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Kinetic study of sphingomyelin hydrolysis for ceramide production

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Abstract

Kinetic study of sphingomyelin hydrolysis catalyzed by *Clostridium perfringens* phospholipase C was, at the first time, conducted for ceramide production. Ceramide has the major role in maintaining the water-retaining properties of the epidermis. Hence, it is of great commercial potential in cosmetic and pharmaceutical industries such as in hair and skin care products. The enzymatic hydrolysis of sphingomyelin has been proved to be a feasible method to produce ceramide. The kinetic performance of sphingomyelin hydrolysis in the optimal two-phase (water:organic solvent) reaction system was investigated to elucidate the possible reaction mechanism and also to further improve the hydrolysis performance. Enzyme in solution had less thermal stability than the enzyme powder and the immobilized enzyme. The thermal inactivation of phospholipase C in all the three forms did not follow the first order reaction at 65 °C. The reactions for both the soluble and immobilized enzymes followed Michaelis–Menten kinetics. K_m's for the soluble and immobilized enzymes were 1.07 ± 0.32 and 1.26 ± 0.19 mM, respectively. The value of V_{max} was markedly decreased by the immobilization without much change in K_m, as if the immobilization functioned as the non-competitive inhibition. Ceramide as product activated the hydrolysis reaction, however, and its addition mainly caused the increase in the affinity of the enzyme–substrate complex.

Keywords: Ceramide; Phospholipase C; Sphingomyelin; Kinetic; Thermal stability

1. Introduction

Ceramide (Fig. 1A) is a key intermediate in the biosynthesis of all complex sphingolipids. Due to its major role in maintaining the water-retaining properties of the epidermis [1–3], ceramide is of great commercial potential in cosmetics and pharmaceuticals such as hair and skin care products. Many ceramide-containing products have already been introduced in the cosmetic market, and the effect of its application is excellent. However, chemical synthesis of ceramide is a costly and time-consuming process for industrial applications. Therefore, developments of alternative cost-efficient and high-yield production methods are of substantial interest. Sphingomyelin (SM) is a ubiquitous component of animal cell membranes and it is one of the major phospholipids in bovine milk. In SM, the ceramide part of the molecule is bound through a phosphodiester bridge to a choline moiety (Fig. 1B). Systematical investigation and optimization have been conducted for the enzymatic production of ceramide from SM hydrolysis [4]. Phospholipase C (PLC, EC 3.1.4.3) from *Clostridium perfringens* shows high activity towards the hydrolysis reaction. The hydrolysis is more efficient in the two-phase (water:organic solvent) system than in the one-phase (water-saturated organic solvent) system [4].

For continuous development of ceramide production, it is necessary to consider the mechanism of the catalytic reactions. Kinetic studies are powerful tools to elucidate the possible reaction mechanisms and improve the hydrolysis in order to obtain the highest reaction rate. Kinetic study for the hydrolysis of SM in monolayer has been focused when sphingomyelinase (EC 3.1.4.12) was used as the catalyst [5]. Kinetic models have been established to illuminate the possible mechanisms for the interfacial activation of the sphingomyelinase. However, due to the need of larger operation area, the monolayer reaction is not quite feasible for ceramide production in industrial applications. Hence, kinetic study is required for the reaction system specific to the ceramide production. In addition, since the hydrolysis catalyzed by the PLC or its immobilized form was better at

