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ORIGINAL PAPER

Immobilization of Phospholipase C for the Production of Ceramide from Sphingomyelin Hydrolysis

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Abstract The immobilization of *Clostridium perfringens* phospholipase C was studied for the first time and the catalytic properties of the immobilized enzyme were investigated for the hydrolysis of sphingomyelin to produce ceramide. Ceramide is of great commercial value in the cosmetic and pharmaceutical industries for use in, for example, hair and skin care products, owing to its major role in maintaining the water-retaining properties of the epidermis. The feasibility of enzymatic production of ceramide through hydrolysis of sphingomyelin has previously been proven. In order to improve the reusability of the enzyme, the present study focused on the immobilization of phospholipase C in the production of ceramide from sphingomyelin. By screening nine different carriers, we found that the enzyme immobilized on Lewatit had the highest catalytic activity towards sphingomyelin hydrolysis. Prewetting Lewatit with ethanol led to higher enzyme fixation on the carrier, but the activity of the enzyme was decreased. Increasing the initial enzyme concentration resulted in more enzyme adsorption on the carrier, where the specific activity was increased. Through optimization of the reaction using the immobilized enzyme, the optimal temperature was around 46 °C and the optimal water volume was 3.5%. The reaction had little dependence on pH. After seven recycles, immobilized enzyme retained around 70% of the initial activity. Immobilized enzyme was deactivated irregularly when stored at room temperature, but followed first-order deactivation when stored at 40 °C.

Keywords Carrier · Ceramide · Hydrolysis · Immobilization · Optimization · Phospholipase C · Response surface methodology · Sphingomyelin

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Introduction

Ceramide (Fig. 1a), the N-acylated derivative of sphingosine, is the key intermediate in the biosynthesis of all complex sphingolipids. Owing to its major role in maintaining the water-retaining properties of the epidermis [1–3], ceramide is of great commercial value in the cosmetic and pharmaceutical industries for use in hair and skin care products, for example. Many ceramide-containing products have already been introduced to the cosmetic market, and the effect of the application of ceramide is excellent. It is claimed that these products dramatically increase the skin's hydration level, repair the cutaneous barrier, prevent vital moisture loss, and contribute to reducing dry flaky skin and aged appearance. However, chemical synthesis of ceramide is a costly and time-consuming process for industrial applications; therefore, the development of alternative cost-efficient and high-yield production methods is 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). Ceramide production from SM hydrolysis has already been de-

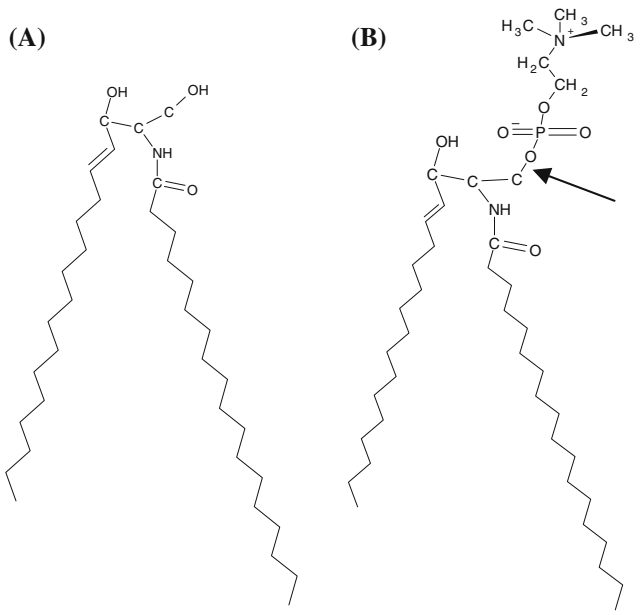

Fig. 1 The structures of ceramide (**a**) and sphingomyelin (*SM*) (**b**). The *arrow* indicates the cleaved bond in SM for producing ceramide

scribed in a few patents [2, 4]. Moreover, systematic investigation and optimization of the enzymatic production of ceramide from SM modification has been conducted in single-batch reactions [5]. The reaction system has been improved, and phospholipase C (PLC, EC 3.1.4.3) from *Clostridium perfringens* shows high activity towards the hydrolysis reaction. It has been verified that SM modification gives a feasible approach to the potential production of ceramide. However, the price of PLC is high, and this would hinder further development and applications at industrial levels; thus, it is necessary to consider the reuse of the enzyme, which would significantly reduce enzyme cost.

Enzyme immobilization is a process to attach an enzyme to an insoluble matrix, while its catalytic activity is still retained. Through immobilization, the enzyme can be physically separated from the bulk reaction medium and, at the same time, is permeable to reactant and product molecules [6]. The advantages of enzyme immobilization can be summarized as follows:

1. The enzyme can be reused and utilized in a more efficient way.
2. Enzyme stability is often increased by immobilization [7].
3. An immobilized enzyme (IE) sometimes offers better process possibilities than a soluble enzyme.
4. Enzyme immobilization avoids the contamination of the products with residual enzymatic activity.

The observed properties of the enzyme are invariably changed by immobilization. The type and the magnitude of these changes depend on the enzyme and the immobilization method used [7]. Many studies of enzyme immobilization have focused on the application of lipase [6]. However, the immobilization of PLC to catalyze SM hydrolysis has not been emphasized and systematically studied.

The aim of present study was to investigate the feasibility of PLC immobilization in the production of ceramide from SM hydrolysis. In order to find the best immobilization process, nine different carriers were screened, and the effects of ethanol as well as the initial enzyme concentration were evaluated. The hydrolysis reaction of IE was optimized for several important factors, individually and together with the assistance of response surface methodology (RSM). Finally the operational and storage stabilities of IE were examined under the optimal conditions.

