Thermoxidation of Substrate Models and Their Behavior During Hydrolysis by Porcine Pancreatic Lipase

Ruth Arroyo[a], Francisco J. Sánchez-Muniz[a], Carmen Cuesta[b], José V. Sinisterra[c], and José M. Sánchez-Montero[c,*]

[a]Departamento de Nutrición y Bromatología I (Nutrición), Sección Lípidos, [b]Instituto de Nutrición y Bromatología (CSIC-UCM), [c]Departamento de Química Orgánica y Farmaceútica, and [a,b,c]Facultad de Farmacia, Universidad Complutense de Madrid, Spain

ABSTRACT: The behavior of thermoxidized triacylglycerols during hydrolysis catalyzed by porcine pancreatic lipase was evaluated using nonpolar triacylglycerols isolated from palm olein (NPTPO), triolein, and *sn*-1,3 diolein substrates. Substrates were thermoxidized at 180ºC for 1 to 4 h. Owing to formation of polymers and dimers of triacylglycerols, the molecular weight of the thermoxidized substrates increased. After 1 h heating, the concentration of polymers and dimers was similar for the *sn*-1-3 diolein and triolein samples but higher in NPTPO samples. Conjugated double bonds were formed in all samples, and α,β-unsaturated carbonyl compounds developed through allylic oxidations. These caused increased ultraviolet absorbance at 232 nm. The hydrolysis of heated and unheated samples by the lipase can be described by a Michaelian equation. The enzyme showed a higher apparent V_{max} and K_M with heated *sn*-1,3 diolein and triolein than with their unheated counterparts. This was due to the generation of polar compounds which acted as emulsifiers and which favored the formation of an oil/water microemulsion. This behavior was not observed in NPTPO, where heating decreased the apparent V_{max} and K_M over the first 2 h. Later, a tendency to increase these values was observed. The results could be explained by a balance between concentration of surfactants and of natural emulsifiers in the thermoxidized samples.
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Lipases (E.C. 3.1.1.3) are characterized by their ability to catalyze the hydrolysis of ester bonds at the interface between an insoluble phase (triacylglycerols) and an aqueous phase in which the enzyme is dissolved (1). Likewise, lipases have the important physiological role of preparing the fatty acids of water-insoluble triacylglycerols for absorption and transport through membranes by converting the triacylglycerols to more polar diacylglycerols, monoacylglycerols, free fatty acids, and glycerol. With the exception of glycerol, these products are absorbed as mixed micelles from the lumen of the small intestine (2) or by lateral diffusion in a continuous water layer extending into the target cell (3).

Porcine pancreatic lipase is extensively used as gastrointestinal tract lipase in experimental systems since it is cheap and widely available. This enzyme shows 86% homology with human pancreatic lipase (4).

Thermoxidized oils, such as those produced by repeated frying, contain a complex mixture of products such as oxidized triacylglycerols, triacylglycerol dimers, and triacylglycerol polymers. These are mainly associated with alterations of the physicochemical properties of the fat (5–7). As a consequence, the oil/water interface can change and the lipolytic activity of the lipase can be altered. However, few studies related to the hydrolysis of thermoxidized oils by pancreatic lipase have been reported (8–10). In a previous paper (11) a study was made of the effects of the complex products originating in palm olein or sunflower oils used in successive frying of potatoes upon the activity of porcine pancreatic lipase. No relevant changes in the enzyme's apparent K_M or V_{max} were seen.

The aim of this work was to study the effect of thermoxidized acylglycerol models upon pancreatic lipase activity, as a tool in understanding lipase hydrolysis of thermoxidized oils. Model acylglycerols such as triolein, *sn*-1,3 diolein, and the nonpolar triacylglycerol fraction of palm olein (NPTPO) were studied as substrate models for porcine pancreatic lipase. These substrates were thermoxidized by heating at 180°C in the presence of oxygen. The complex reaction products made during this process were separated and quantified using various chromatographic techniques. Owing to the great variety of compounds present in thermoxidized oils, these altered compounds were grouped using high-performance size exclusion chromatography (HPSEC) (12). The concentrations of these compounds were then related to changes in the kinetic characteristics of the reaction catalyzed by the pancreatic lipase.

*To whom correspondence should be addressed at Departamento de Química Orgánica y Farmaceútica, Facultad de Farmacia, Universidad Complutense, E-28040 Madrid, Spain. E-mail: jsanchez@eucmax.sim.ucm.es.

