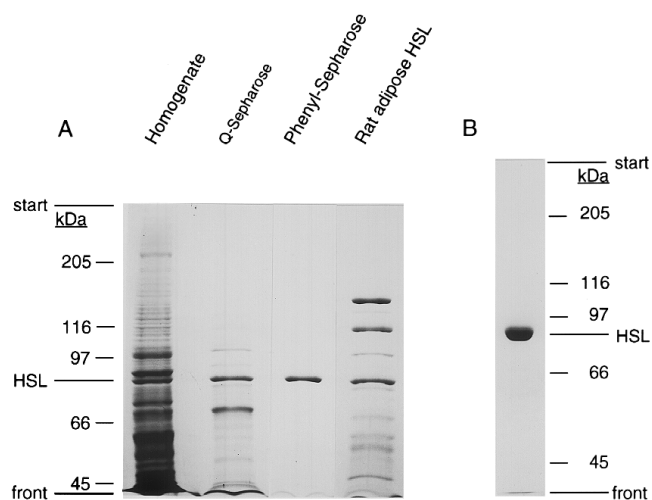


Figure 4 SDS/PAGE analysis of purified recombinant HSL

Samples of pooled material from different purification steps were subjected to SDS/PAGE and Kenacid Bluestaining. (A) shows material corresponding to 1.1 μg of HSL (240 m-units) from purification steps II–IV (Table 1) and the same amount of partially purified rat adipose-tissue HSL. (B) shows 9 μg of HSL (2 units) from purification step V. The mobilities of molecular-mass markers and HSL are indicated.

purification schemes for rat and bovine adipose-tissue HSL, utilizing HPLC and Mono Q [10] and phenyl-silica [11], was employed. The result of such a purification scheme starting with 12 litres of culture (approx. 15 g of total protein), is summarized in Table 1. The purification protocol required 5 days to complete and yielded approx. 220 mg of purified HSL with an overall recovery of 45%. As judged from overloaded Kenacid-stained (Figure 4) and silver-stained (not shown) SDS gels, HSL was purified to homogeneity (99% based on densitometric analysis).

Biochemical comparison of recombinant and rat adipose tissue HSL

To assess the functional state of recombinant HSL, it was compared with the previously characterized rat adipose-tissue HSL [9,10] with regard to specific activity, substrate specificity and phosphorylation and activation by cAMP-PK (Table 2). The specific activity for recombinant HSL was found to be 197–232 units/mg of protein (range from four preparations), based on activity measurements with a long-chain diacylglycerol analogue (MOME) and protein determinations using UV absorption at 280 nm and an absorption coefficient of $50\,490\text{ M}^{-1}\cdot\text{cm}^{-1}$, calculated according to [36] using the amino acid sequence of rat HSL [5,18]. This value is in good agreement with the previously estimated specific activity of rat adipose tissue HSL of 220 units/mg of protein [9]. The specific activity of purified recombinant enzyme was 214 units/mg of protein when protein levels were determined by amino acid analysis. Furthermore, 1 m-unit of recombinant HSL activity from the three different purification steps (I, III and IV; Table 1) and from a rat adipose-tissue homogenate were compared by SDS/PAGE and Western-blot analysis (not shown). Densitometric analysis of the HSL protein (84 kDa) on Western blots and silver-stained SDS/polyacrylamide gels (data not shown) showed that specific activity of recombinant HSL protein from the three different purification steps was virtually identical with that of HSL from a crude rat adipose-tissue homogenate (110000 g supernatant [9]). Therefore