Materials and Methods

Materials

Standard chemicals were of the highest commercial purity available. Bovine brain SM (approximately 99%), standard ceramide (99% or more) from bovine brain, and *Clostridium perfringens* PLC (type I) were purchased from Sigma-Aldrich Denmark (Copenhagen, Denmark). The carriers used for the present study are described in Table 1.

Immobilization Process

The 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 a fume hood overnight. The carrier (200 mg) was suspended in 2 ml enzyme solution [1.14 mg protein/ml 0.01 M tris(hydroxymethyl)aminomethane (Tris)–HCl buffer, pH 8.6]. The mixture was shaken on a tilting mixer shaker (J.P. Selecta, Barcelona, Spain) for 17 h. The incubation mixture was centrifuged at 3,000 rpm in 1 min. A 0.5-ml aliquot of the clear solution was taken out for analysis of the protein concentration according to the Lowry method [8] using bovine serum albumin as the standard. The solids in the mixture were obtained through vacuum filtration and were washed three times with 5 ml buffer solution. IE was dried through 5-min suction with a vacuum pump followed by 5-h in the fume hood. In the study of the effects of ethanol on the

Table 1 The carriers and their characteristics

Carrier name	Abbreviation	Company	General description
Amberlite® XAD7	XAD	Sigma-Aldrich Chemie, Steinheim, Germany	Nonionic weakly polar macroreticular resin (matrix, acylic ester). Particle size, 0.25–0.84 mm (wet)
Supelite™ DAX-8	DAX	Supelco, Bellefonte, USA	Resin with moderate polarity (matrix, acrylic ester). Particle size, 0.25–0.42 mm
Celite 545	Cel	BDH, Poole, UK	Kieselguhr soda ash flux calcined. Particle size, 0.02–0.1 mm
Dowex® 50WX4	Dow	Dow Chemical Company, Midland, USA	Cation-exchange resin (matrix, styrene–divinylbenzene). Functional group, sulfonic acid. Particle size, 0.15–0.30 mm
Lewatit VP OC 1600	Lew	Bayer, Leverkusen, Germany	Divinylbenzene cross-linked polymer (matrix, methacrylate). Particle size, 0.315–1 mm
Duolite A568	Duo	Rohm and Haas, Chauny, France	Polymerized phenol–formaldehyde ion-exchange resin
Sepabeads FP-DA	FP	Resindion, Binasco, Italy	Polymethacrylate (active group, diethylamino). Pore size, >0.1 μm
Sepabeads FP-DA1	FP1	Resindion, Binasco, Italy	Polymethacrylate (active group, diethylamino). Pore size, >0.03 μm
Accurel EP 100	Acc	Akzo, Obernburg, Germany	Macroporous polypropylene support

immobilization process, the same procedure was followed, but 0.15 ml ethanol was added to 200 mg carrier before the carrier was mixed with 2 ml enzyme solution.

Activity Test for Soluble Enzyme

The reaction conditions for the soluble enzyme were as described in a previous study [5]. Briefly, the enzyme was dissolved in 0.01 M Tris–HCl buffer (pH 8.6) containing 25% ethanol, with a final enzyme concentration of 0.219 mg/ml. SM solution (0.1 ml of 20 mg/ml) in chloroform–methanol (2:1) was added to the reactor, and was dried under nitrogen.Then, 1.88 ml ethyl acetate–hexane (50:50) and 0.12 ml enzyme solution were added to the reactor. The reaction was started with the addition of the enzyme solution. The reaction was performed in a sealed reactor at 37 °C using a magnetic stirrer at 450 rpm. Aliquots of 50 μl of the reaction mixture were withdrawn at the desired time intervals using a syringe (Hamilton, Reno, USA). The samples were stored at –20 °C until they were analyzed.

Activity Test for Immobilized Enzyme

Dried IE (50 mg) was put into the reactor, instead of the enzyme solution. Buffer solution containing 25% ethanol (0.12 ml) and 1.88 ml ethyl acetate–hexane (50:50) was added to the reactor. If the reaction mixture was cloudy (depending on the type of carrier), the samples were centrifuged at 3,000 rpm for 2 min prior to sampling. Other procedures were as described above. For the carrier-screening study, 150 mg IE was used in the activity test.

Operational Stability Study

For the soluble enzyme, the lower phase (water phase) was transferred from the last reaction mixture to the new reaction bottle, in the interval between reactions. Then, the new reaction started without further addition of the enzyme. In the case of IE, the supernatant from the last reaction was discarded. Then, IE was washed with 2 ml ethyl acetate–hexane (50:50) and buffer solution containing 25% ethanol, respectively. The wet IE was transferred to a new batch without addition of buffer. The reaction time was 20 min for both the soluble enzyme and the IE. Other procedures were as described above.

Immobilized Enzyme Reaction Optimization Using Response Surface Methodology

For optimization, a central composite design circumscribed with star distance 1.682 was used according to the principle of RSM. Using the three factors, we generated 17 experimental settings. The factors were temperature, water volume and pH. The only response was the initial reaction rate. The water volume was the volume of buffer solution containing 25% ethanol added, and its range was set to be 35.9–204.1 μl. The ranges for other factors were set to 21.9–52.1 °C and pH 7.53–8.87. The variables and the ranges applied are presented in Table 2.

