0273-2289/96/6103—0299$09.00

Kinetic and Stability Characterization of *Chromobacterium viscosum* Lipase and Its Comparison with *Pseudomonas glumae* Lipase

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Received July 21, 1995; Accepted October 5, 1995

ABSTRACT

A kinetic study of *Chromobacterium viscosum* lipase was undertaken, and compared with *Pseudomonas glumae* lipase. Optimum operation conditions were pH 9.0 and 50°C for both enzymes. A substrate specificity study was also developed. Both enzymes showed higher activity on triglycerides with a long chain of fatty acid; the specific activity was always higher for *C. viscosum* lipase. Stability of both enzymes in aqueous medium at 60°C and pH 9.0 was evaluated. *C. viscosum* lipase was three times more stable than *P. glumae* lipase, with a $t_{1/2}$ value of 0.75 h. In addition, the activity of *C. viscosum* lipase with substrate concentration was studied with a triolein emulsion. A dependence of the intrinsic characteristics of the emulsion was observed. Therefore, stability of *C. viscosum* lipase B with reaction products was assayed in a micellar system. Acid products reduced the specific activity of the enzyme. Glycerol and high buffer concentration were stabilizers of enzyme deactivation. Finally, substrate specificity of *C. viscosum* lipase B in a micellar system was developed with tributyrin, tricaprylin, and triolein. Only tributyrin showed an apparent Michaelis-Menten kinetic with V_{max}^{app} = 958 U/mg and K_m^{app} = 75.5 m*M*. Tricaprylin and triolein showed diffusion limitations at low substrate concentration and substrate inhibition at high substrate concentration. Diffusion parameters were calculated for both these

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substrates. Mass transfer coefficients (k_l) were 0.314 Å/min and 1.53 Å/min for tricaprylin and triolein, respectively. Effectiveness factors (η) were 0.536 and 0.768 for tricaprylin and triolein, respectively.

Index Entries: *Chromobacterium viscosum; Pseudomonas glumae;* lipase; triglyceride hydrolysis; emulsion system; reversed micelles.

INTRODUCTION

Lipases (glycerol ester hydrolases, EC 3.1.1.3) catalyze the hydrolysis of esters other than acylglycerols. They are also active and stable in organic solvents, which make them adequate for esterification and transesterification reactions. These properties, together with the commercial availability of a wide range of lipases from different sources (microbial, yeast, fungal, and mammal) at low cost, make them interesting for biotechnological process development. In recent years several works has been reported on lipases in aqueous and organic media.

Particular interest on microbial lipases has increased for the past two decades. The availability of large quantities of microbial enzymes obtained by fermentation has broadened the search for potential industrial uses of bacterial and fungal lipases. Microbial lipases are widely diversified in their enzymatic properties and substrate specificities, and are generally more stable than animal or plant lipases. These properties make them very attractive for industrial applications (*1,2*).

A potent bacterium for lipase production was isolated from soil and identified as *Chromobacterium viscosum* (*3*). Commercial preparations of this lipase are available. The crude lipase preparation contained more than two species of lipases, which showed differences in mol wt and isoelectric point. Several methods have been developed for their purification (*4–8*). In our laboratory the purification of crude extract of *C. viscosum* lipase has been developed by gel filtration (*9*) and by hydrophobic interaction chromatography (*10*). Two fractions were obtained, lipase A, of high mol wt, was an aggregate of lipase B of low mol wt. *C. viscosum* lipase has been employed in lipolytic studies in aqueous (*3,5,11–13*) and micellar systems (*13–16*). Synthetic and transesterification capacities in organic systems have also been developed (*17,18*).

In the present work, triolein hydrolysis in aqueous media by *C. viscosum* lipase B is characterized. The enzyme was purified by gel filtration as previously reported (*10*). Kinetic parameters and optimum conditions were determined and compared with those shown by *P. glumae* lipase. In addition, the stability of both *C. viscosum* and *P. glumae* lipases in aqueous medium was studied. *C. viscosum* lipase stability in the presence of reaction products was carried out in a micellar system. Finally, substrate specificity was also studied with tributyrin, tricaprylin, and triolein in micellar medium, in order to determine the kinetic behavior with substrates with different chains of fatty acids.