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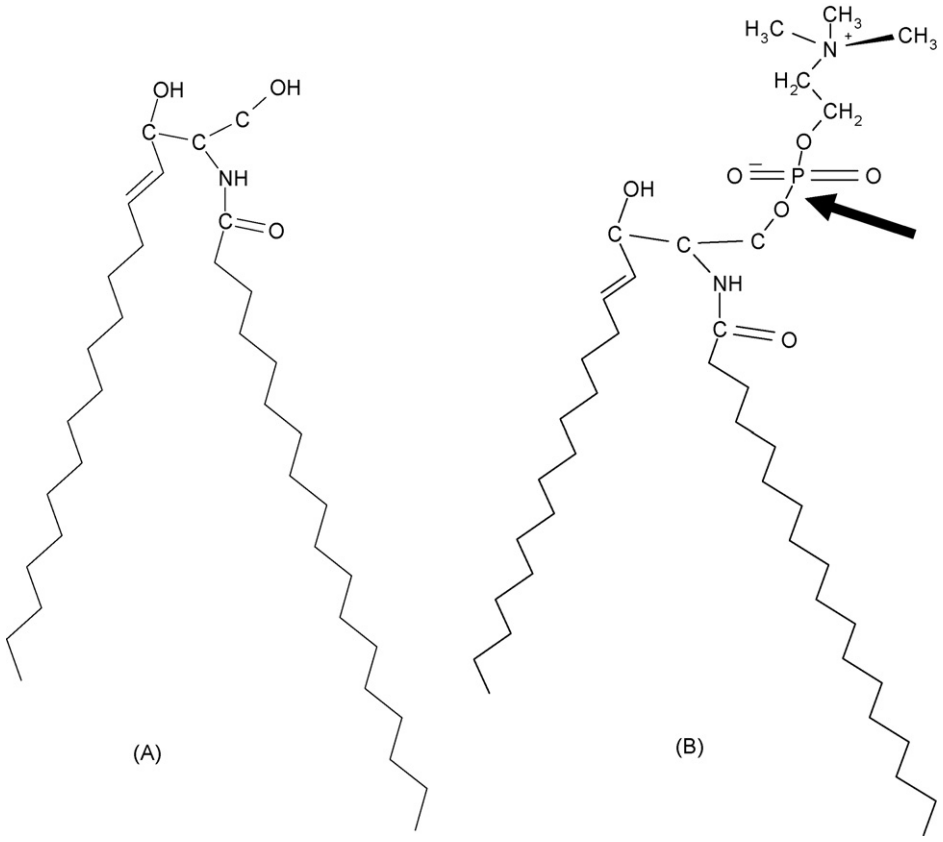

Fig. 1. The structures of ceramide (A) and sphingomyelin (SM) (B). The arrow indicates the bond to be cleaved in SM for producing ceramide.

high temperatures [4,6], thermal stability of the enzymes is also critical.

Present study aims to investigate the kinetic behaviour of SM hydrolysis catalyzed by PLC from *C. perfringens*, with the two-phase reaction system as the model system. Thermal stability was examined for the enzyme powder, the enzyme in solution, and the immobilized enzyme (IE). Enzyme and substrate concentration effects on the hydrolysis were also evaluated. Finally, product activation was found in the current reaction system instead of inhibition.

2. Experiment

2.1. Materials

Bovine brain SM (approx. 99%), standard ceramide (≥99%) from bovine brain and *C. perfringens* phospholipase C (Type I) were purchased from Sigma–Aldrich Denmark A/S (Copenhagen, Denmark). The molecular weight of bovine brain SM is 776 [7]. The carrier, Lewatit VP OC 1600 (Divinyl benzene crosslinked polymer), was from Bayer AG (Leverkusen, Germany). Standard or other chemicals were of analytical and chromatographic purity.

2.2. Immobilization process

The immobilization process of PLC has been described previously [6] with minor change. Carrier (350 mg) was conditioned in 6 ml 96% ethanol for 20 min, followed by washing twice with 6 ml distilled water and drying in fume hood overnight. One-hundred milligrams of carrier was suspended in 1 ml enzyme solution (0.75 mg protein/ml 0.01 M Tris(hydroxymethyl)amino-methane (Tris–HCl buffer, pH 8.6)). The mixture was shaken in a rotary shaker (Bie & Berntsen, Rødovre, Denmark) at 600 rpm for 17 h. Lower clear solution (0.3 ml) was removed for the analysis of protein concentration according to the Lowry method [8] using bovine serum albumin as the standard. The solids in the mixture was obtained through vacuum filtration and washed three times with 3 ml buffer solution. IE was dried through 5 min vacuum followed by 3 h air conditioning in the fume hood.

