CHEMICAL & PHARMACEUTICAL BULLETIN

Vol. 31, No. 12 December 1983

Regular Articles

[Chem. Pharm. Bull. 31(12)4213—4219(1983)]

Kinetic Studies on Pancreatic Lipase Activity in Micellar Systems. II.[1] Effect of Fatty Acid Chain Length of Substrates

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(Received March 14, 1983)

Pancreatic lipase activity toward a series of fatty acid vinyl esters with different chain lengths, solubilized in sodium deoxycholate (NaDC) micelles, was investigated kinetically. The kinetic data could be analyzed on the basis of a fully competitive inhibition mechanism, where substrate-free NaDC micelles act as a competitive inhibitor. The values of inhibition constant (K_4), which is the dissociation constant of lipase–NaDC micelle complex, obtained from the reaction of each substrate were in reasonable agreement with one another. On the other hand, the maximum velocity (V) and the Michaelis constant (K_m) increased with decrease in the fatty acid chain length ($C_{14} \simeq C_{12} < C_{10} < C_8$).

The V/K_m values indicate that vinyl dodecanoate and tetradecanoate are better substrates than vinyl decanoate and octanoate in this micellar system. The K_m/K_4 values suggest that the lipase–substrate-solubilizing NaDC micelle complex is less stable than the lipase–substrate-free NaDC micelle complex. The changes in the V and K_m values with chain length were ascribed to the difference in the interaction between the substrate molecule and NaDC micelles.

Keywords——pancreatic lipase; sodium deoxycholate; fatty acid vinyl ester; micellar system; heterogeneous enzymatic reaction; fully competitive inhibition; substrate specificity; micelle–solubilizate interaction

Since the hydrolysis of a lipid by a lipolytic enzyme is a heterogeneous reaction, the enzyme must bind to the lipid–water interface to exhibit its catalytic activity. Therefore, the enzyme activity is significantly influenced by the interfacial area.[2] In the case of pancreatic lipase, an emulsion system is well known to give a substrate–water interface effective for the enzyme and is commonly used for measuring the enzyme activity. However, since an emulsion system is thermodynamically unstable, and it is not easy to prepare an emulsion with a definite interfacial area, other systems such as substrate monolayer,[3] substrate-covered glass beads,[4] and substrate-solubilizing micelles[1] have been developed. In the previous paper, we investigated kinetically the pancreatic lipase activity in a vinyl dodecanoate (VDD)–sodium deoxycholate (NaDC) micellar system and found that the system offers an effective interface for the enzyme.[1] We have also found that the enzymatic hydrolysis of VDD follows a fully competitive inhibition mechanism where the substrate-free NaDC micelles act as an inhibitor.

In this work, in order to obtain further insight into the pancreatic lipase activity in a micellar system, the effect of fatty acid chain length of the substrate on the enzyme activity has been studied. The changes in the kinetic parameters with the chain length are discussed in relation to the interaction between micelles and substrate molecules.

Theoretical

The hydrolysis of VDD catalyzed by pancreatic lipase in NaDC micellar solutions has been found to follow the mechanism shown in Chart 1:[1] the symbols have the meanings listed in Table 1.

The three limiting cases illustrated in Table II can explain the observed kinetics successfully, where the rate of hydrolysis is given by Eq. (1) or (2).[1]

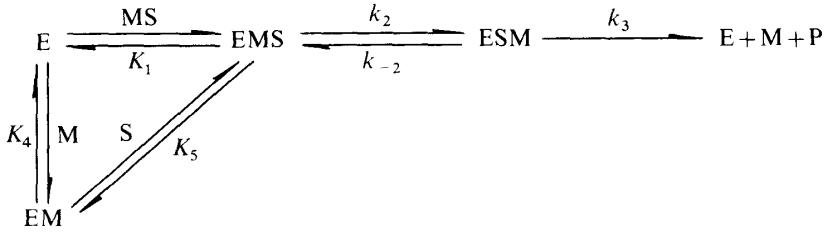

Chart 1

TABLE I. Abbreviations

E	Pancreatic lipase
M	Sodium deoxycholate (NaDC) micelle
S	Substrate: Fatty acid vinyl ester
P	Products
MS	NaDC micelle solubilizing substrate
EM	Lipase–NaDC micelle complex
EMS	Lipase–NaDC micelle solubilizing substrate complex
ESM	Lipase–substrate complex at micelle interface
k_j	Rate constant for forward reaction of step j
k_{-j}	Rate constant for backward reaction of step j
K_j	Dissociation constant of step j (K_4: Inhibition constant)
K_m	Michaelis constant
V	Maximum velocity