Statistical Analysis

The data were analyzed by means of RSM with the software MODDE 6.0 (Umetrics, Umeå, Sweden). Second-order coefficients were generated by regression

Table 2 Experimental setup for the response surface design and the responses obtained from the experiments and analysis

Exp no.	Temperature (°C)	H_2O (μl)	pH	Initial reaction rate (μg/min)
1	28	70	7.8	14.19
2	46	70	7.8	48.25
3	28	170	7.8	18.50
4	46	170	7.8	17.20
5	28	70	8.6	13.06
6	46	70	8.6	59.80
7	28	170	8.6	15.57
8	46	170	8.6	16.78
9	21.9	120	8.2	8.96
10	52.1	120	8.2	27.70
11[a]	37	35.9	8.2	15.28
12	37	204.1	8.2	17.49
13	37	120	7.53	31.78
14	37	120	8.87	34.65
15	37	120	8.2	27.52
16	37	120	8.2	32.87
17	37	120	8.2	23.71

[a] Outlier

analysis with backward elimination. The response was first fitted to the factors by multiple regression. The quality of the fit of the model was evaluated by the coefficients of determination (R2) and the analysis of variances (ANOVA). The insignificant coefficients were eliminated stepwise after examining the coefficients and the model was finally refined. The quadratic response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j, \quad (1)$$

where Y is the response variable, x_i the ith independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadratic coefficient for the variable i, and β_{ij} the model coefficient for the interaction between factor i and j.

Analysis Method

Ceramide concentrations were quantified using high-performance thin-layer chromatography (HPTLC) and in situ densitometry, essentially as described earlier [9]. Briefly, the HPTLC plate (Silica gel 60, E. Merck, Darmstadt, Germany) was prewashed 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 a DESAGA AS30 HPTLC applicator (Sarstedt, Nümbrecht, Germany). Following equilibration and development with heptane–2-propanol–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 it was soaked. Lipids were visualized by heating the plate at 160 °C for 6 min. The intensity of the spots was determined using a DESAGA CD60 HPTLC densitometer (Sarstedt) at 390 nm in the absorbance/reflection mode. The lipid mass of each component was calculated with the software DESAGA ProQuant (version 1.03.200, Sarstedt) using a standard curve run on the same plate. The apparent activity was defined as the initial reaction rate divided by the amount of IE. The specific activity was defined as the initial reaction rate divided by the amount of protein. The fixation level was the percentage of the immobilized protein (estimated through subtracting the protein concentration remaining in the supernatant from the initial protein concentration). The degree of hydrolysis was the percentage of the hydrolyzed SM (ceramide mass multiplied by the molecular weight ratio of SM to ceramide).

Results and Discussion

Carrier Screening

Immobilization can make enzymes reusable and offer both better process possibilities and better process efficiency. There are many factors that can influence the process of enzyme immobilization. Firstly, the type of carrier was studied for optimization of the process. Nine different carriers were used for enzyme immobilization. Their performances were compared on the fixation level (Fig. 2a). The highest fixation level was found for Amberlite® XAD7 (XAD). Based on the same amount of IE, their activities were measured and compared (Fig. 2b). The highest activity was shown by the enzyme immobilized on Lewatit VP OC 1600 (Lew), and the degree of hydrolysis was 98% at 64 h. To enhance the activity of PLC immobilized on XAD, the buffer volume was increased from 120 to 400 μl in the hydrolysis reaction of IE. Adding more buffer increased the ceramide concentration from 0.05 to 0.2 mg/ml after 64-h reaction; however, its activity was still low compared with that of Lew-based products. Therefore, the following work focused on the study of immobilization using Lew as the carrier.

Most of the carriers in the current experiment are commonly used as supports in immobilization processes for lipases and phospholipases. As seen in Ta-

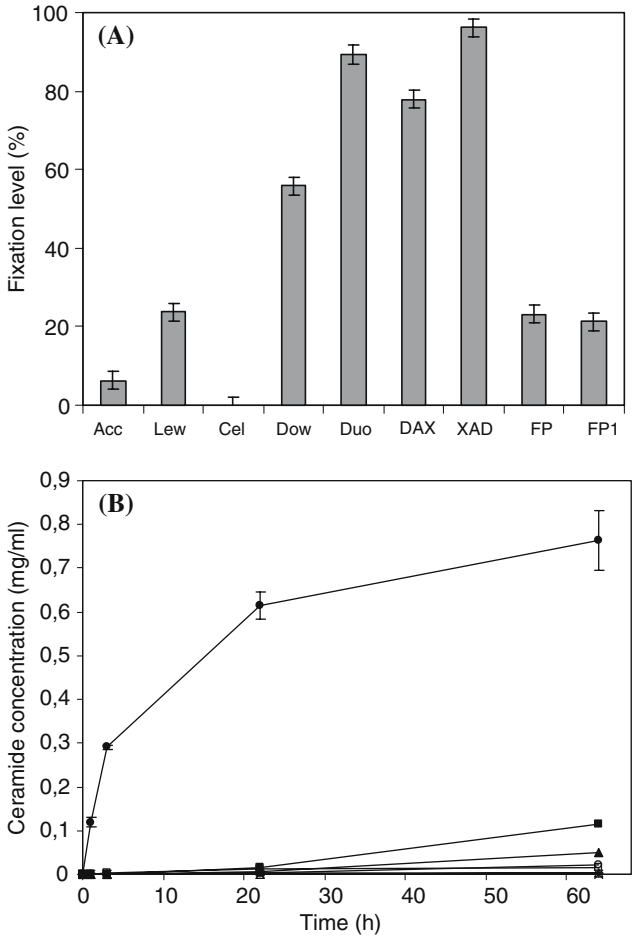

Fig. 2 Comparison of different carriers. The whole name and details of the nine carriers are given in Table 1. **a** Fixation levels of different carriers. **b** SM hydrolysis by differently immobilized phospholipase C based on the same amount of immobilized enzyme (*IE*). The *error bars* are the standard deviation calculated from repeat experiments using Lew. *Open diamonds* Acc, *filled diamonds* Lew, *open triangles* Cel, *crosses* Dow, *horizontal bars* Duo, *filled squares* DAX, *filled triangles* XAD, (*pluses*) FP, *open squares* FP1

