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# PORCINE PANCREATIC LIPASE

## COMPLETION OF THE PRIMARY STRUCTURE

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**The complete primary structure of a lipase (triacylglycerol hydrolase; EC 3.1.1.3) is presented for the first time. The porcine pancreatic enzyme which was investigated is composed of a single chain of 449 amino acids. Upon fragmentation by CNBr, five peptides were obtained. The sequence of four of them (CN I–CN IV) has already been published. The present report deals with the arrangement of the 142 amino acids of the C-terminal peptide CN V, thus completing the analysis of the whole molecule. Special problems resulting from incomplete cleavage of some peptide bonds in CN V and aggregation of large peptides were overcome using Sephadex filtration of succinylated derivatives in 50% acetic acid, automated sequence analysis of peptide mixtures and subdigestion of material which could not be directly resolved. No obvious homology was found when the sequence of porcine lipase was compared with other protein, including pancreatic phospholipase A2 and colipase from the same species. However, a few similarities which might be significant were detected between the environment and relative position of certain half cystines in lipase and colipase, as well as between two tyrosine-rich regions existing in both proteins.**

## Introduction

Pancreatic lipase (triacylglycerol acylhydrolase EC 3.1.1.3) is a key enzyme for the intraluminar digestion of fats. It rapidly hydrolyzes the long chain triacylglycerol derivatives to more polar fragments (mainly monoacylglycerols and free fatty acids) which are able to leave the intestinal lumen and penetrate into the enterocytes by passage across the brush border membrane. One of the interests of this hydrolysis lies in the fact that it is a typical example of heterogeneous biocatalysis [1] due to the complete insolubility of the substrates which form emulsified particles separated from water by an interface. The first step of the catalytic cycle in the case of lipase is an adsorption of the enzyme to the interface followed by a $10^3$-fold activation [1].

The adsorption of lipase, which occurs readily when the particles are composed of triacylglycerols only, is hindered by the accumulation at the interface of various amphiphiles (bile salts, soaps, phospholipids, etc.) present in the duodenum during fat

Abbreviations: dansyl or Dns, 5-dimethylaminonaphthalene-1-sulfonyl; Quadrol, *N,N,N'-N'*-tetrakis (2-hydroxypropyl)ethylenediamine; DMBA, dimethylbenzanthracene.

Supplementary information to this article is deposited with and can be obtained from: Elsevier/North Holland Biomedical Press, B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH, Amsterdam, The Netherlands. Reference should be made to BBA/DD/202/38784/671 (1981) 129. The supplementary information includes: information on the enzymatic digestions and on all the peptides used in the sequence determination. Tables S1–S8 give the purification methods, the amino acid composition and the yield of each peptide. Figs. S1–S6 give the principal elution profiles obtained in peptide purifications.

digestion. Hence, for hydrolysis in vivo, the participation of a pancreatic cofactor called colipase is necessary. Colipase can adsorb to interfaces covered by amphiphiles and then anchor lipase [1,2]. All these phenomena require at the surface of the lipase molecule the presence of, besides the catalytic site proper, specialized areas insuring the necessary interactions with the lipid phase and colipase. Moreover, the three-dimensional structure of the enzyme must be such as to allow the very large activation effect noted upon interfacial adsorption.

This particular behavior, compared to that of other enzymes, prompted us to elucidate the primary structure of the especially abundant porcine pancreatic lipase. This molecule composed of a single chain has already been shown to yield upon CNBr fragmentation five peptides of which four (CNBr I–CNBr IV), including the first 307 amino acids of the molecule, have already been sequenced [3–5]. The sequence of the last C-terminal peptide (CN V; 142 amino acids) is now reported, thus completing for the first time the structure of a lipase. Serious difficulties due to incomplete bond cleavage and peptide aggregation were encountered in the N-terminal region (the first 63 amino acids) of this peptide.

## Materials and Methods

Materials and Methods were essentially the same as in Refs. 4–6. Details on enzymatic digestions and peptide purification are given in the supplementary information section (see footnote on p. 129).

*Peptide nomenclature.* The CNBr peptides are denoted CN I, CN II . . . CN V, the Roman numerals designating their sequential order in the enzyme chain. Trypsin peptides are denoted T, Cit-T, Cyc-T when obtained from unmodified, citraconylated or cyclohexanedione-treated substrates, respectively. An Arabic numeral characterizes each peptide. A small letter after this numeral (for example Cit-T5a) indicates that the peptide results from an atypical splitting due in particular to the chymotrypsin-like activity of trypsin. Peptides issuing from a trypsin subdigestion of decitraconylated Cit-T peptides are denoted Cit-T T. The peptides generated by chymotrypsin, clostripain, thermolysin, pepsin and staphylococcus protease are denoted C, Cl, L, P and G. For pepsin peptides deriving from native lipase, the Roman numeral V following the letter P indicates that these peptides are included in CN V. A succession of symbols each corresponding to an enzymatic attack are used for peptides resulting from several enzymatic digestions. Incomplete digestions led to longer peptides for which a special nomenclature was necessary. For example, the name Cit-T 3.4 reflects the presence in this peptide of the third and fourth arginines of CN V. The symbol G 1.2 indicates the presence of the first and second glutamic acids of the chain, due to poor cleavage of the first glytamyl bond. The location of a peptide is given by two figures between brackets which indicate the position of its N- and C-terminal amino acids in CN V or lipase.