2.3. Enzymatic activity test

Reaction conditions for the soluble and immobilized enzymes were essentially the same as described previously [4,6]. According to substrate concentration planned (e.g. 0.52 mM), certain amount of SM was mixed with ethylacetate:hexane (50:50), and the mixture was placed in an ultrasonic machine (Bie & Berntsen, Rødovre, Denmark) for 5 min at 37 °C. For the soluble enzyme, 200 μl substrate solution was introduced in the reactor, a small glass tube (diameter, 0.9 cm; height, 7.4 cm). The reaction was started by adding 12 μl 0.026 mg/ml enzyme solution (in 0.01 M Tris–HCl buffer (pH 8.6) containing 25% ethanol). The reactor was sealed by a cap, and kept at 37 °C with shaking at 200 rpm. For the IE, 5 mg IE (containing 12.5 μg enzyme) and

7 μl Tris–HCl buffer with 25% ethanol were mixed. Substrate solution (200 μl) was added to start the reaction, which was kept at 46 °C. Other procedures were the same as described above. After reaction, shaking was stopped, and 80 μl reaction mixture from the upper phase (organic solvent phase) was withdrawn and stored at −20 °C until analysis. Reaction time was controlled to make sure that the detected reaction rate was initial reaction rates. All values given were averages from duplicate experiments, and standard deviation was used as error bar in the figures.

2.4. Thermal stability test

The enzyme powder, the enzyme solution (0.104 mg/ml in buffer containing 25% ethanol) and the IE were put in the individual Eppendorf tubes and heated for 10 min at different temperatures. The enzyme solution and the IE were directly used for the activity test. The heated enzyme powder was dissolved into enzyme solution (0.104 mg/ml) before the test. The concentration of the substrate solution used was 0.52 mM. Other procedures were the same as described above.

2.5. Kinetic study

The data were fitted to Michaelis–Menten kinetic model as below:

$$V = \frac{V_{\max}[S]}{[S] + K_m} \quad (1)$$

where V is the initial reaction rate, $[S]$ the initial substrate concentration, $V_{\max}$ the maximum initial reaction rate and K_m the Michaelis constant. The software Prism 5 (Graphpad Software, Inc., San Diego, USA) was used to facilitate the fitting of the data and the calculation of K_m and $V_{\max}$.

2.6. Analysis method

Ceramide concentrations were quantified using high performance thin-layer chromatography (HPTLC) and in situ densitometry, essentially as earlier described [9]. The HPTLC-plate (Silica gel 60, E. Merck, Darmstadt, Germany) was pre-washed through development in chloroform:methanol (2:1 by volume) in a horizontal developing chamber (Camag, Muttenz, Switzerland), and the plate was activated at 120 °C for 30 min. Standards and samples were applied using DESAGA AS30 HPTLC Applicator (Sarstedt Ag & Co., Nümbrecht, Germany). Following equilibration and development with heptane:isopropanol:acetic acid (85:15:1) in the chamber, the plate was dried and sprayed with charring reagent (10% $CuSO_4$ in 8% H_3PO_4) until soaked. Lipids were visualized by heating the plate at 160 °C for 6 min. The intensity of the spots was determined using DESAGA CD60 HPTLC Densitometer (Sarstedt Ag & Co., Nümbrecht, Germany) at 390 nm in the absorbance/reflection mode. The lipid mass of each component was calculated with the software DESAGA ProQuant (1.03.200, Sarstedt Ag & Co., Nümbrecht, Germany) using a standard calibration curve run on the same plate. Initial reaction rate was the ceramide concentration divided by the reaction time.

3. Results and discussion

3.1. Thermal stability

Thermal stability was studied to investigate temperature influence on the catalytic activity of the enzyme. After heating the enzyme in three different forms, i.e. enzyme powder, enzyme in solution, and the IE, their catalytic activities were measured individually in standard conditions. The effect of heating temperature on the catalytic activity was shown in Fig. 2. Enzyme in solution was most vulnerable by the increase of heating temperature. The heat transfer efficiency in solution is much better than in enzyme powder and IE. This could enhance the thermal inactivation of enzyme in solution. In addition, as happened with most proteins, thermal unfolding of the enzyme in solution lead to the aggregation and the loss of the intrinsic structure. Hence, the enzyme in solution became inactivated at higher temperatures.

