A Colorimetric Determination of Fatty Acids as a New Assay of Lipases in Reverse Micelles

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A new simple spectrophotometric method is described for the determination of the free fatty acid content in triglyceride oils or other lipophilic samples. The method utilizes phenol red as fatty acid indicator, which is solubilized in reverse micelles formed by AOT [sodium *bis*(2-ethylhexyl) sulfosuccinate] in isooctane. Fatty acid determinations in vegetable oils can be carried out rapidly with oil samples of less than 100 mg. The acid value of three different oils tested agreed quite well with the acid value obtained for the same samples with another colorimetric determination using cupric acetate.

The method can be extended to a continuous determination of fatty acids which are released during the initial stage of a lipase catalyzed hydrolysis of triglyceride substrates in reverse micelles. This new sensitive lipase assay has been applied for a lipase of *Pseudomonas* bacteria with lipase concentrations as low as 0.1 μg/ml. Using trioctanoylglycerol as substrate in 50 mM AOT/isooctane with $w_o = [H_2O]/[AOT] = 11.1$ (pH 9.0), the apparent overall Michaelis-Menten constant ($K_{m,app,ov}$) is 27 mM and the turnover number (k_{cat}) 44 sec^{-1}.

Although lipases are of growing interest as catalyst in fat technology (1,2) or in organic synthesis (3,4), the mechanism of lipolysis is still not fully understood (5,6). One reason for this may lie in the fact that activity of lipases generally strongly depends on the physical state of the substrate, i.e., on the way the lipophilic substrate is presented to the enzyme (5). In a heterogenous aqueous emulsion system, the apparent K_m value of pancreatic lipase for triglycerides, for example, strongly depends on the physical properties of the emulsion (7).

Reverse micelles (or 'microemulsions' at higher water content) offer, in principle, a unique possibility to overcome the problems caused by the medium heterogeneity. Reverse micelles spontaneously form in an organic solvent (such as alkanes, benzene or chloroform) when certain surfactant molecules and a small amount of water are added. The resulting optically transparent, highly dynamic and thermodynamically stable system is built up of small water droplets, which are surrounded by a layer of surfactant molecules which build the interphase between the dispersed water droplets and the organic solvent (8). The radius of the water droplets mainly depends on w_o, the ratio of molarity of water to the molarity of surfactant ($w_o = [H_2O]/[\text{Surfactant}]$). In the case of AOT [the sodium salt of *bis*(2-ethylhexyl) sulfosuccinic acid] in isooctane or heptane, the radius of the waterpool (in nm) is approximately $0.175 \times w_o$ (9), for $w_o < 30$, 25–200 mM AOT, 25°C.

For a variety of surfactant/solvent systems it has been shown that enzymes can be solubilized in such reverse micelles, and it has been shown that the enzymes generally remain catalytically active (10,11). In the case of lipolytic enzymes, the substrate is mainly localized in the bulk organic phase, whereas the enzyme molecule (which is itself insoluble in the pure solvent) is solubilized within the small water domains of the reverse micellar aggregates. The different studies on lipases in reverse micellar systems which have been carried out so far have recently been summarized (12). These studies include the lipase catalyzed hydrolysis of vegetable oils and synthetic triglycerides (13,14). For these hydrolytic reactions, a simple assay which follows the hydrolysis of triglycerides in reverse micelles continuously by using Fourier transform infrared spectroscopy has been developed (15). The method has already been applied for investigating the activity of lipases in lecithin based organogels (16).

In the present paper we describe another new, simple and sensitive procedure for continuously measuring the activity of lipases in reverse micelles by using solubilized phenol red as reaction indicator. The method is based on the colorimetric determination of the amount of free (non-esterified) fatty acids which are released from the glyceride substrates at the first stage of the reaction.

In addition to the possibility to measure the hydrolytic activities of lipases, the method also allows determination of the free fatty acid content of vegetable oils by using oil samples of less than 100 mg.

