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Homology of lipoprotein lipase to pancreatic lipase

(tryptic peptides/amino acid sequence)

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ABSTRACT **Bovine milk lipoprotein lipase was subjected to amino acid sequence analysis. The first 19 amino-terminal residues were Asp-Arg-Ile-Thr-Gly-Gly-Lys-Asp-Phe-Arg-Asp-Ile-Glu-Ser-Lys-Phe-Ala-Leu-Arg. In addition, reversed-phase high-performance liquid chromatography of a tryptic digest of reduced and alkylated lipase resolved a number of peptides, five of which contained cysteine. Sequence analysis of the tryptic peptides revealed in most instances a close homology to porcine pancreatic lipase. Based on this homology, the relative alignment of the sequenced lipoprotein lipase peptides can be made. In addition, a potential binding site for the triacylglycerol substrate and a carbohydrate-binding domain for lipoprotein lipase are postulated.**

Lipoprotein lipase (LPL, triacylglycero-protein acylhydrolase, EC 3.1.1.34) is a lipolytic enzyme involved in metabolic transformations of triacylglycerol-rich lipoprotein particles (1). The enzyme hydrolyzes the triacylglycerol core of chylomicrons and very low density lipoproteins, generating free fatty acids for tissue assimilation. Although synthesized in and secreted from the parenchymal cells of tissues including adipose, heart, skeletal muscle, and mammary gland, it is bound to the lumenal surface of the capillary endothelium, presumably through an association with heparan sulfate (2).

Extensive research on the physiological roles, regulatory mechanisms, and biological activity of LPL has been reported (3). However, in order to pursue the mechanism of LPL catalysis on a molecular basis, knowledge of the primary structure of the protein is required. In this report, we present results of amino acid sequence analysis on the amino terminus of bovine milk LPL and on tryptic peptides derived from reduced and alkylated enzyme. Our data enable us to identify a putative site for binding to the substrate interface, as well as a carbohydrate-binding domain, on the basis of amino acid homology to a related enzyme, pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3).

MATERIALS AND METHODS

Materials. Bovine skim milk was obtained from the California Polytechnic State University dairy. Bovine pancreatic trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone), acetonitrile (HPLC grade), and 6 N HCl were purchased from Sigma, Baker, and Pierce, respectively. Trifluoroacetic acid was distilled over chromium trioxide and then over alumina.

LPL Purification. All steps were carried out at 4°C. Typically, 24 liters of skim milk, adjusted to 0.4 M NaCl, 2.5 mM benzamidine·HCl, and 1 mM phenylmethylsulfonyl fluoride, was incubated overnight with 200 ml of heparin-Sepharose 4B (5 mg of heparin per ml of wet gel). The gel was collected on a coarse sintered glass funnel and washed with 4 liters of buffer A (20 mM Tris Cl, pH 7.4) containing 0.75 M NaCl and 0.02% (vol/vol) Triton X-100. The lipase was eluted by incubating the gel in the funnel with 150 ml of 2 M NaCl/buffer A for 5 min. The gel was filtered and incubated with a second batch of the same buffer for an additional 60 min. The combined filtrates were diluted to 0.75 M NaCl and applied to a 100-ml column of dextran-Sepharose 4B (5 ml of dextran per ml of wet gel). LPL was eluted with a 500-ml linear gradient of 0.75 to 2.0 M NaCl in buffer A. The major protein peak was concentrated to 1 mg/ml by Amicon ultrafiltration using a UM10 membrane.

Polyacrylamide gel electrophoresis according to Laemmli (4) was run on a slab gel apparatus. The gels were stained with silver nitrate according to Oakley *et al.* (5).

Amino Acid Analysis. The protein (1 μg) was hydrolyzed in 6 N HCl/0.2% 2-mercaptoethanol for 48 hr at 110°C. Hydrolysates were analyzed on a Beckman 121 MB amino acid analyzer (6). Cysteic acid was quantitated by performic acid oxidation of an additional sample. Carboxymethylcysteine was determined by amino acid analysis of S-carboxymethylated LPL.