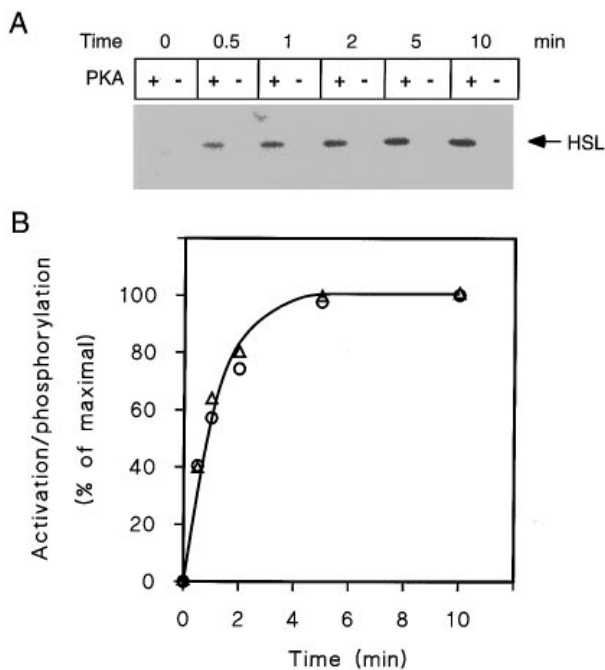
Table 2 Properties of recombinant HSL

| Property | Recombinant HSL* | | | |
|---|------------------|-------------|------------------|-----------------|
| | Homogenate | Q-Sepharose | Phenyl-Sepharose | Rat adipose HSL |
| Specific activity (units/mg of protein) | 6.0 | 37 | 217 | 220† |
| Subunit molecular mass (SDS/PAGE; kDa) | 84 | 84 | 84 | 84 |
| Substrate specificity | | | | |
| Relative V_{max} triacylglycerol, 1,2-diacylglycerol and cholesteryl esters | | | 1:11.3:2.1 | 1:11.5:2.2 |
| Relative V_{max} 1,2-diacylglycerol and PNPB | | | 1:2.0 | 1:1.6 |
| Activation by cAMP-PK‡ | 87 ± 6 | 158 ± 24 | 168 ± 20 | 174 ± 16 |

* The preparation of recombinant HSL was the same as that in Table 1.

† Data are from [11].

‡ Percentage activation over control, $x \pm S.D.$, $n = 3$.

**Figure 5** Time course of phosphorylation and activation of recombinant HSL

(A) SDS/PAGE analysis of recombinant HSL from the phenyl-Sepharose step incubated with and without the catalytic subunit of cAMP-PK and [32 P]ATP as described in the Materials and methods section. (B) The level of phosphorylation (○) was determined by digital imaging of the SDS gel from (A) and compared with activation (▲) determinations at each time point. Phosphorylation/activation is expressed as a percentage of the maximal phosphorylation/activation seen at 10 min.

a specific activity of 215 units/mg of protein has been used in calculations throughout this paper.

In addition, the recombinant HSL had a substrate specificity virtually identical with that of rat adipose tissue HSL [9], including the unique property, among lipases, of hydrolysing cholesteryl ester substrates at approximately the same rate as triglyceride substrates (Table 2). Kinetic studies using PNPB as substrate demonstrated an apparent K_m of 0.59 mM for recom-

binant HSL (results not shown), as compared with a K_m of 0.16 mM previously reported for bovine HSL [41].

cAMP-PK activates rat HSL by phosphorylation of a single serine residue (Ser-563). As shown in Table 2, recombinant HSL from purification steps III and IV (Table 1) was activated by cAMP-PK to the same extent as purified rat adipose-tissue HSL. The time course of the activation correlated with phosphorylation of the enzyme and was rapid, with half-maximal effects within 1 min (Figure 5), as has been shown for rat adipose-tissue HSL [3]. The stoichiometry of the phosphorylation of recombinant HSL by cAMP-PK was 1.04 ± 0.30 ($n = 5$) mol of phosphate/mol of protein. This is in agreement with that found using dephosphorylated rat adipose-tissue HSL as substrate [1,9,42].

Incubation of HSL with protein phosphatase 2A (PP2A) prior to activation with cAMP-PK did not increase the subsequent activation. This indicates that recombinant HSL is obtained in a dephosphorylated form, as in the case of rat adipose-tissue HSL [3].

