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Effects of Stirring and Addition of Chemical Compounds on Glycerolysis of Triglyceride in Reversed Micelles

CHANG, PAHN SHICK AND JOON SHICK RHEE*

Department of Biotechnology, Korea Advanced Institute of Science and Technology
373-1 Kusong-Dong, Yusong-Ku, Taejon 305-701, Korea

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Glycerolysis of triolein by lipase from *Chromobacterium viscosum* lipase was studied batchwise in AOT-isooctane reversed micelles. The reaction mixture was extracted with chloroform and the content of triolein, 1,2-diolein, 1,3-diolein, 1-monoolein, and free fatty acid in the condensed chloroform solution was determined using high performance liquid chromatography (HPLC). The effect of agitation speed on the initial rate of conversion was examined. As the speed of agitation increased up to 700 rpm, the reaction rate increased. However, above 700 rpm, the rate approached maximum and did not increase that much. The glycerolysis activity and the stability of the enzyme were affected by stirring and addition of histidine or copper. Addition of histidine and copper increased the rates of glycerolysis but they are detrimental to the operational stability in reversed micelles.

Among the enzymatic reactions in organic solvents, lipase-catalyzed reactions are very important in the area of biotechnology, especially fats and oils related biotechnology. Currently, very active research is going on in the area of the lipase-catalyzed reactions, because lipase is considered to be relatively stable in organic solvents and may be important in food and synthetic chemistry.

Yamane *et al.* (10) reported that partially purified lipases have shown the enzyme activities for the glycerolysis of triglycerides to produce mono- and/or di-glycerides in several heterogeneous systems (two phase system and hydrophobic membrane bioreactor).

Utilization of the lipase for the glycerolysis reaction was much delayed as compared with those of other ordinary enzymes because catalytic action of the enzyme is accomplished in the heterogeneous system containing water-insoluble substrate and almost all lipase preparations were found to be quite unstable.

To overcome this problem, we employed a relatively new technique of micellar solubilization of enzyme solution in the organic solvents in the presence of surfactant (1, 5, 6, 8).

In our previous work (3), we demonstrated that the reversed micelles are formed by appropriate surfactants and organic solvents and kinetic analysis of the enzymatic fat glycerolysis was feasible in the reversed micellar system.

In the present study of the batchwise glycerolysis of triolein by *Chromobacterium viscosum* lipase in reversed micelles, the effects of triolein concentration, agitation speed, and chemical compounds were investigated to find out the optimal conditions for the operation of glycerolysis reaction.

*Corresponding author

Key words: Glycerolysis, reversed micelles, lipase, histidine, copper

MATERIALS AND METHODS

Lipase and Chemicals

Lipase from *Chromobacterium viscosum* was used because it has high specific activity for the glycerolysis of triglyceride. The specific activity was 15.50×10^{-2} units/mg protein (for definition of the specific activity, see next section "Analysis of lipase-catalyzed glycerolysis").

Partially purified lipase purchased from Toyo Jozo (Shizuoka, Japan) was solubilized and stored in glycerol of molecular biology grade (99.99%, purchased from Sigma).

Bis(2-ethylhexyl) sodium sulfosuccinate (AOT) was purchased from Sigma (St. Louis, U.S.A.), and purified

according to the method of Tamamushi and Watanabe (9) and dried over P_2O_5 vacuum.

Isooctane of HPLC grade (purchased from Burdick and Jackson, Muskegon, U.S.A.) was used as the organic reaction media throughout the experiment, and stored over a Type 4A molecular sieve (purchased from Sigma) and filtered prior to use.

Analysis of Lipase-Catalyzed Glycerolysis

Glycerolysis activity was measured at 37℃ using triolein and glycerol as substrates. A screw-capped vial was filled with 10 m*l* of 50 mM AOT-isooctane solution containing triolein. The desired amount of glycerol containing lipase and water was injected into the vial and the reaction was initiated by vortex-mixing the mixture until clear. After incubation at 37℃, 0.2 m*l* of the sample was taken out from the reaction mixture.

To the sample in a test tube was added 3.0 m*l* of chloroform, and the test tube was shaken vigorously for 2 min. and was then left at least for 1 h to inactivate the enzyme. Then, 0.5 m*l* of water was added, the test tube was again shaken for 1.5 min., and the mixture was centrifuged for 5 min. at 2,400 rpm. The lower chloroform layer was taken out and stored in a round-bottom flask. The upper water layer was re-extracted twice with 3.0 m*l* of chloroform. A blank was prepared by the same procedure as described above except glycerol pool without enzyme being added.