ble 1, their chemical compositions, geometric characteristics, and immobilization principles are diverse, and these characteristics play a crucial role in the apparent catalytic activity of IE. Lew, a divinylbenzene cross-linked polymer (matrix, methacrylate), is hydrophobic. When mixed with the enzyme solution, Lew floated to the top of the solution. With the exception of Accurel EP 100, the other carriers were suspended in the solution. This is probably the explanation for the lower fixation levels in Lew. Though many factors, such as skeletal density, particle size, and porosity, can affect the activity of IE, the high activity of the enzyme immobilized on Lew is probably due to the hydrophobic properties of Lew. Examining the steric view of PLC from *Clostridium perfringens*, we can see that the amino acids involved in catalysis in the active site are hydrophilic [10]. If the carrier is also hydrophilic, there is a large risk of the carrier interacting with the active site of the enzyme. Consequently, once the active site of PLC is blocked by this contact during the immobilization process, the activity of IE will be reduced; therefore, the performance of the IE reaction is highly sensitive to the properties of the carrier, especially its interaction with the amino acid residues in the active site. Furthermore, since both the substrate and the product of the reaction are hydrophobic molecules, the hydrophobic surroundings of the enzyme in the Lew particle will enhance substrate and product transfer to and from the active site, compared with the transfer in the presence of with hydrophilic particles.

Following immobilization, IE was washed five times, and the protein concentration of the effluents (3 ml each time) was measured. After washing twice, we detected no protein in the effluents, indicating that soluble enzyme had no effect on the analysis of the activity of IE. Although the enzyme immobilized on Lew had the highest activity, the specific activity of the IE, 533.9 ± 10.1 μg/mg protein/min (optimal condition), was still low compared with that of the soluble enzyme, 2347.8 ± 125.2 μg/mg protein/min, in the present reaction system. The reduction in catalytic activity of the enzyme after immobilization may have different causes, such as partial denaturation, change of enzyme kinetics, partition effects, and mass-transport limitations [11].

Effect of Ethanol on the Immobilization Process

Since Lew did not mix well with the enzyme solution during immobilization, it would be desirable to find methods to enhance mixing and thereby improve the fixation level of PLC on the carrier. Wetting Lew with ethanol prior to immobilization was an efficient method of increasing the fixation level from 25 to 74% (Table 3). Wetting with ethanol caused the carriers to swell (visual observation), which increased the pore size of the particles and probably improved the penetration of water through the surface. After ethanol treatment, the particles were mixed well with the solution, which probably explains the enhanced adsorption of PLC on the carrier.

However, the specific activity of IE was drastically decreased by prewetting Lew with ethanol. Actually, even the apparent activity of IE was reduced, despite the larger mass of the enzyme immobilized on the carrier (Table 3). Normally, the more enzyme used in the reaction, the higher the reaction rate. The strong

Table 3 The influence of prewetting Lew with ethanol on the immobilization of the enzyme

Procedure (before immobilization)	Fixation level (%)	Protein amount in IE (mg/g)	Apparent activity (μg/g IE/min)	Specific activity (μg/mg protein/min)
Prewetting Lew with 150 μl ethanol	75.0 ± 0.9	8.6 ± 0.1	20.6 ± 2.6	2.4 ± 0.3
Soaking Lew with ethanol, and washing ethanol out	81.8 ± 0.4	9.3 ± 0.1	72.5 + 5.6	7.8 ± 0.6
No ethanol treatment	23.8 ± 2.3	2.7 ± 0.3	139.6 ± 2.2	51.7 ± 0.8

IE immobilized enzyme

inactivation induced by prewetting Lew with ethanol is possibly due to the following:

1. At low loading levels, PLC molecules would have less steric restriction, leading to a more favorable special arrangement for enzyme–substrate complex formation [12]. At high loadings, many active sites of PLC will be blocked and only a fraction of the available PLC would be involved in the catalytic reaction. A higher enzymatic load would increase the limitation of substrate diffusion and therefore decrease enzyme efficiency [12].
2. Ethanol probably has some inhibitive effect on the immobilized PLC. To test this effect, the carrier were soaked in 1 ml ethanol and washed with 4 ml buffer solution three times before immobilization. When the ethanol had been washed out, the fixation level of the enzyme also increased (Table 3); however, the specific activity of the enzyme decreased in a similar manner. As a result, the inhibitive effect of ethanol on PLC activity was not shown. Indeed, the addition of ethanol enhanced SM hydrolysis using soluble PLC from *Clostridium perfringens* in a previous study [5].
3. The carrier was swelled by wetting with ethanol, which increased the pore size of the particles. Consequently, the accessible surface area of the carrier, defined as the fraction of the total surface area the enzyme can approach during immobilization, was increased. Usually, the larger the accessible surface area, the greater the contact between the enzyme and the carrier, and a higher extent of inactivation can be expected, even though the chemical characteristics of the carrier and its affinity for the enzyme are also important [13].

Higher specific activity of IE occurred without ethanol treatment on the carrier, where the fixation level of the enzyme was low. If this characteristic is examined from an engineering point of view, two possible paths can be investigated for potential efficiency gains:

1. Using a larger amount of the carrier while keeping other parameters constant. Hereby, the fixation level will be increased since there are more absorption sites for the enzyme.
2. Reusing the enzyme solution. Since only 25% of the enzyme was immobilized on the carrier, 75% would be left in the solution.

