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A PROTEINACEOUS COMPETITIVE INHIBITOR OF LIPASE ISOLATED FROM *HELIANTHUS ANNUUS* SEEDS

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Abstract—A proteinaceous inhibitor of lipase was isolated and partially purified from confectionery and high-oil type sunflower seed (*Helianthus annuus* L.) by ammonium sulphate fractionation. The 50% of saturation fraction contained most of the proteinaceous lipase inhibitor activity from both seed types. Gel chromatography of dialysed preparations of this fraction indicated that the inhibitor protein has a M_r of *ca* 70000 determined from calculated distribution coefficients (K_d) on G-150 and G-200 Sephadex columns. Complete inhibition of hog pancreas and *C. cylindracea* lipases was achieved when mixed 1:1 (v/v) with the sunflower protein, even though substrate specificity for these two lipases was significantly different on cottonseed and olive oils. The sunflower protein was a competitive inhibitor of *C. cylindracea* lipase during lipolysis of sunflower oil. The apparent V_m determined from the inhibited and uninhibited reactions was 5.5×10^{-6} mol FFA/sec/mg protein. The apparent K_m determined for the uninhibited reaction with sunflower oil was 4.1 M and 6.2 M for the inhibited reaction. The formation of a tightly-bound inhibitor protein-lipase complex probably occurred since free fatty acid composition was unaltered during lipolysis in the inhibited reaction and oil substrate inhibition was approximately the same in both inhibited and uninhibited reactions.

INTRODUCTION

A lipase inhibitor protein has been isolated from soybean seeds and separated into three active fractions. This inhibitor has been shown to be effective on *Aspergillus niger*, *Rhizopus delemar*, castor bean, porcine, and horse pancreatic lipases [1–3] as well as the lipases from corn, cotton, and rapeseeds [4]. The soybean protein was found to be a competitive inhibitor of porcine pancreatic lipase [2], and to be specific for this lipase since inhibition was ineffective on non-lipolytic enzymes [1]. A M_r of 77000 was determined for the fraction with the highest inhibitor activity [2]. A M_r of 70000 has also recently been determined for the soybean lipase inhibitor protein [3]. Lipolytic inhibitor activity in various protein fractions have also been demonstrated in several other oilseeds, including sunflower seed [4]. However, since no kinetic data were given, the exact mode of the sunflower protein inhibition remains unknown.

Currently, the physiological role of lipase inhibitors is not known; although other seed proteinaceous inhibitors are thought to regulate certain metabolic pathways [1, 3, 5]. It has been observed that during germination of certain oilseeds, the *in vivo* lipid degradation does not always correspond to measured lipase activity [6, 7]. Such discrepancy is thought to be caused by the presence of these natural inhibitors [4]. No evidence has been reported in the literature to support a regulatory role these lipase inhibitor proteins may have in lipid mobilization during oil seed germination. However, these lipolytic inhibitors along with other seed proteinaceous inhibitors, could perform a protective-type role that would insure seed survival during adverse storage environments before germination, since lipases from animal, plant, and microbial origins are effectively inhibited by these proteins [1–4].

Earlier attempts to isolate and assay sunflower seed lipase in this laboratory were only partially successful; however, during the process a very active lipase inhibitor protein was found in confectionery and high-oil type sunflower seeds. Although the mechanism of inhibition by the soybean protein is well-documented [3, 8, 9], there is no information currently available in the literature on the type of inhibition which occurs between sunflower inhibitor protein and lipases. Enzyme-inhibitor kinetics could provide information to better understand the interaction between lipase and the inhibitor protein. The purpose of this study was to investigate some of the physical characteristics of sunflower seed inhibitor protein by partial purification and its mode of inhibition by enzyme reaction kinetics using natural oil substrates.

