Kinetics of Enzymatic Synthesis of Isopropylidene Glycerol Esters by Goat Pregastric Lipase

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ABSTRACT: The goat pregastric lipase-catalyzed esterification of isopropylidene glycerol with caproic acid, to form isopropylidene glycerol caproate, followed a ping pong bi bi mechanism incorporating an acyl-enzyme intermediate. The maximum rate was estimated to be 96 μmol min^{-1} mg^{-1} in isooctane at 35°C, and the Michaelis-Menten constants for isopropylidene glycerol and caproic acid were 0.23 and 0.32 M, respectively. The catalyzed rate also correlated well with the partition coefficient of caproic acid between the organic and aqueous phases. The results suggest that the desolvation energy of the substrate from the bulk medium to the active site of the enzyme dominates the reaction rate for the enzyme-catalyzed reaction in organic media.

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KEY WORDS: Caproic acid, enzyme catalysis, esterification, goat, isooctane, isopropylidene glycerol, partition coefficient, ping pong bi bi mechanism, pregastric lipase.

Monoacylglycerols are most widely used as emulsifiers in the food, pharmaceutical and cosmetic industries. Industrial production of monoacylglycerols is by continuous chemical glycerolysis of fats and oils at high temperatures and in an atmosphere of nitrogen gas. More recently, enzyme-catalyzed production of monoacylglycerols or partial acylglycerols has proved promising due to the mild reaction conditions employed and high selectivity obtained. Synthesis of monoacylglycerols *via* enzymatic means has been carried out by hydrolysis (1,2) or glycerolysis (3,4) of fats and oils, or by direct synthesis from glycerol and fatty acids or transesterification from fatty-acid esters (5,6). A comprehensive summary of the recent progress of enzymatic production of monoacylglycerols has been made by Bornscheuer (7).

Use of a protected glycerol derivative, 1,2-*O*-isopropylidene glycerol (IPG), is an alternative method which has been used successfully for catalyzed synthesis of position-specific and enantiomerically-enriched monoacylglycerols in the presence of various enzymes. Upon mild hydrolysis of the protecting group (8,9), a highly purified form of monoglyceride may then be obtained. Over 90% conversion was reported (10) by direct synthesis of IPG-oleate from IPG and oleic acid catalyzed by *Mucor miehei* (Lipozyme®, Fluka, Buchs, Switzerland) and using an oleic acid/IPG ratio >3. Hess *et al.* (11) reported 84% conversion of stearic acid or ethyl stearate to IPG-esters by using *Pseudomonas cepacia* as the catalyst in hexane. Enol esters have also been used as acyl donors which shift the position of the equilibrium because they cause irreversible tautomerization of the vinyl alcohol or isopropenyl alcohol by-product. In a short reaction time, 100% conversion of long-chain fatty-acid vinyl esters was achieved (12,13).

Even though pregastric lipases have not yet been found capable of catalyzing reactions other than hydrolysis of esters or lipids in aqueous solution, we have demonstrated that the native enzyme suspension of goat pregastric lipase (GPGL; EC 3.1.1.3, triacylglycerol acylhydrolase) can catalyze the synthesis of alkyl esters in organic solvents (14). The results suggested that the lipase should be suitable for esterification reactions in anhydrous media. This paper describes a series of kinetic studies which were carried out to further our understanding of the GPGL-catalyzed esterification reaction and to explore the potential use of the enzyme in production of other lipids.

MATERIALS AND METHODS

Materials. Protected glycerols: 1,2(2,3)-IPG (99%, Sigma Chemical Co., St. Louis, MO), 2,3-isopropylidene-*sn*-glycerol (96%, Lancaster Chemicals, Morecambe, United Kingdom) and 1,2-isopropylidene-*sn*-glycerol (98%, Acros Organics, Geel, Belgium) were used as provided. *N*-Morpholine ethanesulfonic acid (Mes) was a Serva product (Heidelberg, Germany). Tris(hydroxymethyl)-aminomethane (Tris) was from United States Biochemical Corp. (Cleveland, OH). All solvents were analytical-grade reagents and were predried against a 4 Å molecular sieve (BDH Laboratory Supplies, Dorset, England). Tributyrylglycerol (Sigma) was used as substrate to determinate the lipase activity of the hydrolysis reaction. The water content of the substrate-stock solution was determined by a Karl-Fisher titration (736 GP Titrino, Metrohm, Herisau, Switzerland) and was equal to or less than the detection limit (0.01% w/w) (14).

