T. P. HILDITCH SYMPOSIUM ON ANALYSIS OF NATURAL FAT TRIGLYCERIDES. IV

conducted by The American Oil Chemists' Society at its 56th Annual Meeting, Houston, Texas April 25-28, 1965

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Specificity of a Lipase from *Geotrichum candidum* for *cis*-Octadecenoic Acid[1]

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Abstract

A lipase from *Geotrichum candidum* released mostly oleic acid from glyceryl 1-elaidate-2,3-dioleate and very little *trans* fatty acid from margarine. When cod liver, Macadamia nut, peanut and safflower oils were substrates, the oleic acid content of the free acids was always in excess of the amount of the acid in the intact triglycerides. Congo palm oil was digested by both *G. candidum* and pancreatic lipases and the fatty acid compositions of the products of hydrolysis compared. The results obtained with the aid of *G. candidum* lipase tend to substantiate existence of some of the triglyceride isomers predicted from pancreatic lipase data.

Introduction

THE SPECIFICITY of pancreatic (P) lipase for the 1- and 3- positions of glycerides, has been used extensively for the study of triglyceride (TG) structure (4, 13) and has focussed attention on existence of different specificities of other lipases. Alford and co-workers described a lipase from the microorganism, *G. candidum*, which released relatively large quantities 18:1 and 18:2 from corn oil and lard (2) and 18:1 from a series of synthetic TG's (3). 18:1 was hydrolyzed from the latter regardless of position. This unique specificity suggested further investigation of differentiation between *cis* and *trans* isomers and structural studies of additional natural oils. In this paper we report the results obtained when synthetic TG's containing elaidic acid, margarine, and natural oils containing a variety of unsaturated fatty acids were partially hydrolyzed by a lipase system from *G. candidum*.

Experimental

The synthetic substrates, glyceryl 1-elaidate-2,3-dioleate (EOO) and glyceryl 1-elaidate -2,3-dilaurate (ELL) were prepared by acylating pure 1- monoelaidin with the appropriate acyl chlorides as described by Mattson and Volpenhein (12). The 1-monoelaidin, pure by thin-layer chromatography (TLC) and by oxidation with periodic acid (8), was synthesized from isopropylidene glycerol and elaidoyl chloride (12). The fatty acids were purchased

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TABLE I

Fatty Acid Composition of Glycerides and Free Fatty Acids Resulting From Lipolysis of Glyceryl 1-elaidate-2,3-dioleate and Margarine by *G. candidum* (GC) and Pancreatic Lipases (P)[a]

Substrate and fatty acid composition	Intact[b] triglycerides		Residual triglycerides		Free fatty acids		Di-glycerides		Mono-glycerides	
Glyceryl 1-elaidate-2,3-dioleate	GC	P	GC	P	GC	P	GC	P	GC	P
M% 18:1 cis	70.0	65.1	73.5	67.1	94.5	52.8	66.0	70.7	30.0	100
18:1 trans	30.0	34.9	26.5	32.9	5.5	47.2	34.0	29.3	70.0	0
Margarine										
M% 12:0	tr		tr	tr	tr	tr	tr	tr	5.1	0.9
14:0	tr		tr	tr	tr	tr	tr	tr	2.3	0.8
16:0	14.0		16.1	14.2	7.2	23.1	25.2	12.2	33.7	5.1
18:0	7.2		10.5	7.9	3.5	8.3	7.4	9.4	12.6	6.7
18:1	61.2		66.9	61.0	74.3	58.3	56.8	64.0	41.7	66.7
18:2	17.6		6.5	16.9	15.0	10.3	10.6	14.4	4.6	19.8
trans as elaidic	26.4		32.2	30.4	5.5	22.0	25.5	28.3	16.8	28.5

[a] Digestion conditions: 5 min for P, 120 min for GC and 37C for both.
[b] Two batches of EOO. P lipase data previously reported.

(Hormel) and halogenated with oxalyl chloride (12). The TG's were purified by crystallization from acetone which was monitored by TLC. The EOO used in this study was prepared from elaidic acid of 96% purity. EOO and ELL were previously analyzed with P lipase (9).

The margarine, consisting of partially hardened cottonseed and soybean oils, was melted and the clear oil decanted and filtered. The Macadamia nut oil, used because of the relatively high 16:1 content, was obtained by initial grinding of the nuts, which were purchased from S. S. Pierce, and subsequent extraction of the ground material with ethyl ether: pentane (1:1) in a Waring Blendor. The peanut, safflower and cod liver oils were purchased commercially. The Congo palm oil was a gift from Dr. G. Jurriens, Unilever Ltd., who provided the TG composition obtained with $AgNO_3$ - TLC and P lipase (10,11). All natural oils were purified by elution from neutral alumina (Brockman activity grade 1) with pentane: ethyl ether (9:1) (5), monitored by TLC.