MATERIALS AND METHODS

Materials

P. glumae lipase was supplied by Unilever (Vlaardingen, The Netherlands). *C. viscosum* lipase (3880 U/mg) was purchased from Finnsugar/Genecor (USA). This lipopytic preparation is a mixture of two components with lipolytic activity. Lipase A is a high mol wt aggregate of low mol wt lipases contained in lipase B fraction. Lipase B, with a high activity, has a mol wt of 33000 D. In the present study, lipase B was employed and it was previously purificated by gel filtration on a Superose 6 column as reported (*10*).

Triolein emulsion 50% (v/v), deoxycolic acid (DOC), and dioctyl sulfosuccinate (AOT) were from Sigma (St. Louis, MO). Triolein, 65% pure, tricaprylin, 97% pure, and tributyrin, 98% pure, were from Fluka (Buchs, Switzerland). Isooctane was from Riedel de Häen (Germany). All other reactives were of analytical grade and were used without further purification.

Triolein Emulsion Preparation

Triolein emulsion was prepared with triolein, in 50 m*M* Tris HCl buffer, pH 9.0, containing 1% arabic gum and 0.2% DOC. The two-phase system obtained was vigorously stirred and sonicated for 2 min, at 1 min intervals, and 250 Hz. The sonicator employed was a Labasonic L-2000, equipped with a Standard probe 5t (108 mm long × 19 mmø) point. Triolein emulsion obtained was stable for at least 48 h.

Activity Measurements

Aqueous System

The standard aqueous enzymatic assay was carried out in a reactor vessel at 37°C with magnetic stirring, using 10% (v/v) triolein in 50 m*M* Tris-HCl, pH 9.0, and 5 mL of reaction volume. The reaction was started by the addition of 200 μL of 0.05 mg/mL enzyme solution. The activity of lipase was assayed by the determination of the oleic acid produced, measured by the Lowry and Tinsley method (*19*). One unit of lipase activity was defined as the amount of enzyme necessary to liberate 1 μmol of product per min.

Micellar System

Reversed micelles with a water content (W_o = $[H_2O]/[AOT]$) W_o = 12, were prepared with 100 m*M* AOT in isooctane, adding the appropriated amount of lipase solution in 50 m*M* Tris-HCl buffer, pH 9.0. The standard micellar enzymatic assay was carried out in a reactor vessel at 37°C with magnetic stirring, using 4 mL of micellar medium and 1 mL triolein. The reaction was started by the addition of substrate. The activity was assayed by the determination of the fatty acid produced, measured by the Lowry and Tinsley method (*19*).

Lipase Stability

Stability was assayed both in aqueous emulsion and reversed micellar systems. The deactivation process was adjusted to a first-order mathematical model, and the half-life time determined.

Aqueous System

Enzyme solutions (0.25 mg/mL) of *C. viscosum* and *P. glumae* lipases were prepared in 50 m*M* Tris-HCl, pH 9.0, and incubated at 60°C. Samples were taken at intervals and the residual enzyme activity was measured under standard conditions in the aqueous system.

Micellar System

Stability of *C. viscosum* lipase in the presence of products (50 m*M* oleic, caprylic, and butyric acids, and 17 m*M* glycerol) was performed in a micellar system. Reversed micelles, with a W_o = 12, were prepared in 100 m*M* AOT in isooctane, adding the appropriated amount of a 0.25 mg/mL lipase solution in 50 m*M* Tris-HCl buffer, pH 9.0. The final lipase concentration was 5.4 μg/mL in the micellar system. Samples were taken at certain intervals, and the residual activity determined under standard conditions in micellar system.

Substrate Specificity

The enzymatic activity of *C. viscosum* lipase was studied, with triolein, tricaprylin, and tributryin in micellar system. The substrate concentration range was 0–300 m*M*. Substrate specificity of *P. glumae* lipase was also determined at 100 m*M* substrate concentration, for the same substrates.

RESULTS AND DISCUSSION

Effect of pH

The activity of both *C. viscosum* and *P. glumae* was assayed at various pHs and at 37°C. This study was carried out in an aqueous system and results are shown in Fig. 1. Although *C. viscosum* lipase always showed higher activity than *P. glumae* lipase, the activity profile as a function of pH values was similar for both enzymes. Optimum activity was measured at pH 9.0 and a significant increase between 8.0 and 9.0 pH values was observed for both enzyme preparations.

