Rapid Paper

Determination of Lipase Activity in AOT-Isooctane Reversed Micelles

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Received September 30, 1986

An assay method in a reversed micellar system, which can be better characterized in physicochemical terms than that in an emulsion system, was investigated. The first-order rate constant when $[S] \ll K_m$ and the zero-order rate constant when $[S] \gg K_m$ for lipase reaction in the system show a linear relationship with enzyme concentration over a twenty-fold range, which is an important criterion for an enzyme assay. Based on the results, a three-point assay of lipase in reversed micelles was established. To ensure the initial rate, the assay time should be limited to the time during which about 4.5% of the substrate is utilized.

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is the enzyme which hydrolyzes the long-chain aliphatic acid esters in acylglycerols. Although this enzyme is one of the most important for digestion, studies on lipase have been relatively rare compared with those on the other ordinary enzymes. One of the main reasons may be that the catalysis of lipase is heterogeneous, since it is active only at the interface.1) Due to this characteristic, a lipase assay is usually carried out in an aqueous emulsion of the insoluble substrate and by determining the rate of formation of the fatty acids.

Many investigators have developed sensitive and convenient methods for the analysis of fatty acids.2~4) However, the principal reason that delayed studies on lipase may not be related to the analytical method of fatty acids but may be related to the reaction system, i.e., an emulsion. It is known that the kinetic constants obtained by the emulsion system are highly dependent on the interfacial area as determined by the size of emulsion droplets, which varies according to the mechanical procedures, and/or the emulsifier used to stabilize the emulsion. These kinetic constants, however, coincided regardless of the degree of dispersion of the same substrate when the substrate concentration was expressed as an area/volume instead of a weight/volume.5) In this case, determining the area/volume is very difficult, since it is not easy to accurately measure the surface area of an emulsion droplet, and the measured value is questionable because the interfacial area occupied by the emulsifier, if used, is unknown.

In this paper, we describe a method for a lipase assay in a single liquid phase, reversed micelles,6'7) that were formed by solubilizing triacylglycerols and an enzyme solution in organic solvent with the help of a surfactant.

MATERIALS AND METHODS

Lipase preparation. Lipase from Candida rugosa purchased from Sigma (St. Louis, MO, U.S.A.) was partially purified as previously mentioned.8* A stock solution of the enzyme was prepared in 0.05m phosphate buffer (pH 7.1) at a concentration of 38.9mg/ml, and a lower concentration was obtained by dilution.

Substrate. Olive oil purchased from Sigma was used as a substrate, the saponification value of which was found to be 191.5.9) It was thus deduced from the value that one gram of live oil was equivalent to 3.114 mmol as an ester-bond concentration.

Other reagents. Bis(2-ethylhexyl) sodium sulfosuccinate (abbreviated as AOT, Sigma) was purified according to the procedure of Tamamushi and Watanabe,10) and iso-octane of HPLC grade (Mallinkrodt, St. Louis, MO, U.S.A.) was used after further dehydration with anhydrous sodium sulfate.

Lipid assay. The hydrolysis of olive oil was carried in a screw-cap culture tube filled with 10ml of 0.05M AOT-isooctane solution containing the olive oil (0.6228 m as an ester-bond concentration, 20% by volume), which was preincubated at 30°C. To the tube, 99μl of the enzyme solution was added, and the reaction was initiated by

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immediately vortex-mixing the mixture until clear (ca. 10 sec). At predetermined time intervals, 0.4 ml of the reaction mixture was transferred to a centrifuge tube containing 4.6 ml of benzene and 1.0 ml of cupric acetate-pyridine solution.11 The mixture was then immediately vortex-mixed to stop the reaction and to determine the content of fatty acids produced. The absorbance of the supernatant after centrifugation was measured at 715 nm and converted to the content of fatty acids from a standard curve plotted by using oleic acid as an internal standard.

All of the data are the average of triplicated samples and were reproducible to within ± 10%.

RESULTS AND DISCUSSION

Optically clear reversed micellar solutions of lipase were secured by vortex-mixing the isooctane-AOT-enzyme solution. The fact that the protein content from any part of the reaction mixture was identical indicates that the lipase was uniformly solubilized and dispersed in the reversed micellar solutions.