Lipases. Partially purified GPGL powder was prepared from

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the commercial pregastric lipase extract (4.2 U/mg in tributyrin/lecithin emulsion at pH 6.5, 35°C; NZ Dairy Meats, Eltham, New Zealand) which followed the method reported previously (14). The crude GPGL enzyme-extract solution [10 g in 400 mL Tris/HCl (50 mM, pH 8.0)], after centrifugation, was fractionated by ion exchange chromatography (Q-Sepharose, 5 cm × 20 cm) in a flow rate of 3.0 mL min^{-1}. The active lipase fractions collected by salt-gradient elution of the ion exchange chromatograph were then precipitated by 2 M $(NH_4)_2SO_4$. The pellet thus obtained was redissolved in Tris/HCl buffer and dialyzed against Tris buffer, and then subjected to an Affi-Gel Blue gel column (1.6 × 20 cm) at pH 8 at a flow rate of 1.0 mL min^{-1}. The lipase fraction was eluted with 2 M NaCl in 50 mM Tris/HCl. The collected fractions were concentrated by ultrafiltration (Amicon Centriprep® 10 and Centricon® 10, Bedford, MA) and the concentrated enzyme solution was finally purified by gel filtration chromatography. The lipase fractions from gel filtration were collected, concentrated, extensively dialyzed against 10 mM Mes buffer (pH 6.5), and freeze-dried. The lipase activity of the final white enzyme powder was determined as 352.5 U/mg in tributyrin/lecithin emulsion at pH 6.5, 35°C (14), an activity corresponding to 65% lipase component and 35% other proteins, mainly albumin.

Two other commercial enzyme sources, porcine pancreatic lipase (PPL, 19 U/mg of solid powder, using olive oil at pH 7.7, 37°C) and *Candida rugosa* lipase (CRL, 1010 U/mg of solid powder, using olive oil at pH 7.2, 37°C) were purchased from Sigma and were used as controls without further purification. All three enzymes were incubated in pure isooctane at 35°C for 24 h and after filtration the powdered enzymes were dried *in vacuo*. No significant loss of activity was detected for any of the enzymes.

Enzymatic esterification of IPG esters. Enzyme powder (2 mg GPGL, 2 mg CRL, or 20 mg PPL), ground molecular sieve (0.2 g), and substrate solution (2 mL in isooctane, various concentrations of IPG and caproic acid) were placed in 4-mL screw-capped vials. The reaction mixture was then incubated at 35°C in a horizontal shaker. Aliquots (100 µL) of the mixture were then removed and placed into a 1.5-mL centrifuge tube, centrifuged at 10,000 rpm for 3 min to remove the enzyme particles and molecular sieves, and the supernatant was then diluted with 20 vol of isooctane. Analysis of the concentration of substrate or product was performed by gas chromatography (14) [Hewlett-Packard 5890 Series II Gas Chromatograph with HP 3345 autosampler (Hewlett-Packard Co. Int., Palo Alto, CA)] and a J&W DB-225 column [(J&W Scientific (Fisons), Folsom, CA)]. The temperature was programmed for 40°C with a 3-min hold, rising at 8°C/min to 220°C. The initial rate of synthesis was calculated from the decrease in concentration of IPG and the increase in concentration of the IPG ester. An amount of bovine serum albumin (equal to the mass of enzyme used in the syntheses) was used in control experiments to determine the background rate of synthesis and to compensate for probable adsorption of the substrate on the particles of protein.

Partition coefficients. Partition coefficients of caproic acid between the organic solvent and the water phase were determined by the method of Martinelle and Hult (15). Various amounts (5–20 mg) of caproic acid were weighed into a 22-mL screw-capped vial, and equal volumes (10 mL) of water and of solvent [isooctane, carbon tetrachloride (CCl_4), or benzene] were added. The mixture was mixed vigorously on a vortex stirrer and placed in a horizontal shaker at room temperature for 24 h. A sample (100 µL) of the organic phase was diluted 10-fold with the appropriate solvent, and a sample (100 µL) of the aqueous phase was similarly diluted with acetone. The concentration of caproic acid in both phases was determined by gas chromatography, as described previously (14).

RESULTS AND DISCUSSION

Solvent effects. It had previously been shown that GPGL was active in catalyzing the synthesis of alkyl esters in various hydrophobic solvents (14). However, in contrast to the requirements for direct esterification of aliphatic alcohols and free fatty acids, the reaction of IPG caproate with a free fatty acid required more stringent conditions. The initial rate of synthesis of IPG was extensively retarded by the addition of a trace amount of water, and thus all experiments were carried out in strictly anhydrous conditions with a water concentration less than 0.01% w/w.