EXPERIMENTAL PROCEDURES

Materials. Palm olein was provided by Agra S.A. (Bilbao, Spain). Triolein, *sn*-1,3 diolein, monoolein, oleic acid, and lipase (E.C. 3.1.1.3) from porcine pancreas (Type II) were obtained from Sigma Chemical Co. (St. Louis, MO). NPTPO were obtained as indicated below. Silica gel 60 F_{250} (63 to 200 µm) for column chromatography and silica gel 60 (20 × 20 cm, 0.2-mm layer thickness) for thin-layer chromatography were obtained from E. Merck (Darmstadt, Germany). All other chemicals used were of the purest grade available. Aqueous solutions were made with double-distilled water.

Thermoxidation of acylglycerol models. Thermoxidation of triolein, *sn*-1,3 diolein, and NPTPO was performed in an open reactor by heating at 180°C in an oil bath. Each lipid sample (500 mg) was placed in 25-mL glass vessels and immersed in a thermostatically controlled oil bath at 180°C. The heating time was chosen depending on the sample: *sn*-1,3 diolein was heated for 1 h, triolein was heated for 1 to 2 h, and NPTPO for 1 to 4 h. All samples were kept at −20°C in a nitrogen atmosphere until use.

Absorbance of samples in the ultraviolet (UV)-visible region was measured using a Shimadzu 2100 spectrophotometer (Kyoto, Japan) with thermostatically controlled cuvettes at 25°C. To keep the absorbance lower than 1.00 (O.D.), samples were dissolved in tetrahydrofuran (THF) at different concentrations (M) depending on the specific extinction coefficient value (ε) of each standard. UV-visible spectra were recorded between 210 and 600 nm. Calibration curves were made at 232 nm for unaltered standard samples. The corresponding equations for sample absorbance were:

$$\text{Triolein: Abs. (O.D.)} = 81.3 \cdot [C\,(M)] - 0.0085 \quad (r = 0.994) \quad [1]$$

$$\textit{sn}\text{-1,3 Diolein: Abs. (O.D.)} = 64.3 \cdot [C\,(M)] + 0.0172 \quad (r = 0.969) \quad [2]$$

$$\text{NPTPO: Abs. (O.D.)} = 278.2 \cdot [C\,(M)] + 0.0125 \quad (r = 0.995) \quad [3]$$

After heating, the components of triolein and NPTPO were analyzed by a combination of adsorption and size exclusion chromatographic techniques.

(i) Determination of polar and nonpolar compounds (polar material refers to the total amount of thermoxidized products formed by heating). This was performed using silica column chromatography following the method of Waltking and Wessels (13) with a modification of the proportion of hexane/diethyl ether used to fill the column and to elute the nonpolar fraction (14). Polar components were eluted with diethyl ether. The efficiency of the separation was checked by thin-layer chromatography to guarantee the absence of nonaltered triacylglycerols in the polar fraction (14).

(ii) Distribution of polar compounds by HPSEC. Polar compounds were analyzed following the method of Dobarganes *et al*. (12), using a Waters 501 chromatograph (Milford, MA) with a 20-µL sample loop. A Waters 410 refractive index detector and two 0.01-µm and 0.05-µm (5 µm particle size) pL gel (polystyrene–divinylbenzene) columns (7.5 × 300 mm) (Hewlett-Packard, Palo Alto, CA) connected in series and at 40°C were used. High-performance liquid chromatographic-grade THF served as the mobile phase at a flow rate of 1 mL/min. Sample concentration was between 10 and 15 mg/mL in THF. All eluents and samples were precleaned by passing them through a 2-µm filter. The concentration of each compound was calculated as previously described (15). Thermoxidized *sn*-1,3 diolein was directly analyzed by HPSEC without previous separation by column chromatography. The average molecular weights of substrates were calculated from a calibration curve (11,15) using the HPSEC technique with triolein, diolein, monoolein, and oleic acid as standard samples. The calibration curve equation obtained was:

$$\log \text{MW} = 6.87 - 0.3\ \text{rt} \quad (r = -0.998) \quad [4]$$

where MW is molecular weight; rt is retention time (min).

Enzyme assays. Triolein or NPTPO (0.225 mL) and 0.675 mL Tris-HCl buffer 0.01 M (pH 8.3) were emulsified with 2.1 mL emulsification reagent (16) using an ultrasonic generator (sonifier 450; Branson, Danbury, CT) equipped with a 10-mm probe. The probe was placed in the bulk of the solution to be emulsified. Output was set at 55 W for 2 min. The preparation was then left to stand for 1 min. This operation was repeated twice. In enzyme assays using *sn*-1,3 diolein, the substrate was prepared by emulsifying 0.105 mL of this lipid and 0.795 mL Tris-HCl buffer 0.01 M (pH 8.3) with 2.1 mL emulsification reagent for 2 min, twice. The emulsification reagent (1 L) contained glycerol (540 mL), arabic gum (10 g), NaCl (17.9 g), KH_2PO_4 (0.41 g), and bidistilled water.