To reduce the total enzyme cost, it is necessary to recycle the supernatant if the activity of the enzyme is not lost during the immobilization process. In the recycling step, the volume of the solution, the enzyme concentration, and the amount of the carrier can be adjusted to fulfill the specific interest.

Effect of Initial Enzyme Concentration

The initial enzyme concentration is also an important factor that not only influences enzyme immobilization, but also affects the retention activity of IE. After the carrier is saturated with loaded enzyme, the immobilization efficiency will decrease with increasing initial enzyme concentration. In the present study, the fixation level was kept almost constant within the whole range of initial enzyme concentration (Fig. 3a), and the amount of the enzyme fixed on Lew continually increased at higher initial enzyme concentration (Fig. 3a). This indicates that the capacity of Lew to bind the enzyme is high, i.e., Lew has the potential to immobilize more enzyme.

In order to see the effect of enzyme loading on the activity of IE, the catalytic activity of the immobilized PLC was measured, and the result is shown in Fig. 3b. The specific activity of IE was essentially unchanged when the amount of enzyme in the carrier was low, but increased linearly when the mass of enzyme in the carrier was above 11 μg/mg. Generally, when the amount of enzyme immobilized on the carrier is increased, the specific activity of the enzyme will decrease owing to the increased steric restrictions of the enzyme and the mass-transfer limitation of substrates and products [12]. Therefore, the current result is abnormal, and only one similar case has been found, in the immobilization of lipase [14]. This phenomenon can be explained in two ways:

1. Certain ‘‘dead pores’’ exist in the carrier, and the enzyme can be inactivated by contacting with these dead pores. Since ‘‘nondead pores’’ exist in the

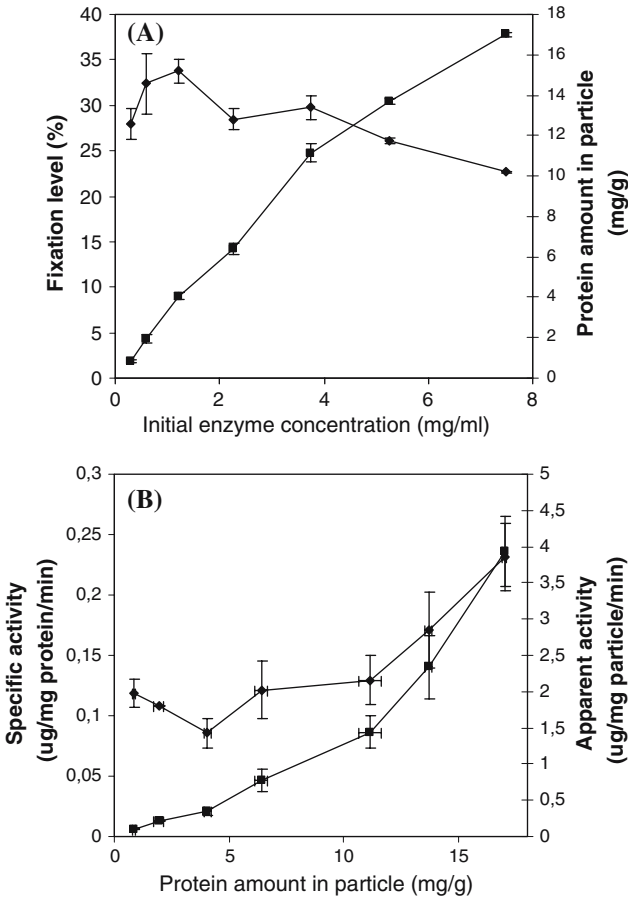

Fig. 3 The influence of initial enzyme concentration on the immobilization process. The immobilization procedure and the activity test of IE are described in the "Materials and Methods." **a** *Diamonds* the fixation level and *squares* the amount of protein immobilized on Lew for different initial enzyme concentrations. **b** *Diamonds* specific activity and *squares* apparent activity on different protein loading

carrier too, enzyme activity can still be detected at low enzyme loading. When the amount of the enzyme in Lew was increased, these dead pores were saturated by the enzyme. After the saturation point, the new adsorbed enzyme exhibited the intrinsic activity. Therefore, the whole specific activity of IE was increased when more enzymes were immobilized on the particles.

2. Since the amount of the carrier was unchanged, the accessible surface area of the carrier was fixed. The increase of the amount of the enzyme in Lew decreased the accessible surface area per enzyme molecule, leading to less contact of individual enzyme molecules with Lew. As a result, the extent of inactivation caused by this contact was reduced, and the specific activity of IE was increased with more enzymes adsorbed in Lew.

By controlling the initial enzyme concentration, we can obtain the same amount of enzyme on the carriers as when prewetting Lew with ethanol; however, both the specific activity and the apparent activity of IE were high compared with those for the ethanol-treated Lew (Fig. 3b). This shows that the inactivation of IE, induced by the ethanol treatment on Lew, is not due to the increased amount of the enzyme on the carriers. Therefore, the first explanation for the inactivation of IE caused by ethanol treatment can be rejected, and the mass-transfer limitations as well as the steric restrictions of the enzyme do not seem to play a vital role in catalytic reaction from the current enzyme loading. The catalytic activity of IE depends more on the accessible surface area of the carrier, which mainly governs how the enzyme is distributed in the pores of the carrier [13]; hence, the geometric characteristic of the carrier is an important factor in determining the catalytic activity of IE.

Optimization of Immobilized Enzyme Reaction Using Response Surface Methodology

So far, the immobilization process was optimized with the considerations of the fixation level and the catalytic activity of IE. By evaluating the influence of carrier type, ethanol treatment, and initial enzyme concentration, we selected the following immobilization conditions for further characterization: Lew as the carrier, no ethanol treatment and initial enzyme concentration of 0.75 mg/ml.

