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Selective production of 1-monocaprin by porcine liver carboxylesterase-catalyzed esterification: Its enzyme kinetics and catalytic performance

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ABSTRACT

Porcine liver carboxylesterase (PLE) belongs to carboxylesterase family (EC 3.1.1.1) as a serine-type esterase. The PLE-catalyzed esterification of capric acid with glycerol in reverse micelles was investigated on the catalytic performance and enzyme kinetics. The most suitable structure of reverse micelles was comprised of isooctane (reaction medium) and bis(2-ethylhexyl) sodium sulfosuccinate (AOT, anionic surfactant) with 0.1 of *R*-value ([water]/[surfactant]) and 3.0 of *G*/*F*-value ([glycerol]/[fatty acid]) for the PLE-catalyzed esterification. In the aspect of regio-selectivity, the PLE mainly produced 1-monocaprin without any other products (di- and/or tricaprins of subsequent reactions). Furthermore, the degree of esterification at equilibrium state (after 4 h from the initiation) was 62.7% under the optimum conditions at pH 7.0 and 60 °C. Based on Hanes–Woolf plot, the apparent K_m and V_{max} values were calculated to be 16.44 mM and 38.91 μM/min/mg protein, respectively.

1. Introduction

Medium chain fatty acids (MCFAs) including caprylic (C8) and capric (C10) acids has unique metabolic pathways distinct from long chain fatty acids (LCFAs). In the intestinal mucosa, MCFAs are absorbed directly into the portal circulation and transported to the liver for rapid metabolism by β-oxidation. LCFAs, on the other hand, are incorporated into chylomicrons and transported via the lymphatic system, allowing for expensive accumulation in adipose tissue [1]. Medium-chain glycerides, which are comprised of a glycerol backbone and one, two, or three MCFAs, have been used as components of infant feeding formulas and nutritional supplements for patients with malabsorption caused by digestive diseases or intestinal disorder [2,3]. In terms of additional functionalities for food-processing, 1-monocaprin (glyceryl monocaprate), a GRAS (generally recognized as safe) food additive, has garnered much attention because of its preservative effect against broad spectrum of microorganisms and emulsifying ability [4–6].

Industrial-scale production of medium-chain glycerides has been performed through chemical glycerolysis between glycerol and medium-chain fatty acids, employing inorganic catalysts (*e.g.*, calcium hydroxide) at high temperatures (220–250 °C) [7]. This process, however, requires high energy consumption and also may cause unexpected changes in physicochemical properties of the products, which has encouraged the development of alternative methods using enzymes under mild conditions without aforementioned disadvantages.

It has been reported enzyme-catalyzed synthesis for the efficient production of various medium-chain glycerides, such as 1-monocaprin and 1,3-dicaproyglycerol [8,9]. The previous approaches were based on the sequential process which needs separation (and/or purification) steps for certain substance (*i.e.*, desired product) after synchronous synthesis of medium-chain glycerides including mono-, di-, and triacylglycerols. In this process, however, it is extremely difficult to obtain abundant monoacylglycerols because of subsequent conversions of the produced monoacylglycerols into di- and triacylglycerols, which has been regarded as a

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major obstacles in the aspect of selective synthesis of monoacylglycerols (*e.g.,* 1-monocaprin production).

On the other hand, because of the unique characteristics of lipid-catalyzing enzyme reaction between hydrophilic enzymes and hydrophobic substrates, the selection of reaction medium is a main factor affecting the conversion yield and efficiency. Among several proposed systems, reverse micelles have been received attention as a favourable approach. The structure of reverse micelles consists of an aqueous micro-domain (polar phase, core) facing the polar heads of the surfactant that surrounds this core and interacts with the bulk organic solvent (non-polar phase), which is supported by hydrophobic interactions [10]. This reaction system provides a variety of advantages, such as simple control on reaction variables and enormous interfacial area where the enzymatic reaction occurs [11,12]. Furthermore, it was recently revealed that the encapsulation of enzymes in reverse micelles increased the deactivation energy (E_{de}) and led to the enhancement of thermal stability (*i.e.,* resistance to heat-induced denaturation) [13].

Porcine liver carboxylesterase (PLE) has the consensus sequence motif Gly–Glu–Ser–Ala–Gly in its lipolitic active site, which belongs to a serine-type esterase [14]. For decades, PLE has been mainly utilized as a hydrolase to produce various carboxylic acids, alcohols, and diol derivatives, and showed high regio-selectivity and stereo-specificity which make them attractive biocatalysts for the production of pure organic compounds [15–17]. Based on the preliminary screening for several commercial enzymes, such as carboxylesterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), PLE produced 1-monocaprin to the much higher level among all enzymes tried, indicating that PLE was the most suitable enzyme for the selective production of 1-monocaprin. Therefore, the primary purpose of this study was to determine optimum conditions and kinetic parameters of the PLE-catalyzed esterification for the selective production of 1-monocaprin.