Titrimetric assay was used to determine the enzyme's lipolytic activity. This activity was monitored in a Metrohm pH-stat [Impulsomat 614, Dosimat 665 (with microstirrer), pH-meter 691] (Herisau, Switzerland). Several NaOH solutions (5 to 50 mM) were used as titrating agents. The reaction mixture consisted of different volumes of substrate emulsion (varying from 10 to 150 µL), 1 mL of lipase solution (10 mg/mL) and 0.01 M Tris-HCl buffer (pH 8.3), up to a total volume of 10 mL. In a typical procedure, Tris-HCl buffer (pH 8.3) and substrate emulsion were placed in the thermostatically controlled cuvette of the pH-stat and maintained at 37ºC. The mixture was shaken vigorously for 10 min. Stirring rate was adjusted to approximately 1300 rpm. Then, 1 mL of lipase solution (10 mg/mL) in Tris-HCl buffer (pH 8.3) was added.

In all cases, lipase activity was measured as the initial reaction rate to avoid the possible inhibition that might take place owing to the appearance of reaction products. The slopes of the initial linear stretch of the kinetic curves were determined graphically. Experiments were performed in triplicate. Maximum deviations from the mean were less than 5%. Specific lipase activity was defined as the µmol of free fatty acids released per min and per mg of crude enzyme.

Statistical analysis. Data from the different samples were compared by one-way analysis of variance (ANOVA) of re-

peated measurements, followed by multiple-range analysis. Curve fitting and statistical treatment of the data were performed using the SIMFIT statistical package (17). The kinetic behavior of the thermoxidized and nonthermoxidized oils were considered statistically different when their confidence limits did not overlap.

RESULTS AND DISCUSSION

UV-visible spectra and absorbance at 232 nm of thermoxidized and nonthermoxidized acylglycerol models. Figures 1–3 show the UV-visible spectra of heated and unheated triolein, *sn*-1,3 diolein, and NPTPO. The parent samples showed maximum UV absorption at approximately 210 nm owing to the presence of the chromophores CH_2–CH=CH–CH_2– and –COO– from the oleic acid chain of the acylglycerol molecules. The ε obtained at 232 nm were 81.3 $cm^{-1}M^{-1}$, 64.3 $cm^{-1}M^{-1}$, and 278.2 $cm^{-1}M^{-1}$ for unheated triolein, *sn*-1,3 diolein, and NPTPO. The ε_{232} value for 1,3 diolein is lower than the ε_{232} value for triolein because 1,3 diolein has four chromophores of two different types (2 C=C and 2 C=O from two chains of oleic acid) whereas triolein presents six chromophores of the same type (3 C=C and 3 C=O from three chains of oleic acid). For NPTPO, the high ε_{232} value obtained might be due to the presence in the NPTPO of some minor compounds such as tocopherols, tocotrienols, and β-carotenes, which have conjugated groups (–CH=CH–CH=CH–) (18). These compounds belong to the unsaponifiable matter and elute together with the NPTPO in column chromatography, giving a yellowish color to the sample.

When samples were heated at 180°C, there was an increase of UV absorbance at 232 nm owing to formation of conjugated double bonds and α,β-unsaturated carbonyl compounds (19). Figures 1–3 show the absorption spectra of triolein heated for 1 and 2 h, 1,3 diolein heated for 1 h, and NPTPO heated for 1 to 4 h. Heating produced a shift of their absorption bands toward higher wavelengths. Consequently, the terminal absorption of unheated triolein at 210 nm was shifted to 211.5 nm and 217 nm after 1 h and 2 h of heating, respectively. In the case of thermoxidized NPTPO, the absorption band showed a maximum at 225 nm.

TABLE 1
Ultraviolet Absorbance at 232 nm Before and After Thermoxidation at 180°C in Open Oil Bath

Sample	Heating time (h)	Concentration ($M \cdot 10^3$)	Absorbance at 232 nm	$\varepsilon_{heated}/\varepsilon_{nonheated}$ [a]
Triolein	0	2.934	0.237	1 [b]
	1	0.648	0.398	7.55
	2	0.368	0.452	15.11
sn-1,3 Diolein	0	5.640	0.432	1 [b]
	1	0.814	0.453	8.65
NPTPO	0	1.15	0.310	1 [b]
	1	0.62	0.638	3.70
	2	0.41	0.665	5.83
	4	0.49	0.667	4.89

[a]Specific extinction coefficient, ε.
[b]Reference value. NPTPO, nonpolar triacylglycerol fraction of palm olein.