These results are comparatively higher than those reported at pH 7.0–8.0 at 37°C for crude extract of *C. viscosum* lipase (*3*), and 6.5 at 37°C for purified lipase B of *C. viscosum* (*5*), with olive oil emulsion as substrate. In the second case a high activity range for pH 4.0–9.0 was described. In a previous research work, the optimum pH value obtained for *C. viscosum* lipase B was around 7.0 at 30°C (*13*). On that occasion the enzyme was purified by liquid–liquid extraction in a reverse micellar system, with the

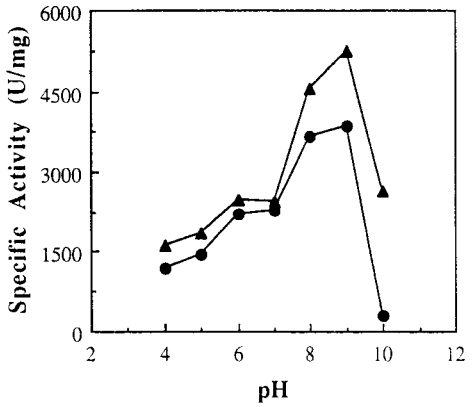

Fig. 1. Effect of pH on specific activity of *C. viscosum* (▲) and *P. glumae* (●) lipases at 37°C, in aqueous system.

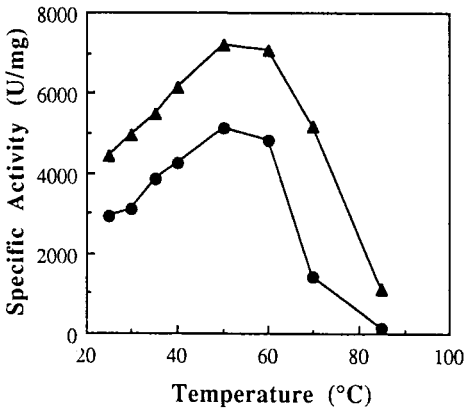

Fig. 2. Effect of temperature on specific activity of *C. viscosum* (▲) and *P. glumae* (●) lipases at pH 9.0, in aqueous system.

subsequent re-extraction to an aqueous phase. The extraction yield of lipase B at basic pH values was very poor. Therefore, no assays were performed at pH values higher than 8.0. The main difference between the previous results and those reported here was the purification process. During the liquid–liquid extraction, enzyme deactivation by organic solvent or surfactant could occur differently at different pH values. The gel filtration purification system gave an aqueous solution of concentrated lipase (≈2.5 mg/mL), which was easily used in aqueous solution at different pH and concentration values.

The optimum pH value obtained (pH 9.0) with the *P. glumae* lipase was the same as reported by another author using soybean triacylglycerol in aqueous emulsion as substrate (*20*). A basic optimum pH (8.0–9.5) for different *Pseudomonas* genus lipases has been also reported (*21*).

Effect of Temperature

The optimal temperature for both *C. viscosum* and *P. glumae* lipases was determined at pH 9.0. Results are shown in Fig. 2. As occurred with

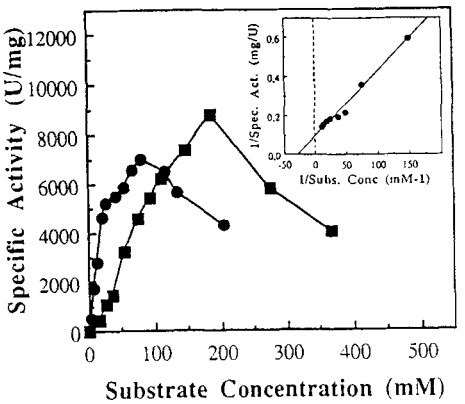

Fig. 3. Effect of substrate concentration on the activity of *C. viscosum* lipase, using commercial (●) and prepared (■) triolein emulsions (pH = 9.0 and 37°C). Inserted: Lineweaver-Burk representation for Sigma triolein as substrate.

pH, the profile of activity with temperature was similar for both enzymes. Optimum temperature was at 50°C for both, showing also high activity at 60°C. *C. viscosum* lipase B always displayed a higher lipolytic activity.

In previous work, 42°C was determined as optimum temperature for triolein emulsion hydrolysis by *C. viscosum* lipase B at pH 7.0, with high activity between 42 and 50°C (*13*). Other authors reported an optimum temperature at 65°C (3) and 170°C, at pH 7.0 (*5*), for native *C. viscosum* lipase and purified *C. viscosum* lipase B, respectively, using an olive oil emulsion as substrate. In these two works, the activity was determined after incubation for 20 min, and a stabilization by substrate at high temperature (70–80°C) was described. In the present work, the initial velocity was determined during the first 5 min of reaction, and low enzyme stability was observed at 60°C and 70°C (data not reported). Thus, the differences in optimum temperature could be determined by the different experimental procedures.