The effect of heating period on the catalytic activity was evaluated at heating temperature 65 °C. The catalytic activity of the enzymes in all three forms decreased with the prolongation of the heating time (Fig. 3A). As the reason discussed above, the enzyme in solution was denatured very quickly by heating. The enzyme powder was less affected by heating time than the IE. This implied that the enzyme powder had high thermal stability comparing with the IE. Due to a more rigid external backbone for enzyme molecules, the IE should be stable at higher temperatures [10]. In this study, the protein purity in the enzyme powder from the company was 0.52 mg protein/mg powder, as measured by the Lowry method [8]. Therefore, protein stabilizing substances, e.g. ions or sugars, might exist in the enzyme powder. These substances can protect the enzyme from the thermal denaturation. However, they might be lost during the immobilization process of the enzyme. As a result, the IE had less thermal stability than the enzyme powder.

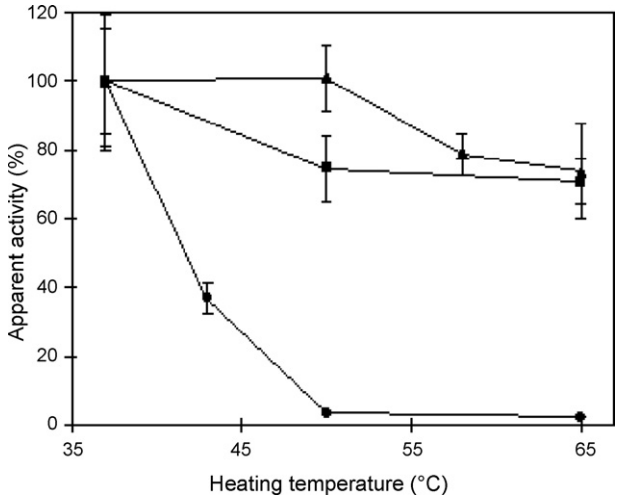

Fig. 2. The effect of heating temperature on the catalytic activity of enzymes. After heating the enzyme powder (■), the enzyme in solution (●) and the immobilized enzyme (▲) for 10 min at different temperatures, their catalytic activities were measured as described in Section 2. Apparent activity was expressed as a percentage of the initial reaction rate of enzymes heated at 37 °C.