Table 1 shows the values of absorbance at 232 nm against the concentrations of unheated and heated models. Sample concentrations were different depending on the degree of alteration; this determines absorbance at 232 nm. The $\varepsilon_{heated}/\varepsilon_{nonheated}$ ratio increased with heating time for triolein and *sn*-1,3 diolein. For NPTPO, the $\varepsilon_{heated}/\varepsilon_{nonheated}$ ratio increased over the first 2 h of heating to reach an almost steady state during further heating. This is related to the establishment of an equilibrium between the formation rate of conjugated dienes and polymers. This occurs *via* the Diels-Alder reaction (20) .

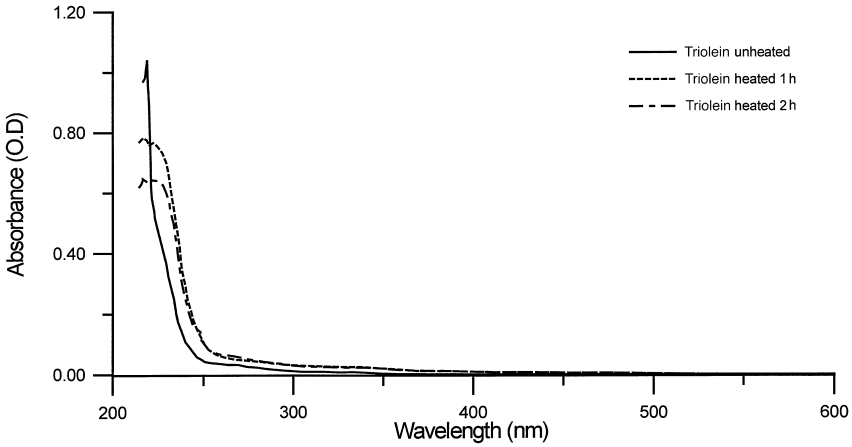

FIG. 1. Ultraviolet (UV)-visible spectra of nonthermoxidized triolein and of triolein when thermoxidized at 180°C for 1 and 2 h. Conditions: the solvent was tetrahydrofuran (THF), sample concentrations were $2 \cdot 10^{-3}$ M, $0.65 \cdot 10^{-3}$ M, and $0.37 \cdot 10^{-3}$ M for triolein, and triolein was heated for 1 h and for 2 h. UV-visible spectra were recorded between 210 and 600 nm in a Shimadzu 2100 spectrophotometer (Kyoto, Japan) with thermostatically controlled cuvettes at 25°C.

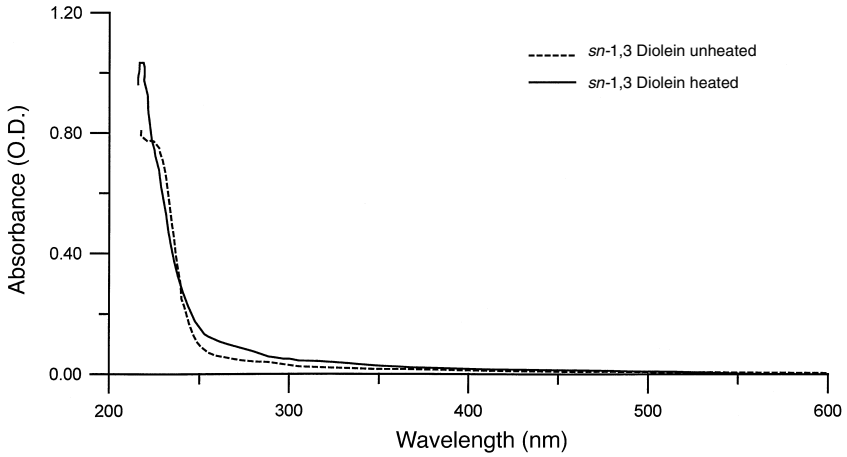

FIG. 2. UV-visible spectra of nonthermoxidized *sn*-1,3 diolein and of *sn*-1,3 diolein after thermoxidation at 180°C for 1 h. Conditions: sample concentrations in THF were $2.4 \cdot 10^{-3}$ M for unheated *sn*-1,3 diolein and $0.81 \cdot 10^{-3}$ M for the same when heated for 1 h. For conditions and abbreviations see Figure 1.

Composition of thermoxidized and nonthermoxidized acylglycerol models. During the heating of triolein and NPTPO in the presence of air, both thermal and oxidative alteration may take place, resulting in the formation of the polar compounds mentioned above. Tables 2 and 3 show the changes produced in the polar content of thermoxidized triolein and NPTPO. For the same thermoxidation period, the polar content increased more rapidly for triolein than for NPTPO. This was due to (i) the greater percentage of unsaturated fatty acids in triolein than in NPTPO since oxidation rate is roughly proportional to the degree of unsaturation of the fatty acids in the sample (7); and (ii) the presence of natural antioxidants (<0.1%) from the unsaponifiable fraction of the palm olein; these are present in NPTPO (18) and would prevent the oxidation of NPTPO fatty acids. These antioxidants are not present in pure triolein.