Reduction and Alkylation. LPL (200 μg) was reduced by incubation in 6 M guanidine·HCl/0.5 M Tris Cl, pH 8.5/1 mM EDTA/0.3 mM 2-mercaptoethanol for 24 hr at room temperature. The sample was then alkylated in the same buffer with 0.1 μmol of iodo[^{14}C]acetic acid (5 μCi; 1 Ci = 37 GBq) at room temperature in the dark for 1 hr, followed by an excess of unlabeled iodoacetic acid (20 μmol) for an additional 1 hr. The S-carboxymethylated protein was recovered from the product mixture by gel-permeation HPLC fractionation on a Waters Protein Pak 300SW column developed with 0.1% CF_3COOH at a flow rate of 0.2 ml/min.

Tryptic Digestion and Reversed-Phase HPLC. S-carboxymethylated LPL (50 μg) was treated with 1 μg of trypsin in 0.2 M NH_4HCO_3 (pH 8) for 18 hr at room temperature. The tryptic digest was directly fractionated on a Vydac C_4 reversed-phase column (4.6 mm × 25 cm, 5-μm particle size, 300 Å pore size), using a 90-min linear gradient from 100% solvent A (0.1% CF_3COOH) to 70% solvent B (CF_3COOH/H_2O/CH_3CN, 0.1:9.9:90, vol/vol) at a flow rate of 1 ml/min. Undigested protein, dissolved in 4 M guanidine·HCl, was eluted in a similar manner.

Microsequence Analysis. A gas-phase peptide/protein microsequencer (built at City of Hope) was used for automated Edman chemical degradations (7). The phenylthiohydantoin (>PhNCS) amino acid derivatives were identified by reversed-phase HPLC on an Ultrasphere ODS column (4.6 mm × 25 cm; Altex, Berkeley, CA) and were quantitated by

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Abbreviations: LPL, lipoprotein lipase; >PhNCS, phenylthiohydantoin.

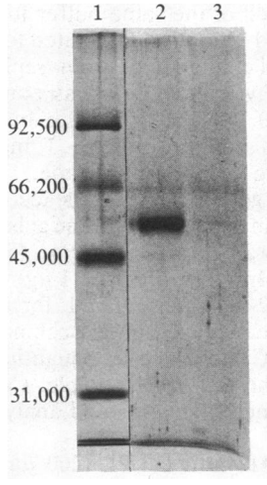
an integration program on a Spectra Physics 4000 computer (8).

Mass Spectral Analysis. Fast atom bombardment mass spectra were taken with a JEOL HX-100HF mass spectrometer utilizing a 6-kV xenon atom primary beam. Mass assignments of 0.1- to 1-nmol samples were accurate within 0.2 atomic mass units, and values for the molecular ion clusters are reported as the nearest integer value of the monoisotopic mass.

RESULTS

Sample Purity. Bovine milk LPL was analyzed for purity by $NaDodSO_4$/polyacrylamide gel electrophoresis and reversed-phase HPLC. Electrophoresis gave a band (M_r 57,000) characteristic of the intact protein (Fig. 1 *Upper*), and HPLC gave one major elution peak comprising >95% of the total UV-absorbing material (Fig. 1 *Lower*).

Amino Acid Analysis. Compositional analysis was performed on a trichloroacetic acid-precipitated sample of LPL. The results (Table 1) compare favorably with the data reported elsewhere (1). In addition, 2.1 mole % cysteic acid was determined by performic acid oxidation of the protein and 1.8 mole % carboxymethylcysteine was found in an S-carboxymethylated sample.