Due to the limited solubility of IPG in hydrocarbon solvents, a series of solvents was used to examine the lipase activity. It was found that the rate of synthesis of the IPG ester was very dependent on the hydrophobicity of the solvent. The rates of synthesis of IPG caproate in CCl_4 and benzene were only 22 and 16% of that in isooctane under the same experimental conditions, and no significant activity was detected in ether (diethyl ether or methyl *t*-butyl ether), dioxane, or acetonitrile. The rate of catalyzed synthesis of IPG ester was slightly dependent on the carbon chain length of the hydrocarbon solvent, with 98% activity occurring in *n*-heptane and 95% in *n*-hexane compared with the activity in isooctane. Thus isooctane was the solvent of choice, since its use also eliminated any error that might have been caused by evaporation of a more volatile solvent. A similar dependency of activity on solvent has also been found in the *P. cepacia*-catalyzed reaction between a fatty acid or fatty-acid ester with IPG (11). In that study, *n*-heptane was reported to be a better solvent with a threefold enhancement of the initial rate compared with those in other hydrocarbon solvents. Thus, the results in this investigation suggested that the synthesis of IPG esters might have some fundamental differences from the reaction between aliphatic alcohols and acids.

The native or tertiary structure of enzymes is usually maintained by several interactions, e.g., hydrogen-bonding, ionic interactions, hydrophobic attraction, and van der Waal's interactions. The addition of a water-miscible solvent may disrupt the enzyme structure itself or change the conformation of the active site of the catalyst or its environment, with the result that there is little or no activity in the lipase-catalyzed

reaction in a polar solvent-medium. Monitoring of the structural change in enzymes caused by the addition of a solvent has been carried out (16,17) and the results confirmed the conclusion that water may condense in the active site and increase its polarity (P), and that the active site is thereby shielded from a bulk, water-immiscible solvent but greatly perturbed by a polar solvent.

It has been suggested that the initial rate of synthesis might be related to the log *P* value of the solvent used, and that the rate should decrease as the solvent is changed from hydrophobic to hydrophilic [i.e., as log *P* of the solvent is decreased (11,18)]. However, the exact nature of the effect of solvent on enzyme kinetics has yet to be confirmed. An explanation for the dependency of enzyme catalysis on the solvent has been formulated by considering the thermodynamic properties of the substrate in different organic systems (19). In different solvent systems, the rate of catalysis is strongly dependent upon the extent of desolvation of the substrate from the solvent. The thermodynamic model of the solvent effect has also been applied by Martinelle and Hult (15) to explain the effect of solvent on the esterification and transesterification reactions between octanol and octanoic acid or ethyl octanoate, catalyzed by *C. antarctica* lipase B. Their model for the substrate-desolvation effect was based on the hypothesis that the substrate specificity of an enzyme arises from its utilization of the free energy of binding with the substrate, which varies from one solvent to another.

It was assumed that the solvation effect on the enzyme and on the enzyme-substrate complex was minimal, since the surface area per unit mass of enzyme is far less than that of substrate. The relationship between k_{cat} and K_m can then be related to the partition coefficient of the substrate between the organic solvent and aqueous solution (19):

$$\log\left[\frac{(k_{cat}IK_m)_A}{(k_{cat}IK_m)_B}\right]_{solvent} = \log\frac{P_B}{P_A} + \log\left[\frac{(k_{cat}IK_m)_A}{(k_{cat}IK_m)_B}\right]_{water} \quad [1]$$

Thus, at a fixed value for the concentration of substrate B, the value of the ratio of K_m/k_{cat} could be related to the partition coefficients, P_A, of the substrate *A* in a given solvent system.

Since IPG has low solubility in isooctane, we need only to consider the solvation effect on caproic acid in different media. Table 1 shows the values of K_m for the catalyzed synthesis of IPG caproate for three different solvent systems. The large standard error of K_m for the reactions was caused by the relatively limited sampling of the reaction in the possible range of study. The increased values of K_m in CCl_4 and benzene, however, compared with the value in isooctane, can then be related to the increased partition coefficient in these two solvents. For more polar solvents, such as CCl_4 and benzene, the free energy of substrate solvation for caproic acid is larger than that in a nonpolar medium, such as a hydrocarbon, and thus substrate desolvation from the bulk solvent medium into the active site of the enzyme will require more energy if it is to achieve an efficient substrate binding. A similar argument may also be applied to the acyl acceptor, IPG, because, with its high polarity, it will interact more strongly with a polar solvent, and thus the GPGL-catalyzed reaction can then only take place in a nonpolar hydrocarbon solvent, where the effect of substrate solvation is minimized.

TABLE 1
The Values of the Michaelis-Menten Constants, K_m and k_{cat}, and the Partition Coefficients, P_s, for Goat Pregastric Lipase (GPGL)-Catalyzed Synthesis of Isopropylidene Glycerol (IPG) Caproate in Different Solvent Systems

Solvent	K_m[a] (mM)	k_{cat}[a,b] (μmol min^{-1} mg^{-1})	Partition coeff. (P_s)[c]
Isooctane	60 ± 5	15 ± 0	3.03
CCl_4	530 ± 70	2.4 ± 0.1	6.82
Benzene	650 ± 100	2.4 ± 0.2	10.0

[a]K_m and k_{cat} values were determined in 50 mM IPG at 35°C.
[b]The k_{cat} values are presented in units of per mg of lipase content rather than the total weight of protein.
[c]P_s is the molar ratio of caprioc acid distributed between solvent and water.