MATERIALS AND METHODS

Reagents. Isooctane (for UV spectroscopy), phenol red (phenolsulfonephthalein), caprylic acid (octanoic acid, purum), palmitic acid (hexadecanoic acid, puriss.), oleic acid (*cis*-9-octadecenoic acid, puriss.), tricaprylin (trioctanoylglycerol, puriss.), tricaprin (tridecanoylglycerol, puriss.), glycerol (puriss.), and tris(2-amino-2-hydroxymethyl-1, 3-propandiol) were from Fluka, Switzerland. Cold pressed vegetable oils from sunflower seeds, soybeans and sesame seeds were products from the local market (Morga, Switzerland). AOT [sodium *bis*(2-ethylhexyl) sulfosuccinate] was from Sigma (St. Louis, MO) and used as obtained (15). Porcine pancreatic lipase (type VI-S) was also from Sigma and the lipase from *Pseudomonas* sp. from Boehringer-Mannheim, Germany. Upon analyzing the two lipases with SDS-polyacrylamide gel electrophoresis, the pancreatic lipase appeared in three different bands in a molecular weight range of 10'000 to 55'000 D, while the lipase from *Pseudomonas* was homogeneous with a molecular weight of 30'000 D, in agreement with other reports on lipases from different strains of *Pseudomonas* bacteria (17–19). Due to the heterogeneity of the commercial porcine pancreatic lipase sample used, the activity measurements were mainly carried out with the enzyme from *Pseudomonas* sp. This lyophilized bacterial lipase sample was used as received, the enzyme content of the lyophilisate was 20 w/w%. According the specifications given by the supplier (Boehringer), the specific activity was 200 units per mg protein, when assayed at 37°C and pH 7.5 with olive oil as substrate and Rhodoviol (a polyvinyl alcohol) as detergent. One unit hydrolyzes 1 μmole of the emulsified olive oil per minute.

In the case of the pancreatic enzyme, the specific activity given by Sigma was 400 units per mg protein at 37°C and pH 7.7 (olive oil as substrate).

UV/VIS spectroscopy. Absorption spectra were recorded at 25°C either on an Uvikon 810 spectrophotometer from Kontron, or on a HP 8452A diode array UV/VIS spectrophotometer from Hewlett Packard with thermostatted cuvette holders and quartz cells of 1 cm pathlength.

Solubilization of phenol red in AOT reverse micelles. Fifteen μl 0.1 M tris/HCl, pH 9.0 and 5 μl 2 mM phenol red in 0.1 M tris/HCl, pH 9.0 were added to 2 ml 50 mM AOT in isooctane. A clear red solution was obtained after vortexing for 5-10 seconds. The resulting solution contained 50 mM AOT with an overall phenol red concentration of 5 μM (concentration in the water pool: 0.5 mM), w_o = 11.1. The intensity of the red color (λ = 560 nm) may be lowered upon using AOT samples which contain high amounts of carboxylic acid impurities (20).

Effect of fatty acids on the absorption of solubilized phenol red; determination of fatty acids in vegetable oils. A few μl of a 50 mM solution of fatty acid in isooctane was added to 3 ml solutions containing 50 mM AOT in isooctane and 5 μM phenol red overall, w_o = 11.1 (0.1 M tris/HCl, pH 9.0) and the optical density measured at 560 nm. As a control, glycerol was used as a reagent which could possibly interfere with the fatty acid analysis (see below). No change in the absorption of phenol red at 560 nm was detected in the presence of 3.5 mM glycerol (overall).

A procedure for the determination of non-esterified fatty acids in vegetable oils is given under the Results and Discussion section. If oils or other fatty acid containing samples are to be analyzed which absorb at 560 nm, appropriate corrections have to be made (absorption in the absence of phenol red). In the case of the three oil sample tested, such a correction was not necessary.

As a comparison, the determination of fatty acids in vegetable oils were carried out following a titration procedure described in the literature (21) using 96% ethanol:diethyl ether (1:1 v/v) as solvent, phenolphthalein as indicator, and NaOH as titration solution. The colorimetric fatty acid determination using cupric acetate (22) was carried out by D. Han in our laboratory by utilizing standard curves made with oleic acid. In all determinations, the mean molecular weight for the fatty acid was taken as 282 g/mol.