The two lipases assayed have a similar behavior, with temperature and pH showing the same optimum operational conditions, pH 9.0 and 50°C, and with some differences in specific activity. This coincidence could reflect a similarity between the enzymes, as suggested by their biochemical characterization (22). The differences observed in the specific activity of both lipases may be caused by the different residual activation displayed by the lipases as a result of storage conditions.

Effect of Substrate Concentration

Two different emulsions of triolein were assayed as *C. viscosum* lipase B substrate. A commercial triolein emulsion from Sigma, and a triolein emulsion prepared in our laboratory, as reported in Materials and Methods. The activity profile with substrate concentration was different for both triolein emulsions, as shown in Fig. 3. Higher specific activity at

low substrate concentration was observed for commercial triolein; the maximum activity was obtained for prepared triolein at 180 m*M* substrate concentration. In both cases, there was a substrate inhibition effect, which was evident at different substrate concentrations. Enzyme was inhibited above 80 m*M* in commercial triolein, while with prepared triolein, inhibition effect was detected over 180 m*M*.

The apparent Michaelis-Menten kinetic parameters were determined from Lineweaver-Burk plot, for the Sigma substrate, omitting the inhibitory substrate concentrations (Fig. 3). K_m^{app} and V_{max}^{app} values of 35.7 m*M* and 10733 U/mg, respectively, were obtained. Diffusion limitations were also observed with prepared triolein, as can be observed from Fig. 3 for the low substrate concentrations. This condition, along with the substrate inhibition effect, made it impossible to determine the apparent kinetic parameters with this substrate.

The interpretation of lipases kinetic behavior in emulsion systems is more complex than the other hydrolases. Lipase action occurs at the interface of two phase systems. Lipolytic activity is determined by lipase adsorption and catalysis in a heterogeneous system. Lipase activity is related with the physicochemical properties of the interface and the amount of interfacial area. In addition, emulsions are dynamic systems, which physicochemical properties are constantly changing in time, as a result of, for example, product formation. Consequently, the droplet-size distribution and therefore the interfacial area change during the course of the reaction, which poses an obstacle to proper interpretation of lipase kinetics (*23*). Thus, the kinetic behavior of *C. viscosum* lipase B obtained in the present work, can be explained by the differences between both substrate emulsions employed. On the one hand, the assay system was different at every substrate concentration because of the different amount of emulsion added in each case. In this way, the ratio organic phase, aqueous phase, and the interfacial area increased when substrate concentration increased. On the other hand, the two emulsions had some intrinsic differences: The laboratory-prepared triolein was obtained in the same buffer used for enzyme activity assay; the commercial triolein emulsion was not buffered. Besides, it may be supposed that the droplet size was lower for the Sigma triolein, which means a high interfacial area, as can be concluded from results reported in Fig. 3, at low substrate concentrations.

Therefore, assuming that the interfacial area of the prepared triolein emulsion was lower than that of the Sigma emulsion, the displacement of both substrate inhibition and maximum activity with substrate concentration could be a function of interfacial area available for catalytic activity. The higher maximum activity obtained with the prepared emulsion could be a consequence of a better buffering system, or of the intrinsic characteristics of this emulsion, fresh prepared and free of other components of the commercial emulsion. Thus, the characteristics of a substrate emulsion was a determining factor in the kinetic behavior of *C. viscosum* lipase B, with triolein as substrate.

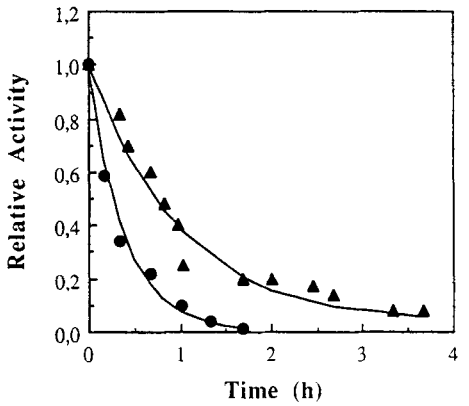

Fig. 4. Stability of *C. viscosum* (▲) and *P. glumae* (●) lipases at 60°C and pH 9.0, in aqueous system. Experimental results adjusted to a first-order deactivation.