RESULTS AND DISCUSSION

The results of gel chromatography indicated the lipase inhibitor isolated from sunflower seed (confectionery and high-oil type) to be a medium-size protein. The inhibitor protein eluted behind two major protein peaks on the G-150 column, but was retained almost twice as long on a similar size G-200 column at the same elution rate (Fig. 1). Distribution coefficients (K_d) for the inhibitor protein were calculated to be 0.27 on the G-150 column and 0.41 on the G-200 column, based on elution volume at maximum inhibitor activity. The K_d values corresponded to M_r of 69000 and 71000, respectively, and yielded an

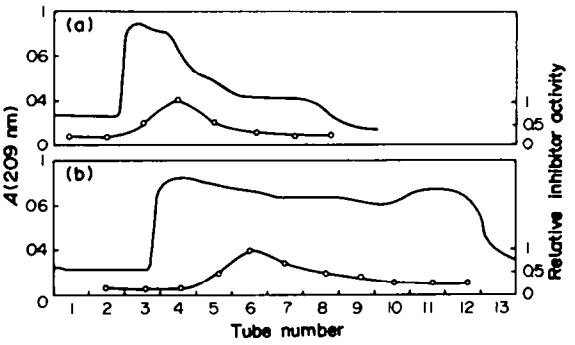

Fig. 1. A Elution profile of dialysed confectionery sunflower seed protein measured at 209 nm (solid line) and inhibitor activity (O——O) on 2.7 (i.d.) × 28 cm Sephadex G-150. Elution rate was 0.5 ml/min with 0.1 M Tris–HCl (pH 8), with 8 ml eluant collected per tube. B, Same as above except on Sephadex G-200.

estimated M_r 70 000 for the inhibitor protein [10–12]. The sunflower inhibitor protein M_r is similar to that of the soybean inhibitor protein which is reported to be 77 000 [2] and more recently 70 000 [3].

Preliminary efforts to reduce oil substrate concentration by dilution with organic solvents in determining reaction velocities were found to inhibit the action of *C. cylindracea* lipase. Chloroform, hexane, toluene, and ethylacetate reduced lipolysis about 80–90% while ethylcaprylate and cyclohexane were about 50–60% inhibitory. Heptane and tetradecane were the least inhibitory, but reduced lipolysis by 30% (Table 1). Consequently, oil substrate concentration could not be reduced by these solvents and, therefore, reciprocal plots could not be obtained in unstirred reactions. Tween-20 and Tween-40 also significantly reduced lipolysis, and higher levels of free myristic and palmitic acids were obtained with Tween-20, indicating possible esterase activity in the lipase preparation. Carbowax 400 had no inhibitory effect on this enzyme and also made a good emulsifier, hence the reason for its use in this study.

When sunflower lipase inhibitor protein was preincubated 20 min with hog pancreas and *C. cylindracea* lipases (1:1, v/v), no free fatty acids (FFA) were produced, even after some reactions were incubated for 72 hr, which indicates that complete inhibition had been achieved. In reactions using hog pancreas lipase, only cottonseed and olive oils were suitable substrates, because sunflower oil was hydrolysed much too slowly to obtain reliable kinetic data. However, some significant variation in lipolytic activity was observed between the two lipases and their action on cottonseed and olive oils. These results show a significant difference in the composition of free fatty acids liberated during lipolysis (Table 2). Hog pancrease lipase tended to hydrolyse more saturated TGs from both oil substrates which produced higher levels of saturated FFA than did *C. cylindracea*, while *C. cylindracea* lipase hydrolyses more unsaturated TG's yielding higher levels of unsaturated FFA. Hog pancreas lipase released 28.5 and 21.1% of the total free acids as 16:0+18:0 from cottonseed and olive oil substrates, respectively; while *C. cylindracea* lipase released only 19.7 and 14.8% of these two acids. However, oil hydrolysis by *C. cylindracea* produced 79.9% and 85.2% of unsaturated free acids as

Table 1. The effect of organic solvents on *C. cylindracea* lipase activity*

Solvent	% FFA	Solvent	% FFA
No solvent	1.12	*n*-Heptane	0.80
n-Tetradecane	0.76	Ethylcaprylate	0.49
Cyclohexane	0.37	Tween 20	0.22
Hexane	0.19	Tween 40	0.17
Ethylacetate	0.15	Toluene	0.14
Chloroform	0.10		

*Solvent (0.1 ml) mixed with 835 mg sunflower oil then 1 ml lipase solution added to initiate reaction. Values are the average of two determinations after 40 min reaction at 40°.

16:1+18:1+18:2+18:3 from cotton seed and olive oils, while hog pancreas lipase produced only 71.5 and 78.6% of these acids (Table 2). These results suggest the two lipases have different TG substrate specificities under the same reaction conditions. Yet, both lipases could be completely inhibited by the sunflower protein.