Preparation of lipase-containing micellar reaction mixtures. The reaction mixtures were prepared in an analogous way as described earlier (15). A typical procedure is carried out as follows: First, an aqueous *Pseudomonas* sp. lipase stock solution was prepared (1 mg lyophilisate in 1 ml 0.1 M tris/HCl buffer, pH 9.0; corresponding to 0.2 mg lipase/ml). One ml of tricaprylin substrate in isooctane (e.g., 122 mM), 10 μl 0.1 M tris/HCl, pH 9.0, and 5 μl 2 mM phenol red in 0.1 M tris/HCl, pH 9.0 were added to 1 ml 100 mM AOT/isooctane. After mixing and thermostatting at 25°C for 15 min, the reaction was started by adding 5 μl of the lipase stock solution. The enzyme was solubilized by mild shaking for 10 seconds and the time course of the reaction at 25°C was monitored either by recording the entire visible spectrum (diode array instrument), or alternatively, the reaction was followed at a fixed wavelength of 560 nm (Uvikon instrument). The overall phenol red concentration was 5 μM and the initial w_o-value 11.1. A w_o-value around 10 was used, because in reverse micellar solution maximal enzyme activity is often observed at this rather low water content (10,11,13,15).

Control experiments in the absence of lipase showed no measurable substrate hydrolysis.

Note that it is required to use purified triglyceride substrates, because protonations of solubilized phenol red can already occur to a great extent with a fatty acid concentration of less than 1 mM (see below). Tricaprylin in 'practicum' purity degree (from Fluka, for example) could not be used as substrate for the lipase assay because the free fatty acid content was too high (we estimated the fatty acid content to be 1.4 mol%, compared with 0.18 mol% for 'puriss.' grade tricaprylin from Fluka).

RESULTS AND DISCUSSION

Description of the reverse micellar system used. If not otherwise stated, all measurements in the present work have been carried out with an isooctane solution which contained per ml 50 μmoles AOT and 10 μl of a 0.1 M tris/HCl buffer solution of pH 9.0. The concentrations of AOT and water in this reverse micellar solution was therefore 50 mM (corresponding to 2.2 w/v% or 3.2 w/w%) and 555 mM (corresponding to 1.0 w/v% or 1.4 w/w%), respectively. The w_o-value of the system was 11.1 (w_o = $[H_2O]$/[AOT]), the concentration of micelles approximately 0.4 mM, the mean waterpool radius of a single reverse micelle 20 Å ca, and the total interfacial area covered by the AOT head groups 14 m^2 per ml ca (8,9,15).

Solubilization of phenol red in AOT reverse micelles. Phenol red is an acid-base indicator belonging to the class of sulfonephthaleines with a pK_{a1}-value of 7.8 (23), (Fig. 1) (24). In the alkaline region, the phenol red molecule bears two negative charges and is red (λ_{max} in aqueous solution: 558 nm). Decreasing the pH of the solution, the phenolate group is first protonated (pK_{a1}) resulting in the formation of a cyclic γ- sultone with a transformation of the central sp^2-C-atom to sp^3 geometry, which leads to a decrease in the number of conjugated double bonds (24). The resulting color of phenol red (in weakly acid or neutral solution) is yellow (λ_{max} = 430 nm). Additional protonations of phenol red are possible on decreasing the

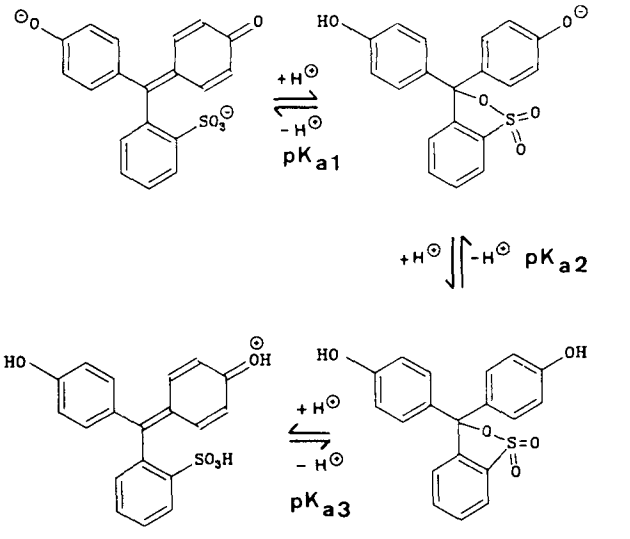

FIG. 1. Chemical structure of phenol red and its protonation equilibria.

pH of the solution further: (i) protonation of the second phenolate group without changing the color ($pK_{a2} \leq pK_{a1}$), and (ii) protonation of the sulfonate group under strong acidic conditions ($pK_{a3} < 1$, $\lambda_{max} \approx 505$ nm, 24).