The use of HPSEC allows the separation and quantification of the different compounds that constitute the polar component of triolein and NPTPO (triacylglycerol polymers, triacylglycerol dimers, and oxidized triacylglycerol monomers) which increased in both triolein and NPTPO samples as a result of heating (Tables 2 and 3). However, during heating, oxidized triacylglycerols were formed in greater proportion than triacylglycerol polymers and dimers. More thermoxidative alterations took place in triolein than in NPTPO for the same heating time. The concentration of total polar compounds of triolein was around double that in NPTPO after 2 h of heating. Triolein and NPTPO, after 2 and 4 h heating, respectively, reached a rather similar degree of polymerization: 15.9 vs. 16.6 mg/100 mg sample of triacylglycerol polymers and 19.6 vs. 14.8 mg/100 mg sample of triacylglycerol dimers.

Table 4 shows the composition of the *sn*-1,3 diolein sam-

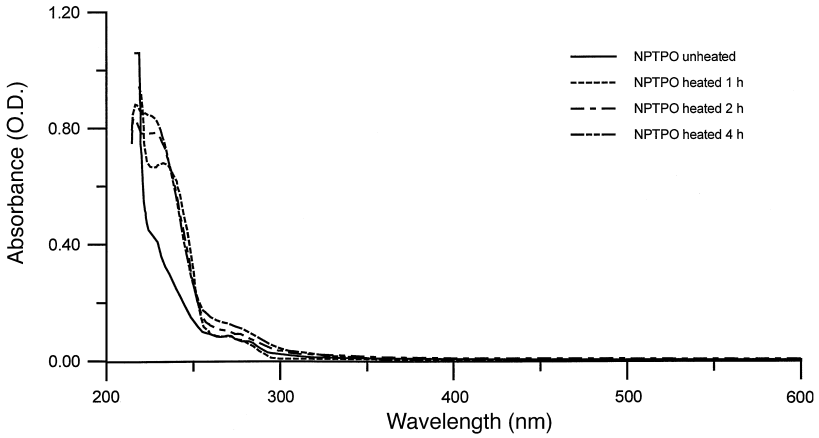

FIG. 3. UV-visible spectra of nonthermoxidized nonpolar triacylglycerol fraction of palm olein (NPTPO) and of NPTPO following thermoxidation at 180°C for 1, 2, and 4 h. Conditions: the solvent was THF, sample concentrations were $1.1 \cdot 10^{-3}$ M, $0.62 \cdot 10^{-3}$ M, $0.41 \cdot 10^{-3}$, and $0.49 \cdot 10^{-3}$ M for unheated NPTPO, and NPTPO heated for 1, 2, and 4 h, respectively. For conditions and abbreviations see Figure 1.

TABLE 2
Composition of Triolein Before and After Thermal Oxidation at 180°C in Oil Open Bath

	Heating time (h)		
Compounds[a]	0	1	2
NPTPO	100	58.39	22.31
Total polar content	—	41.61	77.69
Triacylglycerol polymers	—	2.90 ± 0.03[a]	15.9 ± 0.02[b]
Triacylglycerol dimers	—	10.1 ± 0.04[a]	19.7 ± 0.03[b]
Oxidized triacylglycerols	—	28.6 ± 0.08[a]	42.1 ± 0.01[b]

[a]Values are means of two samples ± SD expressed in mg/100 mg sample except for NPTPO and total polar content, where one determination was made. Values in the same row bearing a different superscript letter (a,b) are significantly different. For abbreviation see Table 1.

TABLE 4
Composition of *sn*-1,3 Diolein Before and After Thermal Oxidation at 180°C in Oil Open Bath

	Heating time (h)	
Composition[a]	0	1
Diacylglycerol polymers	0	3.80 ± 0.24
Diacylglycerol dimers	0	12.1 ± 0.42
Diacylglycerol monomers (oxidized + nonoxidized)	100	83.8 ± 0.58
Monoacylglycerols	0	0.25 ± 0.05
Fatty acids	0	0.11 ± 0.02

[a]Values are means of two samples ± SD expressed in mg/100 mg sample. Values measured after 1 h heating are all signicantly different from those of unheated samples.

ple after 1 h at 180°C in an open oil bath. Column chromatography separation of thermoxidized and nonthermoxidized *sn*-1,3 diolein was not performed because all the components that constitute this sample elute into the polar fraction. For this reason, the thermoxidized *sn*-1,3 diolein was directly analyzed by HPSEC. The chromatographic peak of diacylglycerol monomers corresponds to nonaltered diacylglycerols plus oxidized diacylglycerols. It is possible that alteration in *sn*-1,3 diolein occasioned by heating was similar to that produced in triolein, since polymer and dimer contents of both samples were rather similar (Tables 2 and 4).