Limited proteolysis and denaturation

Purified recombinant HSL was used to test the proposed model of the domain structure (Figure 2). Pure recombinant protein was subjected to limited proteolysis, using three different proteinases, namely trypsin, chymotrypsin and endoproteinase Glu-C. The proteolytic fragments were analysed by electrophoresis (Figure 6), and the effect of cleavage by trypsin and chymotrypsin was monitored by five different activity assays (Figure 7). Results from representative experiments are shown. Early in the proteolysis, bands between 48 and 68 kDa appeared and, later, two bands at approx. 30 kDa and two, or more, at approx. 18 kDa were generated (Figure 6). All three proteinases can potentially recognize more than 40 sites in HSL. Since essentially the same proteolysis pattern occurred, it is likely that the three proteinases recognize similar exposed regions in the protein and in intermediate cleavage products. The breakdown pattern also indicates that HSL has modules and/or domains that are resistant to proteolysis. Figure 7 shows the impact of the tryptic and chymotryptic digestion on five different HSL activities. The curves are very similar for the two proteinases, which supports the suggestion that similar peptides are generated by each. Activities measured with the two water-soluble substrates, PNPB and TB, were the same, and they were affected much less than activities measured with the lipid substrates TO, CO and MOME. This suggests that, despite proteolysis, capacity to hydrolyse