Two methods are suggested to assay the lipase in reversed micelles. The first is to calculate the first-order rate constant from the reaction data and the other is the initial rate method. The linear relationship between \( v \) and [S] when [S] \( \ll Km \) can be derived from the Michaelis–Menten equation.

\[
v = \frac{k_{cat}[E_0][S]}{Km + [S]}
\]

When [S] \( \ll Km \), the equation reduces to Eqs. (2) or (3).

\[
v = k_{cat}[E_0][S]/Km
\]

or

\[
v = k[S]
\]

From the relationship between Eqs. (2) and (3), a plot of the first-order rate constant \( k \), vs. the initial enzyme concentration \([E_0]\) should be linear. The apparent \( Km \) value which was obtained under these experimental conditions was \( 3.11 \times 10^{-2} \text{ M} \). In a range when [S] \( \ll Km \), the lipase-catalyzed reaction in reversed micelles was found to follow first-order kinetics. Figure 1 shows a plot of \( k \) vs. enzyme concentration at a substrate concentration of 3.11 \( \times 10^{-3} \text{ M} \). A linear relationship was obtained over a twenty-fold range of enzyme concentration, which is one of the important criteria for enzyme assays. The value of \( k/[E_0] \) was found to be \( 3.2 \times 10^{-3} \text{ (mg/ml·sec)}^{-1} \) from the slope of Fig. 1.

The fatty acid formation as a function of the reaction time at different enzyme concentrations and at a fixed substrate concentration \( (0.6228 \text{ M}) \) is shown in Fig. 2, in which only three curves are plotted to avoid complication. The progress curve of the lipase-catalyzed reaction shows a typical hyperbolic form, starting off linearly (the initial-rate phase) but falling off with increasing time. For example, the product formation was linearly proportional to the reaction time up to 22 min at an enzyme concentration of 4.4 mg/ml, but as the reaction continued the initial rate became shorter as the enzyme concentration increased. In this reaction, therefore, the assay time has to be limited to the time during which about 4.5% of substrate is utilized.

The relationship between the enzyme concentration and the initial rate of lipase-catalyzed hydrolysis of olive oil is shown in
Determination of Lipase Activity

Fig. 2. Fatty Acid Formation as a Function of the Reaction Time at Different Lipase Concentrations.
The reaction conditions were the same as those described in Fig. 1, except that the substrate concentration was 0.623 M. Enzyme concentration: ■, 21.9 mg/ml; ○, 8.8 mg/ml; ▲, 4.4 mg/ml.

Fig. 3. Initial Rate of Lipase in Reversed Micelles as a Function of the Enzyme Concentration.
The reaction conditions were the same as those described in Fig. 1, except that the substrate concentration was 0.623 M. ■, initial rate obtained from the slope of Fig. 2; ○, initial rate obtained by the three-point assay.

The rate of fatty acid formation obtained from the slopes of Fig. 2 is proportional to the enzyme concentration over a twenty-fold range of enzyme concentration, which is another important criterion for enzyme assay.12)

Based on these findings, a three-point assay of lipase in reversed micelles was established using the same conditions as already mentioned. To a screw-cap culture tube filled with 10 ml of reaction solution is added 99 µl of lipase solutions, and the content of fatty acids produced is determined at predetermined time intervals (2 times). The initial rate can be calculated from the slope of the line passing through the two points and the original point. Some examples are shown in Fig. 3, where one unit of enzyme is defined as the amount of lipase that liberated 1 µmol of fatty acids per min under the assay conditions. The lipase reaction under these assay conditions follows zero-order kinetics, since the substrate concentration (0.6228 M) is within the range of [S] > Km, and the ratio of the zero-order rate constant to enzyme concentrations was found to be $1.0 \times 10^{-4} \text{ M} \cdot \text{ml/min} \cdot \text{unit}$ from the slope of Fig. 3. Therefore, it is recommended that a lipase solution of over 140 units/ml should be diluted for the assay because 4.5% of the substrate will be utilized within 2 min at enzyme concentrations over 140 unit/ml.

An assay of lipase in reversed micelles has several different characteristics from those in conventional methods using an aqueous emulsion. First, lipase was found not to be inhibited by the substrate at concentrations up to 1.25 M (40%, v/v), and thus the condition for enzyme assaying that the velocity does not change during the period when a small fraction of the substrate is utilized was satisfactorily met, because in a reversed micellar system, the lipase can be assayed within a substrate range of [S] > Km. However, in an emulsion system, it cannot be assayed under such a condition because the enzyme has been found to be inhibited at a substrate concentration over approximately 3% (v/v).13) Second, the kinetic results obtained by the reversed micellar system are comparable among the studies because the system introduced a single liquid phase by solubilizing the water-insoluble substrate and
enzyme solution in an organic solvent with the help of a surfactant, and the system was chemically well defined.\textsuperscript{14,15} Third, a spectrophotometric analysis of fatty acids is more sensitive and time-saving than the procedures based on mannotometry\textsuperscript{16} or titrimetry.\textsuperscript{17} One can thus assay 3 units (as defined in this study) of lipase per 10 ml in the reaction solution. Finally, an assay in reversed micelles is convenient because the procedure excludes the tedious step of making a fresh emulsion for every experimental run, and many samples can be simultaneously treated.

\textit{Acknowledgment.} This work was supported in part by the Korea Science and Engineering Foundation (KOSEF).

\textbf{REFERENCES}