## Results

*General aspects*

As shown by previous data [3], the peptide CN V should be composed of about 140 amino acids with a glycine at the N-terminus and a carboxymethyl cysteine at the C-terminus. Most of the peptides used for sequence determination were obtained by trypsin digestion of citraconylated or cyclohexanedione-treated CN V and also by incubation of citraconylated CN V with the staphylococcal protease. If needed, other peptides deriving from digestion of CN V by chymotrypsin and clostripain were taken into consideration. Pepsin digestion of native lipase primarily performed for the determination of disulfide bridges also supplied useful peptides. All hydrolysates were first passed through Sephadex G-50. In most cases, the fractions thus obtained were further chromatographed on DEAE-cellulose or SP-Sephadex. When necessary, further purification was achieved by high-voltage paper electrophoresis, or by chromatography and/or electrophoresis on thin-layer cellulose plates. The sequence of the 142 amino acids of CN V is reproduced in Figs. 1 and 2. Detailed and yields of automated peptide degradations are given later in the text.

*Sequence of the first 63 residues in CN V (Fig. 1)*

As mentioned in the Introduction, serious difficulties had to be overcome before this region could be fully sequenced. These difficulties were mainly caused by the poor splitting of two arginyl bonds (Arg-Trp (30-31) and Arg-Tyr (32-33)) by trypsin