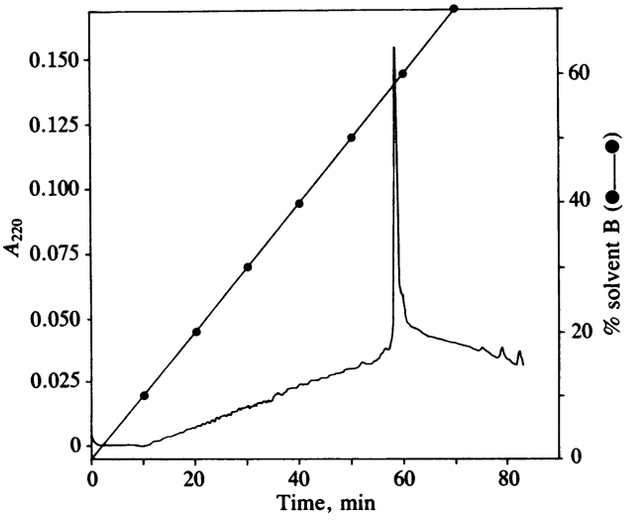


FIG. 1. (*Upper*) $NaDodSO_4$/PAGE of bovine milk LPL. Purified enzyme was electrophoresed in a 12% polyacrylamide gel and stained with $AgNO_3$. Lanes: 1, molecular weight markers (values at left); 2, 0.5 μg of LPL; 3, Tris buffer blank. (*Lower*) Reversed-phase elution profile of LPL. Fifteen micrograms of protein was adjusted to 4 M guanidine·HCl and 0.1% CF_3COOH and chromatographed on a Vydac C_4 column. The enzyme was eluted with a linear gradient from 100% solvent A (0.1% CF_3COOH) to 100% solvent B $CF_3COOH/H_2O/CH_3CN$, 0.1:9.9:90, vol/vol).

Table 1. Amino acid analysis of bovine milk LPL

Amino acid	Mole %
Cya*	2.1
Cys†	1.8
Asx	9.7
Thr	5.5
Ser	9.3
Glx	9.6
Pro	5.6
Gly	7.8
Ala	6.2
Val	6.1
Met	1.5
Ile	3.8
Leu	8.2
Tyr	4.2
Phe	6.2
His	3.1
Trp‡	0.1
Lys	7.8
Arg	4.9

Approximately 1 μg of trichloroacetic acid-precipitated bovine milk LPL was hydrolyzed in 6 N HCl/0.2% 2-mercaptoethanol for 48 hr at 110°C. The sample contained ≈8% glucosamine by weight.
*Cysteine was determined in separate analyses as cysteic acid (Cya) following performic acid oxidation.
†Cysteine was determined as its S-carboxymethyl derivative after acid hydrolysis of 0.4 μg of S-carboxymethylated LPL.
‡Tryptophan recovery is generally low due to the conditions of hydrolysis.

Amino-Terminal Sequence Analysis. LPL (1 nmol) was precipitated in 10% trichloroacetic acid and dissolved in an equal volume of 0.1% CF_3COOH/acetonitrile. This corresponds approximately to the percentage of acetonitrile required for elution of LPL in the HPLC solvent gradient (Fig.

Table 2. Amino-terminal sequence of bovine milk LPL

Cycle	Amino acid	pmol
1	Asp	321
2	Arg	186
3	Ile	190
4	Thr	140
5	Gly	515
6	Gly	506
7	Lys	711
8	Asp	299
9	Phe	545
10	Arg	172
11	Asp	244
12	Ile	513
13	Glu	352
14	Ser	264
15	Lys	507
16	Phe	438
17	Ala	446
18	Leu	441
19	Arg	62

Approximately 1 nmol of trichloroacetic acid-precipitated bovine milk LPL was applied to the microsequencer. Amino acids were determined as >PhNCS derivatives. Picomole yield is based on the recovery of >PhNCS amino acid derivative. For serine, the quantitation is based on the sum of the pmol yield of the >PhNCS derivative of serine and the pmol yield of the dithiothreitol adduct of the >PhNCS derivative.