The dependency of the substrate-desolvation effect may well explain why two different enzymes (GPGL and microbial lipase) act similarly in different solvent systems. It has been suggested that the desolvation model is independent of the enzyme and of the catalyzed reaction because the contribution of the enzyme-substrate binding is accounted for only by the substrate-affinity in water. Thus in enzymatic catalysis in an organic medium, the thermodynamic properties of the substrates play a more important role than they do in an aqueous medium.

Stereoselectivity. A set of chiral substrates, 1,2-IPG and 2,3-IPG, was then used and the initial rates with each were first determined with the two lipases, PPL and CRL (Table 2). Both PPL and CRL showed a preference to catalyze the synthesis of the 1,2-IPG ester and the initial rate of 2,3-IPG ester, synthesis was *ca.* 70% of that for the 1,2-IPG ester. However, when purified GPGL was used, it was found that it had a strong preference to catalyze the synthesis of the 2,3-IPG

TABLE 2
Values for the Initial Rate of Synthesis of IPG Caproate Catalyzed by GPGL, Porcine Pancreatic Lipase (PPL), and *Candida rugosa* Lipase (CRL) in Isooctane at 35°C[a]

Enzyme	Substrate	Initial rate (μmol h^{-1} mg^{-1})
Purified GPGL[b]	1,2(2,3)-IPG	7.0 ± 0.3
	1,2-IPG	2.8 ± 0.2
	2,3-IPG	8.4 ± 0.3
Crude GPGL extract[c]	1,2(2,3)-IPG	$(1.6 \pm 0.1) \times 10^{-2}$
	1,2-IPG	$(1.8 \pm 0.1) \times 10^{-2}$
	2,3-IPG	$(1.8 \pm 0.1) \times 10^{-2}$
PPL[c]	1,2(2,3)-IPG	1.9 ± 0.1
	1,2-IPG	2.1 ± 0.1
	2,3-IPG	1.3 ± 0.1
CRL[c]	1,2(2,3)-IPG	4.0 ± 0.2
	1,2-IPG	4.6 ± 0.2
	2,3-IPG	3.4 ± 0.2

[a]For other abbreviations see Table 1.
[b]The initial rates of reaction catalyzed by GPGL are presented in units of per mg of lipase content rather than the total weight of protein.
[c]The initial rates are presented in units of per mg of enzyme powder used.

ester, and the initial rate of synthesis was almost three times that for the 1,2-IPG ester. A slight preference for synthesis of the 2,3-IPG ester was also observed when crude GPGL extract was used as catalyst, but the ratio of the initial rates for the 2,3-IPG and 1,2-IPG esters was significantly decreased. The loss of stereoselectivity might be due to contamination by other constitutional components in the crude enzyme extract. Thus, to achieve a high degree of stereoselectivity, a highly purified enzyme is required.

There is no literature evidence for stereoselectivity of IPG by PPL and CRL. A slight preference for reactivity with 1,2-IPG has been reported for the immobilized lipases, *C. antarctica* and *M. miehei*, in the direct synthesis of ester from IPG and methyl oleate in the absence of solvent (20). However, the reported results were for *ca.* 70% synthesis, which is insufficient to determine the true selectivity of a lipase. High stereoselectivity for the isomer 2,3-IPG was also observed in the transesterification reaction between IPG and alkyl esters, catalyzed by *P. cepacia* lipase (11). Stereoselectivity was also found to increase with an increase in both substrate concentrations, but no explanation was given for the relationship between substrate concentration and stereoselectivity.

Kinetics of synthesis of IPG esters. The study of the kinetics of GPGL-catalyzed synthesis of IPG esters from IPG and free fatty acid was best achieved by measuring the initial rate in the presence of different concentrations of substrate. At low concentrations of substrate, determination of the initial rate would simplify interpretation of the kinetics and eliminate the effect of such factors as mass transfer, product inactivation and thermo-inactivation. The relationship between the initial rate of synthesis of 2,3-IPG caproate and the concentrations of IPG and caproic acid, catalyzed by GPGL, is illustrated in Figure 1.