Stirring the mixture of lipase and oil substrate was very critical for enzyme kinetic results. Although unstirred reactions of *C. cylindracea* lipase and sunflower oil could not be used for the kinetic studies, a lag-phase was observed in the product vs time curve which is indicative of enzyme-substrate complex formation (Inset Fig. 2). This lag-phase was never detected in stirred mixtures because of the much higher reaction velocities. When reactions were stirred only once for six sec at the beginning of the reaction, a good Lineweaver–Burk plot was obtained with the data ($r^2 = 0.98$), and the calculated V_m was 1.4×10^{-6} mol FFA/sec/mg prot. and $K_m = 1.7$ M (Fig. 2). However, reaction mixtures stirred for six sec and every five min during the course of the reactions (10–60 min), yielded V_ms four-fold higher (Fig. 3). Thus, reaction velocity is very dependent on the length and duration of stirring time. Therefore, inhibited and uninhibited reactions were conducted under these conditions so that kinetic data could be interpreted with a higher degree of reliability.

Most of the reciprocal data points of reaction velocity vs substrate concentration fit a straight line curve ($r^2 = 0.98$), and were extrapolated to the $1/V$ axis to determine V_m, K_m and the mode of inhibition (Fig. 3). These Lineweaver–Burk plots show sunflower protein to be a competitive inhibitor of *C. cylindracea* lipase under the assay conditions used. Maximum reaction velocity (V_m) was calculated to be 5.5×10^{-6} mol FFA/sec/mg protein for both inhibited and uninhibited reactions. Michaelis constant (K_m) for *C. cylindracea* lipase was calculated to be 4.1 M and the K_m for the inhibited reactions was 6.2 M using sunflower oil substrate.

As oil substrate concentration approached about 0.8 M, there was a significant drop in reaction velocity as indicated by the sharp upward bend in both curves (Fig. 3). The reaction velocity dropped about 33% with increased oil concentration in reactions with lipase only and indicate substrate inhibition of *C. cylindracea* lipase under these reaction conditions (lower curve Fig. 3). In the inhibited reaction, the velocity dropped about 41% as substrate oil was increased in the same concentration range (top curve Fig. 3). Competitive inhibition reactions

Table 2. Free fatty acid composition of oil substrates after lipolysis by different lipases*

Fatty Acid	Hog pancreas†		*C. cylindracea*‡	
	Cottonseed	Olive	Cottonseed	Olive
16:0	25.93 ± 0.74	17.81 ± 0.33	18.87 ± 0.09	13.72 ± 0.43
16:1	—	1.48 ± 0.07	1.14 ± 0.21	3.02 ± 0.01
18:0	2.60 ± 0.06	3.31 ± 0.06	0.80 ± 0.10	1.05 ± 0.01
18:1	14.20 ± 0.39	63.43 ± 0.41	14.30 ± 0.55	66.53 ± 0.07
18:2	57.26 ± 0.44	10.14 ± 0.06	63.52 ± 0.39	12.49 ± 0.29
18.3	—	3.59 ± 0.37	0.91 ± 0.09	3.17 ± 0.10

*Weight % FFA of the total weight % FFA. Values are the average of three determinations ± s.d.

†Produced 0.77% FFA in 60 min.

‡roduced 0.50% FFA in 10 min.

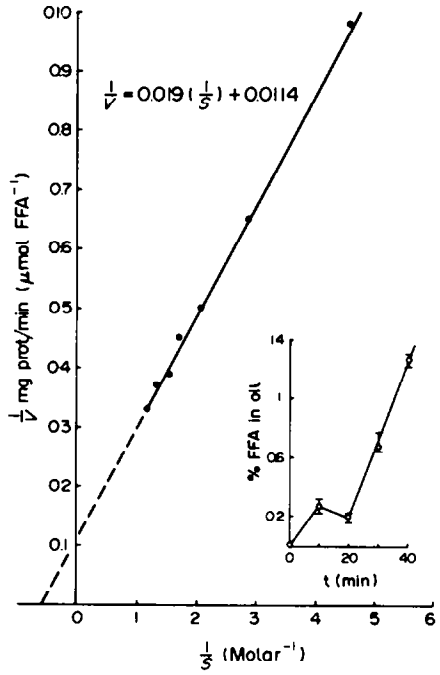

Fig. 2. Lineweaver–Burk plot of sunflower seed oil hydrolysis by *C. cylindracea* lipase. Reactions run at pH 8/40° but stirred only once for 6 sec. Data points are the average of two determinations. Inset, FFA production vs reaction time during lipolysis with *C. cylindracea* lipase and sunflower oil at the oil at pH 8/40°.