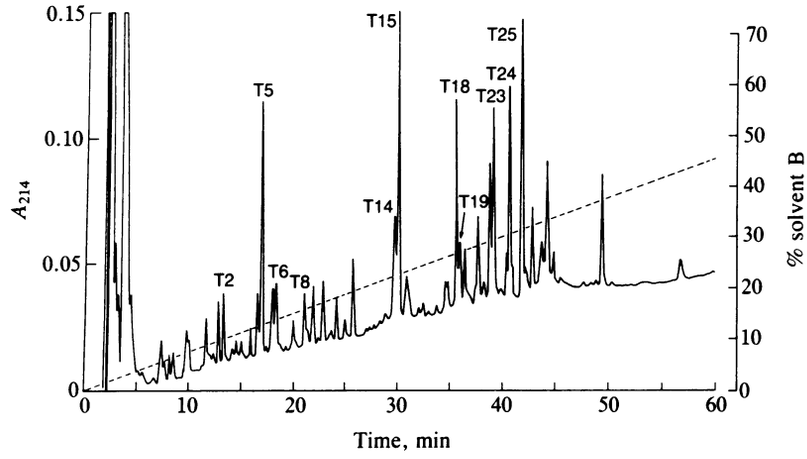


FIG. 2. Tryptic peptide HPLC map of S-carboxymethylated lipoprotein lipase. Approximately 1 nmol of reduced and alkylated protein was digested with 1 μg of trypsin and fractionated on a Vydac C_4 reversed-phase HPLC column. The amino acid sequences of the designated peaks are shown in Table 3.

1 *Lower*). The amino-terminal sequence analysis resulted in an unambiguous sequence for 19 amino acid residues (Table 2).

Tryptic Digestion. Approximately 1 nmol of S-[^{14}C]carboxymethylated LPL, purified by gel-permeation HPLC, was subjected to tryptic digestion. Reversed-phase HPLC fractionation of the digest revealed a complex peptide map suggesting extensive proteolysis (Fig. 2).

Microsequence Analysis of Tryptic Peaks. The major tryptic HPLC peaks were assayed for radioactivity and seven radiolabeled fractions were identified. The results of microsequence analysis are shown in Table 3.

Consistent with the chromatographic profile of peak T6, this fraction revealed three amino acid sequences, one of which was identical to that found for peak T2. The other two sequences for T6 differ only by an amino-terminal lysine residue.

Peak T15, although radioactive, did not contain carboxymethylcysteine. Since the amino acid sequence of T14 was found as a minor component in the sequence analysis of T15, we assume that the radioactivity in this fraction was due to contamination by the chromatographically unresolved peak T14 (Fig. 2). Moreover, the mass spectral analysis of peak T15 showed several molecular ions, including one with a value of 2006 mass units, which corresponds to the predominant sequence found for this tryptic fraction. Similarly, peak T18 was radioactive but did not reveal a carboxymethylcysteine residue on sequence analysis. This peak gave a mass spectral molecular ion at 3296 mass units, which is significantly larger than the calculated mass of the observed amino acid sequence. This implies that the actual amino acid sequence extends beyond the last residue assignment made, locating a carboxymethylcysteine residue in the carboxyl-terminal portion of the peptide. The seventh radioactive peptide, peak T19, showed identical amino acid sequence and mass fragmentation pattern to peak T18.

Four additional major tryptic peptides, T5, T23, T24, and T25, were also sequenced, and the data are shown in Table 3.

DISCUSSION

The amino-terminal sequence analysis of bovine milk LPL constitutes our initial primary structural study on this enzyme. Our microsequencing results agree with an observation by Olivecrona and Bengtsson (9) identifying aspartic acid as the amino-terminal residue and thus differ with the report (10) identifying that residue as glycine.