Given the nature of enzyme-catalyzed reactions, the case of a single substrate–single product reaction is very rare. Most enzyme-catalyzed hydrolyses may be treated as a single substrate reaction because the excess of water which is present usually remains at a constant concentration over the full course of the reaction. However, most cases of enzyme-catalyzed reactions involve more than one substrate and produce two or more products, and simple Michaelis-Menten kinetics do not apply. If one considers the production of esters from alcohols and acids in an organic solvent, then the rate of reaction will depend on the concentration of both substrates. Since a lipase-catalyzed reaction involves an acyl-enzyme intermediate, the model for a "substituted-enzyme mechanism" is better at explaining the kinetic behavior. More strictly, a mechanism in which a product is released between the addition of two substrates is called "ping pong bi bi" (i.e., bi-substrate, bi-product, nonsequential kinetics), in which one substrate adds to the enzyme and forms the enzyme-substrate complex, and then a second substrate is added after the first product is ejected. Ping pong bi bi mechanisms are common in group transfer or substituted enzyme-catalyzed reactions, and for an enzyme-catalyzed hydrolysis of an ester under conditions of limited water concentration, the reaction scheme

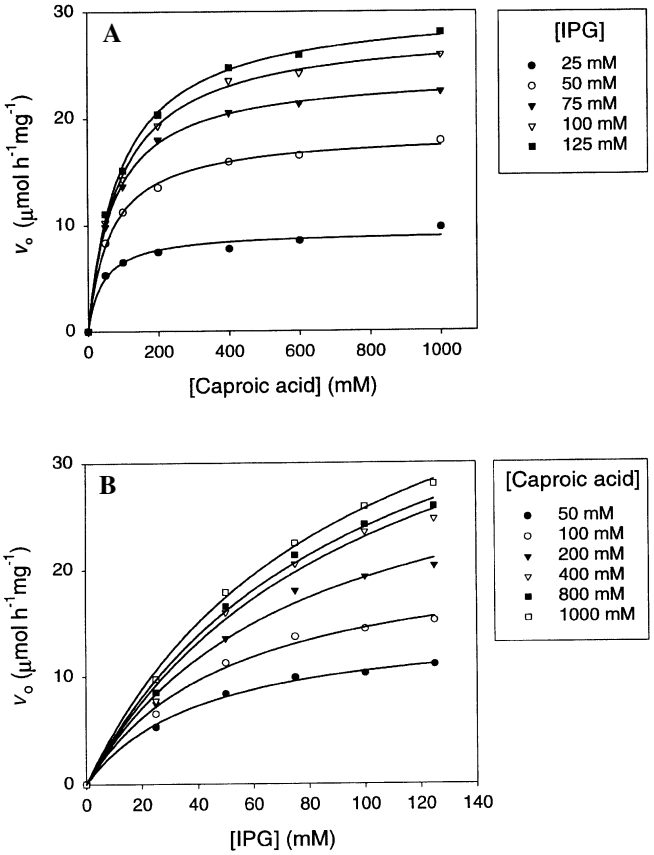

FIG. 1. The initial rate of direct synthesis of 2,3-isopropylidene glycerol (IPG) caproate catalyzed by goat pregastric lipase (GPGL) in isooctane, 35°C. Effect of the concentration of caproic acid (A) and 2,3-IPG (B). (The initial rates of reaction catalyzed by GPGL are presented in units of per mg of lipase content.)

can be described by Equation 2:

$$v = \frac{[A][B]V_{max}}{K_m^A[B] + K_m^B[A] + [A][B]} \quad [2]$$

where V_{max} is the maximum velocity and K_m^B, K_m^A are the Michaelis-Menten constants for the substrates A and B, respectively. Equation 2 can be rearranged to:

$$\frac{1}{v} = \frac{K_m^A}{V_{max}}\frac{1}{[A]} + \frac{1}{V_{max}}\left(1 + \frac{K_m^B}{[B]}\right) \quad [3]$$

$$\text{or } \frac{1}{v} = \frac{K_m^B}{V_{max}}\frac{1}{[B]} + \frac{1}{V_{max}}\left(1 + \frac{K_m^A}{[A]}\right) \quad [4]$$

At a given concentration of B, Equation 2 can be treated as an analog of the Michaelis-Menten equation, with the apparent V_{max}^{app} and K_m^{app} being equal to:

$$V_{max}^{app} = \frac{[B]V_{max}}{K_m^B + [B]} \quad [5]$$

and

$$K_m^{app} = \frac{K_m^A[B]}{K_m^B + [B]} \quad [6]$$

Thus, an alternative method for calculating the values of K_m^A and K_m^B is to use a "Michaelis-Menten relationship" which is restricted by the values of V_{max}^{app} and K_m^{app} to a range of concentrations of substrate B.

Interpretation of the kinetics of most enzyme-catalyzed synthetic reactions is hampered because the behavior predicted by a mathematical formula is only valid in an ideal system in which the concentration of both substrates is small. However, the enzymic catalysis of a two-substrate reaction can be simplified, as described previously, by maintaining constant the concentration of one substrate, and a plot of initial rate against the other substrate concentration will follow a Michaelis-Menten type of relationship (Eq. 2), provided that there is no obvious inhibitory effect from the substrate at high concentration. Another way of presenting the rate data is to plot the reciprocal of initial rate against the reciprocal of the concentration of substrate. Double reciprocal plots for the data shown in Figure 1 are given in Figure 2, and it is clearly seen that there are two sets of parallel lines, especially in the range of low substrate concentrations. This distinctive pattern of parallel lines is characteristic of a nonsequential kinetic mechanism, or the ping pong bi bi mechanism.