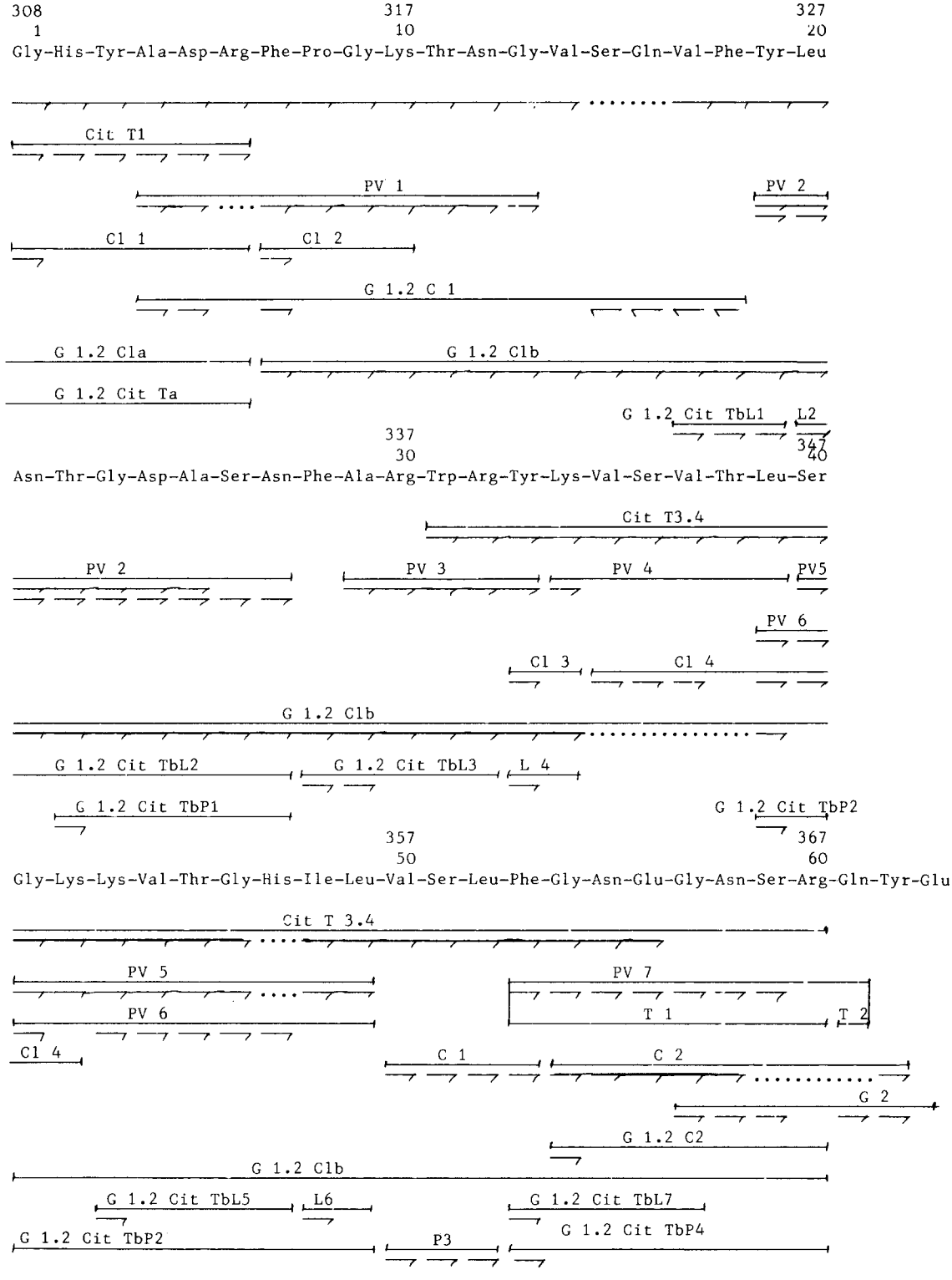
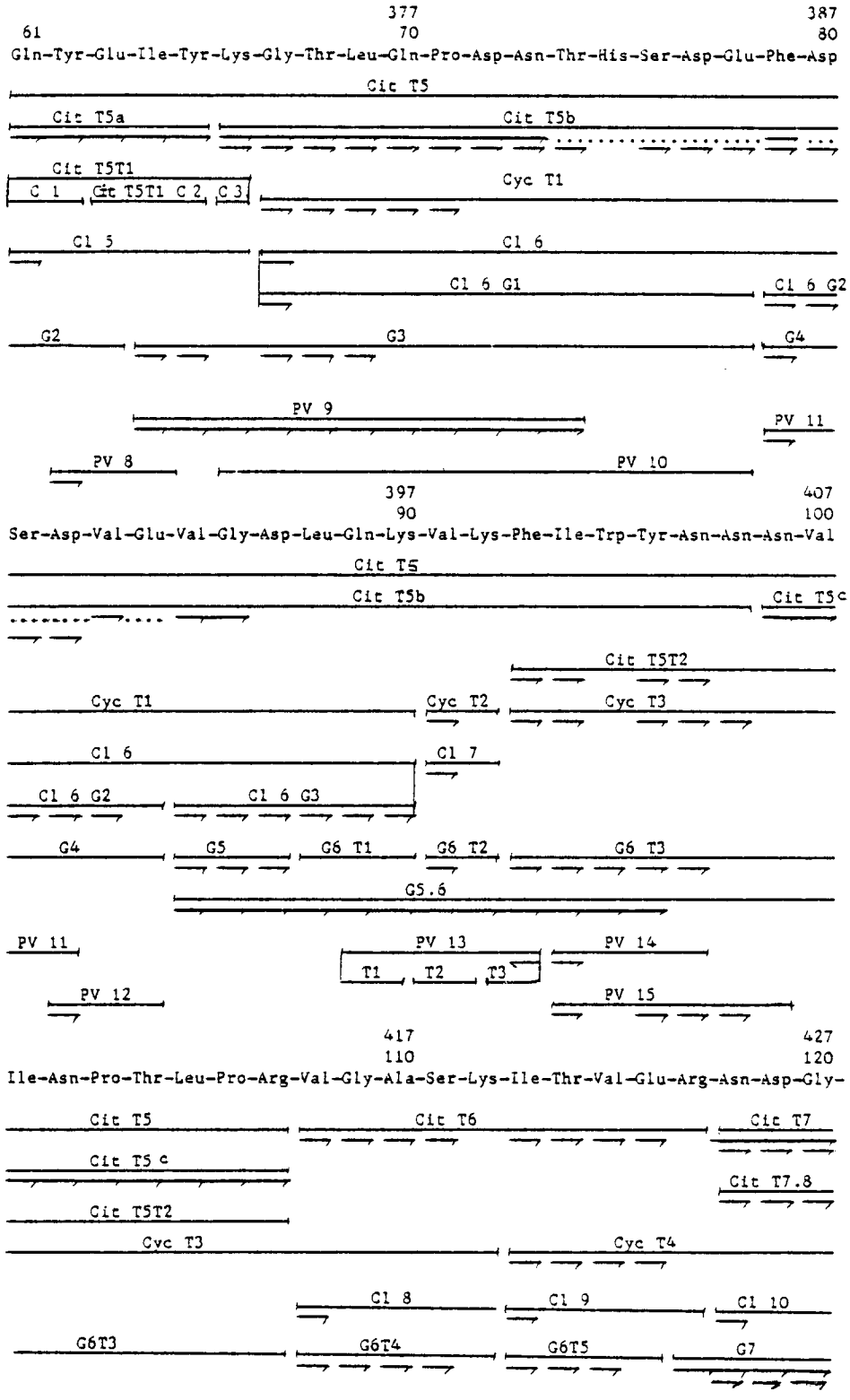



Fig. 1. Sequence of the first 63 amino acids of CN V. ——→, sequencer analysis; ——→ - - - - ——→, dotted line indicates that the phenylthiohydantoin amino acid was not identified; ——→ ——→, Edman-dansyl analysis; ←—— ←——, carboxypeptidase digest analysis. The double numbering indicates the amino acid positions in CN V and in lipase, respectively. The peptides Gl, G 1.2 and G 1.2 Cit-Tb are omitted as they were not obtained in pure form.