The amino acid sequences determined for tryptic peaks T2, T6, T8, T23, and T24 represent full-length peptides. Peptides T5 and T25 were sequenced two residues short of their respective carboxyl termini. However, mass spectral analysis revealed the identity of those residues in both cases. A

Table 3. Microsequence analysis of tryptic peptides

	Amino acid (pmol)												
Cycle	T2	T5	T6a	T6b	T6c	T8	T14	T15	T18	T19	T23	T24	T25
1	Cys (227)	Ala (329)	Cys (86)	Lys (111)	Val (313)	Gly (221)	Thr (100)	Ile (543)	Ser (103)	Ser (30)	Glu (184)	Ser (194)	Leu (178)
2	Asn (262)	Gln (275)	Asn (132)	Val (238)	Ile (300)	Leu (197)	Pro (151)	Thr (111)	Ile (62)	Ile (68)	Pro (122)	Val (416)	Ser (84)
3	Asn (246)	Gln (380)	Asn (159)	Ile (273)	Phe (289)	Cys (294)	Glu (264)	Gly (876)	Gly (159)	Gly (81)	Asp (197)	His (192)	Pro (149)
4	—	His (84)	—	Phe (203)	Cys (194)	Leu (192)	Asp (122)	Leu (948)	Ile (38)	Ile (81)	Ser (68)	Leu (270)	Asp (119)
5	Gly (122)	Tyr (203)	Gly (84)	Cys (140)	Ser (122)	Ser (151)	Thr (73)	Asp (1088)	Gln (184)	Gln (111)	Asn (262)	Phe (535)	Asp (173)
6	Tyr (138)	Pro (125)	Tyr (95)	Ser (59)	(Arg) (22)	Cys (159)	Ala (248)	Pro (764)	Lys (127)	Lys (108)	Val (103)	Ile (297)	Ala (138)
7	Glu (122)	Val (289)	Glu (59)	Arg (14)		Arg (51)	Glu (122)	Ala (659)	Pro (135)	Pro (73)	Ile (70)	Asp (286)	Asp (138)
8	Ile (122)	Ser (167)	Ile (59)				Asp (78)	Gly (386)	Val (97)	Val (143)	Val (68)	Ser (97)	Phe (59)
9	Asn (103)	Ala (148)	Asn (76)				Asp (103)	Pro (348)	Gly (135)	Gly (70)	Val (81)	Leu (186)	Val (84)
10	Lys (20)	Gly (86)	Lys (22)				Cys (103)	Asn (564)	His (154)	His (43)	Asp (130)	Leu (194)	Asp (97)
11		Tyr (103)					(His) (23)	Phe (370)	Val (100)	Val (149)	Trp (89)	Asn (265)	Val (111)
12		(Thr)					Leu (57)	Glu (446)	Asp (176)	Asp (81)	Leu (65)	Glu (157)	Leu (95)
13		(Lys)					Ile (38)	Tyr (356)	Ile (49)	Ile (73)	Ser (14)	Glu (132)	His (59)
14							Pro (86)	Ala (389)	Tyr (132)	Tyr (51)	Tyr (22)	Asn (159)	Thr (24)
15							Gly (46)	Glu (324)	Pro (105)	Pro (35)	(Lys) (22)	Pro (103)	Phe (46)
16							(Val)	Ala (346)	Asn (24)	Asn (35)		Ser (16)	(Thr)
17							(Val)	Pro (219)	Gly (14)	Gly (24)		(Lys) (65)	(Arg)
18							(Glu)	(Ser)	(Gly)	(Gly)			
19								(Arg)					

Approximately 1 nmol of S-carboxymethylated bovine milk LPL purified by gel-permeation HPLC was treated with trypsin and fractionated by reversed-phase HPLC, and peptide fractions were subjected to gas-phase microsequence analysis. Numbers in parentheses refer to pmole yields of >PhNCS derivatives. Amino acid assignments enclosed in parentheses denote sequence ambiguities of those cycles. Cysteine was identified as its S-carboxymethyl derivative on >PhNCS amino acid analysis and by radioactivity.