Table 1
Deactivation Parameters of both *C. viscosum* and *P. glumae* Lipases Incubated at 60°C and pH 9.0 in Aqueous Medium

Lipase	$t_{1/2}$ (h)	k (h^{-1})
P. glumae	0.27	2.60
C. viscosum	0.75	0.92

Lipase Stability

Lipase stability was assayed at pH 9.0 and 60°C, in aqueous medium. Both *C. viscosum* and *P. glumae* lipases were studied. Evolution of relative activities with the times are shown in Fig. 4, where the respective deactivation mathematical models are also shown. Half-life time ($t_{1/2}$) and deactivation constant (k) were calculated following a first-order decay model. These parameters are reported in Table 1. *P. glumae* lipase was less stable under the assay conditions, *C. viscosum* lipase B being three times fore stable than *P. glumae* lipase, with a half-life time of 0.75 h. The initial specific activity was 5800 U/mg for *C. viscosum* lipase B, while it was 3900 U/mg for *P. glumae* lipase. *C. viscosum* lipase B always displayed more lipolytic activity than *P. glumae* lipase. Therefore, *C. viscosum* lipase B was more active and stable than *P. glumae* lipase, under the conditions tested.

High stability of *C. viscosum* crude lipase has been reported at 60°C (3). However, the incubation time was only 10 min and the deactivation parameters were not calculated. For lipase B of *C. viscosum*, a high stability below 40°C has been described, but with a complete loss of activity at

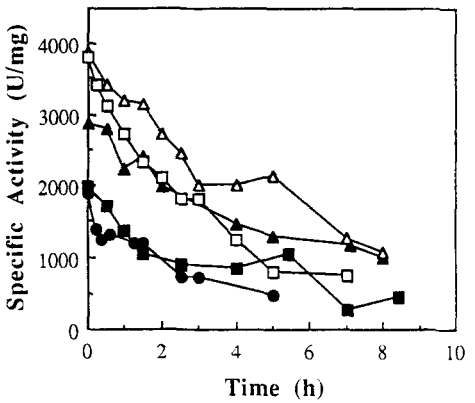

Fig. 5. Stability of *C. viscosum* lipase B at 60°C, pH 9.0, and 50 m*M* buffer concentration, in a micellar system, with products: reference (□), 17 m*M* glycerol (△), 50 m*M* caprylic acid (●), 50 m*M* oleic acid (■), and 50 m*M* butyric acid with 500 m*M* buffer concentration (▲).

60°C within 20 min (*5*). Our results showed a higher stability of this enzyme, which had a residual activity of 450 U/mg after 3 h incubation at 60°C and pH 9.0. Presumably, the stability would be increased by decreasing the temperature and the pH values. This would agree with previous reported data, where a half-life time of 21 d at 30°C and pH 7.0 for *C. viscosum* lipase B in aqueous system was obtained (*13*).

On the other hand, the stability of *P. glumae* lipase was very low, with a strong fall of activity in the first 20 min of incubation at 60°C. Another author studied the stability under different conditions, and reported a completely loss of activity at pH 9.0 and room temperature (*20*); however, the enzyme was more stable in the presence of some metal ions, even at high pH values (8.0, 9.0). For other *Pseudomonas* lipases, high stability has been reported, even at high temperatures (40–70°C) (*21,24*).

Lipase Stability in Presence of Reaction Products

C. viscosum lipase B stability was also studied in the presence of reaction products: 17 m*M* glycerol and 50 m*M* butyric, caprylic, and oleic acids. Glycerol and butyric acid are soluble in aqueous phase, but caprylic and oleic acids are not. It was not possible to obtain stable aqueous emulsions of these two latter products. Therefore, to use comparable systems, the stability tests with products were assayed in a reversed micellar system, AOT-isooctane (W_o = 12). An AOT-isooctane system has been described as the optimum for *C. viscosum* lipase activity in reversed micelles (*15*).

The results obtained are shown in Fig. 5. Enzyme solution (0.25 mg/mL) was prepared in 50 m*M* Tris-HCl buffer pH 9.0. With butyric acid, no activity was obtained at that buffer molarity. Butyric acid is water-soluble and could be located inside the reversed micelles, with a subsequent decrease of the local pH. Thus, the loss of activity was probably caused by

Table 2
Deactivation Parameter of *C. viscosum* Lipase B Incubated with Different Reaction Products in Reverse Micellar System at 60°C and pH 9.0

Assay	$t_{1/2}$ (h)	k (h^{-1})
Reference	2.23	0.31
Glycerol 17 m*M*	4.07	0.17
Butyric acid 50 m*M*	4.33	0.16
Caprylic acid 50 m*M*	1.69	0.41
Oleic acid 50 m*M*	3.01	0.23

a low pH value in the water pool. Buffer molarity was increased 10 times, to 500 m*M*, for butyric acid assay. This change determined an increase in enzyme stability, as will be reported below.