Temperature and pH optima are two basic properties of an enzyme. The optimization of temperature and pH for soluble enzyme has already been addressed [5]. The immobilization of an enzyme often changes the temperature and pH profiles. In addition, the water content in the reaction medium is also an important factor whose optimum is often subjected to the change induced by immobilization. These issues can be better solved through an optimization operation assisted by RSM. RSM is an effective statistical technique for the optimization of complicated systems, and enables the evaluation of effects from multiple parameters, alone or in combination, on response variables. The main advantage of RSM is the reduced number of experiments needed to provide sufficient information for statistically acceptable results [15]. The three parameters and their ranges in Table 2 were decided on the basis of previous studies. The responses were first fitted to the parameters. The best-fitting quadratic model was determined by regression and backward elimination. The model coefficients and the P values for the regression variables are given in Table 4. All P values

Table 4 Regression coefficients describing the influence of different parameters on initial reaction rate (μg/min)

Variables	Coefficient	Standard error	*P* value	Conf[a]
Constant	29.16	1.57	1.17×10^{-9}	3.45
Temperature	6.83	1.17	1.12×10^{-4}	2.57
H_2O volume	–6.87	1.17	1.04×10^{-4}	2.57
Temperature × temperature	–3.91	1.17	6.65×10^{-3}	2.58
Temperature × H_2O volume	–8.13	1.27	5.24×10^{-5}	2.81

[a] The 95% confidence interval (±) on the coefficient value

of the coefficients were below 0.01 after the model had been refined. The coefficient of determination (R2), which is a measure of fit, was 0.92. In the normal probability plot of residuals, there was a good linear relationship for all the points within a narrow range of standardized residues (–1.4 to 1.7) (Fig. 4). This shows that the model generally represented the actual relationships between the response and the reaction parameters within the ranges selected.

Figure 5a is a summary of the effects on the reaction based on the model defined. Since the pH and its interactions with the other factors had little effect on the catalytic reaction of IE, they were eliminated from the model. The effect of pH on the catalytic reaction is discussed in the next section. Temperature and water volume had a significant influence on the SM hydrolysis. The interaction between these two factors also had a big negative effect on the reaction. With the aim of providing a clear evaluation of the two significant parameters, the main effects of each parameter are given in Fig. 5b and c. Theoretically, the higher the temperature, the greater the reaction rate that can be reached when other aspects, such as thermal inactivation of enzyme and thermal degradation of substrates and products, can be ignored. From the result, the optimal temperature was around 46 °C, and the initial reaction rate was not increased beyond 46 °C (Fig. 5b). When the optimal temperature of the IE is compared with that of the soluble enzyme, which has the highest SM hydrolysis around 37 °C [5], we can conclude that the immobilization process increases the optimal temperature of the reaction. Because immobilization provides a more rigid external backbone for enzyme molecules, the proper and catalytically active structure of the enzyme becomes more stable at higher temperatures [6]. Accordingly, the inactivation of PLC at higher temperature becomes less significant, and the optimal temperature will increase.

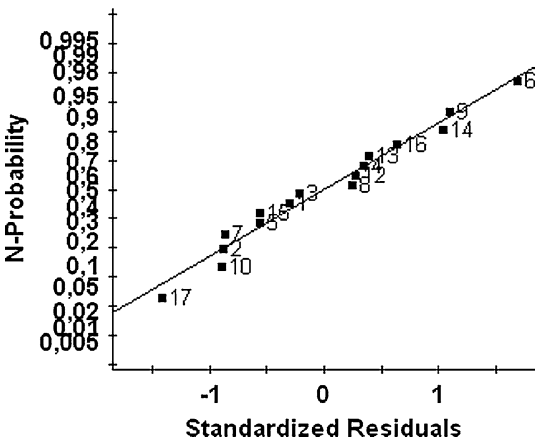

Fig. 4 The normal probability plot of residuals. The *numbers* represent the number of the experiment

Water not only plays the role of a nucleophile in the reaction, but also contributes to the active state of the enzyme [16]; therefore, the amount of water has a large impact on SM hydrolysis catalyzed by soluble PLC [5]. In present study of IE, the initial reaction rate and the water volume exhibited a negative linear relationship (Fig. 5c), when the other factors in the design were set to their average. An excess amount of water possibly assists the thermal inactivation of IE, leading to the decrease in the catalytic activity at high temperatures. In order to examine this assumption, the relationship and the interaction between temperature and water volume are illustrated by the contour plot (Fig. 5d) when the other parameter in the design was set to its average. At lower temperatures, the water volume had little effect on the reaction rate, while at higher temperatures, increasing water volume was strongly inhibitory. This indicates that the negative effect of water volume was temperature-dependent. Furthermore, when the water volume was low, increasing temperature enhanced SM hydrolysis, and thermal inactivation was reduced. This was reversed when the water volume was high. In conclusion, it is verified that a high water volume makes the IE reaction more vulnerable to thermal deactivation, which is responsible for the negative effect of the water volume on SM hydrolysis.

Through iterative calculation to determine the maximum initial reaction rate, the results recommend the general optimal conditions as water volume 70 μl and temperature 46 °C. Under these conditions, the initial reaction rate should be 52.2 μg/min according to the prediction of the model.