Average molecular weights of thermoxidized and nonthermoxidized acylglycerol models. As mentioned earlier, heating of the acylglycerol models produced changes in their composition that in turn modified their respective molecular weights (Table 5). To carry out a study of pancreatic lipase activity in several thermoxidized and nonthermoxidized acylglycerol models, we needed a way to establish the substrate concentration in the reactor. Because of the absence of acylglycerol models with higher molecular weights than triacylglycerols (≈900), an extrapolation to the Equation 4 (see the Experimental Procedures section) was performed. In a previous paper we demonstrated that errors in molecular weight calculations were lower by using equations such as 4 than by applying other calibration curve equations obtained with different chemical compounds (11). Table 5 shows the estimated average molecular weight of thermoxidized and nonthermoxidized acylglycerol models. Values in brackets correspond to real molecular weights of triolein and 1,3 diolein. The validity of this methodology is given by the lower deviations (<2%) of estimated molecular weight with respect to real values in the case of triolein and *sn*-1,3 diolein. As can be observed, average molecular weights of acylglycerols increased in parallel with the formation of oligomers.

Activity of pancreatic lipase on thermoxidized and nonthermoxidized acylglycerol models. In an attempt to determine if kinetic behavior of the enzyme was influenced by compounds produced during heating, hydrolysis rate vs. substrate concentration ($V - [S]$) studies were performed and the corresponding kinetic parameters were calculated. The concentration of each substrate was determined using its respective average molecular weight (Table 5). Figures 4 and 5 show the enzymatic hydrolysis of heated and nonheated acylglycerol models, respectively. Figures 4A and 5A show that, in all cases, the reaction can be described by

TABLE 3
Composition of NPTPO Before and After Thermal Oxidation at 180°C in Oil Open Bath

	Heating time (h)			
Composition[a]	0	1	2	4
NPTPO	100	83.72	63.92	39.52
Total polar content	0	16.28	36.08	60.48
Triacylglycerol polymers	0	1.30 ± 0.01[a]	5.88 ± 0.03[b]	16.6 ± 0.12[c]
Triacylglycerol dimers	0	5.16 ± 0.02[a]	10.7 ± 0.02[b]	14.8 ± 0.03[c]
Oxidized triacylglycerols	0	9.82 ± 0.04[a]	19.5 ± 0.05[b]	29.1 ± 0.10[c]

[a]Values are means of two samples ± SD expressed in mg/100 mg sample except for NPTPO and total polar content, where one determination was made. Values in the same row bearing a different superscript letter (a,b,c) are significantly different. For abbreviation see Table 1.

TABLE 5
Average Molecular Weights (determined by high-performance size exclusion chromatography) of Triolein, *sn*-1,3 Diolein, and NPTPO Before and After Thermal Oxidation at 180°C in an Open Oil Bath

Heating time (h)	Average molecular weight
Triolein	
0	903.6 (855.4)[a]
1	974.5
2	1202
sn-1,3 Diolein	
0	623.7 (621)[a]
1	722.6
NPTPO	
0	851.8
1	919.1
2	1000
4	1109

[a]True molecular weight values are in parentheses. For abbreviation see Table 1.