On the other hand, the enzyme was inhibited by acid products, as reflected by the loss of initial specific activity. With 50 m*M* butyric acid, the initial activity decreased about 25%; 50% of initial lipolytic activity was lost with 50 m*M* oleic and 50 m*M* caprylic acid. However, 17 m*M* glycerol did not affect the lipase initial activity.

The deactivation parameters, calculated following a first-order decay model, are reported in Table 2. The half-life time and deactivation constant values have to be interpreted along with the specific activity results reported in Fig. 5. The lower stability obtained was for the caprylic acid, which also displayed the lower specific activity. In this way, caprylic acid had a double negative effect, as enzyme inhibitor and as nonprotector of enzyme stability. On the other hand, although the system with 50 m*M* oleic acid led to good stability parameters, even higher than that obtained for the reference assay, the specific activity was also very low. Oleic acid stabilized the enyzme, but a high product inhibition was also detected.

As was expected, and independent of the enzymatic inhibition by product, the system with 50 m*M* butyric acid and 500 m*M* Tris-HCl buffer was the most stable among the fatty acids tested. The enzyme was protected from pH deactivation by using a high buffer concentration. From the assays performed at 50 m*M* buffer concentration, the highest stability was obtained with 17 m*M* glycerol, which showed higher specific activity than the reference. Glycerol was not mixed with isooctane, so it was introduced inside the micelles by increasing the micellar size. The higher stability could be a consequence of this phenomenon, or glycerol could operate as a lipase stabilizer, or a combination of both factors may occur. Thus, enzyme was stabilized both by high buffer concentration and by glycerol.

Lipase Stability in an Aqueous System vs a Micellar System

Figure 6 shows the stability of *C. viscosum* lipase B in aqueous and reversed micellar systems. This study was developed at 60°C and pH 9.0.

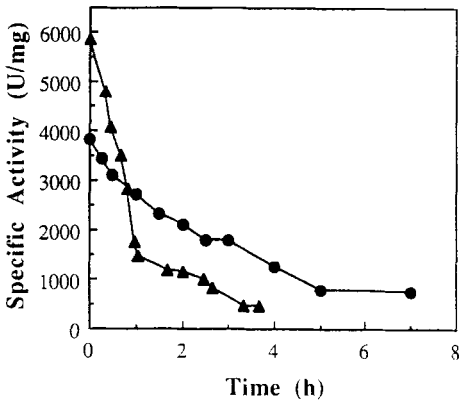

Fig. 6. Stability of *C. viscosum* lipase B at 60°C, pH 9.0, in reversed micellar (●) and aqueous (▲) systems.

Higher initial specific activity was obtained in aqueous medium, but a strong decrease of activity occurred in the first hour of incubation. The major stability was obtained for the micellar system, where the loss of activity was less pronounced. The half-life time obtained through a first-order deactivation equation, were 0.75 h and 2.23 h for aqueous and micellar systems, respectively. Therefore, the enzyme in the micellar system was three times more stable than in the aqueous system. In a previous work, a higher stability of *C. viscosum* lipase B in micellar system was also reported (*13*).

Substrate Specificity

A substrate specificity study was undertaken with *C. viscosum* lipase B using tributyrin, tricaprylin, and triolein as substrates. Preliminary assays were developed in aqueous medium, with aqueous emulsions of these three substrates prepared in our laboratory. Results were difficult to compare. The physical properties of each emulsion were different and it would be necessary to refer a specific activity to an interfacial area. Thus AOT reversed micelles were used again. The specific activity values determined at every substrate concentration are reported in Fig. 7.

The highest activity and affinity were for triolein, which gave a maximum specific activity of 4022 U/mg at 106 m*M* substrate concentration. The maximum activity with tricaprylin was 3565 U/mg at 179 m*M* substrate concentration. Tributyrin showed lower affinity and activity, and it was necessary to increase the enzyme concentration five times in the reaction vessel to obtain a measurable amount of product under the experimental conditions tested. Only tributyrin showed an apparent Michaelis-Menten kinetic, with kinetics parameters of K_m^{APP} = 75.5 m*M* and V_{max}^{APP} = 958 U/mg. With the other substrates, tricaprylin and triolein, the enzyme showed diffusion effects at low substrate concentrations, and substrate inhibition at high substrate concentrations.