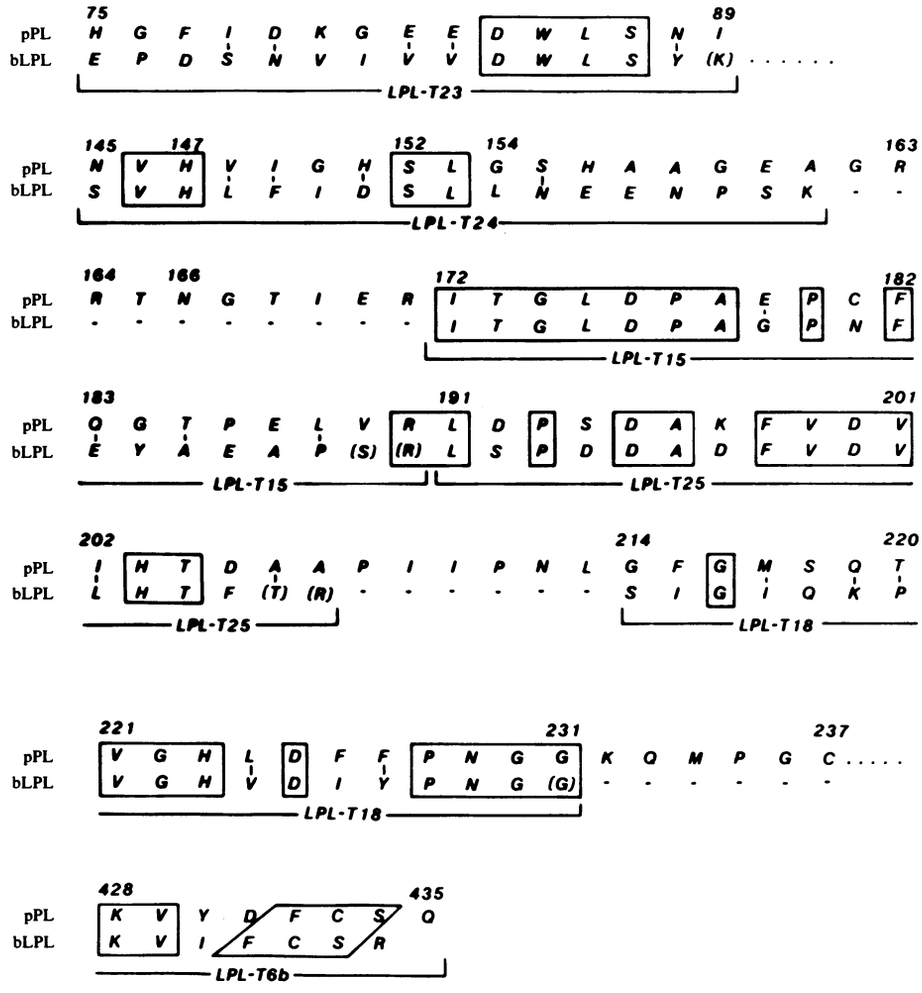


FIG. 3. Amino acid sequence homology between bovine milk LPL (bLPL) tryptic peptides and porcine pancreatic lipase (pPL) and alignment of peptides. The amino acid sequences are shown in one-letter code (17). Amino acid identities are boxed. Vertical lines show single-base changes at the nucleotide level. Dashes signify gaps in the LPL sequence and dots represent an extended region in the pancreatic lipase sequence. Position numbers refer to the pancreatic lipase sequence. Identities of LPL residues in parenthesis were ambiguous (see legend to Table 3) but were consistent with mass spectral analysis (see *Results*).

molecular ion of 1450 mass units determined for T5 accounts for the identification of threonine and lysine at the carboxyl terminus. Similarly, 1949 mass units found for T25 account for threonine and arginine at its carboxyl terminus. Peptides T14, T18, and T19 were incompletely sequenced due to their low yields of >PhNCS amino acid derivatives.

Our identification of five distinct cysteine-containing tryptic peptides does not correspond to the actual number of cysteine residues present in the protein. Amino acid analysis of S-carboxymethylated LPL suggests the presence of seven cysteines [based on a sedimentation equilibrium molecular weight of 42,000 (11)], whereas performic acid oxidation of native LPL revealed eight cysteine residues. Thus, despite the high efficiency of cysteine modification under our reduction and alkylation reaction conditions, we have so far not been able to identify any additional cysteine-containing peptides.