TABLE II. Assumptions in Deriving Eq. (1) or (2) and Concomitant Meanings of V and K_m

Case	Assumption			V	K_m
	Rapid equilibrium	Steady state	Rate determining step		
1	Steps 1, 4, 5	[ESM]	Step 2 or 3[a]	$\frac{k_2k_3[E]_0}{k_2+k_{-2}+k_3}$	$K_1 \cdot \frac{k_{-2}+k_3}{k_2+k_{-2}+k_3}$
2	Steps 1, 2, 4, 5	—	Step 3	$\frac{k_2k_3[E]_0}{k_2+k_{-2}}$	$K_1 \cdot \frac{k_{-2}}{k_2+k_{-2}}$
3	Steps 1, 4, 5	—	Step 2	$k_2[E]_0$	K_1

a) The "Step 3" in our previous paper (ref. 1) is incorrect.

$$v=\frac{V[\mathrm{MS}]}{K_m(1+[\mathrm{M}]/K_4)+[\mathrm{MS}]} \quad (1)$$

$$\frac{1}{v}=\frac{K_m}{K_4V[\mathrm{MS}]}\cdot[\mathrm{M}]+\frac{1}{V}(1+K_m/[\mathrm{MS}]) \quad (2)$$

In addition to the processes in Chart 1, the solubilization equilibrium (3) must also be considered.

$$\mathrm{M}+\mathrm{S}\underset{K_0}{\rightleftharpoons}\mathrm{MS} \quad (3)$$

However, the concentrations [MS] and [M] can be approximated by Eqs. (4) and (5), respectively, provided that the solubilization proceeds quantitatively, *i.e.*, $[\mathrm{MS}]\gg[\mathrm{S}]$, and that the solubilization of the substrate does not modify the critical micellar concentration (cmc) and the aggregation number of NaDC micelles;

$$[\mathrm{MS}]=[\mathrm{S}]_t \quad (4)$$

$$[\mathrm{M}]=[\mathrm{M}]_t-[\mathrm{MS}]=\frac{[\mathrm{D}]_t-\mathrm{cmc}}{N}-[\mathrm{MS}] \quad (5)$$

where $[\mathrm{S}]_t$, $[\mathrm{M}]_t$, and $[\mathrm{D}]_t$ represent the total concentrations of the substrate, NaDC micelles, and NaDC, respectively, and N is the aggregation number.

The kinetic parameters V, K_m, and K_4 can be estimated by the following procedures; measure the initial rates for various NaDC concentrations at several constant substrate concentrations; plot the reciprocal of initial rate against [M] (Eq. (2)) and estimate the slopes ($K_m/K_4V[\mathrm{MS}]$) and the intercepts ($(1+K_m/[\mathrm{MS}])/V$) of the lines; replot the slope values against 1/[MS] and estimate the slope (K_m/K_4V) of the line, and replot the intercept values against 1/[MS] and estimate the slope (K_m/V) and the intercept ($1/V$) of the line; calculate V, K_m, and K_4 from the last three estimates.

The values of these kinetic parameters for several substrates with different fatty acid chain lengths may lead to a better understanding of the individual steps involved in the proposed mechanism.

Experimental

Materials——Pancreatic lipase was a product of Sigma Chemical Co. (lipase Type VI from porcine pancreas). According to the supplier, the specific activity is 340 unit/mg protein using olive oil at pH 7.7 and 37 °C. Vinyl hexanoate (VH), vinyl octanoate (VO), vinyl decanoate (VD), vinyl dodecanoate (VDD), and vinyl tetradecanoate (VTD) from Tokyo Kasei Kogyo Co. were of high purity (>99%) and were used without further purification. NaDC from Merck Co. and water were purified as described previously.[1] All other chemicals were of reagent grade.