377
387
61
70
80
Gln-Tyr-Glu-Ile-Tyr-Lys-Gly-Thr-Leu-Gln-Pro-Asp-Asn-Thr-His-Ser-Asp-Glu-Phe-Asp
Cit T5
Cit T5a
Cit T5b
Cit T5T1
C 1
Cit T5T1 C 2
C 3
Cyc T1
C1 5
C1 6
C1 6 G1
C1 6 G2
G2
G3
G4
PV 9
PV 11
PV 8
PV 10
397
407
90
100
Ser-Asp-Val-Glu-Val-Gly-Asp-Leu-Gln-Lys-Val-Lys-Phe-Ile-Trp-Tyr-Asn-Asn-Asn-Val
Cit T5
Cit T5b
Cit T5c
Cit T5T2
Cyc T1
Cyc T2
Cyc T3
C1 6
C1 7
C1 6 G2
C1 6 G3
G4
G5
G6 T1
G6 T2
G6 T3
G5.6
PV 11
PV 13
PV 14
T1
T2
T3
PV 12
PV 15
417
427
110
120
Ile-Asn-Pro-Thr-Leu-Pro-Arg-Val-Gly-Ala-Ser-Lys-Ile-Thr-Val-Glu-Arg-Asn-Asp-Gly-
Cit T5
Cit T6
Cit T7
Cit T5 c
Cit T7.8
Cit T5T2
Cyc T3
Cyc T4
C1 8
C1 9
C1 10
G6T3
G6T4
G6T5
G7

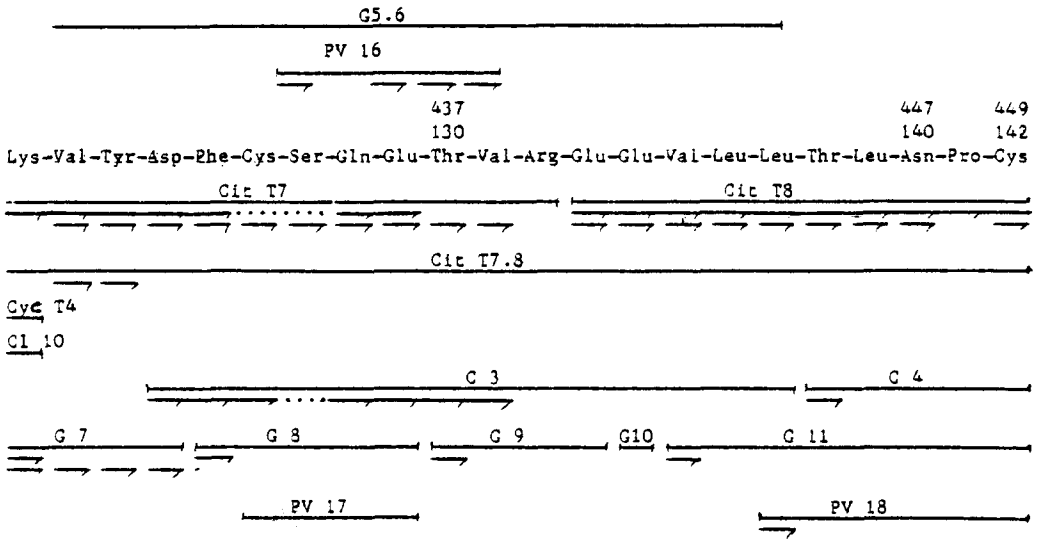



Fig. 2. Completion of the CN V sequence from the 61st to the 142nd, which is the final amino acid. Explanations of the symbols are given in the legend of Fig. 1. The peptide G6 is omitted as it was not obtained in pure form.

and clostripain. Likewise, the glutamyl bond Glu-Gly (56-57) was not fully split by the staphylococcal protease and the large peptides (20–60 amino acids) originating from the 1–63 area of CN V were excluded from Sephadex G-100 in 60 mM ammonium bicarbonate (pH 8.5), suggesting aggregation. The same peptides could not be eluted from DEAE-cellulose, presumably because of strong interactions with the ion exchanger. Attempts to separate these peptides by HPLC in an organic solvent [7] were unsuccessful.

Automated sequential degradation of CN V gave data up to Leu-20 except for Ser-15 and Gln-16, which were identified after carboxypeptidase A digestion of G 1.2 Cl and automated sequencing of G 1.2 Clb.