The enzyme was obtained from *G. candidum* as previously described (2, 3). The TGs or oils were emulsified in a Waring Blendor to which was added (for each flask) 8 ml of phosphate buffer (pH 6.7) containing 1% gum arabic, 0.5 ml 1% $CaCl_2$ and 200 mg of TG or 300 mg of oil. After agitation for about 5 min, 8 ml of the emulsions were transferred to 50 ml Erlenmeyer flasks and 2 ml of buffer containing 5 mg of enzyme were added. The contents were incubated with shaking in a water bath for 2 hr at 37C. Three 10 ml replicates plus two controls, one without enzyme and the other without substrate, were prepared. To stop the reaction, 0.5 ml of 20% H_2SO_4 was added to the flasks, followed by extraction of each with 250 ml of $CHCl_3$:CH_3OH (9:1) (7).

One aliquot was titrated with 0.05 N alcoholic KOH to obtain the concentration of FFA liberated. The solvent was removed from the two other aliquots and the products of digestion separated and obtained by preparative TLC. The glycerides or FFA were converted to methyl esters by acid catalyzed methanolysis and estimated by gas-liquid chromatography (GLC). The digestion products from EOO and margarine were analyzed by infrared spectrophotometry (Perkin-Elmer Infracord) to estimate both carbonyl and *trans* absorption. Appropriate standard curves were prepared from a number of glycerides containing elaidate and from methyl elaidate. All of these procedures have been described (9). The replicates were analyzed separately and the results averaged. Agreement between replicates was close. The palm oil and margarine were also digested with P lipase (9), at 37C for 5 min and the glycerides and free fatty acids analyzed as described above. Equivalent chain lengths of fatty acids (1,6) were used to provide tentative identifications of some of the acids found in cod liver and Macadamia nut oils.

Results and Discussion

The net microequivalents of FFA liberated from the substrates by *G. candidum* after 2 hr of incubation were: EOO, 110; ELL, 6; margarine, 103; safflower, 192; peanut, 201; Macadamia nut, 139; cod liver, 68; and palm, 136. Similar data from 5 min P lipase digestions of margarine and palm oil were: 205 and 343. The oils containing larger quantities of oleate supported greater GC lipase activity. With respect to palm oil and margarine, the GC lipase was less active than P lipase. When ELL was the substrate, digestion was insignificant. Therefore, the GC lipase did not remove elaidate from an otherwise saturated TG.

The fatty acid compositions of the intact TG's and of the products resulting from the lipolysis of EOO and margarine by GC and P lipases are presented in

TABLE II

Fatty Acid Composition of Several Oils and of the Fatty Acids Liberated by *G. candidum* Lipase[a]

		Fatty Acid M%																			
		10:0	10:1	12:0	14:0	15:0	16:0	16:1	17:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:5	22:0	22:1	22:2	22:5	22:6
Cod liver	TG				7.9		14.3	15.6	0.5	0.9	32.0	1.3	....		15.5	....	3.1		5.7		....	3.2
	FFA				3.2		9.1	19.6	1.5	0.8	47.5	3.0	2.5		2.5	3.8	....		3.4		3.1	....
Macadamia nut	TG	0.2	0.1	0.3	1.6	0.1	10.1	19.7		1.8	60.2	1.5	1.5		1.4					1.5		
	FFA	0.1	....	0.2	0.3	....	2.3	17.8		0.1	77.7	1.0	0.3		0.2					....		
Peanut	TG				0.2		11.2			1.3	58.0	25.7	1.0	1.1				1.5				
	FFA				....		5.4			0.4	83.7	10.5	....	....				....				
Safflower	TG						8.3			1.3	9.8	80.6										
	FFA						0.8			tr	20.8	78.4										

[a] Incubated for 2 hr at 37C.

TABLE III

Fatty Acid Composition (M%) of Congo Palm Oil and of the Products of Digestion with *G. candidum* (GC) and Pancreatic (P) Lipases[a]

Fatty acid	Intact TG		Residual TG		DG		MG		FFA	
	GC	P	GC	P	GC	P	GC	P	GC	P
10:0	tr		0.2	tr	....	....	....	....	....	tr
12:0	0.4		0.7	0.4	tr	0.4	0.1	0.3	tr	0.5
14:0	0.8		2.0	1.8	1.2	1.5	1.8	0.9	0.1	1.8
16:0	46.5		58.4	55.1	68.6	49.7	69.1	12.5	7.1	62.2
16:1	tr		tr	0.9	tr	1.7	....	0.5	0.2	tr
18:0	5.4		1.9	6.5	8.2	6.1	10.2	2.4	0.1	8.5
18:1	38.9		31.2	31.5	18.3	36.4	16.6	66.7	82.0	23.6
18:2	8.1		5.3	3.8	3.7	4.2	2.2	16.7	10.5	3.4
18:3	tr		0.3	....	....	....	....	....	....	....