The content of triolein, 1,2-diolein, 1,3-diolein, 1-monoolein, and oleic acid in the condensed chloroform layer was determined by a HPLC as described in our previous article (2).

One unit of lipase was defined as the amount of the enzyme that reacted 1 micromole of triolein/min. under the assay conditions.

For this glycerolysis study, the degree of glycerolysis was calculated using the following formula:

Degree of glycerolysis (%) =

$$\left[1-\frac{\text{Residual concentration of triolein}}{\text{Initial concentration of triolein}}\right]\times 100$$

All of the data are the average of triplicate samples and are reproducible within ±10%.

RESULTS AND DISCUSSION

Effect of Triolein Concentration on the Degree of Glycerolysis

Fig. 1 shows the degree of glycerolysis of triolein as function of reaction time at various triolein concentrations. At the triolein concentration of 6.67 mM, the degree of glycerolysis was about 80% after 80 h reaction time.

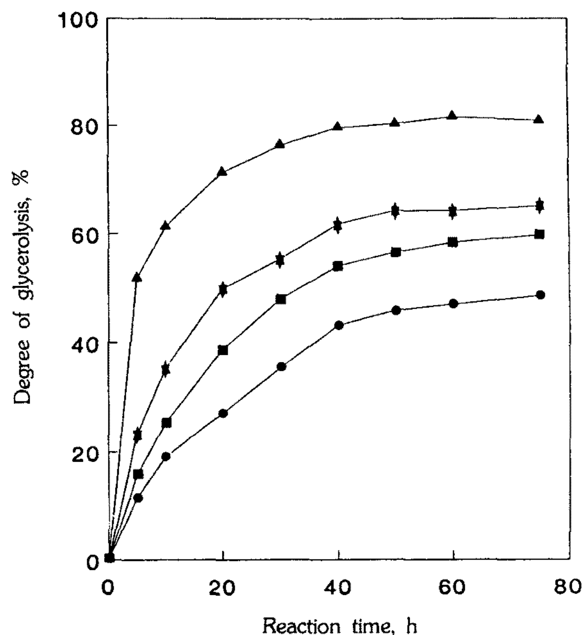

Fig. 1. Time course of the glycerolysis of triolein at a different concentration of triolein.
Glycerol, 200 mM; ▲, triolein, 6.67 mM; *, triolein, 10 mM, ■, triolein, 20 mM; ●, triolein, 40 mM.

A substantially higher rate for the removal of triolein at higher triolein concentration indicates that volumetric productivity (micromoles of triolein reacted/m*l*/h) can be increased using high triolein concentration in batchwise glycerolysis of triolein in reversed micelles. This is possible because lipase in reversed micelles was not inhibited at triolein concentration up to 40 mM. Fig. 1 also shows that the reaction progressed rapidly approximately for the first 20 h, and then slowed down thereafter. This might be due to the denaturation of lipase and chemical equilibrium between substrate (triolein) and products (mono- and di-olein).

Effect of Agitation Speed on Glycerolysis Rate

Since the reversed micellar system which contains triolein and glycerol is not an authentic monophase, a vigorous agitation might be required to achieve a substantial reaction rate. Therefore, to elucidate the nature of the lipase-catalyzed glycerolysis of triolein in the reversed micellar system, experimental data should be taken at the various agitation speeds which render the sufficient interfacial areas so that the observed reaction rate may not be limited by the interfacial area.

Effect of agitation speed on the initial rate of glycerolysis is shown in Fig. 2. As the agitation speed increased up to 700 rpm, the reaction rate increased. However, above 700 rpm, the rate approached maximum and

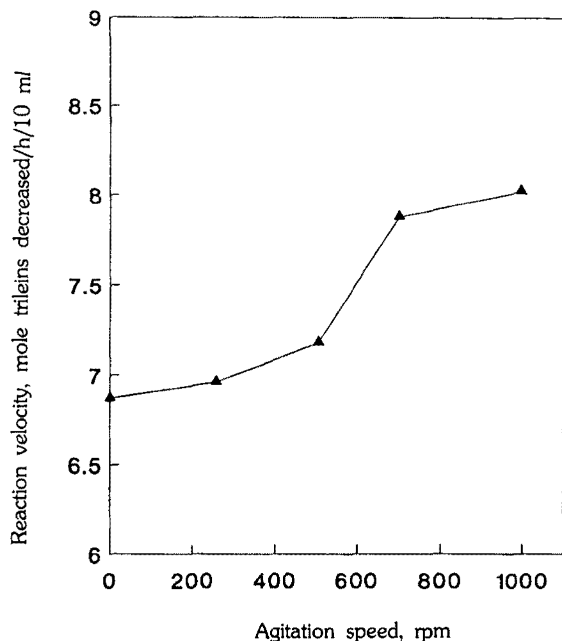

Fig. 2. Effect of stirring on lipase-catalyzed glycerolysis of triolein in reversed micelles.