Equations 5 and 6 are used for calculating the Michaelis-Menten constants, K_m^A and K_m^B, and the theoretical value of V_{max}. A double-reciprocal plot (Fig. 2) of the initial rates against caproic acid concentration can be further used to generate plots of the dependency of the values of the *x*- and *y*-intercepts for the different concentrations of IPG to yield the values of K_m^A, K_m^B, and V_{max}. However, the use of a linear regression of the reciprocal values overweights the values of the rates at low concentrations of substrate. Moreover, these values usually have a relatively large error, engendered, in part, by the method of analysis, traces of impurities in the solvent, and possible evaporation of substrates and/or product. Errors in K_m^A and K_m^B which were >100% were obtained by using a linear regression analysis of the double-reciprocal plots.

Thus, only those rate data which lay within the concentration ranges of 50–400 mM caproic acid and 25–100 mM IPG were used to calculate the Michaelis-Menten constants for both substrates. At a given concentration of IPG, the initial rate of synthesis of IPG caproate by GPGL was plotted against the concentration of caproic acid, and the data were then fitted by Equation 2 using a nonlinear regression performed by SigmaPlot™. The values of the apparent V_{max}^{app} and K_m^{app} were then plotted against the individual concentrations of IPG (Fig. 3) and fitted with another Michaelis-Menten relationship (Eqs. 5 and 6). The values of V_{max} and K_m^B were then calculated from Equation 5, and those of K_m^A and K_m^B from Equation 6. The values are listed in Table 3.

The values of K_m^A and K_m^B are very similar, and lie within the range of 0.2–0.3 M in isooctane, implying that the affinities between caproic acid and the free enzyme, and IPG and the acyl-enzyme complex are very comparable. The net rate of synthesis of the IPG ester should depend on both the rates of acylation and deacylation, without either one acting as the rate-determining step. The theoretical value of V_{max} is only valid if the kinetic analysis remains valid when the substrate concentration approaches its maximum. In an industrial situation, the increase in the concentration of IPG would cause phase separation and the kinetic mechanism would no longer be applicable, However, an increase in the concentration of fatty acid would change the viscosity of the medium, and mass diffusion would then be the rate-determining step.

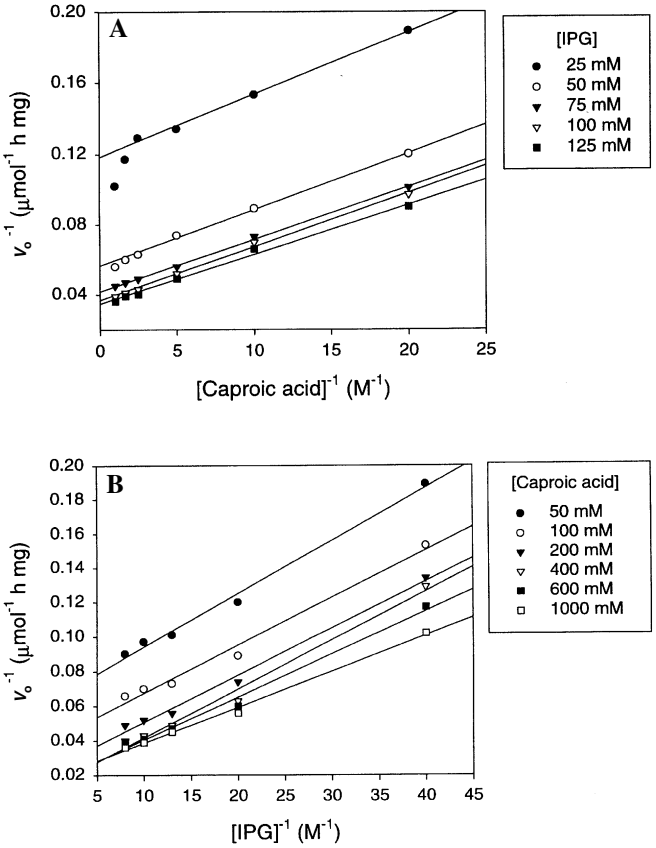

FIG. 2. Double reciprocal plots of the initial rate of synthesis of IPG caproate against the concentration of caproic acid (A) and IPG (B) catalyzed by GPGL in isooctane at 35°C. (The initial rates of reaction catalyzed by GPGL are presented in units of per mg of lipase content.) For abbreviations see Figure 1.