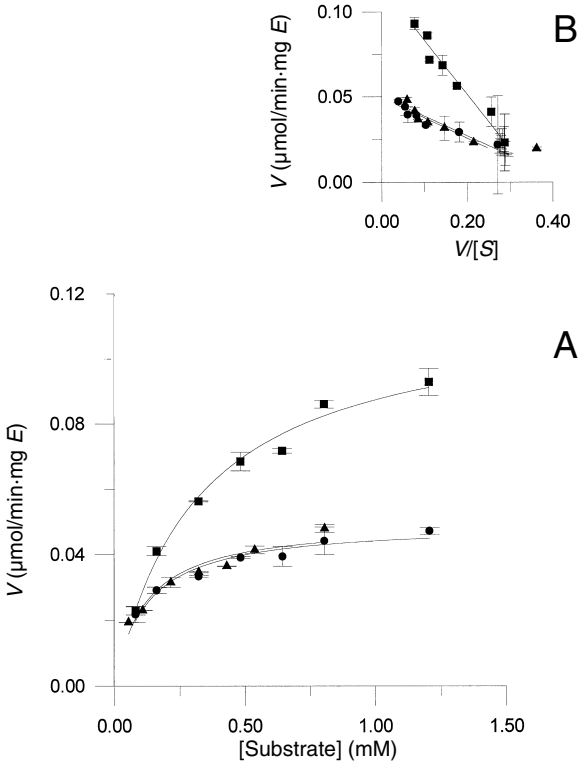

FIG. 4. (A) Activity of porcine pancreatic lipase on nonthermoxidized acylglycerol models: triolein (●), *sn*-1,3 diolein (▲), and NPTPO (■). Conditions were: [Substrate] ranging from $0.5 \cdot 10^{-1}$ mM to 1.2 mM, [Enzyme] = 1 mg/mL, 0.01 M Tris-HCl buffer (pH 8.3), temperature = 37°C. The solid line corresponds to computer fitting of the data to the Michaelis–Menten equation. Error bars refer to 95% confidence limits using the corresponding Student's *t*-test. (B) The same results expressed using the Eadie–Hofstee plot. *E* = enzyme; *V* = hydrolysis rate; *S* = substrate. For other abbreviation see Figure 3.

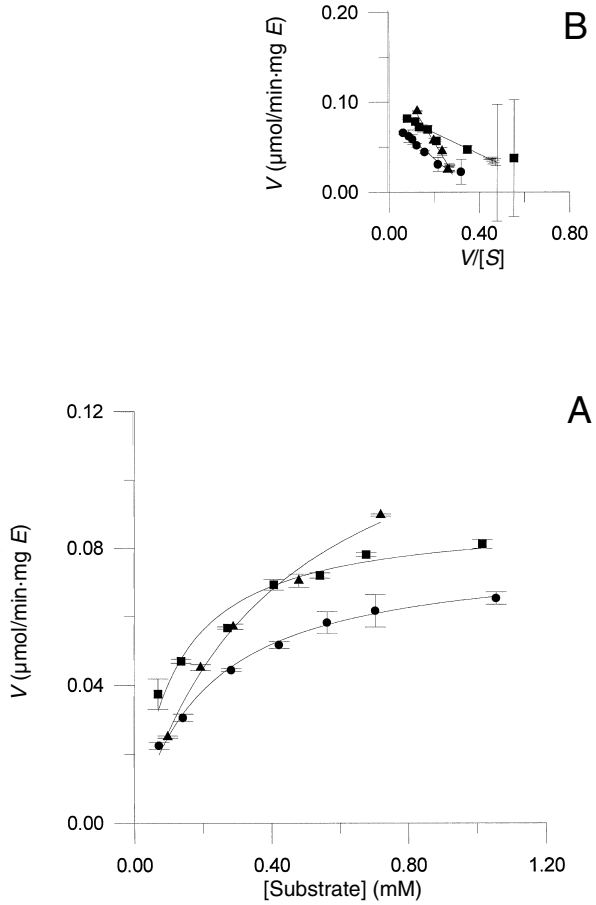

FIG. 5. (A) Activity of porcine pancreatic lipase on thermoxidized acylglycerol models: triolein 1 h (●), *sn*-1,3 diolein 1 h (▲), and NPTPO 2 h (■). For conditions see Figure 4. (B) The same results expressed using the Eadie-Hofstee plot. For abbreviations see Figures 3 and 4.

Michaelis–Menten saturation kinetics, in agreement with the straight line observed in the Eadie–Hofstee plot (Figs. 4B and 5B). Nevertheless, a true Michaelis–Menten mechanism, cannot be accepted for this interfacial catalysis as was explored by Fersht (21). Each data set was fitted to the formal Michaelis–Menten equation by nonlinear regression and the corresponding apparent V_{max} and K_M were determined. In this interfacial mechanism, K_M has the meaning of substrate concentration.

The effect of acylglycerol thermoxidation on the Michaelis–Menten parameters is shown in Table 6. For the enzymatic hydrolysis of nonheated triolein and *sn*-1,3 diolein, the apparent V_{max} and K_M obtained were very similar. This is as expected, since porcine pancreatic lipase acts in both cases on the same type of linkage and at the same position in the acylglycerol molecule. However, the enzymatic hydrolysis of nonheated NPTPO presented higher values for apparent V_{max} and K_M than both unheated triolein and *sn*-1,3 diolein. This is probably more related to physicochemical differences in the substrate emulsion than to differences in the composition of acylglycerols. The NPTPO sample contains natural emulsifying compounds (18) that increase the stability of the oil/water interface. Thus, the apparent V_{max} appears higher with NPTPO when these compounds are present. Apparent K_M values can be similarly explained taking into account that they represent the interface concentration at which the rate is apparent $V_{max}/2$ (21).