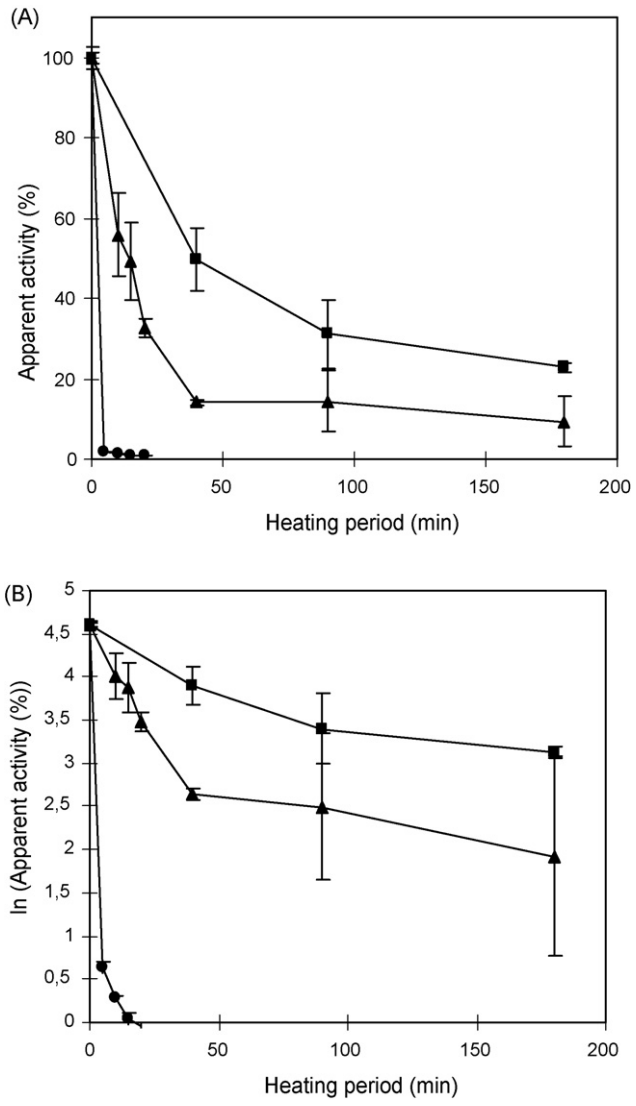

Fig. 3. The effect of heating time on the catalytic activity of enzymes at heating temperature 65 °C. The legend was the same as Fig. 2. Apparent activity was expressed as a percentage of the initial reaction rate of enzymes without heating. (A) Normal plot and (B) the logarithm plot.

Usually in thermal inactivation process, the activity plot against heating time followed the equation of the first order reaction:

$$[E_t] = [E_0]e^{-k_D t} \quad (2)$$

where $[E_t]$ is the enzyme activity at heating time t, $[E_0]$ the original enzyme activity without heating and k_D is the first order inactivation rate constant. Below is the logarithm form of Eq. (2), which can be used to calculate k_D.

$$\ln[E_t] = \ln[E_0] - k_D t \quad (3)$$

Thus, if the plot of $\ln[E_t]$ against t is linear, the inactivation follows the first order reaction, and k_D can be determined from the slope. From Fig. 3B, all plots were not linear, indicating that the thermal inactivation of enzymes in all three forms did not follow the first order reaction at 65 °C. In the previous study [6], the thermal inactivation of immobilized PLC is irregular at the room temperature and follows the first order reaction well at 40 °C. Therefore, heating temperature has a crucial effect on the performance of the thermal inactivation of the immobilized PLC. When we evaluated the outcome in different heating periods, the plot of the immobilized PLC was linear at heating time from 0 to 40 min. After 40 min, the line turned to be flat until 180 min. Hence, its property of the thermal inactivation also depended on heating period. During heating, the microenvironment of the enzyme molecule in the IE could change, or this change might accumulate. Accordingly, the decrease of the catalytic activity of the enzyme became slow in certain heating time. Consequently, k_D value was higher in early stage than in late stage for the IE.

3.2. Enzyme concentration effect

When substrate concentration is much higher than enzyme concentration and other factors such as mass transfer limitation can be ignored, the increase of enzyme amount will make the reaction faster. The effect of enzyme concentration for the soluble enzyme was evaluated with the fixed SM concentration (0.52 mM). The initial reaction rate was proportional to enzyme concentration in the test range (Fig. 4). Therefore, the current reaction system could be a new method for the catalytic activity assay of PLC. The general activity assays for PLC use aqueous system. Only one assay of *B. cereus* PLC works in the two-phase (chloroform:buffer) system, where phosphatidylcholine is the substrate [11]. In that assay, two-phase system provides high efficiency for the hydrolysis reaction. However, when chloroform was used with buffer in the present two-phase system, *C. perfringens* PLC had little catalytic activity. Therefore, we concluded to use the organic solvent mixture, ethylacetate:hexane (50:50), for the two-phase system.

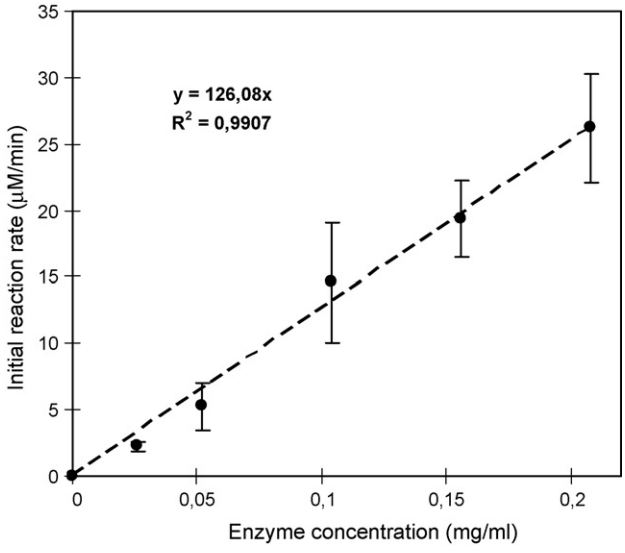

Fig. 4. Enzyme concentration effect on the hydrolysis reaction. The soluble enzyme was used to catalyze the reaction at a fixed sphingomyelin concentration (0.52 mM). The *X*-axis was the enzyme concentration in the water phase.