The characterization of the ping pong bi bi mechanism for the GPGL-catalyzed synthesis of the IPG ester confirmed that the reaction follows an acylation-deacylation mechanism. The ratio of K_m^{app}/V_{max}^{app} remained constant for IPG concentrations within the range of 25–125 mM, suggesting that neither substrate showed any significant inhibition of the lipase activity within this limited concentration range. The parallel relationship is seen in the double-reciprocal plots of the initial rate vs. concentration of caproic acid (Fig. 2A). At concentrations of caproic acid >400 mM, the corresponding double reciprocal plots using 1/IPG values on the *x*-axis become coincident at high concentrations of IPG. The relationship suggests that the kinetics approach a single substrate mechanism, since at high concentrations of the acyl donor, most of the enzyme will then be in the form of the acyl-enzyme intermediate. The rate of synthesis will be less dependent upon the increasing

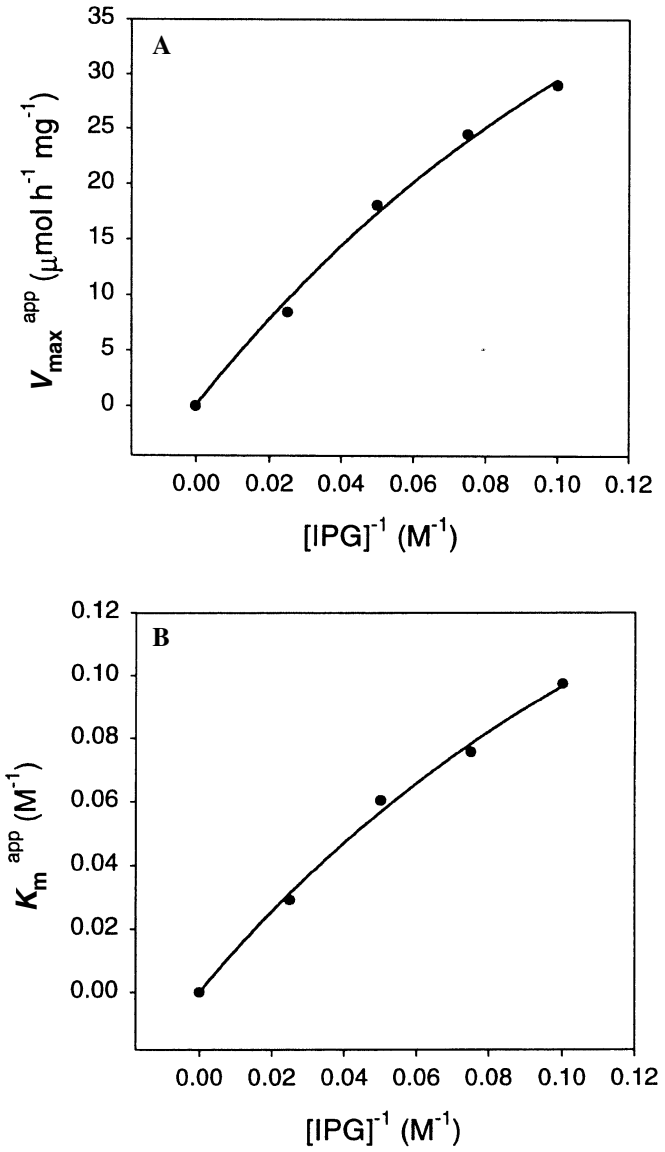

FIG. 3. The dependency of the apparent V_{max}^{app} value (A) and Michaelis-Menten constant, K_m^{app} (B), on the reciprocal concentration of IPG. (The initial rates of reaction catalyzed by GPGL are presented in units of per mg of lipase content.) For abbreviations see Figure 1.

TABLE 3
Kinetic Parameters for the Ping Pong Bi Bi Mechanism for Synthesis of 2,3-IPG Caproate Catalyzed by GPGL in Isooctane at 35°C

	96 ± 25	
V_{max}	μmol h^{-1} mg^{-1} [b]	Turnover number[c]
K_m^A	0.32 ± 0.09 M	Michaelis-Menten constant for binding of caproic acid[d]
K_m^B	0.23 ± 0.08 M	Michaelis-Menten constant for binding of 2,3-IPG[c]
K_m^B	0.23 ± 0.09 M	Michaelis-Menten constant for binding of 2,3-IPG[d]

[a]For abbreviations see Table 1.
[b]The unit of V_{max} is based on units of per mg of lipase content rather than the total weight of protein.
[c]Data calculated from Equation 5 and Figure 3A.
[d]Data calculated from Equation 6 and Figure 3B.

concentration of caproic acid. Thus the decrease in activity at high concentrations of caproic acid is probably due to the mass diffusion of the substrates and products in and out of the active site of the enzyme.

Even in a pure caproic acid medium (8 M), there was *ca.* 30% activity compared with that in 1 M caproic acid. However, when IPG was used as the solvent, no formation of IPG ester could be detected. A similar behavior was also found when other aliphatic alcohols, butanol and hexanol, were used as the acyl acceptor. The inhibition of enzymic activity in the presence of alcohol is caused by denaturation of the lipase by the polar solvent which forms a dead-end complex with the lipase.