[a] Digestion conditions: 5 min for P, 120 min for GC and 37C for both.

Table I. For comparative purposes, the P lipase results on EOO as reported before (9) are also tabulated in Table I. Based upon the products of digestion of EOO and margarine, GC lipase hydrolyzed oleic acid and not the *trans* isomer. The small quantity of impurity in the elaidic acid used to prepare the EOO for GC lipase hydrolysis, causes the TG figures in Table I to depart slightly from theoretical values. Complete data on margarine are included because such information has not, to our knowledge, heretofore been reported. This specificity, unique for lipases, raises intriguing questions about enzyme-substrate geometry. Since the *cis* double bond appears to be involved, the enzyme may have a relatively large active site binding both the carbonyl carbon and the double bond. The length of the last nine carbons in 18:1 may be important because, based upon the data of Table II, the 16:1 ester is hydrolyzed at a slower rate than oleate. This could be studied by synthesizing TG's containing various combinations of oleate and other positional isomers of 18:1 for use as substrates. In the case of margarine and the other oils (Tables II and III) at least 18:1 and possibly 16:1 were preferred over polyunsaturates. The *trans* acid contents in Table I are probably too high because they were reported as elaidic acid and isomers of linoelaidate were undoubtedly present.

Table II contains the fatty acid composition of intact cod liver, Macadamia nut, peanut and safflower oils and of the FFA's released therefrom by GC lipase. Again 18:1 was preferentially released, the quantities in the FFA being greater than the quantities in the TG's. Other unsaturated acids were liberated, particularly from cod liver oil. Some difficulty was encountered in GLC analysis of the cod liver oil perhaps due to the instability of highly unsaturated fatty acids. Several of the identifications here were based upon only equivalent chain lengths (1,6). The specificity of the lipase for 18:1 is most strikingly illustrated by the safflower oil data, 9.8% in the TG compared to 20.8% in the FFA and almost equal quantities of 18:2 in both components. Therefore it is obvious that 18:2 was released at a much slower rate than 18:1. Since Alford and Pierce (2) noted that the FFA from a GC lipase digestion of corn oil at −7C, contained 95% 18:2, this particular lipase may require a liquid substrate. We had previously assumed that 16:1 ester would also be hydrolyzed at the same rate as oleate, but the data in Table II do not allow this assumption. The 16:1 contents of TG and FFA in both cod liver and Macadamia nut oils did not differ greatly.

In Table III, the results for Congo palm oil, selected because it has been thoroughly analyzed (10,11), are given. Since M% of 18:1 in the FFA was 82.0, GC lipase preferentially removes this from the glycerides. The MG composition represents primarily the TG which originally contained at least two oleate residues. Thus, based upon the relatively large amount of 16:0 in the MG's GPO_2 was the major dioleoyl TG. Also, the original fat apparently contained some triolein since 18:1 was present in the MG. Similar reasoning applied to the DG data from GC lipolysis implies that these fatty acids were originally derived from TG which contained at least one 18:1 residue. If we assume that only 18:1 was liberated from the TG to form DG and that no particular oleate TG was attacked preferentially, a calculation of oleate glyceride types can be made by distributing the DG fatty acids at random among two of the three TG positions. The random proportions can be derived by expanding the polynomial $(P+S+O+L)^2$ where P is the proportion of palmitate, myristate, and laurate combined and S, O, and L are respectively the proportions of stearate, oleate and linoleate, in the DG. These calculations are presented in Table IV as are similar values from P lipase data and a strict random distribution. The random calculations were derived by distributing the fatty acid composition of the intact TG according to probability, i.e. $(P+S+O+L)^3$ and the P lipase figures were obtained by the method of Coleman (4). Only those glycerides containing oleate are tabulated in Table IV and represent 78.3% (pancreatic lipase) and 76.2% (random) of the total TG. The values have been scaled up to 100% for comparison with the GC lipase calculations. The calculations from the GC data agree more closely with the P lipase values than with those calculated from strict random predictions.