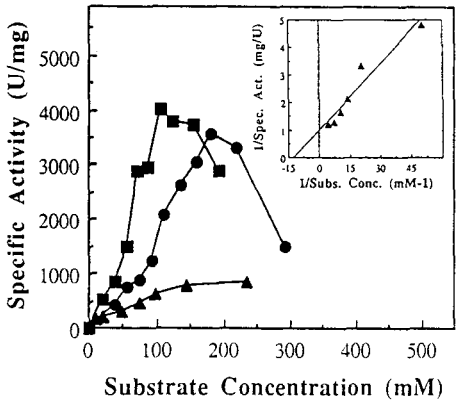

Fig. 7. Substrate specificity of *C. viscosum* lipase B with tributyrin (▲), tricaprylin (●), and triolein (■), at different substrate concentrations. Micellar system at 37°C and pH 9.0. Inserted: Lineweaver-Burk representation for tributyrin as substrate.

In reversed micellar systems, a Michaelis-Menten kinetic behavior has been reported for several lipases (*25*), with different substrates. In a previous work (*13*), triolein hydrolysis in micellar medium has also been reported. The system was different in W_o, AOT concentration, pH, and temperature, whereas the profile of activity with substrate concentration was similar to that described here. Product inhibition was detected also, as approx 110 m*M* triolein concentration.

Chang and Rhee (*15*) reported the triolein glycerolysis by *C. viscosum* lipase in AOT-isooctane at 37°C. The apparent kinetic parameters obtained were 0.87 m*M* and 0.029 U/mg for K_m^{app} and V_{max}^{app}, respectively, and were determined in a substrate concentration range from 0 to 40 m*M*. These authors employed a different reaction system: *C. viscosum* lipase was employed without further purification; the specific activity was defined as μmol of triolein, and not as a function of product formation; this means to reduce comparatively three times the activity values and consequently the V_{max} value. In addition, the initial velocity was determined after 5 h of reaction in the presence of glycerol. Measurement of initial velocity at long time could have determined an inherent error in specific activity, and diffusion effects could be omitted. Thus, the results obtained by Chang and Rhee (*15*) are difficult to compare with those reported in the present work.

In order to establish a comparison between lipolysis of tricaprylin and triolein, a mathematical treatment of the experimental data was developed, and the diffusion parameters obtained (Table 3). For this mathematical treatment, only the results adjusted to a first order kinetics were considered (from 0 to 88 m*M* for triolein and from 0 to 160 m*M* for tricaprylin). Results were adjusted to Eq. (1):

$$1 / V_{ef} = (K_m / V_{max} + 1 / k_l \cdot a)\, 1 / S_b \qquad (1)$$

Table 3
Diffusion Parameters of C. *viscosum* Lipase B, with Two Substrates, in Micellar System at 37°C and pH 9.0

Substrate	k_l (Å/min)	η
Tricaprylin	0.314	0.536
Triolein	1.530	0.768

where V_{ef} was the measured velocity, K_m and V_{max} were the intrinsic kinetic parameters, k_l was the mass transfer coefficient, "a" was the superficial area, and S_b the substrate concentration in bulk.

The mass transfer coefficient (k_l) for tricaprylin and triolein substrates can be calculated from a modification of this equation.

$$1 / k_l = a\,(S_b / V_{ef} - K_m / V_{max}) \tag{2}$$

K_m / V_{max} were calculated as the slope of $1/V_{ef}$ vs $1/S_b$ representation in the range of high substrate concentrations. $a = 0.15\,Å^{-1}$ was determined from the reversed micelles radii corresponding to $W_o = 12$ (*26*).

The effectiveness factor (η), defined as $\eta = V_{ef} / V_{kin}$, where V_{kin} is the initial velocity when the reaction is controlled kinetically, was also determined. For a first order kinetics, the velocity can be expressed as in Equation 3:

$$V_{ef} = k_l \cdot a\,(S_b - S_s) = V_{max}\,S_b / K_m = \eta\,V_{max}\,S_s / K_m \tag{3}$$

where S_s is the substrate concentration in the microenvironment of the enzyme.