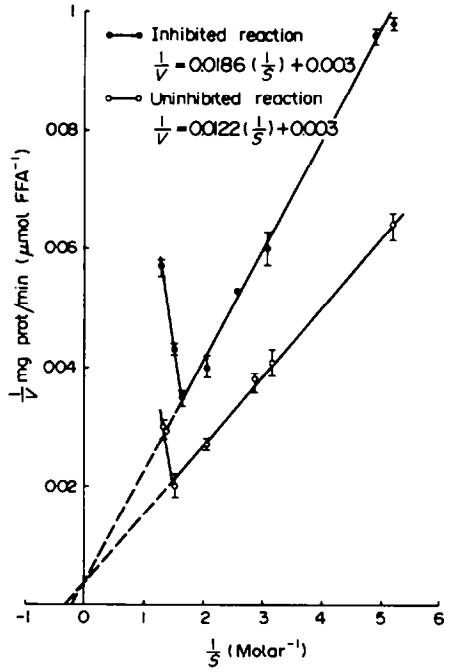

Fig. 3. Lineweaver–Burk plots of sunflower oil substrate hydrolysis by *C. cylindracea* lipase with (●——●) and without (○——○) sunflower inhibitor protein. Most data points are the average of three determinations, with at least two of the determinations using lipase + inhibitor preincubated at 5 and 20 min (Top curve).

can usually be overcome by increased substrate concentration if the enzyme and inhibitor forms a loosely bound complex. However the results (top curve, Fig. 3) suggest that the inhibitor and lipase may have formed, a complex that could not be dissociated by increased oil substrate; otherwise, the effect of substrate inhibition would have been much less in the inhibited reaction (less sharp upward bend) due to the release of additional lipase molecules.

Competitive inhibition suggest some interesting possibilities about the interaction between two protein molecules. The M_rs determined for lipases from different sources are quite varied. A lipase isolated from *C. lipolytica* was reported to be only 5×10^3 [13], while the lipases isolated from rat adipose tissue [14], *Vernonia* seed [15] and rat and porcine pancreas [16] reported M_r of 84×10^3, 200×10^3, and 3000×10^3, respectively. Based on the results of gel chromatography (Fig. 1), the sunflower inhibitor protein is perhaps larger than *C. cylindracea* lipase, assuming a similar M_r as *C. lipolytica*, and would be much smaller than hog pancreas lipase. That the sunflower protein is a competitive inhibitor of lipase (Fig. 3), means the active site on the lipase must come into direct contact with the active site on the inhibitor protein,

regardless of lipase M_r and TG substrate specificity (Table 2). This would involve a protein–protein interaction at specific sites and could be rapid for these large proteins since the same level of inhibition occurred when preincubation time of lipase + inhibitor protein was either five or 20 min (Top curve, Fig. 3). Competitive type inhibition was also observed for the soybean inhibitor protein; although, no V_m and K_m values for inhibited and uninhibited reactions were reported [2]. More recent studies, however, indicate the inhibition is due to the soybean inhibitor protein binding to lipid substrates and not to lipases [3, 4, 8, 9].

Inhibitor activity was also tested on a non-lipolytic enzyme. Sunflower inhibitor protein preincubated 20 min with yeast invertase had no effect on the hydrolysis of sucrose. The increase in reducing sugars was measured with dinitrosalicylic acid [17] and yielded identical results with and without inhibitor (data not shown). An invertase inhibitor protein has, however, been found in potato tubers [18]. It has been suggested that because soybean lipase inhibitor did not effect the activities of amylase and ribonuclease, it may be specific for lipases [1]. Therefore, the proteinaceous inhibitor from sunflower seeds may also be specific for lipases.

There was little change in the free fatty acid composition during lipolysis when reaction velocities were increased three-fold. These results suggest that substrate specificity of *C. cylindracea* lipase is unaltered by reaction velocity. Also, the proteinaceous inhibitor had no effect on lipase specificity since little variation in FFA composition was observed between inhibited and uninhibited reactions during oil hydrolysis (Table 3). Had the inhibitor protein first bound randomly with substrate TG molecules, as reported for the soybean protein [3, 4, 8, 9], then the composition of liberated FFA in the inhibited reactions would have been expected to be significantly different each time the reaction was run; such was not the case (Table 3). Because the FFA composition did not change during lipolysis and because enzyme kinetic results show competitive inhibition, indicate that only those lipase molecules bound to the proteinaceous inhibitor were effectively removed from the reaction. Other evidence presented here suggest the sunflower seed inhibitor protein may form a tightly bound complex with *C. cylindracea* lipase since the percentage drop in reaction velocity with added substrate was about the same in both reactions (Fig. 3). Results of the study also demonstrate that if meaningful kinetic data are to be obtained, it is absolutely necessary to have inhibited and uninhibited reaction mixtures stirred precisely the same. The exact mole:mole ratio of inhibitor to lipase is not known at the present time and additional studies will be needed to determine these properties.