Solubilization of the water soluble phenol red inside AOT reverse micelles has been carried out by adding an alkaline, buffered aqueous solution of indicator to an isooctane solution containing 'dry' AOT (see above). The resulting overall concentration of phenol red and tris were 5 μM and 1 mM, respectively. The visible spectrum of phenol red in AOT reverse micelles under these conditions is characterized by an absorption band with peak maximum at 560 nm, similar to the spectrum in aqueous solution (see above). Using a slightly acidic sodium acetate buffer (0.1 M, pH 5.6) for the reverse micellar solutions instead of the alkaline tris/HCl buffer, the absorption at 560 nm disappears and a new band at 430 nm appears, again in analogy to the situation in water (see above). The molar absorption of the indicator at 560 nm in the micellar solution (5 μM phenol red overall, w_o=11.1, 0.1 M tris/HCl, pH 9.0) is 53'600 ± 600 $M^{-1}cm^{-1}$; the corresponding value in the aqueous solution 62'400 ± 600 $M^{-1}cm^{-1}$. These differences probably reflect small changes in the pK_{a1} value of phenol red in the reverse micelles and a lower apparent pH of the waterpools (23).

Based on the measured molar absorption at 560 nm and on the pK_a-values of phenol red in water and in AOT reverse micelles [7.8 and 7.7, respectively, (23)], the pH inside the micelles is estimated to be 8.3 if the added aqueous solution was a 0.1 M tris/HCl buffer of pH 9.0 (w_o 11.1). Similar pH changes have been observed with the help of comparative NMR studies under slightly different experimental conditions (23,25).

Effect of fatty acids on the visible spectrum of phenol red in reverse micelles. The effect of three different fatty acids on the visible absorption spectrum of phenol red solubilized in AOT reverse micelles has been investigated. The fatty acids used were caprylic acid (C 8:0), palmitic acid (C 16:0) and oleic acid (C 18:1). In all three cases a linear decrease of the absorption at 560 nm was observed on increasing the fatty acid concentration up to 0.7 mM ca (Fig. 2). Linear regression analysis of the three curves in the insert of Figure 2 gave slopes of -0.262, -0.261 and -0.260 for caprylic acid, palmitic acid, and oleic acid, respectively, with a mean value of -0.261. Based on this observation, one can conclude that it is possible to use solubilized phenol red as an indicator for the quantitative determination of free fatty acids.

Colorimetric determination of free fatty acids in vegetable oils. With the calibration curves of Figure 2 (insert), the amount of free fatty acids (present, for example, in vegetable oils) can easily and rapidly be determined by the following steps: (i) Solubilize 5 μM (overall) phenol red in 50 mM AOT/isooctane (w_o=11.1, 0.1 M tris/HCl, pH 9.0) and read the absorbance at 560 nm (let the total volume be X ml); (ii) add a small volume (Y ml) of the free fatty acid containing oil to the reverse micellar solution (e.g., 0.020 ml oil added to 3 ml solution) and read the absorbance at the 560 nm again; (iii) repeat step (ii) two or three times, as long as the total volume does not change markedly, and as long as the absorbance is above 0.1 ca (limit of linearity in Fig. 2); (iv) calculate from the measured optical density changes $|\Delta OD_{560nm}$ (l=1 cm) |the molar fatty acid concentration in the oil sample by applying the following formulae:

[Free fatty acid] (mM) =

$(|\Delta OD_{560\,nm}$ (l=1 cm $|) \times (X + Y)/(0.261 \times Y)$; and (v) if desired, calculate the free fatty acid content in the oil in wt% by knowing the density of the oil and by assuming a certain reasonable mean molecular weight for the fatty acids (which, of course, depends on the oil analyzed).