2. Materials and methods

2.1. Chemicals and reagents

The lyophilized powder of PLE with a reported catalytic activity of >15 units/mg solid (one unit is equivalent to the hydrolysis of 1 μmole of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25 °C) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Standard compounds for the quantitative analysis of the HPLC (JASCO LC-2002, Tokyo, Japan) such as capric acid, 1-monocaprin, 2-monocaprin, 1,2-dicaprin, 1,3-dicaprin, tricaprin, and glycerol were purchased from Sigma–Aldrich Co., Bis (2-ethylhexyl) sodium sulfosuccinate (AOT), dimyristoyl phosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), cetyltrimethylammonium bromide (CTAB), Triton X-100 (Sigma–Aldrich Co.), and polyethylene glycol 6000 (Wako Pure Chemical Industry Ltd., Osaka, Japan) were used as surfactants to form reverse micelles. AOT was purified according to the method of Tamamushi and Watanabe [18], and dried over P_2O_5 under reduced pressure. Acetone, benzene, cyclohexane, heptane, *n*-hexane, and isooctane, all of HPLC-grade (Honeywell Burdick & Jackson International, Inc., Muskegon, MI, USA), were stored over a Type 4 Å molecular sieve (8–12 mesh, Sigma–Aldrich Co.), filtered prior to use, and tested for selection as a proper organic reaction medium in reverse micelles with 50 mM of surfactant. Water content was determined with a Karl–Fisher moisture meter (CA-200, Norwood, NJ, USA). All other chemicals were of analytical grade.

2.2. Preparation of reverse micelles

Predetermined amounts (0.16 units/mL of reactant) of PLE in buffer solution (10 mM Clark and Lubs buffer, pH 7.0) and capric acid (0–100 mM as the final concentration) were added to the organic solvent containing glycerol (100 mM) and surfactant (50 mM), and then the mixture was vortex-mixed for 60 s to form clear micellar solution. The desired initial water content and glycerol concentration were defined as the *R*-value, which indicates the molar ratio of water to surfactant ([water]/[surfactant]), and the *G*/*F*-value ([glycerol]/[fatty acid]), respectively. Clear micellar solution without turbidity could be generated under the experimental conditions within the range of 0 to 200 mM glycerol. The Type 4 Å molecular sieve with a concentration of 20% (w/v) against the reactant was subsequently added to remove water during the esterification.

2.3. Analysis of PLE-catalyzed esterification

The esterification by PLE in reverse micelles was conducted using capric acid and glycerol as two kinds of substrates. A screw-cap vial was filled with 5 mL of selected surfactant-organic solvent solution containing capric acid, and the final concentrations of surfactant and capric acid were adjusted to 50 mM and 33 mM, respectively. The desired amount of glycerol containing water and PLE was injected into the screw-cap vial, and the PLE-catalyzed esterification was initiated by vortex-mixing until the mixture became clear. The final concentrations of buffer, surfactant, capric acid, and glycerol were adjusted to 10, 50, 33, and 100 mM, respectively (unless otherwise specified).

A sample of 0.4 mL was taken from the reaction mixture at predetermined intervals. The equal volume of acetone was added to the sample in a test tube, and the test tube was shaken vigorously for 2.0 min. Thereafter, an aliquot of 10 μL was applied to HPLC for further analysis. A blank was prepared by the same procedure as described above except the use of thermal-deactivated PLE. Esterification-quenched samples were analyzed with a HPLC (Jasco LC-2002, Tokyo, Japan) equipped with a silica-based Luna C18 column (5 μm, 4.6 × 150 mm; Phenomenex Inc., Torrance, CA, USA) and a refractive index detector (RID-2031, JASCO corp., Tokyo, Japan) at a 0.8 mL/min flow rate. The mobile phase was acetonitrile/2-propanol/acetic acid (15/3/1, v/v/v). Each peak in the HPLC chromatogram was identified by comparison of its retention time (R_t) with those of 2-monocaprin (R_t = 1.64 min), 1-monocaprin (1.71 min), capric acid (2.36 min), 1,3-dicaprin (4.65 min), 1,2-dicaprin (5.31 min), and tricaprin (12.36 min) standards. One unit of enzyme for the esterification was defined as the amount of PLE that reacted 1 μmol of capric acid/min under the assay condition. All of the data were the average of triplicate samples and were reproducible within ±10%. The degree of esterification was defined by the following equation, and calculated from the peak areas integrated by Borwin software (ver. 1.21, JASCO Corp.):

$$\text{Degree of esterification (\%)} = \frac{\text{Concentration of capric acid reduced at termination of esterification}}{\text{Concentration of capric acid at initial time}} \times 100$$

The amount of protein was determined using the method described by Bradford [19].