Determination of the cmc of NaDC——The cmc of NaDC was determined by the spectral shift method with Rhodamine 6G.[5] Under the present experimental conditions (Tris-HCl buffer 40 mM, NaCl 0.18 M, pH 8.0, and 25 °C), the value was determined to be 1.75 mM.

Assay Method and Kinetic Measurement——Stock solutions of vinyl esters solubilized by NaDC micelles were prepared prior to the kinetic runs by the procedures described previously[1] except that the concentration of VTD in acetonitrile, which was used to prepare the stock solution, was 0.2 M instead of 0.5 M and that vinyl ester–NaDC mixture was sonicated to achieve the solubilization equilibrium within shorter time. The reaction was followed by monitoring acetaldehyde, a hydrolysis product, by the MBTH method as described previously.[1]

Results

The pancreatic lipase activities toward some vinyl esters in NaDC micellar systems were measured for various NaDC concentrations at several constant substrate concentrations. Representative plots (for VO) of initial rate (v) *vs.* total concentration of NaDC are shown in Fig. 1. When the reciprocal of initial rate was plotted against [M] calculated from Eqs. (4) and (5) with cmc = 1.75 mM and $N=17$,[6] straight lines intersecting in the left upper quadrant were

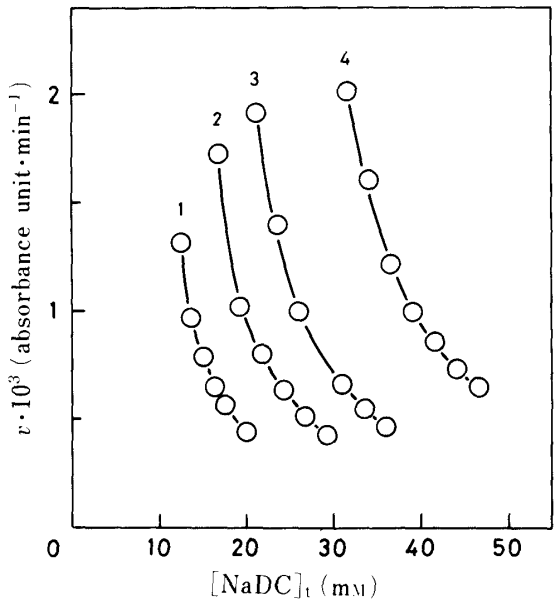

Fig. 1. Plots of Initial Rate *vs.* Total Concentration of NaDC at Several Concentrations of VO

Concentration of VO: 1, 0.50 mM; 2, 0.75 mM; 3, 1.00 mM; 4, 1.50 mM.
Conditions: Tris-HCl buffer 40 mM, NaCl 0.18 M, pH 8.0, temperature 25°C.

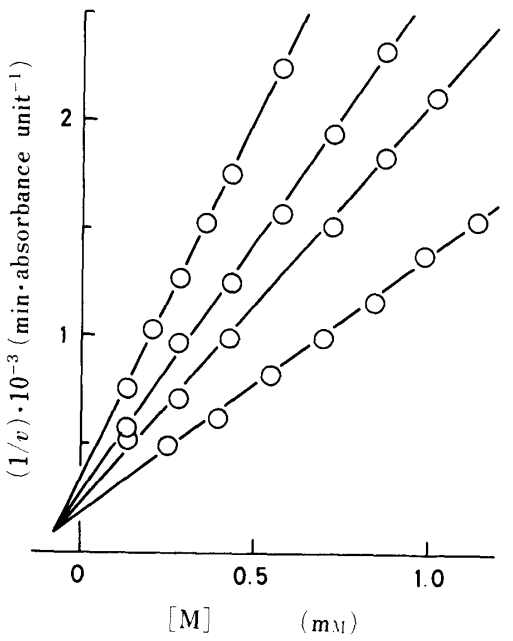

Fig. 2. Plots of $1/v$ *vs.* [M] for VO at Several Constant [MS]s

Solid lines are regression lines. [MS] values are 0.50, 0.75, 1.00, and 1.50 mM from top to bottom, respectively.