Compared with other colorimetric methods for the determination of free fatty acids (22,26-28), in the

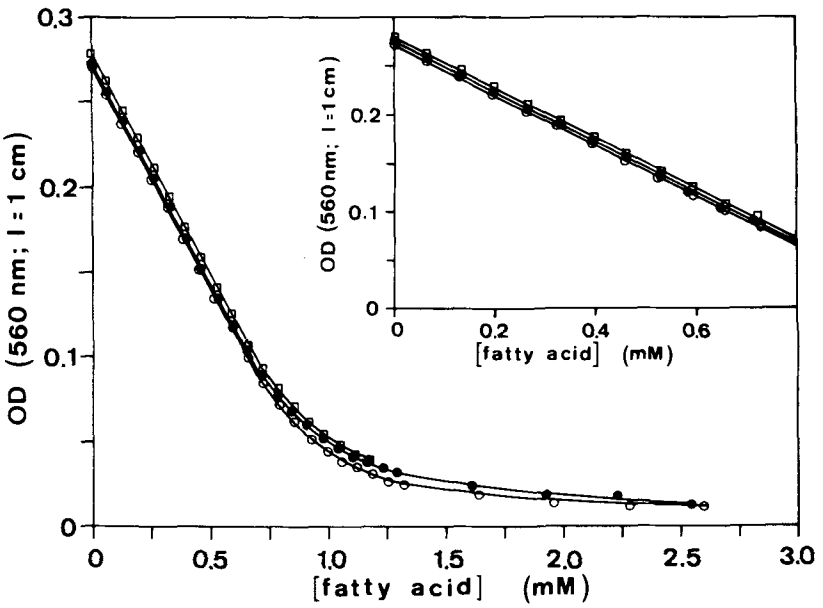

FIG. 2. Effect of fatty acids on the absorption at 560 nm of solublized phenol red in 50 mM AOT/isooctane reverse micelles, w_o=11.1 (0.1 M tris/HCL, pH 9.0). [Phenol red]$_{ov}$= 5 μM.
○: caprilic acid (C 8:0); ●: palmitic acid (C 16;0); □: oleic acid (C 18:1). Path length: 1 cm, 25°C. Insert: Linear section between 0 and 0.7 mM fatty acid.

present method no extraction process is needed and the amount of oil sample required is quite low [samples containing less than 3 μmoles (or 0.8 mg ca) free fatty acids can be analyzed]. We did not attempt to optimize the method further with respect to an increase in sensitivity, e.g., using a higher phenol red concentration. It is likely, however, that this could be done.

The procedure described above has been applied for the analysis of three different vegetable oils, soybean oil, sunflower oil and sesame oil. The determined free fatty acid content in the three samples is given in Table 1, together with the results obtained by using a standard titration procedure (21) and a colorimetric method using cupric acetate to form blue cupric soaps which are soluble in benzene (22). Generally, the phenol red method gives values for the amount of free fatty acids, which are comparable to the values obtained by the cupric acetate method; these values are 10-15% below those obtained by titration (Table 1). We did not investigate the reason for these differences further.

Determination of lipase activity. The observations made above on the linear relation between the absorption at 560 nm of solubilized phenol red and the fatty acid concentration permits the researcher to continuously follow the lipase catalyzed hydrolysis of triglycerides in a reverse micellar system. We have tested this possibility by using a purified lipase from *Pseudomonas* sp. and tricaprylin as substrate. A typical time scan of the lipase catalyzed hydrolysis of tricaprylin (initially 61 mM) is shown in Figure 3. The overall phenol red concentration was 5 μM and the starting w_o-value ($w_{o,st}$) 11.1 (used buffer 0.1 M tris/HCl, pH 9.0). During the reaction, the peak at 560 nm disappears and upon protonation of the dye molecule, the expected new band with a lower extinction appears at 430 nm; the isosbestic point lies at 480 nm (Fig. 3). Although the entire visible spectrum of the reaction mixture is shown, it is enough to analyze the progress of the reaction by following the absorption at 560 nm as function of incubation time. If the initial substrate concentration (61 mM tricaprylin) and $w_{o,st}$ (11.1) is kept constant, and if the overall enzyme concentration is varied, one obtains the kinetic behavior shown in Figure 4—the higher the enzyme concentration, the faster the reaction. If the initial velocity is plotted as a function of the overall lipase concentration, a straight line is obtained (not shown). On varying the initial substrate concentration and keeping the overall enzyme concentration and $w_{o,st}$ constant, it is possible to determine the overall apparent Michaelis-Menten constant ($K_{m,app,ov}$) and the maximal velocity v_{max} (or the turnover number k_{cat}) for the enzyme-substrate system under the experimental conditions (Fig. 5). In the case of *Pseudomonas* sp. lipase, we obtained for $K_{m,app,ov}$ 27 mM and for k_{cat}44 sec^{-1}. The K_m-value is quite high because the substrate (the triglyceride) is highly soluble in the continuous organic phase. In principle, one should consider only the interfacial substrate concentration (5) which, unfortunately, is unknown in our case. Similar high values for $K_{m,app,ov}$ of lipase-triglyceride systems in AOT reverse micelles have been obtained in the case of porcine pancreatic lipase (29), *Candida rugosa* lipase (13) and *Rhizopus delemar* lipase (30).