Trypsin digestion of citraconylated CN V yielded Cit-Tl (1–6), Cit-T 3.4 (31–60), Cit-T4 (33–60) and a dipeptide Cit-T3 (31–32). Cit-T2 could not be eluted from Sephadex G-50 at pH 8.5. The behavior of Cit-T 3.4 and Cit-T4 was as previously described for the large peptides from this area. Both emerged from Sephadex G-50 at the void volume of the column as shown by the dansyl method and amino acid analysis. Nevertheless, the automated Edman degradation of this mixture provided the sequence (31–56). The position of His-47 was ascertained by the manual sequencing of PV 6 and further confirmed by the amino acid composition of several peptides. The rest of the sequence from position 57 to 63 was given by analysis of G2, C2 and PV 7.

The staphylococcal protease digestion of Cit-CN V provided two large peptides Gl (1–56) and G 1.2 (1–63), and a short peptide G2 (57–63) in the sequence area investigated. Gl and G 1.2 were excluded from Sephadex G-50 and G-100. They were contaminated by G 5.6 (85–116) (Fig. 2). The following operations were performed on this mixture.

1. Automated sequencing, which provided no further information about the 1–63 area but yielded the sequence of the first 12 amino acids of G 5.6 (Fig. 2. see later).

2. Enzymatic digestions of the citraconylated peptide complex yielded several subpeptides among which only those included in the 1–63 area were taken into consideration. The chymotryptic peptide G 1.2 C1 (4–18) was pure. The still impure tryptic peptide G 1.2 Cit-Tb (7–60) excluded from Sephadex G-100 was further digested by thermolysin or pepsin, thus yielding two groups of peptides G 1.2 Cit-Tb L1–L7 and G 1.2 Cit-Tb P1–P4 which served for obtaining a final confirmation of the sequence.

3. Sephadex G-50 filtration using 50% acetic acid as eluant was performed on the peptide mixture

which was previously decitraconylated and succinylated. Two peaks were obtained: one which eluted at the void volume of the column contained still impure Gl and G 1.2. The other (elution volume, 1.3 $V_0$) contained pure G 5.6. Attempts to improve the accessibility of the arginyl bonds Arg-Trp (30-31) and Arg-Tyr (32-33) in Gl and G. 1.2 towards clostripain by dissolving the mixture in 8 M urea and performing the digestion in 2 M urea were unsuccessful. However, this experiment permitted full purification of G 1.2 Clb (7–60) by filtration through Sephadex G-50. Automated degradation on the latter peptide (28 productive cycles) confirmed the amino acid arrangement at positions 7–20 and also elucidated the sequence in the 20–34 area.

*Sequence from the 61st to the 142nd and last amino acid (Fig. 2)*

The tryptic digestion of Cit-CN V yielded four peptides covering the whole investigated sequence; Cit-T5 (61–107), Cit-T6 (108–117), Cit-T7 (118–132) and Cit-T8 (133–142). Moreover, four additional peptides were analyzed; Cit-T 7.8 (118–142) resulting from incomplete splitting of the Arg-Glu bond (132–133) and Cit-T5a, -T5b and -T5c deriving from the extra splitting by trypsin of Tyr-Lys (65–66) and Asn-Asn (98-99).

*Amino acid assignment to position 61–86.* The necessary overlap between the sequences reproduced in Figs. 1 and 2 was supplied by Cit-T5a (61–65). The peptides PV 9 and G3 gave proof of the alignment between Cit-T5a and Cit-T5b. The manual and automated sequencings of Cit-T5b furnished data up to Gly-86. The analyses of the subpeptides Cl6 (Cl6 Gl-G3) and of the peptides G3, G4, G5 and PV 10 allowed the assignment of His-75 and Glu-84 and confirmed the considered sequence (61–86).

Since Edman degradation failed on Cit-T5, the location of this peptide was determined as follows: decitraconylated Cit-T5 was digested with trypsin. One of the resulting peptides, Cit-T5 T1, also had its N-terminus blocked. From the analytical results of the chymotryptic subpeptides Cit-T5 T1 Cl–C3, it was concluded that Cit-T5 T1 was located at position 61–66. This indicates that Cit-T5 T1 forms the N-terminal sequence of Cit-T5. Glutamine, which is the N-terminus of Cit-T5, Cit-T5 T1 and Cit-T5a, was not cyclized in Cit-T5a.

*Amino acid assignment to position 85–107.* The sequence was extended up to the 96th amino acid by sequencing Cl6 G3 and G 5.6. It was not possible to sequence Cyc-T3 beyond Asn-98 and Cit-T5 T2 beyond Asn-97. This difficulty was probably caused by the three adjacent asparagine amino acids at position 97–99. But the amino acid composition of Cit-T5 T2, Cyc-T3 and G6 T3 allowed the positioning of Cit-T5c and the extension of the known sequence to Arg-107.