Table 3. The composition of FFA liberated from sunflower oil at different lipolytic rates with and without sunflower seed inhibitor protein*

	Without inhibitor			With inhibitor		
	(rates × 10^7)†					
FFA	2.56	5.37	7.57	1.73	3.08	4.90
16:0	3.77	3.40	3.27	3.19	3.24	3.45
18:0	0.74	0.71	0.56	0.71	0.67	0.68
18:1	17.41	16.77	15.41	16.43	15.96	15.72
18:2	76.64	77.69	78.72	78.05	78.37	78.28
18:3	1.45	1.44	2.05	1.62	1.75	1.86

*Weight % FFA of the total wt % FFA.
†Mol FFA/sec/mg protein with *C. cylindracea* lipase.

EXPERIMENTAL

Seed material and enzymes. High-oil type sunflower seed (*Helianthus annuus* L.) used in this study were grown in North Dakota (1983 crop) and confectionery seed were purchased locally (Clarke Co. Milling, Co., Athens, GA).

Hog pancreas (16 U/gm) and *Candida cylindracea* (640 U/mg solid) lipases (EC 3.1.1.3) were purchased from Sigma. Yeast invertase (EC 3.2.1.26) (100 U/mg) was purchased from ICN Nutritional Biochemicals. Enzymes were stored desiccated at 3° until needed.

Isolation of lipase inhibitor protein. Clean, whole sunflower seed (7% moisture; 25–30 g) were ground to a fine meal in a Krups grinder. The meal was Soxhlet extracted for 20 hr with petroleum ether (30–60°) to remove lipids and the defatted meal stored at −20°. The sunflower oil obtained from several petrol extracts were pooled and saved after flash-evapn of solvent. Defatted meal (6–7 gm) was extracted with 100 ml 0.1 M Tris–HCl/0.02 M mercaptoethanol (pH 8) buffer, and stirred (magnetic) at slow speed for 2 hr. The extract was allowed to stand overnight at 1°, then filtered through 4-layers of cheesecloth and centrifuged at 20 000 *g*/3° for 10 min. The clear supernatant was made 30% satn with $(NH_4)_2SO_4$ and allowed to stand for 30 min packed in ice. The soln was centrifuged at 13 500 *g* for 10 min and the pellet discarded. The supernatant was made 50% of satn with $(NH_4)_2SO_4$, and after 30 min, centrifuged at 13 500 *g* for 10 min. The supernatant was discarded and the pellet dissolved in *ca* 15 ml of 0.1 M Tris–HCl/0.02 M mercaptoethanol (pH 8). Previous test revealed that the 50% fraction contained most of the lipase inhibitor activity. The crude inhibitor soln was dialysed overnight against 30 vols of 0.1 M Tris–HCl/0.02 M mercaptoethanol (pH 8) at 1°, then centrifuged at 20 000 *g* for 10 min, and stored at 1° until needed. Protein content of this preparation was 3.5 mg/ml. Confectionery and high-oil type sunflower seed lipase inhibitor proteins were stable for more than 6 months when stored under these conditions and were used for the kinetic studies without further purification.

Chromatography on G-150 and G-200. Gel chromatography was performed using Sephadex G-150 and G-200 (Sigma). About 2 ml of the dialysed protein inhibitor soln was applied to the columns (2.7 i.d. × 35 cm) and eluted with 0.1 M Tris–HCl (pH 8) at a flow rate of 0.5 ml/min. Void vols (determined with Blue Dextran, M_r = 2 M) were collected in a graduated cylinder, then 8 ml fractions were collected using a fraction collector equipped with an electric-eye drop counter (LKB). Protein was monitored with a V4 Absorbance Detector (ISCO, Inc., Lincoln, NE) at 209 nm with a chart speed of 6 cm/hr. Inhibitor activity was monitored in each tube by TLC and the assay described below. All gel chromatography procedures were conducted at 20°/50% RH. Distribution coefficients (K_d) were computed from chromatographic data to estimate the M_r of the inhibitor protein [10–12].