Use of GC lipase for structural studies has several limitations. *Cis*-unsaturates other than 18:1 are attacked. The preparation would be more useful if the activity and specificity could be increased. In conjunction with $AgNO_3$-TLC, GC lipase can be used

TABLE IV

Comparison of Some Palm Oil Oleoyl Triglycerides Calculated from *G. candidum* and Pancreatic Lipase Data

Glyceride types [a]	Random [b]	*G. candidum* lipase [c]	Pancreatic lipase [d]
	M%		
O_3	7.7	3.3	3.8
PO_2	28.3	25.6	24.8
SO_2	3.1	3.0	2.7
LO_2	4.9	1.4	2.7
P_2O	35.0	48.7	42.3
S_2O	0.4	0.7	0.5
L_2O	0.9	0.1	0.6
PSO	6.6	11.4	9.1
PLO	11.9	5.2	12.1
SLO	1.2	0.6	1.4

[a] Fatty acids distributed without positional consideration. P=laurate + myristate + palmitate; S=stearate; O=oleate; L=linoleate.
[b] Calculated from proportions by expansion of $(P+S+O+L)^3$, using fatty acid composition of the intact fat. Scaled up to 100%.
[c] Calculated from proportions by expansion of $(P+S+O+L)^2$ using *G. candidum* DG data and assuming that 18:1 was released from each TG to produce DG.
[d] Calculated from proportions according to Coleman. Scaled up to 100%.

to confirm P lipase data on oils containing predominantly 18:1 as the unsaturated fatty acid. More information is needed concerning the specificity of GC lipase, especially as to the relative rates of lipolysis of TG's containing other *cis* unsaturated acids and of TGs containing 18:1 in different positions. However, present evidence indicates that GC lipase is highly specific for *cis* 18:1 and that in conjunction with other methods the enzyme can be used to study the structure of TG's.

ACKNOWLEDGMENT

The sample of Congo palm oil and an analysis of the component TG's were the gift of Dr. G. Jurriens, Unilever, Ltd.

Supported in part by Public Health Service Research Grant AM-02605-07 from the Institute of Arthritis and Metabolic Diseases.

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Application of Computer Methods to the Calculation of Triglyceride Structure[1]

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Abstract

A digital computer method for the calculation of triglyceride structure using a FORTRAN program has been developed. The results of several methods of calculation and hypothesis of glyceride structure were compared with values determined experimentally. The comparison obtained with the random, restricted random, 1,3-random-2-random distribution hypotheses, as well as other proposed hypotheses, indicated that the 1,3-random-2-random hypothesis best approximated the values obtained experimentally by other investigators.

Introduction

EXPERIMENTS DESIGNED to examine the effect of dietary fat on the glyceride structure of carcass or depot fat yield large amounts of data which must be transformed into a form suitable for interpretation. Computer methods of rapid calculation become especially desirable where a long or relatively complex series of calculations must be performed repeatedly on a small amount of data. Such is the case in many of the methods for the estimation of triglyceride structure, especially when comparisons between theories of triglyceride distributions are to be made. In this report we wish to describe a FORTRAN program for the calculation of triglyceride distribution.

Procedure

A digital computer program (FORTRAN) may be written for the purpose of performing straightforward calculations since the FORTRAN machine language allows algebraic formulas to be represented in familiar form. A computer program translates the formulas, when punched in correct form, into the actual computer instructions which govern the calculations.

The FORTRAN method provides for five basic operations: Each of these operations is represented by a distinct symbol (1):

Addition	+
Subtraction	−
Multiplication	*
Exponentiation	**
Division	/

In addition, provisions are also made for certain mathematical functions. Every function has a preassigned name. In order to make use of any function (square root or exponentiation), it is only necessary to write the name of the function followed with an expression enclosed in parenthesis. The computer will then carry out the named operation.

Several versions of FORTRAN are available. The version employed in the work reported used the Control Data Corporation 1604 computer (which is capable of storing about 32,000 items).

Before one can solve a problem using this type of computer it must be outlined using a number of statements. These statements control and outline the necessary arithmetic operations, the input of data, and the final printing out of results according to a predetermined format. Statements concerning the order of execution and statements which provide additional information about the problem are included. This information in FORTRAN language is then punched out on tape using the FORTRAN 60 compiler. The program, which is now on punched tape, contains the complete instructions necessary for the solution of the problem. Sub-programs containing instructions not in the main program for the detailed solutions of the glyceride distribution equations are then written.[2] The main program is reproduced in Table I.

Mathematical equations[3] for the calculation of a random distribution are shown in Table II. The

[1] Presented at the AOCS meeting, April 1965, Houston.

[2] A very limited number of the complete computer programs are available from the authors.

[3] S = saturated fatty acids; U = unsaturated fatty acids. S_3, S_2U, SU_2 and U_3 represent the four possible types of glycerides in terms of their S and U content without regard to position. SUS, SSU, USU and UUS represent structurally the varieties possible in terms of S and U content when the position is indicated in the sequence. SUS and SSU are therefore isomers comprising S_2U.