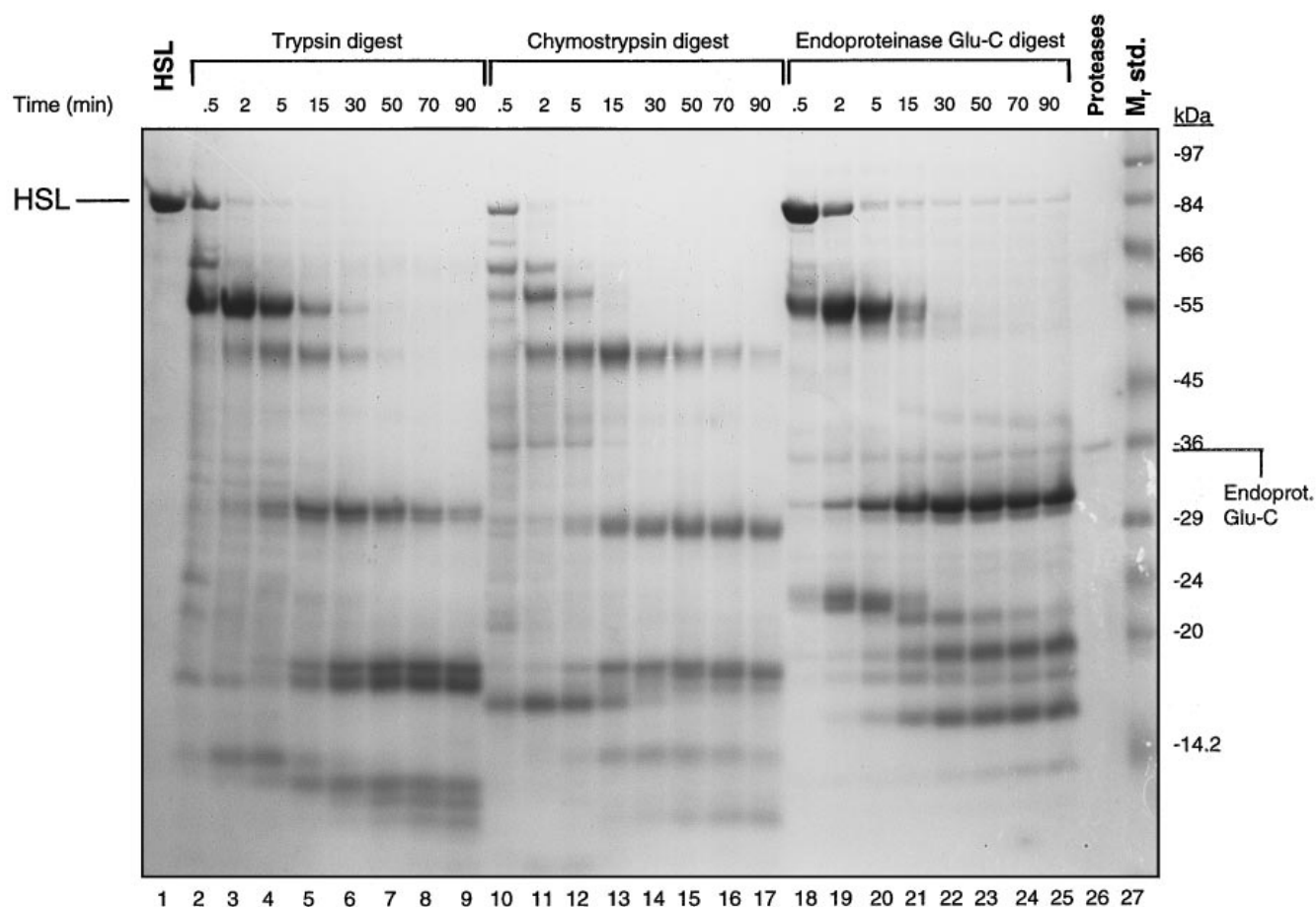


Figure 6 SDS/PAGE analysis of HSL during limited proteolysis

HSL fragments were analysed after limited proteolysis on Tricine/SDS/PAGE stained with Coomassie Brilliant Blue. HSL was digested with trypsin (lanes 2–9), chymotrypsin (lanes 10–17) and endoproteinase Glu-C (lanes 18–25). Proteolysis was stopped at the indicated time points. Molecular-mass markers are shown in lane 27 ('M_r std.'). HSL (2.5 μg) is shown in lane 1 and the three different proteinases, in the same amounts as in the respective proteolytic aliquots, in lane 26 (only endoproteinase Glu-C can be seen). Data are representative of five experiments.

substrates is retained in smaller fragments, whereas recognition and hydrolysis of the phospholipid emulsified lipid substrates depends on a more intact HSL molecule. Analyses of both lipoprotein lipase (LPL) [43] and gastric lipase (GL) [44] during proteolysis, have similarly shown that lipase activity is affected more than esterase activity during proteolysis. The fact that TO activity declines more rapidly than CO and MOME activities, can be explained by differences in substrate accessibility at the interface of the different lipid emulsions. Considering the lower hydrophobicity of MOME as compared with TO and the much lower ratio CO/PL as compared with TO/PL (see the Materials and methods section) it is reasonable to assume that both MOME and CO are more readily accessible at the interface than TO. CO and MOME molecules may even be found to some extent in the BSA-containing aqueous phase.

It has also been shown that cleavage of bovine HSL by trypsin results in a more extensive loss of hydrolytic activity against a phospholipid-stabilized TO emulsion than against the water-soluble PNPB [45]. In an attempt to identify the minimal catalytically active peptide, the active-site serine of HSL was labelled with di-isopropyl fluorophosphate (DFP) after proteolysis. A 17.6 kDa labelled peptide, spanning the amino acids 333 to 499 (numbering by the rat sequence), which was highly

stable to further proteolysis, was identified and suggested to retain ability to hydrolyse PNPB [45], though no evidence was shown. This would suggest that the catalytic triad is to be found within these 167 amino acids, leaving only one possible active-site histidine residue (His-493), which, however, is not conserved in the bacterial lipase (Figure 1 and [19]). On the other hand, a catalytic fragment that contains both Ser-423 and His-733 would contain more than 400 amino acids. Since no peptides above 40 kDa can be seen after 70 and 90 min of cleavage by trypsin (Figure 6), the remaining hydrolytic activity seen is probably due to interaction of peptides, containing the active-site serine, aspartic acid and histidine residues, though they may have been separated by cleavage of peptide bonds. It is also possible that one or more of the generated peptides actually have higher specific activity toward PNPB and TB than HSL itself. In several of the proteolytic analyses, an increase (though not always statistically significant) in activity was measured with PNPB and TB up to 15 min after initiation of proteolysis (see Figure 7).