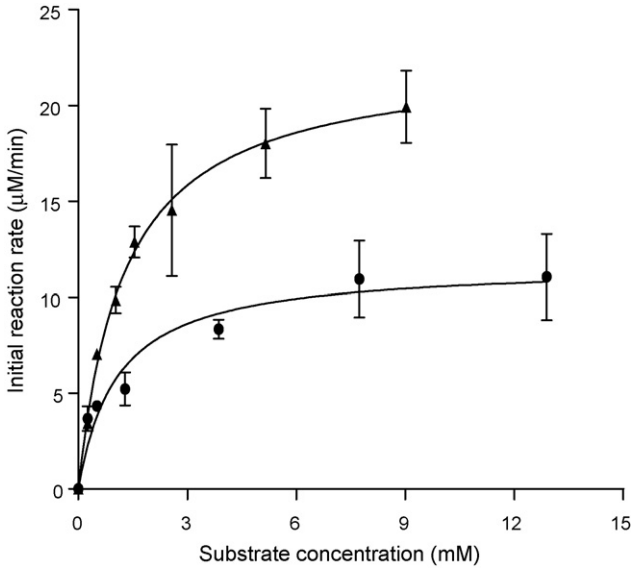

Fig. 5. Substrate concentration effect on the hydrolysis reaction. The plots were drawn using the Michaelis–Menten kinetic model assisting with the software Prism 5. (●) Soluble enzyme and (▲) immobilized enzyme. The amount of enzyme used was 0.31 μg (for the soluble enzyme) and 12.5 μg (for the immobilized enzyme).

3.3. Substrate concentration effect

Since water molecule is abundant in the hydrolysis reaction, its concentration has no significant effect on the kinetic behaviour of the reaction. As a result, we can focus on the effect of SM concentration, and the hydrolysis can be simplified as one-substrate reaction for kinetic study. Consequently, Michaelis–Menten kinetics can possibly be applied in the enzymatic reaction. SM concentration effect on the hydrolysis reaction was examined with the fixed enzyme amount. The reactions in both the soluble and immobilized enzymes followed Michaelis–Menten kinetics (Fig. 5). Kinetic parameters, K_m and V_{max}, were summarized in Table 1 for both enzymes. The amount of the enzyme used was different between the soluble enzyme (0.31 μg) and the IE (12.5 μg). Therefore, in table we used V_{max} divided by the amount of the enzyme in order for a clear comparison.

When an analogue of lysophosphatidylcholine was the substrate, the hydrolysis reaction catalyzed by *C. perfringens* PLC also followed Michaelis–Menten kinetics in the micellar reaction system [12]. K_m was 36 μM from the study and was different from the present study (1.07 ± 0.32 mM for the soluble enzyme). The Michaelis constant, K_m, is normally interpreted as the dissociation constant of the enzyme–substrate complex, and a low K_m value represents a high affinity of the enzyme to the substrate. Therefore, the big difference in K_m indicated that the affinity of the enzyme–substrate complex was lower in the present study. This is possibly caused by the difference in the substrate between the two studies. The other possible reason is the discrepancy in the reaction system. Enzyme molecule in buffer can directly bind the substrate micelle in the micellar system. In contrast, enzyme molecule had to stay in the interface between two phases to catch the substrate in the two-phase system used for the present study. However, two-phase system is superior to micellar system for phosphatidylcholine hydrolysis catalyzed by *B. cereus* PLC [11]. Hence, it has no conclusion on which reaction system is the best for the catalytic activity of the enzyme in current applications. In plant scale productions, the separation of ceramide from the micelle and the requirement of high-speed stirring to prevent aggregate formation make the practice more difficult in micellar systems. Thus, two-phase system would be preferable for the ceramide production in practical operations.