Homology to Pancreatic Lipase. A sequence comparison[¶] of the LPL tryptic peptides to porcine pancreatic lipase (13) shows considerable homology. Fig. 3 illustrates the sequence homology and the alignment of the LPL peptides.

Peptides T23 and T24 show limited homology to their respective sequences in pancreatic lipase. Peptide T15, on the other hand, shows complete amino acid identity to pancreatic lipase residues 172–178. A single nucleotide base change at codon 179 increases the linear homology to nine identical amino acid residues. The sequence of peptide T25 displays 10 out of 17 identical amino acid residues with pancreatic lipase (positions 191–207). The overall homology for this peptide increases when the single nucleotide base changes are considered.

A similar analysis for peptide T18 also reveals extensive homology, in this case to pancreatic lipase positions 214–231. As pointed out in *Results*, the cysteine residue of T18 has been tentatively assigned to the carboxyl-terminal portion of this peptide. The corresponding cysteine in this region of pancreatic lipase occurs at position 237, six residues carboxyl-terminal to the last amino acid determined in LPL peptide T18. Therefore, we might expect the position of the cysteine in peptide T18 to correspond to position 237 of pancreatic lipase. Lastly, peptide T6 shows a high degree of homology to pancreatic lipase positions 428–434 if a deletion at either residue 430 or 431 is considered.

Peptide Alignment. The alignment of six LPL peptides along the length of the LPL molecule can be inferred from the homology to pancreatic lipase. Fig. 3 depicts the relative alignment of these peptides. Tryptic peptide T23 corresponds to the amino-terminal region, whereas peptide T6 can be positioned at the carboxyl-terminal portion of the protein. The four remaining LPL peptides can be aligned within a

[¶]Protein sequence homology search was made using the program PROSCAN by the method of Lipman and Pearson (12) modified for incorporation into the DNAStar (Madison, WI) program.

block of 87 amino acids found in pancreatic lipase from residue 145 to 231. Peptide T24 is amino-terminal to T15, which is amino-terminal to and contiguous with T25. Peptide T18 follows T25 after a stretch of six amino acid residues in accord with pancreatic lipase residues 208–213.

Binding Domains. Peptide T24 shows limited homology to that portion of pancreatic lipase containing an essential serine residue at position 152 (Fig. 3). This serine is thought to convey a crucial structural feature involved in binding the enzyme to the substrate interface (14). The identity of serine at this position in T24 raises the possibility of a similar role for this residue in the LPL molecule.

The asparagine residue at position 166 in pancreatic lipase (Fig. 3) was identified as the glycan-moiety binding site in that enzyme (15, 16). Although the homologous LPL peptide located in this region between T24 and T15 has not yet been identified, we assume that the glycan portion of LPL also occurs as an asparagine-linked carbohydrate in such a peptide. The presence of arginine residues in pancreatic lipase at positions 163 and 164, and also at position 171, which represent tryptic cleavage sites, strengthens the likelihood of the presence of a trypsin-labile LPL peptide in this region.

Our amino acid sequence data have enabled us to pursue the structure–function relationship of bovine milk LPL. Additional peptide sequencing will undoubtedly facilitate this task, and we anticipate that it will also reveal further homologies between LPL and pancreatic lipase.

In general, lipolytic enzymes may bear extensive sequence homology at functionally relevant domains. This assumption, based on the LPL/pancreatic lipase homology, derives additional support from a recent finding of homology between rat hepatic lipase and bovine LPL. A tryptic peptide obtained from hepatic lipase shows identity to a portion of LPL peptide T15 in 11 out of 12 residues (unpublished data). The similar catalytic function of pancreatic lipase, LPL, and hepatic lipase would suggest a similar amino acid sequence at critical domains, and this appears to be the case. It is likely that these lipases form a multigenic family involved in absorption and transport of lipids. Perhaps these enzymes arose from a common ancestral gene and diverged into distinct evolutionary pathways.

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