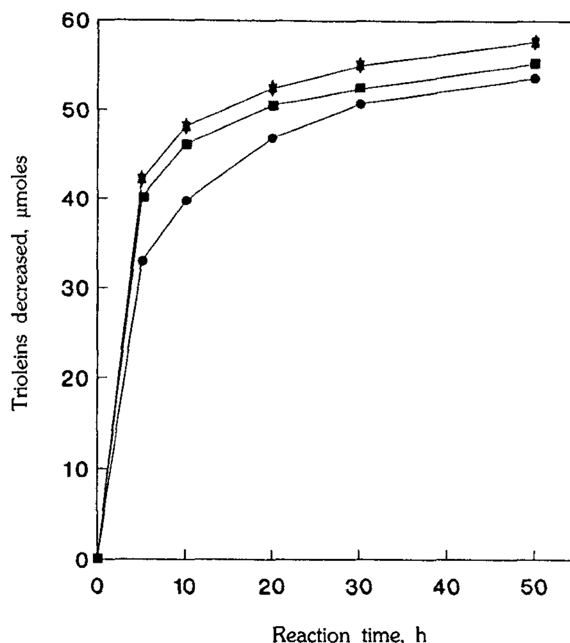

Fig. 3. Effect of addition of histidine.
●, control; ■, 12.5 mM; *, 25.0 mM.

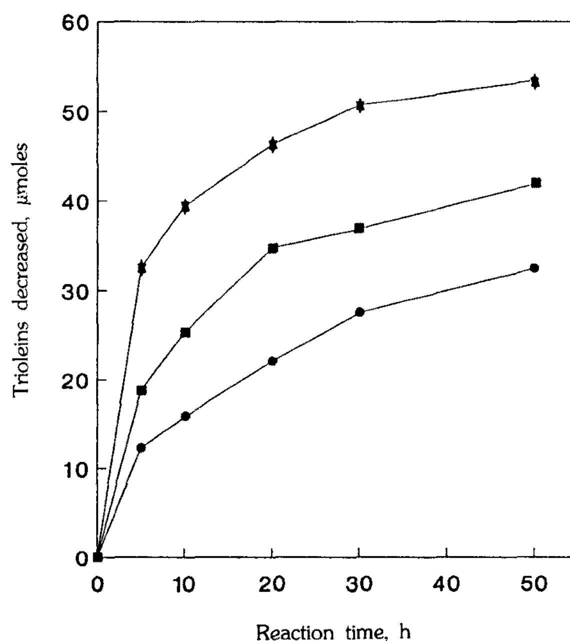

Fig. 4. Effect of addition of $FeCl_3$ on lipase-catalyzed glycerolysis of triolein in reversed micelles.
●, control; ■, 12.5 mM; *, 25.0 mM.

did not change that much. Stirring substantially increased the initial rate of lipase-catalyzed glycerolysis of triolein in reversed micelles, but the effect was slight. This result is indicative of the high mass transfer rate in the reversed micellar system. Mass transfer coefficient of triolein into the reversed micelles (or enzyme in glycerol pool) is very large (4). Therefore, progress of batchwise glycerolysis of triolein in reversed micelles was hardly affected by stirring.

Effect of Chemical Compounds on Glycerolysis Reaction Rate

Effects of addition of histidine, Fe^{3+}, Cu^{2+} to reversed micelles on batchwise glycerolysis are shown in Fig. 3, 4, and 5, respectively. The lipase in glycerol containing phosphate buffer (pH 7.), whose final concentration was made to 10 mM by adding 4.0% (v/v) of 250 mM phosphate buffer, was incubated with inorganic salts (Cu^{2+}, Fe^{3+}) and the amino acid (histidine) at 37℃.

Addition of histidine somewhat increased the rates of lipase-catalyzed glycerolysis of triolein in reversed micelles, but the effect was reduced when the glycerolysis was carried out over longer periods of time. This result was related to the stability of the enzyme. That is, by addition of histidine, the enzyme denatured more rapidly than that of control (Fig. 6). This explains why enhancing the reaction rates at initial period disappeared as the reaction time was extended.