Table 1
Comparison of kinetic parameters for the soluble and immobilized enzymes

Enzyme type	K_m (mM)	V_{max} (μM/(μg enzyme min))
Soluble enzyme	1.07 ± 0.32	37.68 ± 2.91
Immobilized enzyme	1.26 ± 0.19	1.80 ± 0.09

When kinetic parameters were compared between the IE and the soluble enzyme, only V_{max} had significant difference (Table 1). Small variation in K_m indicated that the affinity of the enzyme to the substrate was little affected by the immobilization. The reduction of V_{max} for the IE without much change in K_m implied that the immobilization probably functioned as non-competitive inhibition. A non-competitive inhibitor binds the enzyme at a site distinct from the substrate, and the binding is independent on the enzyme–substrate binding [13]. Therefore, it could be deduced that the carrier contacted the enzyme molecule not mainly through the active site of the enzyme. The decrease of the reaction rate in the IE might be caused by the reduced moving capability of the enzyme due to the immobilization. However, the reduction of V_{max} for the IE without much change in K_m can also be from the other reason. After immobilization, some enzymes might have the same values of K_m and V_{max} as the soluble enzyme, while other enzymes could lose most of the catalysis capability by immobilization. Therefore, the apparent K_m only showed the K_m of the former enzymes, where the value would be similar to the soluble enzyme. Since the total catalytic activity was reduced by immobilization, V_{max} was significantly decreased.

According to data in Table 1, the catalytic efficiency, V_{max}/K_m, was decreased to 4.1% by the immobilization of the enzyme. This seems to be unfavourable for the practical use of the IE. However, the soluble enzyme is difficult to be reused, and the IE retains around 70% of the initial activity after seven cycles [6]. Other benefits of the immobilization is that the reaction catalyzed by the IE has little dependence on pH and has a higher temperature optimum than the reaction catalyzed by the soluble enzyme [6]. The price of PLC is high. Therefore, using the immobilization method to improve the reusability of the enzyme would considerably reduce enzyme cost in industrial applications.

3.4. Product effect

Product effect on the hydrolysis catalyzed by the soluble enzyme was evaluated through the addition of ceramide (0.33 mM) before the reaction in different SM concentrations. Obviously but surprisingly, ceramide activated the hydrolysis

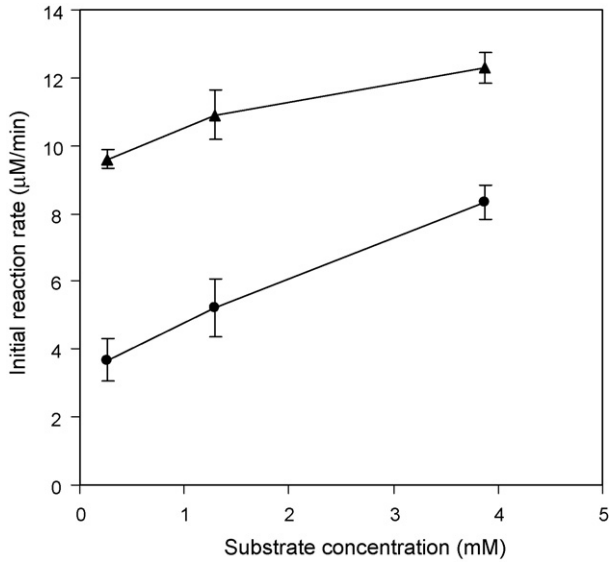

Fig. 6. Ceramide effect on the hydrolysis reaction in different sphingomyelin concentrations. (▲) Ceramide was added (0.33 mM) before the reaction. (●) Without addition of ceramide.