One drawback of the phenol red lipase assay presented here lies in the requirement of pure substrates. For obvious reasons (see above), only triglycerides (or other fatty acid esters) which do not contain free non-esterified

TABLE 1

Determination of the Content of Free (Non-Esterified) Fatty Acids in Vegetable Oils

Oil analyzed	Fatty acid content (w/w %)[a]		
	Phenol red - reverse micelle method[b]	Titration method[c] (21)	Cupric acetate method[d] (22)
Soybean oil	0.33 ± 0.02	0.40 ± 0.01	0.32; 0.32
Sunflower oil	0.77 ± 0.07	0.91 ± 0.01	0.79; 0.82
Sesame oil	0.94 ± 0.03	1.03 ± 0.01	0.88; 0.91

[a]Mean molecular weight for the fatty acids taken as 282 g/mol.
[b]Mean value and standard deviation of 4-8 determinations.
[c]Mean value and standard deviation of three determinations.
[d]Carried out in duplicates.

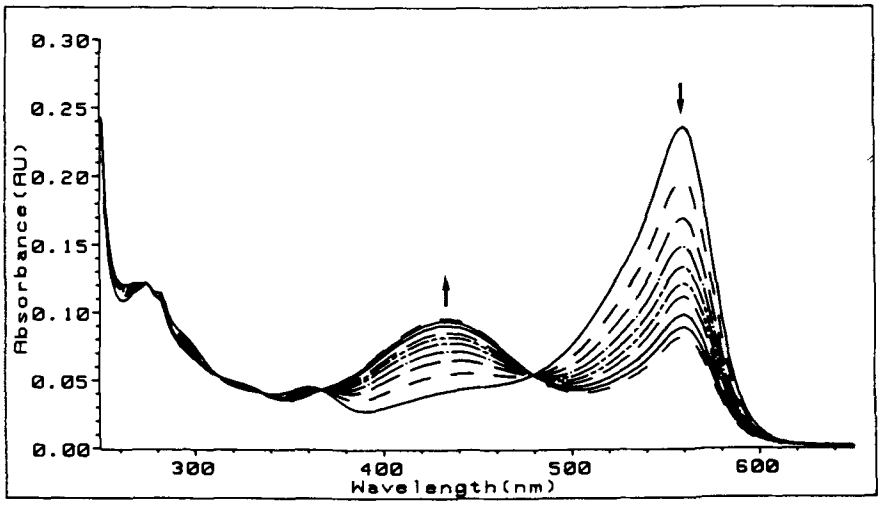

FIG. 3. *Pseudomonas* sp. lipase catalyzed hydrolysis of tricaprylin (inititally 61 mM) at 25°C in 50 mM AOT/isooctane, $w_{o,st}$=11.1 (0.1 M tris/HCl, pH 9.0). $[Phenol\ red]_{ov}$= 5 μM, path length: 1 cm. Absorption spectra measured after 1, 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes. $[lipase]_{ov}$=0.51 μg/ml.