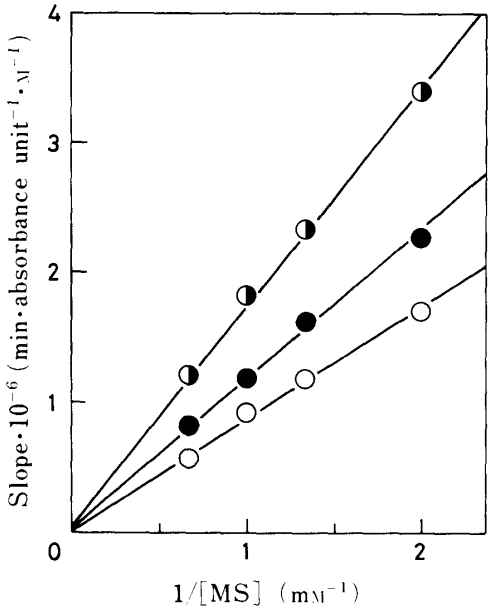

Fig. 3. Replots of Slopes Obtained from $1/v$ *vs.* [M] Plots against 1/[MS]

○, VTD; ●, VD; ◑, VO. Solid lines are regression lines.

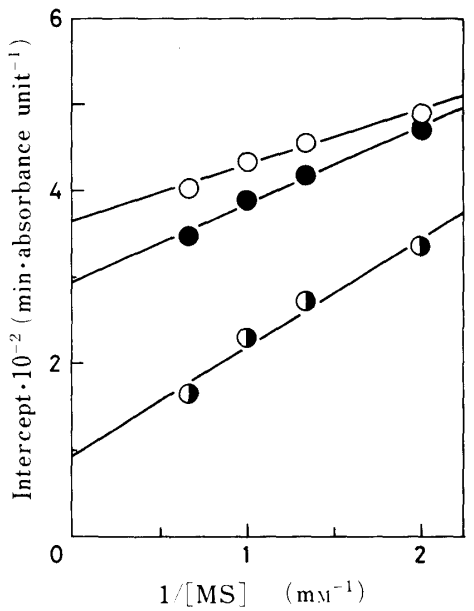

Fig. 4. Replots of Intercepts Obtained from $1/v$ *vs.* [M] Plots against 1/[MS]

○, VTD; ●, VD; ◑, VO. Solid lines are regression lines.

obtained (Fig. 2) as in the case of VDD reported previously:[1] from Eq. (2), the intersection is $(-K_4, 1/V)$. For VD and VTD, similar results were obtained.[7] Replots of the slopes and the intercepts against 1/[MS] also gave straight lines (Figs. 3 and 4). Thus, the kinetic parameters V, K_m, and K_4 could be calculated as described in Theoretical, and the results are listed in Table III together with those for VDD obtained before.[1]

TABLE III. Kinetic Parameters Obtained for Several Vinyl Esters

Vinyl ester	$V \times 10^3$ (au[a]·min^{-1})	$K_m \times 10^4$ (M)	$K_4 \times 10^5$ (M)	V/K_m (au[a]·min^{-1}·M^{-1})	K_m/K_4
Octanoate	10.5 ±2.4	13.1 ±3.4	7.72±1.01	8.00±1.00	17.0 ±3.8
Decanoate	3.38±0.11	3.00±0.26	8.21±0.78	11.3 ±0.9	3.65±0.22
Dodecanoate[b]	2.60±0.06	1.60±0.17	8.50±0.87	16.3 ±1.6	1.88±0.07
Tetradecanoate	2.72±0.07	1.71±0.19	7.52±0.87	15.9 ±1.7	2.28±0.11

a) Absorbance unit.
b) Values are cited from ref. 1, but the V value is corrected on the basis of the experimental data with the enzyme stock solution used in this work.

If the present enzymatic reaction follows the mechanism shown in Chart 1, K_4 should be independent of the substrate because it is the dissociation constant of lipase–substrate-free NaDC micelle complex (EM). The K_4 values obtained for the four substrates were in agreement with one another within the limits of experimental error, supporting the validity of the mechanism. On the other hand, the V and the K_m values increased with decrease in the fatty acid chain length (VTD $\simeq$ VDD $<$ VD $<$ VO).