From this equation, Eqs. (4) and (5) can be obtained:

$$\eta = S_s / S_b \tag{4}$$

$$S_s = [k_l \cdot a / (V_{max} / K_m + k_l \cdot a)]\,S_b \tag{5}$$

There, η can be calculated as a function of known parameters.

$$\eta = k_l \cdot a / (V_{max} / K_m + k_l \cdot a) \tag{6}$$

The values determined are reported in Table 3.

The diffusion of triolein was higher than diffusion of tricaprylin, which led to a higher effectiveness factor. The calculated k_l and η allowed the determination of the theoretical reaction velocity at every substrate concentration. Figure 8 shows both the theoretical and experimental velocity values obtained for those substrate concentrations in which kinetic behavior was of first order.

As can be seen when substrate concentration was low, until 55 m*M* and 75 m*M* for triolein and tricaprylin, respectively, there was a coincidence of experimental and theoretical results. Thus, at low substrate con-

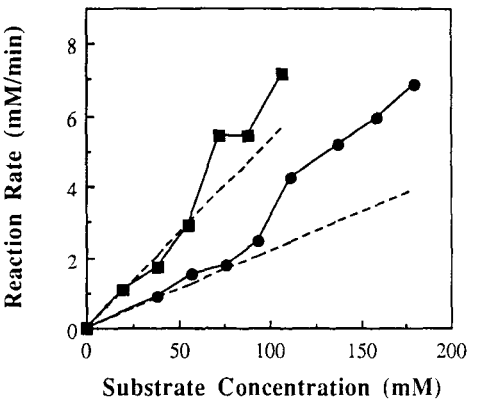

Fig. 8. First-order kinetic showed by *C. viscosum* lipase B, with tricaprylin (●) and triolein (■) as substrates. Comparison between theoretical (---) and experimental (—) reaction rate. Micellar system at 37°C and pH 9.0.

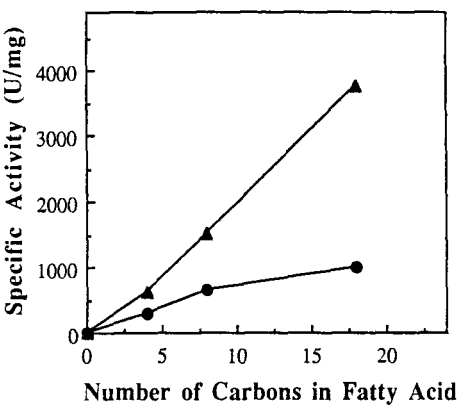

Fig. 9. Specific activity with number of carbons in fatty acid showed by *C. viscosum* (▲) and *P. glumae* (●) lipases.

centration, the system was under diffusion control. When substrate concentration increased, the kinetic control was more important, resulting in a reaction rate controlled by the combination of both parameters.

Finally, a substrate specificity study was performed with *P. glumae* lipase in reversed micellar system. The substrates, tributyrin, tricaprylin, and triolein, were assayed at 100 m*M*. The results, as a function of the chain length, were compared with those obtained for *C. viscosum* lipase B at the same substrate concentration (Fig. 9). *P. glumae* lipase continued to display lower specific activity than the *C. viscosum* lipase at every assay.

Both enzymes showed increasing activity with the chain length of the fatty acids. Although the *P. glumae* lipase displayed lower lipolytic activity, the substrate affinity was close to that shown by *C. viscosum* lipase B. This also supports the similarity between the two enzymes.

CONCLUSIONS

Lipolytic activity of *C. viscosum* lipase B and *P. glumae* lipase were studied. Similar behavior was observed for both enzymes, which showed the same optimum pH (9.0) and temperature (50°C) values. Similar substrate specificities were also obtained. These results seem to reflect a similarity between the two enzymes, but specific activity and stability were always higher for *C. viscosum* lipase B.

The kinetic behavior of *C. viscosum* lipase B in an aqueous system was influenced by the intrinsic characteristics of the substrate emulsion employed. Also, this enzyme was stabilized by glycerol and at high buffer concentration; an inhibitory effect by the acid products was observed. On the other hand, *C. viscosum* lipase B was more stable in the micellar system than in the aqueous system.

This work contributes to lipase knowledge, and could be a base for further applications or research of bacterial lipases.

ACKNOWLEDGMENTS

The award of a fellowship to M. R. Castellar from the M.E.C. of Spain in support of this work is gratefully acknowledged.

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