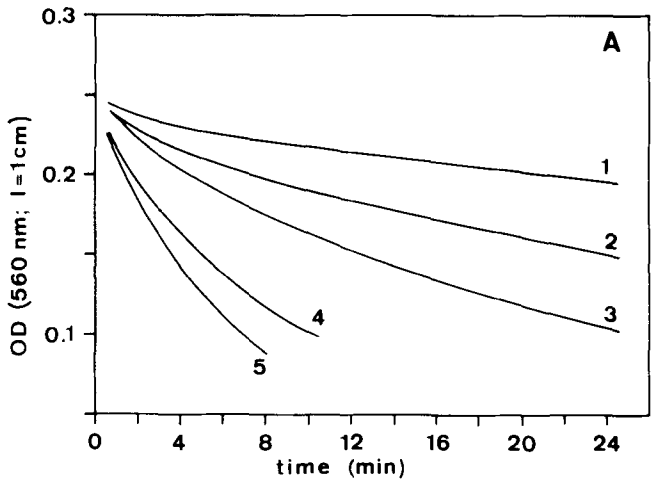

FIG. 4. *Pseudomonas* sp. lipase catalyzed hydrolysis of tricaprylin (initially 61 mM) at 25°C in 50 mM AOT/isooctane reverse micelles, $w_{o,st}$=11.1 (0.1 M tris/HCl, pH 9.0). [Phenol red]$_{ov}$= 5 μM. Continuous time scan until at a maximum 0.7 mM caprylic acid have been released. [lipase]$_{ov}$: 0.10 (1), 0.26 (2), 0.51 (3), 1.03 (4) and 1.54 μg/ml (5). Path length: 1 cm.

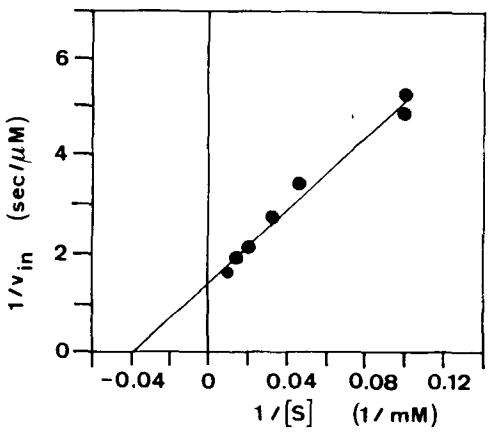

FIG. 5. Lineweaver-Burk plot for the *Pseudomonas* sp. lipase catalyzed hydrolysis of tricaprylin at 25°C in 50 mM AOT/isooctane reverse micelles, $w_{o,st}$=11.1 (0.1 M tris/HCl pH 9.0), [Phenol red]$_{ov}$= 5 μM, [lipase]$_{ov}$= 0.51 μg/ml.

fatty acids can be used. Cheap vegetable oils should therefore first be purified before being used as substrates. The advantage of the method is, however, the relative high sensitivity with respect to enzyme concentration. We have measured this with overall lipase concentrations between 0.1 and 1.5 μg/ml, corresponding to 0.02 and 0.3 units/ml.

The assay is not limited to the lipase from *Pseudomonas* sp. We have also carried out a few measurements with porcine pancreatic lipase and could also follow the initial hydrolysis of substrate; in this case, we used tricaprin (tridecanoylglycerol) instead of tricaprylin (data not shown). However, a detailed kinetic analysis was not meaningful in this case because we did not have a pancreatic lipase which was pure enough (see the Materials and Methods section). It is known, for example, that the presence of colipase may affect the enzyme activity (6). The requirement for the lipase is its capability of being catalytically active at the pH used (pH of the added buffer was 9.0, see above). In those cases where the requirements are fulfilled, the phenol red lipase assay can be useful for detecting low amounts of lipases in biological material, regardless of whether it is water soluble or not. The presence of polar (water), apolar (organic solvent) and amphiphilic compounds (AOT) in the optically transparent compartmentalized reverse micellar reaction medium helps solubilizing different compounds possibly present in a crude extract. The advantage of having this property in reverse micellar solution has already been utilized in the case of a simultaneous extraction of proteins and oil from crude, oily sunflower seed cakes (31). Therefore, it is possible that an application of the new assay could be used during enzyme purification, where in the case of lipases, lipophilic impurities are often present, or where the enzyme itself is sparingly soluble in an entirely aqueous medium.

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