It has been shown that k_{cat}/K_m (k_{cat}, the catalytic constant) is the most meaningful kinetic parameter for comparing specificity for different substrates.[9] A good substrate will have a large k_{cat}/K_m value. Since, in our experiments, the enzyme molar concentration is unknown (see "Experimental"), the V/K_m value was calculated; the latter value at a constant enzyme concentration is a relative measure of k_{cat}/K_m value because $V = k_{cat}[E]_0$. As can be seen in Table III, the partial mutual compensation of changes in V and K_m resulted in a smaller change in the V/K_m value among the substrates as compared with the individual changes in V and K_m values, and hence the differences in substrate specificity were not very pronounced, but one can say that VDD and VTD are better substrates than VO and VD in the micellar system employed.

It is of interest to compare the values of K_4 and K_1, whose reciprocals are related to the affinities of lipase for substrate-free NaDC micelles (M) and for substrate-solubilizing NaDC micelles (MS), respectively. Although the K_1 value cannot be evaluated, K_m represents the lower limit of K_1 as can be seen in Table II: if Case 1 or 2 is a valid approximation, $K_1 > K_m$, and if Case 3 is so, $K_1 = K_m$. The K_m/K_4 values calculated for the four esters (Table III) are more than unity, and thus K_1/K_4 should also be more than unity, indicating that the lipase–substrate-solubilizing NaDC micelle complex (EMS) is less stable than the lipase–substrate-free NaDC micelle complex (EM).

Discussion

Substrate Specificity

For a series of fatty acid vinyl esters (C_8—C_{18}) in an emulsion system, no difference was observed in the maximum velocity of lipase-catalyzed hydrolysis;[10] the K_m value was not determined because it is difficult in an emulsion system to estimate a meaningful value. On the other hand, for the present NaDC micellar system, changes in the values of V and K_m with fatty acid chain length were observed. The specificity of an enzyme is normally defined in terms of the chemical structure of its substrates. However, in the case of a lipase which acts in a heterogeneous system, the physicochemical state of substrates at the interface must also be considered.[11,12] Since it has now been established that the specificity of pancreatic lipase with respect to fatty acid chain length is mainly attributable to the physicochemical state of the substrates,[11] the changes in V and K_m values with fatty acid chain length in the present

TABLE IV. Requirements for K_1, k_2, K_2, and k_3 for Increasing V and K_m

	Parameter	Meaning	Requirement for increasing V and K_m			
			K_1	k_2	K_2	k_3
Case 1	V	$\frac{k_2k_3[E]_0}{k_2+k_{-2}+k_3}$	—	—[a]	Decrease	Increase
	K_m	$K_1 \cdot \frac{k_{-2}+k_3}{k_2+k_{-2}+k_3}$	Increase	—[a]	Increase[b]	Increase
Case 2	V	$\frac{k_3[E]_0}{1+K_2}$	—	—[a]	Decrease	Increase
	K_m	$K_1 \cdot \frac{K_2}{1+K_2}$	Increase	—[a]	Increase[b]	—
Case 3	V	$k_2[E]_0$	—	Increase	—	—
	K_m	K_1	Increase	—	—	—

a) Consideration of the requirement for K_2 is more practical.
b) This requirement is not met (see the text).

micellar system may reflect the difference in the strength of interaction between the micelles and the substrate or in the location of the substrate in the micelles.

Since it has been reported that, for the NaDC micelles–fatty acid *p*-nitrophenyl ester system, increase in the fatty acid chain length of esters causes an increase in the interaction between the micelles and the ester, and that the location of solubilization is correspondingly shifted from the surface to the interior of the micelles,[13] similar factors were expected to operate in the present NaDC micelles–fatty acid vinyl ester system. Based on these considerations, the changes in V and K_m with fatty acid chain length will be discussed below.