Temperature and pH

The hydrolysis reaction of IE was optimized using RSM, including three important factors, temperature,

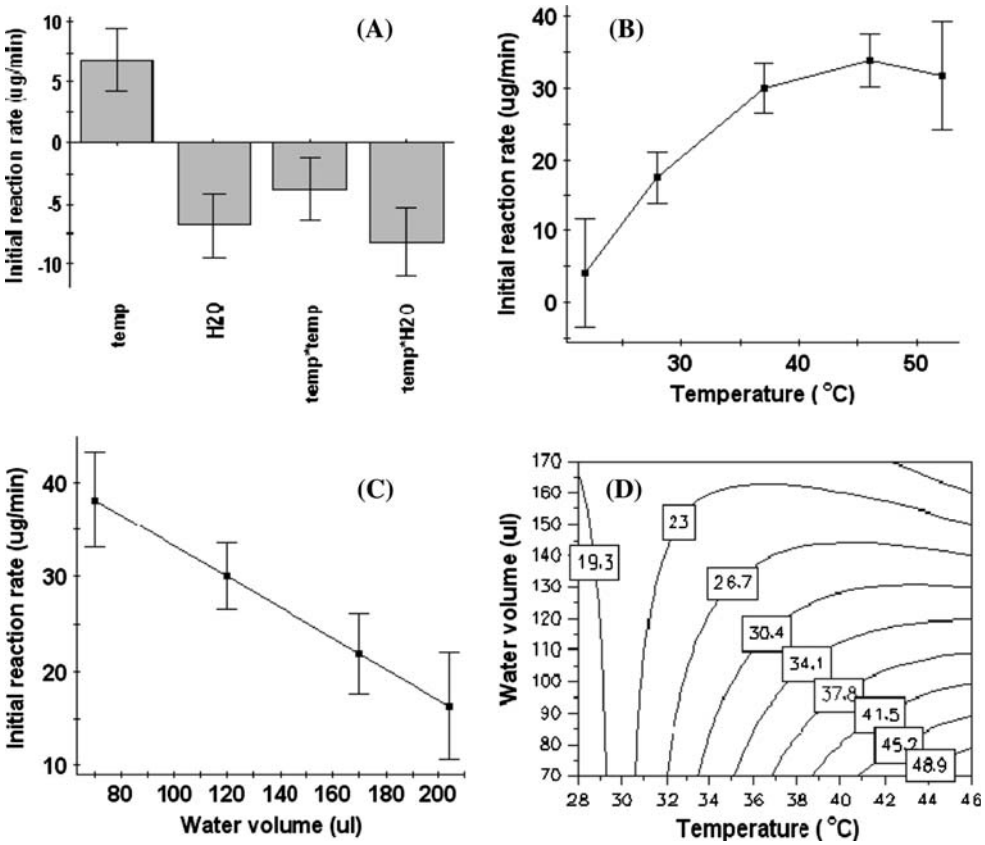

Fig. 5 The results from the optimization study for IE reaction using response surface methodology. Three factors, temperature, water volume, and pH, were selected for optimization. The only response was the initial reaction rate. **a** Summary of the effects on SM hydrolysis. Owing to their having little effect, the pH and its interactions with other factors were eliminated from the model. **b**, **c**, Main effect of temperature and water volume, respectively. The plot of the main effect displays the predicted change in the response when the factor varies from its low to its high level, all other factors in the design being set to their average. **d** The contour plot between temperature and water volume. The *numbers* indicate the initial reaction rate (μg/min)

pH, and water volume, in the analysis. Optimal reaction conditions were generated through the model. However, the model evaluates the effects on response variables through multiple parameters, and evaluation of the effects of each factor will influence the evaluation of the other factors, even though in a good model the predicted value is almost same as the observed value. To get the exact profile of the effect, the factor has to be optimized, with other parameters fixed to optimal values; therefore, the two basic factors, pH and temperature, were further evaluated individually.

In the present study, the effect of pH was tested in a range from 7.4 to 9. The initial reaction rate varied insignificantly within the selected pH range (Fig. 6a); therefore, the enzymatic reaction of IE has little dependence on pH. The negligible effect of pH on SM hydrolysis was concurrent with the result from the model described above, and this concurrency indicates the model is valid. The result differed from what was observed in the soluble enzyme, where the reaction is sensitive to pH shifts [5]. Similar pH tolerance, induced by enzyme immobilization, has been reported in a study of phospholipase D immobilization [11]. The possible explanation is that adsorption of the enzyme in the carrier can shield the charge state of the substrate or the enzyme, so pH changes in the medium are less effective in altering protonation [11]. Because of this pH tolerance, we can conduct the reaction within a broad pH range, and the choice of pH can be subjected to the specific preference. Therefore, the small pH effect is advantageous for utilization of PLC. The selected temperature range for evaluation was from 21 to 60 °C. The optimal temperature was between 46 and 53°C, and the initial reaction rate was dramatically decreased beyond 53 °C (Fig. 6b). The decrease in the reaction rate at higher temperature is possibly due to the thermal inactivation of the enzyme.

Operational Stability Test

In the present optimization study using IE for the reaction, the optimal conditions included a temperature of 46 °C and a water volume of 70 μl; therefore, these conditions were applied in the following stability study. The operational stabilities were compared among reactions with the soluble enzyme and the IE at 46 and 37 °C. In the soluble enzyme catalyzed reaction, only the water phase of the reaction mixture was recycled, meaning that some water might be dissolved in the organic solvent and therefore lost. After the fourth batch, the water phase disappeared in the reaction mixture, and no ceramide was detected