To investigate HSL domain structure further, the effect of denaturation, using guanidinium chloride (GdnCl), on HSL activities was analysed (Figure 8). The effect of GdnCl is not restricted to specific sites, but affects all non-covalent bonds within the enzyme. The effect of GdnCl is seen already at low

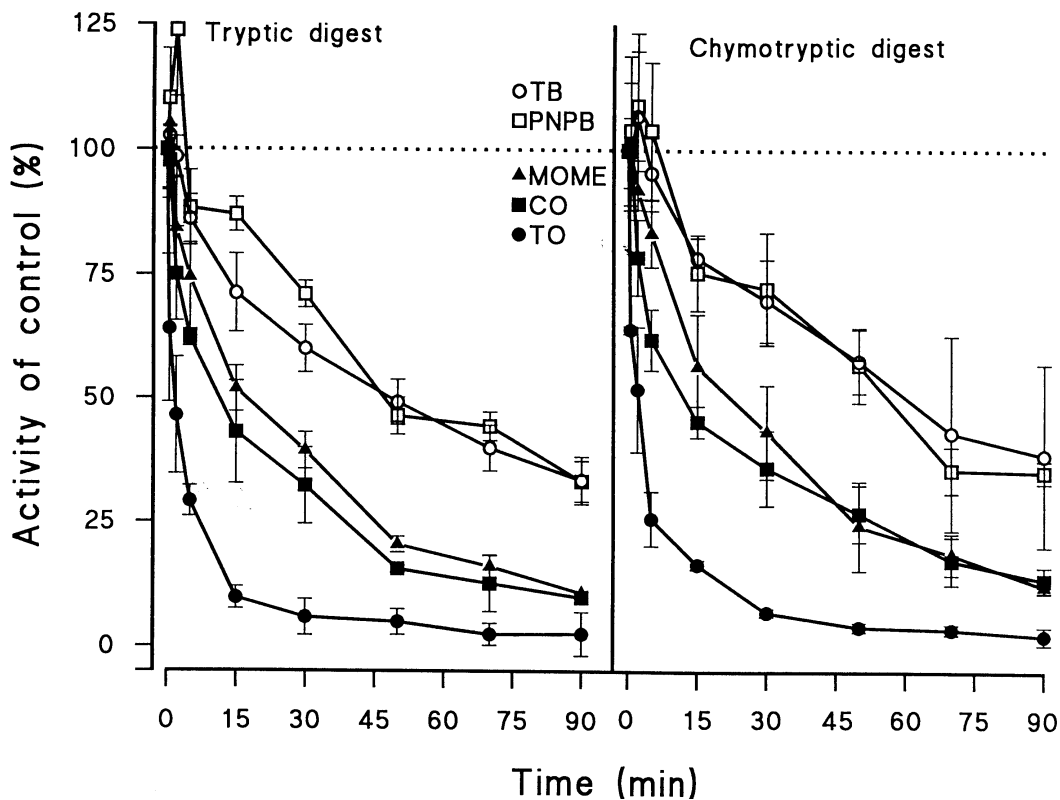


Figure 7 HSL activities during tryptic and chymotryptic digests

Activities of HSL during limited proteolysis with trypsin (A) and chymotrypsin (B) was measured using TO (●), CO (■) and MOME (▲) in phospholipid emulsions and TB (○) and PNPB (□). The results are from the same experiment shown in Figure 6; determinations are means of triplicates; error bars represent S.D. values. The results are related to activity of uncleaved HSL (100%).

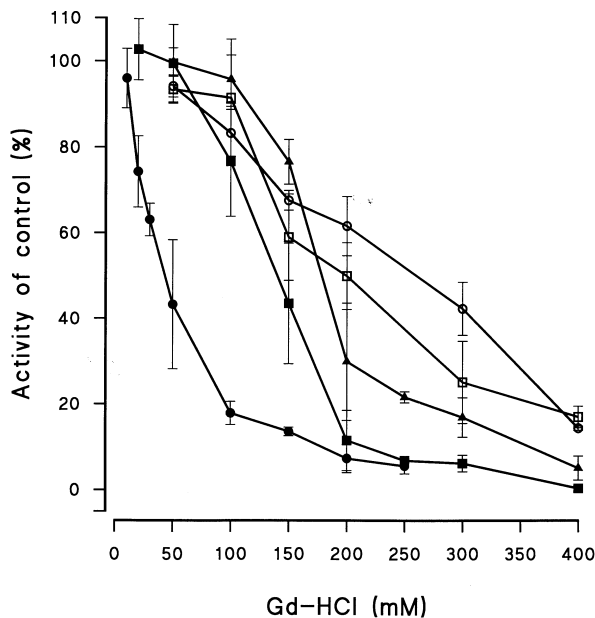


Figure 8 HSL activities during denaturation with GdnCl

HSL activities were measured after incubation with different concentrations of GdnCl ('Gd-HCl'). Assays are TO (●), CO (■), MOME (▲), PNPB (□) and TB (○). Results are representative of four experiments; determinations are means of triplicates; error bars represent S.D. values. The results are related to activity of native HSL (100%).