Changes in V and K_m with Chain Length of Substrates

It should be noted here that pancreatic lipase has two kinds of binding sites, *i.e.*, that to the interface, and the active site. The binding to the interface, which precedes the active complex formation, is a hydrophobic interaction. In contrast, the active complex formation does not involve hydrophobic bonding between the enzyme and the aliphatic chain of the substrate.[14] Accordingly, it has been suggested that the binding site to the interface is separated from but is near to the active site so as to allow the enzyme to orient favorably at the interface for active complex formation.[11]

The strength of interaction between NaDC micelles and vinyl esters, and/or the location of solubilization may affect the rates and equilibria of individual steps (Chart 1) and thereby may cause the V and K_m values to vary. The significance of these values depends on the relative rate of each step: three limiting cases account for the observed kinetics (Table II). Unfortunately, it is not clear at present which case is the best approximation, and hence all of them must be taken into account. The requirements of the individual parameters K_1, k_2, K_2, and k_3 for increasing V and K_m are summarized in Table IV: the present results indicate that decrease in the fatty acid chain length increases V and K_m.

Change in V Value——In order to increase V, increases in k_2 and/or k_3 and/or a decrease in K_2 are required. Since, as described in the preceding section, a vinyl ester with a shorter fatty acid chain is expected to interact less strongly with NaDC micelles and to be solubilized at the outer regions of the micelles, it will have higher mobility within the micelles and will interact more easily with the active site of the enzyme (located near the surface of the micelles). Therefore, decrease in the chain length can give rise to an increase in k_2 or a decrease in K_2, both of which increase V.[15] On the other hand, the effect of fatty acid chain length on

the rate constant k_3 is expected to be small unless the location of the enzyme–substrate binding part of the active complex[14] at the micelle interface depends significantly on the chain length.

Change in K_m Value——In order to increase K_m, increases in K_1 and/or K_2 and/or k_3 are required. Since, from the above discussion, the possibility of an increase in K_2 with decrease in the chain length is ruled out, and the increase in k_3 seems to be less important, the remaining possibility of the increase in K_1 must be considered. The EMS complex is less stable than the EM complex ($K_1 > K_4$, see above), which suggests that the substrate competes for the micelles with the enzyme. If the affinity of the substrate for the micelles were higher than that of the enzyme, the increase in the fatty acid chain length would facilitate the dissociation of EMS complex to MS and E *via* the backward reaction of step 1, which increases K_1: this situation does not explain the actual results. On the other hand, if the affinity of the enzyme for the micelles is higher than that of the substrate, the decrease in the fatty acid chain length will facilitate the dissociation of EMS complex to EM and S *via* the backward reaction of step 5, which will result in the increase in K_1. Although the K_m value seemed to increase with decrease in the fatty acid chain length, the difference in K_m value between VDD and VD was small and there was no difference between VTD and VDD (Table III). Thus, for VO, the affinity of the enzyme for the micelles may be higher than that of the substrate but the latter may become progressively comparable to the former with increase in the fatty acid chain length.

In summary, the increases in V and K_m with decrease in the fatty acid chain length of vinyl esters appear to be due to the increase in k_2 and/or the decrease in K_2, and to be due to the increase in K_1, respectively.

It is suggested above that the fatty acid chain length significantly affects the relative stability of the EMS complex with respect to the EM complex, which may also be influenced by the properties of the micelles (particularly micellar size). We are now working with other surfactant micelles of different sizes.

References and Notes

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6) The aggregation number was estimated to be 17 from the kinetic analysis in our previous work (ref. 1). This value was in agreement with the literature value (ref. 8).
7) Similar measurements were also carried out on vinyl hexanoate (VH), but, in contrast to the case of other vinyl esters, the plots of $1/v$ *vs.* [M] did not give straight lines intersecting in the left upper quadrant, probably because the solubilization of VH by NaDC micelles is not quantitative, that is, Eq. (4) is not valid in this case. Hence, further analysis was not performed.
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14) It was suggested that the binding of the enzyme to the substrate is an interaction between –OH of the serine residue and –C–O– of the ester group (ref. 11).
15) If hydrophobic interaction participated in the formation of active complex, it would have the inverse effect of decreasing k_2 or increasing K_2 with decrease in the chain length. However, since the binding of the enzyme to the substrate is a polar interaction (see note 14), such an effect can be neglected.