In the mean time, addition of Fe^{3+} and Cu^{2+} substantially increased the rate. Fe^{3+} and Cu^{2+} were found to be very toxic to the progress of batchwise glycerolysis of triolein in reversed micelles. For example, initial rate of the reaction was below 40% of its original rate when

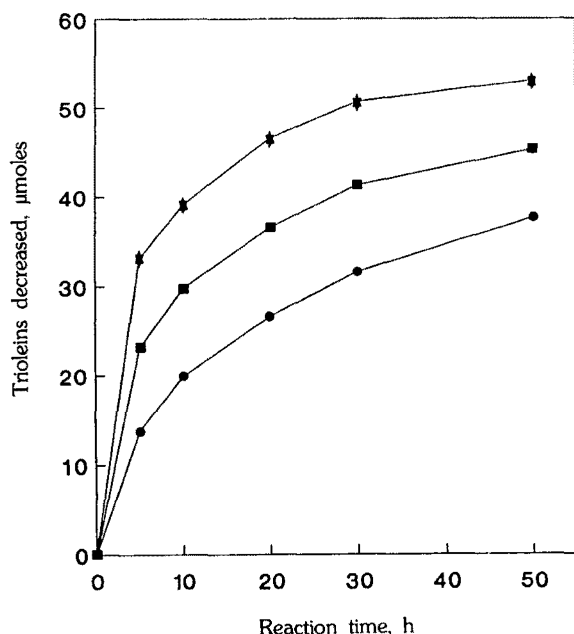

Fig. 5. Effect of addition of $CuCl_2$ on lipase-catalyzed glycerolysis of triolein in reversed micelles.
●, control; ■, 12.5 mM; *, 25.0 mM.

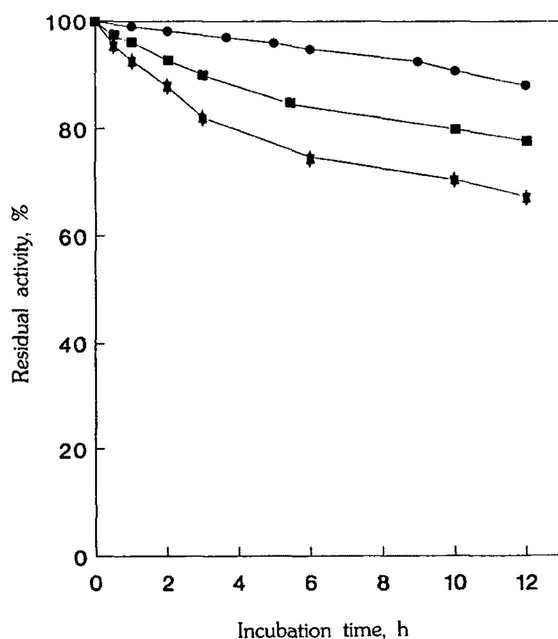

Fig. 6. Effect of stirring and histidine on lipase stability in reversed micelles.
●, control; ■, 1000 rpm of stirring; *, histidine 25.0 mM.

Cu^{2+} (25 mM) or Fe^{3+} (25 mM) was added and it was already reported that lipase was inhibited by addition of Fe^{3+} and Cu^{2+} to reversed micellar solutions (3).

Effecft of Stirring and Histidine on Lipase Stability

One serious problem for the biotechnological application of lipase to the fat glycerolysis is the stability of the enzyme during the reaction in isooctane micellar phase. After incubation of the enzyme, aliquots were taken out at the indicated time and the residual activity was measured.

Effect of addition of histidine on the enzyme stability in reversed micelles as shown in Fig. 6 seems to be contradictory to the results of batchwise glycerolysis activity.

Stirring (1,000 rpm) was found to be also detrimental to the stability. Stirring seems to increase the collision frequency between enzyme and triolein. For this reason, stirring denatured the enzyme structure and thereby stirring decreased the stability of the enzyme in reversed micelles.

AOT-isooctane reversed micellar system (control in Fig. 6) had the best operational stability and it can be explained by the contribution of glycerol and AOT molecules to the enzyme stabilization as described in our previous article (7).

CONCLUSION

Lipase solubilized in a glycerol phase by reversed micelles was found to catalyze the glycerolysis of triolein. It was of interest that stirring and addition of histidine and copper could facilitate the glyerolysis reaction but the stirring and addition of histidine were detrimental to the enzyme stability in the reversed micelles.

The field of enzyme-catalyzed organic reaction in reversed micelles is still in its infancy but shows promise for implementation on the technical application of enzymes acting at a phase interface.

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