In agreement with the results observed for the nonthermoxidized triolein and *sn*-1,3 diolein, Michaelian behavior was observed when thermoxidized triolein and *sn*-1,3 diolein were hydrolyzed by porcine pancreatic lipase. The thermoxidation produced in the substrates causes (i) an increase of conjugated C=C bonds as observed by UV spectroscopy, (ii) an increase in total polar content and polymerization, and (iii) an increase in the average molecular weight of the substrate. These phenomena would explain the change in the enzyme's kinetic behavior. It can be observed that the longer the thermal treatment, the greater the apparent V_{max}, but no strong influence is observed in apparent K_m. This finding may be related to differences in the catalytic process at the interface for thermoxidized and nonthermoxidized models since the chemical process produced was the same in all cases and the presence of C=C bonds in the acid chain far away from the ester group would not be important in the hydrolytic process. Thus,

TABLE 6
Kinetic Parameters for Hydrolysis by Porcine Pancreatic Lipase of Glycerolipids Before and After Thermal Oxidation at 180°C

Substrate[a]	Time (h)	K_M (mM)·10	V_{max} (μmol/min mg *E*)·10^3	V_{max}/K_M (μmol min^{-1} mg E^{-1}/ mM)·10
Triolein	0	1.2 ± 0.2[a]	49.6 ± 2.1[a]	4.2 ± 0.9[a,b]
	1	2.3 ± 0.3[b]	79.3 ± 3.0[b]	3.8 ± 0.6[a]
	2	1.6 ± 0.3[a,b]	92.0 ± 5.1[c]	5.8 ± 1.4[b]
sn-1,3 Diolein	0	1.2 ± 0.2[a]	50.1 ± 2.4[a]	4.2 ± 0.9[a]
	1	4.1 ± 0.4[b,b]	138.3 ± 7.1[b,b]	3.4 ± 0.5[a]
NPTPO	0	3.2 ± 0.4[a,b,c]	115.6 ± 4.8[a,b,c]	3.6 ± 0.6[a]
	1	1.5 ± 0.1[b,b,c]	105.6 ± 2.4[a,b,c]	7.0 ± 0. 6[b,b,c]
	2	1.2 ± 0.1[b]	89.5 ± 2.8[b]	7.4 ± 0.8[b]
	4	2.5 ± 0.4[a]	92.2 ± 5.8[b]	3.7 ± 0.8[a]

[a]Values (means ± confidence limits) for the same substrate bearing a different superscript letter (a,b) are significantly different.
[b]Significantly different from triolein for the same duration of heating.
[c]Significantly different from 1,3 diolein. V_{max} = hydrolysis rate; K_M = Michaelis–Menten constant. For other abbreviation see Table 1.

the different catalytic behavior observed is probably related to the different interface properties of the heated and unheated samples.

The results suggest that the polar compounds formed during thermal treatment could act as polar surfactants favoring the formation of a microemulsion. Blumenthal (22) showed that the more altered the fat, the higher the production of surfactants. The differences observed in the behavior of the samples after 1 h heating (*sn*-1,3 diolein presented higher apparent V_{max} and K_M than triolein) may be explained by the thermoxidation of *sn*-1,3-diolein producing a higher percentage of polar compounds than triolein. These compounds are amphiphilic and can stabilize the microemulsion favoring the enzymatic activity of lipase (V_{max}) and the concentration of substrate molecules at the interface (K_M).

For NPTPO, the presence of thermoxidized acylglycerols seems to exert an opposite effect on the kinetics of lipase hydrolysis compared with that of triolein or *sn*-1,3 diolein. This is not easy to explain but could be a consequence of a balance between the decrease in natural emulsifiers present in NPTPO and the increased production of surfactants. According to Yuki and Ishikawa (23), the concentration of tocopherols significantly decreases as a consequence of heating. Furthermore, thermoxidation of samples has been shown to produce surfactants (22). It can be hypothesized that after 2 h of thermoxidation, the natural emulsifier in NPTPO has more or less disappeared. This would explain the lower apparent K_m and V_{max} at this time. Later the increased production of polar surfactants would explain the increase in the K_m at 4 h with respect to NPTPO samples heated for 2 h.

From these results it can be deduced that under these experimental conditions the formation of polar compounds in the thermoxidation of substrate models affects the kinetic behavior of porcine pancreatic lipase. The presence of natural tensoactives in the NPTPO samples, but not in triolein and *sn*-1,3 diolein, explains the differences in the kinetic behavior of porcine pancreatic lipase in the hydrolysis of these thermoxidized and nonthermoxidized substrate models.

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