In addition to using free caproic acid as the acyl donor, methyl butyrate and tributyrin were also examined for their ability to undertake a transesterification reaction catalyzed by GPGL. However, no activity was detectable for either substrate, even when the reaction was subjected to a high concentration of enzyme and a low concentration of substrate. The reason for the absence of activity may be due to the poor interaction between tributyrin and methyl butyrate. Moreover, in an environment of low water concentration, the rate of acylation of an enzyme by triacylglycerols is largely retarded by the limited concentration of water, which is required to remove the alcohol product (arising from the reaction of the triacylglycerol) from the active site of the enzyme.

This mechanism also accounts for the inability of triacylglycerol to undergo a transesterification reaction with fatty-acid esters in the presence of GPGL. Free fatty acids inhibit the activity of the enzyme in aqueous solution (data not shown), and the affinity of the fatty acid for the active site of the enzyme may be far greater than that of triacylglycerols. The low interaction between tributyrin and PGL renders impossible any transesterification reaction. Meanwhile, chiral selectivity of the enzyme does not depend on the acylation step, but is determined by the nucleophilic reaction between alcohols and the acyl-enzyme intermediate, which is adjusted by the microenvironment around the active site.

Effect of carbon chain length of free fatty acids. In the GPGL-catalyzed synthesis of esters, the relative rate of reaction not only was dependent upon the carbon chain length of the acid substrate but was also affected by the size of the acyl acceptor. The longer the carbon chain length of the aliphatic alcohol, the more dependent was the rate on the chain length of the fatty acid (14). The maximum rate was seen for a composite of alkyl moieties, from the two individual substrates, with a combined carbon chain number equaling eight to ten. Therefore it is not surprising that the rate of synthesis of the IPG-ester decreased significantly as the carbon chain length of the fatty acid increased in length, and no significant activity could be detected at chain lengths greater than $C_{12:0}$. Since the IPG acts as a bulky primary alcohol, the long carboxylic acid chain of the acyl-enzyme intermediate may hinder access of IPG to the active site of the enzyme.

The dependency of the initial rate of synthesis of IPG esters on the fatty acid concentration of different fatty acids at a fixed concentration of IPG is illustrated in the Lineweaver-

Burk plot shown in Figure 4. The increase of the carbon chain length caused a decrease in the apparent V_{max} value and an increase in the apparent K_m value, an effect which is similar to that found for the catalyzed hydrolysis of monoacid triacylglycerols (21). Thus we may conclude that the dependency of the catalyzed rate of synthesis on the different fatty acids is largely determined by the affinity between the acyl donor and the pregastric lipases themselves, as verified by the short-chain preference seen in Figure 4.

However, when the solvation energy of the substrate is taken into account, we then expect that the dependence of initial rate of catalyzed synthesis of IPG esters, from IPG and fatty acids, on the carbon chain length of fatty acids could be related to the desolvation of the free fatty acids. Provided the acyl acceptor remains the same and the solvent medium remains constant, then the longer the carbon chain length of the fatty acid, the higher the value of the partition coefficient, and the greater the energy required for desolvation of the substrate. Thus, interpretation of the results of chain-length dependency for pregastric lipase-catalyzed synthesis of esters in organic solvents should include parameters for both the substrate selectivity of the enzyme and the thermodynamic properties of the substrates themselves.

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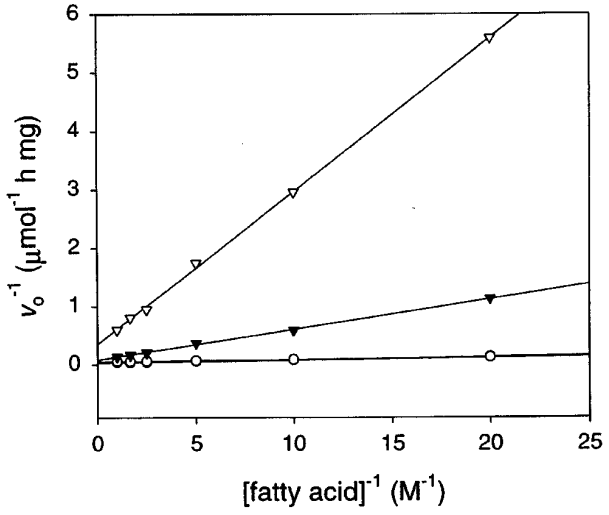

FIG. 4. Lineweaver-Burk plots for GPGL-catalyzed synthesis of IPG esters in isooctane, 35°C. Effect of carbon chain length of fatty acids: $C_{4:0}$ and $C_{6:0}$(○); $C_{8:0}$(▼); $C_{10:0}$(▽). (The initial rates of reaction catalyzed by GPGL are presented in units of per mg of lipase content.) For abbreviations see Figure 1.

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