reaction (Fig. 6). This observation was not reported before. At lowest SM concentration (0.26 mM), the effect of ceramide concentration on the initial reaction rate was shown in Fig. 7. The effect of ceramide concentration on the hydrolysis was not linear, and certain ceramide concentrations were required to get the significant activation effect on the reaction. Since SM is an amphiphilic molecule, it is easy to congregate in the organic solvent. SM solution was cloudy with the concentration above 5 mM. In contrast, ceramide was dissolved in the organic solvent. Ceramide can interact with SM through their hydroxyl groups that are able to form hydrogen bonding between them. Therefore, the interaction between SM and ceramide result in the increase of SM solubility and diffusion rate. As a result, the addition of

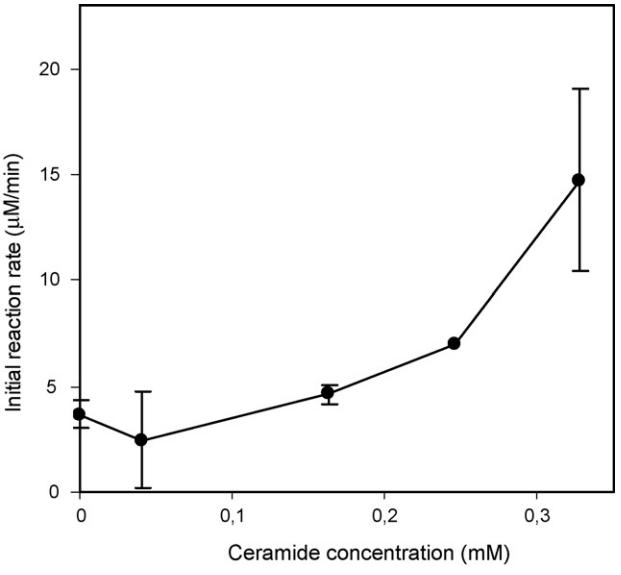

Fig. 7. Ceramide concentration effect on the hydrolysis reaction. Ceramide was added at its various concentrations before the reaction. The substrate concentration used was 0.26 mM.

Table 2
Kinetic parameters in different initial ceramide concentrations

Initial ceramide concentration (mM)	K_m (mM)	V_{max} (μM/(μg enzyme min))
0	1.07 ± 0.32	37.68 ± 2.91
0.16	0.21 ± 0.05	27.9 ± 1.97
0.33	0.06 ± 0.03	38.94 ± 1.38

ceramide can assist SM molecule approach to the enzyme in the interface, and the hydrolysis rate could be increased. The other possible reason is that ceramide might stabilize the enzyme in the interface, where PLC from buffer contacted SM from organic solvent to catalyze the reaction. PLC has a hydrophobic surface area near the active site [14], and ceramide is a hydrophobic compound. Therefore, through interacting with the hydrophobic surface area of PLC, ceramide might stimulate the aggregation of PLC molecule in the interface. Thus, more PLC could stay in the interface to contact SM, and the hydrolysis was consequently enhanced.

Kinetic parameters were compared in different initial ceramide concentrations (Table 2). With increasing initial ceramide concentrations, K_m decreased rapidly, and V_{max} did not change significantly. This seems to be a reversal of the effect from competitive inhibition. The big change in K_m pointed out that the addition of ceramide mainly caused the increase in the affinity of the enzyme–substrate complex. However, the specific mechanism for the product activation cannot be deduced from the result. More study is required to understand the exact principle of the activation. In large-scale productions, the product activation can be utilized to maximize the reaction rate in engineering level. In continuous stirred-tank reactor or fed-batch reactor, certain amount of the product will stay in the reactor before the new substrate joins the reaction. This will increase the reaction rate due to the product activation. Therefore, these reactors could be favoured for selection in industrial applications.

4. Conclusion

In general, the kinetic phenomenon of the selected two-phase system for the hydrolysis of SM was found quite different from previous studies. Thermal inactivation did not follow the common first order kinetics and the powder form of the enzyme showed better thermal stability than the immobilized form as commonly regarded. K_m for the two-phase system was not low comparing to the early work. However, immobilization did not change much of the K_m. Surprisingly, ceramide did not show product inhibition; instead it showed higher activation for the reaction system. The existence of ceramide reduced the K_m values of the system. This phenomenon is most likely due to the two-phase system used.

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