concentrations when measuring the activity with TO; the inhibitory concentration at which 50% activity is measured (IC_{50}) is approx. 40 mM. However, activity toward the other substrates is not markedly affected until concentrations above 100 mM. The activity toward CO and MOME decreases dramatically between 100 mM and 200 mM GdnCl. By contrast, activity toward PNPB and TB declines much more gradually with increasing GdnCl. Thus the IC_{50} is approx. 140 mM and 170 mM for CO and MOME and around 200 and 260 mM in the case of PNPB and TB respectively. This suggests that, at concentrations above 100 mM GdnCl, the ability of HSL to recognize the lipid interface is disrupted, whereas its ability to hydrolyse soluble substrates, which bind presumably mainly at the active site, is much less affected. Binding of long-chain triacylglycerols to the active site probably puts higher demands on the protein structure than any of the other substrate molecules, explaining the dramatic effect of GdnCl on TO hydrolysis.

In summary, both limited proteolysis and denaturation support a model for HSL with at least two domains, of which one constitutes the catalytic domain containing the catalytic triad, and a separate domain that, either alone or through interaction with the rest of HSL, is responsible for recognition of the lipid interface.

The subunit composition of HSL is not known. It is possible that HSL exists as a homodimer. The fact that denaturation by GdnCl affects lipase activity more than esterase activity could be explained by a dimer model of HSL similar to the one suggested for LPL, i.e. a 'head-to-tail' model where the C-terminal domain

of one monomer is proposed to co-operate with the N-terminal domain of the other monomer in substrate binding [46].

The only lipases known to consist of more than one structural domain are the closely related LPL, pancreatic (PL) and hepatic lipase (HL). These lipases (only distantly related to HSL, see above) have a large N-terminal catalytic domain and a smaller C-terminal domain [47–50]. Studies of chimaeras of LPL and HL have demonstrated the importance of the C-terminal domain for lipid binding and substrate specificity [48,49]. However, it is also clear, from studies of PL, that the two domains co-operate in interacting with the substrate. In the absence of substrate, colipase binds exclusively to the C-terminal domain of PL [50,52]. In the presence of a lipid/water interface, however, the colipase also binds to the 'lid' region of the catalytic domain, and thereby stabilizes an open activated conformation [50,51]. The catalytic domain and colipase together form a hydrophobic surface that presumably interacts with the lipid/phospholipid substrate surface [51]. It is not clear from our results if HSL has a separate lipid-binding region or domain, or if interaction with the lipid interface is distributed on several surface sites, as in the case for PL.

In conclusion, the HSL protein sequences have been analysed and compared with *Moraxella* TA144 lipase 2. On the basis of these data, as well as on previous alignments, a model for HSL with two major domains and a regulatory module has been suggested. In order to examine the domain structure experimentally, recombinant rat HSL was expressed using a baculovirus/insect-cell system and purified to more than 99% purity. The recombinant protein was compared with the partially purified HSL from rat adipose tissue [9] with respect to specific activity, substrate specificity and phosphorylation and activation by cAMP-PK, revealing no differences between the two enzymes. Examination of the domain structure has been initiated through limited proteolysis and denaturation of the recombinant HSL. The results support the hypothesis that HSL contains more than one domain, as similar analyses have shown for LPL, PL and HL.

To our knowledge, we have presented the first model on functional and structural domains of HSL. The recombinant HSL will be invaluable for further investigations on structure–function relationships, as well as for determination of the three-dimensional structure by X-ray crystallography, which will provide the ultimate confirmation of the domain structure.

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