*Amino acid assignment to position 108–142.* The overlap between Cit-T5 and Cit-T6 was given by PV 16 and Cyc-T3. The peptide G6 was not pure, but the products resulting from its tryptic subdigestion could be purified and sequenced. As the amino acid composition of the five subpeptides G6 T and peptide G5 matched with that of G 5.6, it was obvious that G6 T4 directly followed Arg-107. The rest of the sequence was easily deduced.

*Automated sequence analysis*

Peptides were taken up in 300 µl 10% formic acid, or in 75% formic acid when less soluble (CN V, Cit-T 3.4 + Cit-T4 and G 1.2 Clb). Most analyses were carried out on 200–300 nmol peptides with the aid of a Socosi Sequencer Model PS 100 using the DMBA buffer program in the presence of 3 mg apocytochrome *c* as carrier [8]. The phenylthiohydantoin amino acids were identified by gas chromatography, thin-layer chromatography and, when necessary, by amino acid analyses after post-regeneration of the parent amino acids. Sequencing of CN V was performed in the same manner but using 1 M Quadrol and no carrier. Cit-T 3.4 (18 nmol), PV 2 (6 nmol) and G 1.2 Clb (200 nmol) were sequenced in a Beckman Sequencer Model 890 C with the 0.1 M Quadrol program and 3 mg poly(β-alanine) as carrier. The phenylthiohydantoin amino acids were identified by HPLC.

The initial yields were 85–90% for PV 3 and PV 5, 40–60% for Cit-T 3.4 + Cit T4, PV 2 and PV 9; 30–35% for CN V, Cit-T5c and PV I; 10–20% for Cit-T5b, Cit-T7, Cit-T8, G7, G 5.6, G 1.2 Glb and C3. Where calculations were possible, repetitive yields averaged 92%. This yield was not appreciably lowered by the Asn-Gly bond existing in CN V and G 1.2 Clb. Relatively large quantities of PV 5 were obtained, thus permitting systematic assays which led to technical improvements [8].

*Amide determination*

The amide assignments were made either by direct identification of the phenylthiohydantoin derivatives (thin-layer or HPLC) or by the electrophoretic mobility of the peptides at pH 6.5. The two negative net charges of the peptides G3, PV 11 and PV 12 characterized Asp-77, Glu-78, Asp-80, Asp-82 and Glu-84. The single negative net charge of G5 indicated the presence of Asp-87. The neutral behavior of Cl6, G3, PV 14, PV 15 and Cl9 was consistent with Gln-89 and the three adjacent Asn-97–99.

*The complete sequence of lipase*

The entire amino acid sequence of porcine pancreatic lipase (449 amino acids) (Fig. 3) could be unambiguously deduced from the data reported in the