Oil substrate preparation. Once-refined cottonseed (Goldkist, Inc., Valdosta, GA), olive (purchased locally), and crude sunflower (from above) oils were used as substrates for the lipases. About 125 ml of each oil was extracted with 300 ml of spectrophotometric grade MeOH (Burdick and Jackson Laboratories, Inc., Muskegon, MI) in a large separatory funnel to remove

endogenous free fatty acids (FFA) and phospholipids. The oil layer was removed and the extraction procedure repeated × 3. Residual MeOH was removed from the oil by flash evaporation at 35° under vacuum (40 mm Hg) for 1 hr. Only small amounts of FFA (0.07%) and no phospholipids were detected in these oil substrates by titration and 2D-TLC methods [19, 20]. The average M_r of triglyceride (TG) in each oil substrate was determined based on average fatty acid M_r computed from total FA composition of oils [21]. The average M_r of FA for sunflower. cottonseed and olive oil substrates were 284.10, 276.16, and 273.62 and their TG M_r were calculated to be 890.25, 866.52, and 861.93 respectively. Oil substrate concentration was based on mmol TG/ml reaction emulsion, assuming the oil to be 100% TG by wt. The vol. of each oil substrate was computed based on weight and density at 25° [22] since accurate oil vols cannot be delivered with pipettes. The maximum oil substrate concn that could be obtained under the reaction conditions was *ca* 0.8 M. All enzyme-inhibitor kinetic data was obtained using *C. cylindracea* lipase and sunflower oil substrates because hog pancreas lisase hydrolysed this oil much too slowly under the assay conditions used.

Lipase and inhibitor activity determination. About 60 mg of solids containing *C. cylindracea* lipase was dissolved in 10 ml of 0.1 M Tris–HCl (pH 8), centrifuged at 2000 *g* for 5 min and mixed 1:1 (v/v) with carbowax 400 (Supelco, Inc., Bellefonte, PA). The carbowax-enzyme soln was stirred slowly with a glass rod while cooled under tap water. Hog pancreas lipase (20 mg) was prepared in the same manner except centrifugation was omitted. Enzyme solns were prepared fresh every 2 days. Oil samples were carefully pipetted into 15 × 150 mm culture tubes and weighed (450 mg–2.5 g). To initiate the reaction, 0.5–2 ml of lipase soln was added to the oil substrate, stirred 6 sec with a Vortex mixer and placed in a water bath (40°). During the course of the reaction, the mixture was stirred *ca* every 5 min for 6 sec to maintain the reaction in emulsified form. Reactions were stopped at different times (10–60 min) by mixing (Vortex) with 2 ml hexane, followed by several rinses with hexane and H_2O into a separatory funnel. The hexane layer was washed twice with H_2O and once with saturated NaCl to break the emulsion. The hexane layer was collected over solid Na_2SO_4, filtered and then removed from the oil under vacuum. Oil substrate recovery was almost 100% with this procedure.

Weight % FFA were determined on aliquots (about 235 mg) of this oil by GC [23] except oven temp was increased to 205°. The GC method was modified by using 3 mm (o.d.) × 5 cm s.s. guard columns packed with 60/80 mesh Chromasorb W (acid washed-DMCS). After subtracting endogenous FFA (0.07%), weight % was converted to mol FFA produced per reaction time.

Inhibitor activity was assayed in the same manner, except lipase and inhibitor were pre-incubated 5 and 20 min in a ratio of 20:1 (*v/v*) at room temp, prior to mixing with the oil substrate.

Protein, V_m, and K_m determinations. Protein concn of lipase-carbowax solns were determine by the biuret and 280 nm *A* methods using purified egg albumin (J. T. Baker, Phillipsburg, NJ) as standard [24]. Lipolytic rate data was based on linear production of FFA as function of time (10–20 min) and sp. act. reported as mol FFA/sec/mg protein. Rates were determined in triplicate for each substrate concn. Least squares linear regression analysis was performed on the data to determine the best possible equations for the Lineweaver–Burk plots. From the equations derived, V_ms were computed as reciprocal of *y*-intercepts, and K_ms by V_m × slopes of the inhibited and uninhibited reaction curves.

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