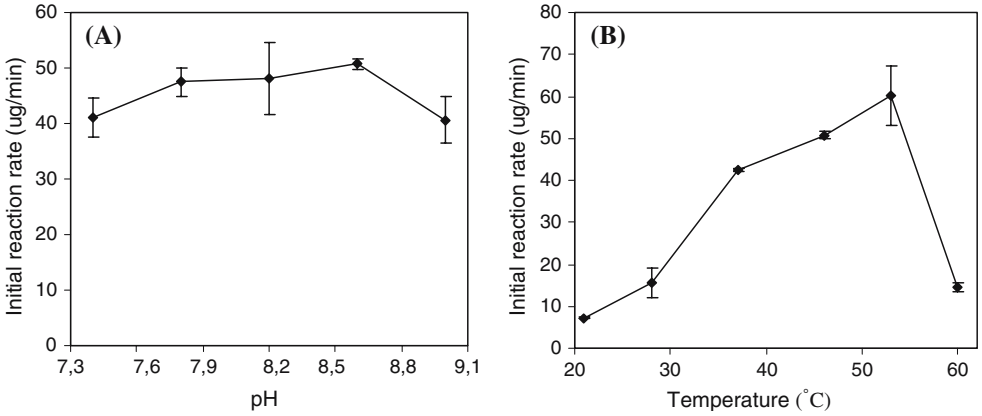

Fig. 6 Influence of pH (**a**) and temperature (**b**) on SM hydrolysis using immobilized phospholipase C as a catalyst

(Fig. 7a). The decrease in the volume of the enzyme solution due to solubility of water in the organic solvent contributes the activity loss of the enzyme in each cycle. Therefore, PLC in soluble form is difficult to reuse under the present conditions. For the operational stability test of IE, the reusability of the enzyme was reaction temperature dependent (Fig. 7a). After recycling, IE remained more active at 37 °C than at 46 °C, verifying that the thermal inactivation of IE plays an important role in the reuse of the enzyme. Since the initial reaction rate was higher at 46 °C from the optimization study, a compromise has to be made between the reaction rate and the stability of IE. With a reaction temperature of 37 °C, the activity of IE was even enhanced by the recycling; thus, the activity was higher in the second and third batches than in the initial batch. This activation is probably due to the conformation change of the enzyme in the enzyme-conditioning step. In the eighth batch, IE retained around 70% of the initial activity; therefore, the reusability of the enzyme is greatly improved by immobilization.

Storage Stability Test

The storage stability test was conducted under three different storage conditions: PLC dissolved in buffer solution at room temperature, IE at room temperature, and IE at 40 °C. The activities of the enzyme at different storage times were irregular when both PLC was dissolved in the solution and IE was stored at room temperature (Fig. 7b). The catalytic activities increased for some days at the beginning, and this phenomenon probably has the same principle as the IE activation induced by recycling in the operational stability test study. When IE was stored at 40 °C, the activity of the enzyme decreased with the storage time. The plot of activity against storage time followed the equation of the first-order reaction of thermal inactivation:

$$[E_t] = [E_0]\mathrm{e}^{-k_\mathrm{D}t}, \tag{2}$$

where $[E_t]$ is the enzyme activity at time t, $[E_0]$ the original enzyme activity without heating, and k_D the

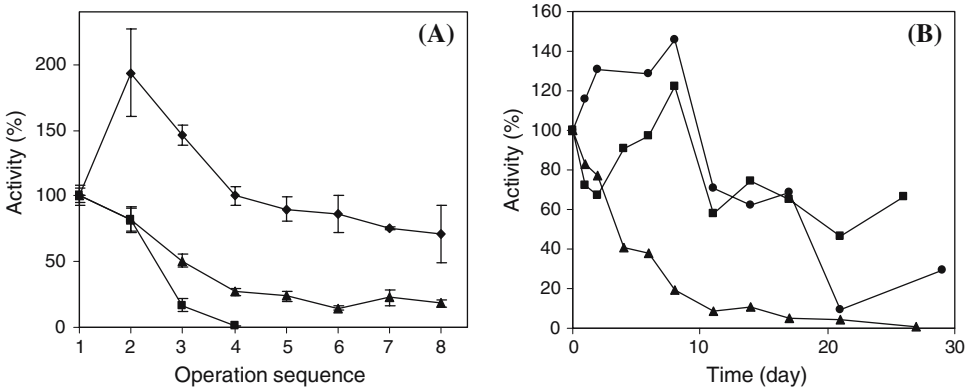

Fig. 7 Stability test for immobilized phospholipase C. The *y*-axis is the percentage of the original catalytic activity that was calculated from the initial reaction rate. **a** Operational stability test. *Squares* soluble enzyme reaction, *Diamonds* IE reaction at 37 °C, and *triangles* IE reaction at 46 °C. **b** Storage stability test. *Squares* soluble phospholipase C stored in buffer solution at room temperature, *circles* IE stored at room temperature, and *triangles* IE stored at 40 °C

first-order inactivation rate constant. When the data were fitted to the equation with the intercept set to 100%, k_D was calculated to be 0.17 per day, and the coefficient of determination (R2) was 0.97. Through this equation, the characteristic half time for thermal inactivation was $\ln(2/k_D) = 4.1$ days. The rate constant, k_D, which is characteristic for the reaction conditions at the given temperature, depends on many factors, such as temperature, pH, substrates, and enzymes. The effect of temperature on k_D can be examined by tests with different temperatures, with other parameters fixed. However, since the results from the storage stability tests at room temperature were irregular in the present study, it is difficult to obtain the profile of the influence of temperature on k_D.

In summary, PLC immobilized on Lew had the highest catalytic activity towards SM hydrolysis. The geometric characteristics of the carrier are a crucial factor that influences the observed catalytic activity of IE. From the results of the optimization study and the stability test of IE reaction, immobilization of the enzyme improved the performance of the SM hydrolysis reaction. Application of the immobilized PLC to produce ceramide from SM modification seems to be attractive and will be the objective of future studies.

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