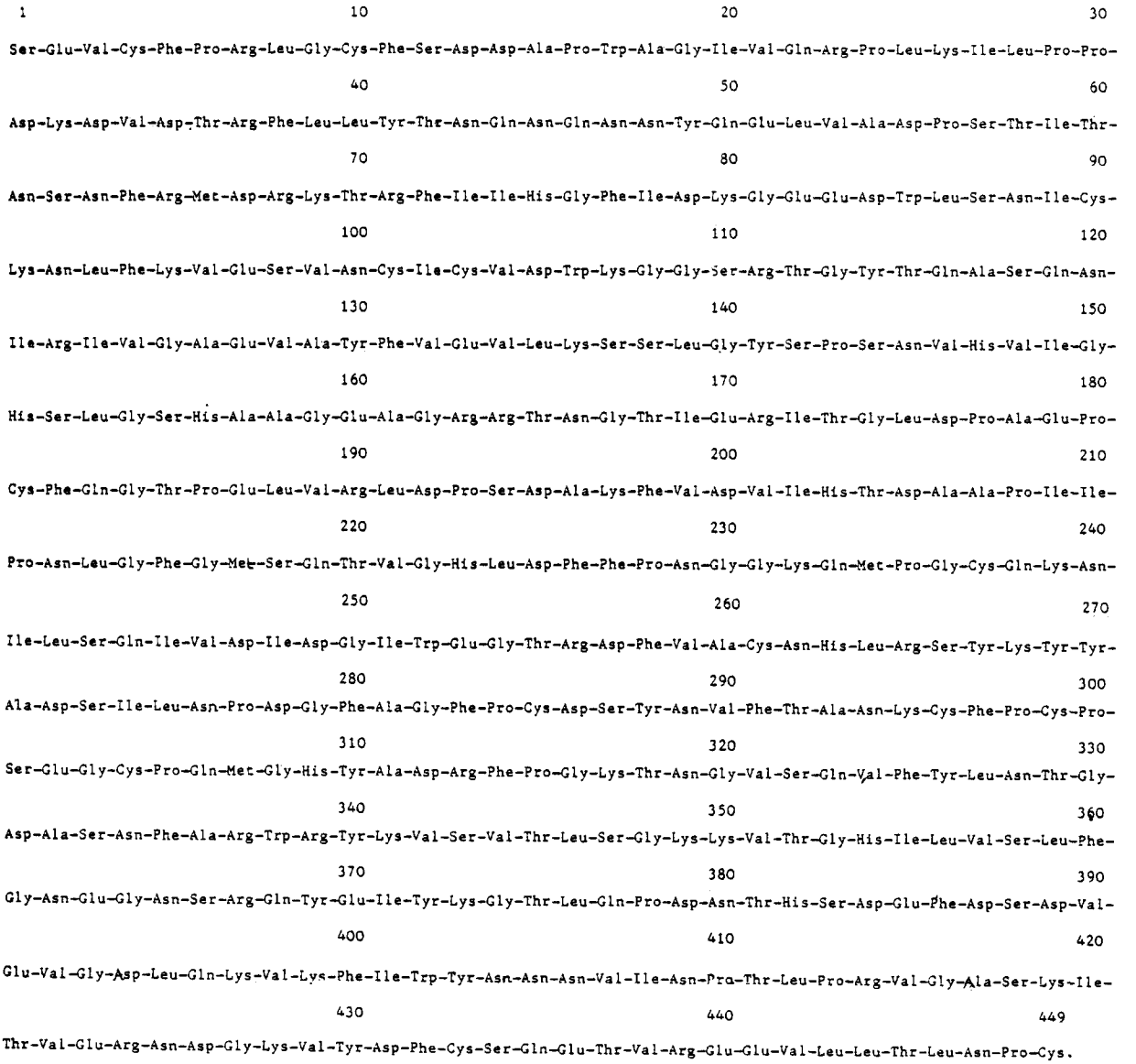



Fig. 3. Total sequence of porcine pancreatic lipase.

present and previous publications [4,5]. It must be noted that modifications have been made at positions 302 and 305 of the previous published sequence [5]. The peptide at position 296–307 of the lipase sequence, deriving from pepsin and trypsin digestions was reduced and *S*-carboxymethylated with [14C]-iodoacetic acid and was automatically sequenced. Instead of two successive cysteines at positions 304 and 305 as previously found by Edman dansyl determination, Cys-304 and Pro-305 were characterized. The radioactivity counting results demonstrated that the CM-cysteine previously found at position 305 was actually an overlap of CM-Cys-304. Pro-305, which for some unknown reason could not be identified as a Dns derivative, was unambiguously characterized as phenylthiohydantoin derivative by HPLC. The results obtained by the latter technique were also consistent with a glutamic acid at position 302 instead of a glutamine.

Table I shows the amino acid compositions, molecular weights and polarities of the five CNBr peptides and of the complete lipase chain.

## Discussion

The primary structure of a lipase has been fully elucidated for the first time. The porcine pancreatic enzyme is composed of a single chain with 449 amino acids. The calculated molecular weight of the protein moiety is 49 859, to which that of the glycan moiety

TABLE I

AMINO ACID COMPSITIONS, MOLECULAR WEIGHTS AND POLARITIES OF CN I–CN V AND OF LIPASE

These results are deduced from the amino acid sequences of the CN peptides. Values are given as mol amino acid per mol peptide or lipase. The two figures between brackets indicate the respective position of the N- and C-terminal amino acids of the compound in the entire polypeptide chain of lipase.

| Amino acid | CN I (1–66) | CN II (67–217) | CN III (218–234) | CN IV (235–307) | CN V (308–449) | Lipase (1–449) |
|---|---|---|---|---|---|---|
| Cys | 2 | 4 | | 6 | 2 | 14 |
| Asp | 6 | 10 | 1 | 6 | 9 | 32 |
| Asn | 6 | 6 | 1 | 5 | 12 | 30 |
| Thr | 4 | 8 | 1 | 2 | 10 | 25 |
| Ser | 4 | 11 | 1 | 5 | 10 | 31 |
| Glu | 2 | 9 | | 2 | 8 | 21 |
| Gln | 4 | 3 | 2 | 3 | 5 | 17 |
| Pro | 6 | 7 | 1 | 6 | 5 | 25 |
| Gly | 2 | 16 | 3 | 6 | 12 | 39 |
| Ala | 3 | 10 | | 4 | 4 | 21 |
| Val | 4 | 12 | 1 | 3 | 15 | 35 |
| Met | 1 | 1 | 1 | 1 | | 4 |
| Ile | 3 | 13 | | 5 | 5 | 26 |
| Leu | 6 | 9 | 1 | 3 | 10 | 29 |
| Tyr | 2 | 3 | | 4 | 7 | 16 |
| Phe | 4 | 7 | 2 | 5 | 7 | 25 |
| His | | 5 | 1 | 1 | 3 | 10 |
| Lys | 2 | 7 | 1 | 3 | 9 | 22 |
| Arg | 4 | 8 | | 2 | 7 | 21 |
| Trp | 1 | 2 | | 1 | 2 | 6 |
| Total residue | 66 | 151 | 17 | 73 | 142 | 449 |
| Weight of the residues ($X10^{-3}$) | 7.533 | 16.379 | 1.843 | 8.097 | 15.989 | 49.841 |
| Molecular weight ($X10^{-3}$) | | | | | | 49.859 |
| Polarity [a] | 48.5 | 45.7 | 47.0 | 39.7 | 51.4 | 47.0 |

[a] The polarity of a peptide chain is defined as the sum of the amino acid mol percentages of polar amino acids. Asp, Asn, Glu, Gln, Thr, Ser, His, Lys, Arg are classified as polar amino acids [9].

(about 2000) [10] should be added, leading to a total of about 52 000. This value is in excellent agreement with previous sedimentation equilibrium data (50 000–52 000 daltons [11,12]). Likewise, the amino acid composition of the protein determined some years ago in this laboratory [3,11] is largely confirmed by the sequence. Although unfractionated mixtures of several isolipases [4] were used, no allotypic replacement could be detected, suggesting that variability occurred in the glycan chain only.

The lipase from *G. Candidum* has been reported to be devoid of sulfur-containing amino acids [13], whereas the pancreatic enzyme possesses 14 half cystines (six disulfide bridges and two free sulfhydryls [11]) and four methionines. The α-helix content of the proteins has also been found to be widely different [13,15]. These large structural variations within the same enzyme class are unexpected, in spite of the known divergences between the respective specificities (positional and chemical) of the two lipases.

As shown in Fig. 3, the 14 half cystines of porcine pancreatic lipase are unevenly distributed in the chain, the highest proportion being found in the 285–304 area (Fig. 3) (4 half cystines out of a total of 20 amino acids); Phe-Pro-Cys-Asp . . . Cys-Phe-Pro-Cys-Pro . . . Gly-Cys-Pro. Preliminary results seem to show that these 4 half cystines form two S-S bridges. This structure may be compared to that of colipase [6,16] Cys-Pro-Cys (59–61) linked to Cys-Cys (27-28) by two adjacent disulfide bridges. In the same region of colipase exists the sequence Tyr-Gly-Val-Tyr-Tyr-Lys (53–58), the adjacent Tyr-56 and Tyr-57 being involved in the interface recognition site of the cofactor [17–20]. A similar structure is found in lipase in the sequence Tyr-Lys-Tyr-Tyr (267–270). It might be postulated that, in both cases, an aromatic-hydrophobic site for interface recognition [19] is stabilized at the surface of the molecules by a disulfide-bridge knot. An alternative hypothesis, however, is that this site pre-exists inside both molecules in aqueous solution and that it is driven to the surface by a trans-conformation due to the approach of a hydrophobic interface [21]. These types of structure are certainly more important for the function of many lipolytic enzymes than a low polarity of the entire chain. As shown in Table I, the lipase polarity is 47, compared to 47 ± 6% for most soluble proteins [9]. Nevertheless, two segments of the chain are worth mentioning; the region (206–217) in which the succession of 11 hydrophobic amino acids is only interrupted by an asparagine, and the region (336–349) in which a regular alternation of polar and apolar amino acids is noted.

The position of some particular amino acids in the lipase chain has already been discussed. The non-essential tyrosine which reacts with concentrated diisopropylphosphofluoridate [22] is Tyr-49 [4]. As shown recently, the essential serine which selectively reacts with micellar diethyl *p*-nitrophenyl phosphate [23–24] and participates in the interfacial fixation of lipase is Ser-152 [25] in the sequence His-Ser-Leu-Gly (151–154). Other amino acids involved in lipase activity (the histidine of the catalytic site [24], the acylation site, which permits the transient formation of an acyl lipase derivative [26] and the acidic amino acid which controls the interfacial activation step [24]) have not yet been identified. As reported earlier [4], the short glycan chain of lipase is bound to Asn-166. Alignment of this amino acid with Asn-85 to which a glycan is also bound in the α,1-acid glycoprotein of human plasma [27] reveals an unexpected homology which is reproduced below.

82 Gln-Arg-Glu-Asn-Gly-Thr-Ile-Ser-Arg-Tyr-Glu-Gly-Gly 94

163 Arg-Arg-Thr-Asn-Gly-Thr-Ile-Ser-Arg-Ile-Thr-Gly-Leu 175

Apart from this short segment, no obvious similarity was found when the sequence of lipase was compared with all other known proteins (Dayhoff, M., unpublished results), especially with phospholipase A2 which, like lipase, hydrolyzes ester bonds of insoluble substrates. As already pointed out earlier for the polarity index, similarities in limited areas after chain folding are probably more important for the function of lipolytic enzymes than real sequence homology which would reveal the existence of a common ancestor.

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