2.4. Optimization of the esterification conditions

Various concentrations of capric acid solution in 100 mM glycerol were prepared to provide *G*/*F*-values between 1.0 and 9.0 in the presence of PLE with 0.16 units/mL of reactor. The appropriate amounts of water in the range of *R*-values from 0 to 1.0 were evaluated in the selected organic solvent medium containing 50 mM of surfactant. PLE-catalyzed esterification in reverse micelles was evaluated by varying the enzyme concentration (2–8 mg protein/mL), pH (3–13), and temperature (20–70 °C). The

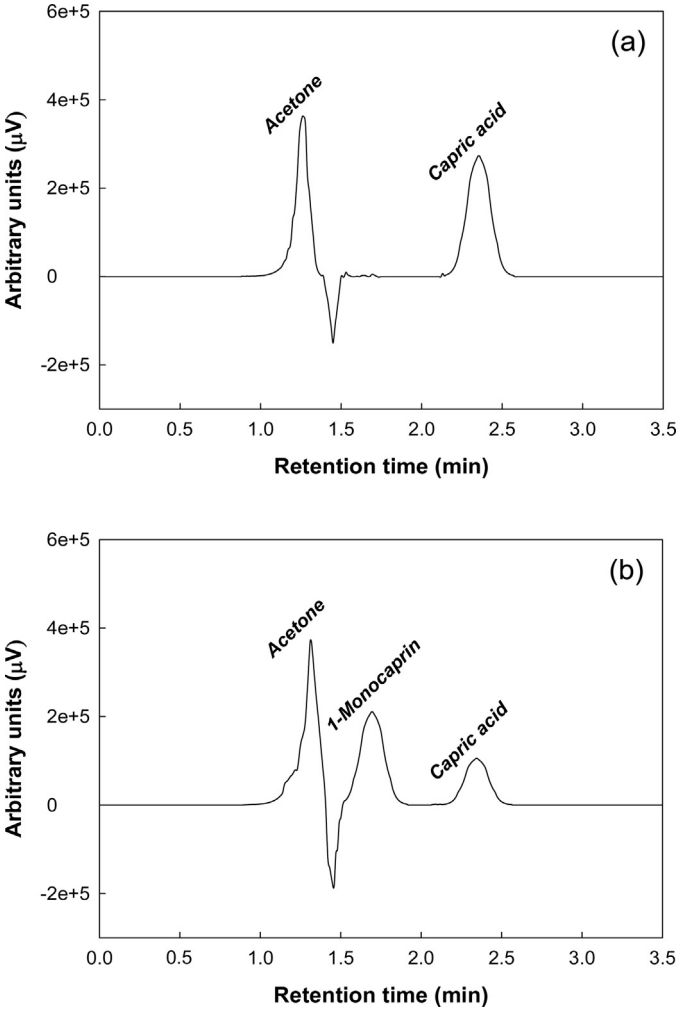

Fig. 1. HPLC chromatograms at initial time (a) and after termination (b) of the PLE-catalyzed esterification employing glycerol and capric acid as substrates.

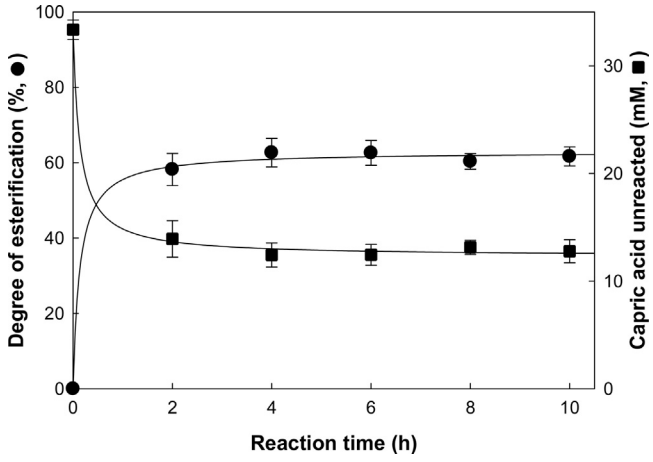

Fig. 2. Time course of PLE-catalyzed esterification exerted by the change of degree of esterification (%) and capric acid unreacted (mM).

kinetic parameters of PLE-catalyzed esterification were calculated from Hanes–Woolf equation by determining the reduction rate of the substrate, capric acid. The substrate concentration used in the determination of kinetic parameters was 10–60 mM. The kinetic study was run under optimum conditions.

Table 1
Relative catalytic efficiency of the PLE-catalyzed esterification in reverse micelles employing different surfactants and organic solvents.

	Chemicals	Relative activity (%)
Surfactants	AOT	100.0
	Dimyristoylphosphattidylcholine	12.0
	Dipalmitoylphosphatidylcholine	8.5
	Triton X-100	6.4
	Polyethylene glycol 6000	0.0
	Cetyltrimethylammonium bromide	0.0
Organic solvents	Isooctane	100.0
	Acetone	18.0
	Benzene	8.4
	Cyclohexane	1.3
	n-Hexane	0.0
	Heptane	0.0

*The PLE reaction was executed for 2 h at pH 7.0 and 60°C in the reverse micelles of *R*-value 0.1 and *G*/*F*-value 3.0.

3. Results and discussion

3.1. Time course of PLE-catalyzed esterification in AOT/isooctane reverse micelles

Based on quantitative analysis using HPLC, the typical time course was shown in Fig. 1 and Fig. 2. The PLE-esterification between glycerol and capric acid produced only 1-monocaprin without any other products such as dicaprins and/or tricaprins during the entire reaction (Fig. 1). Therefore, it was revealed that PLE could be favorable for the selective production of pure 1-monocaprin without further separation process. The degree of esterification proportionally increased to 58.2% for the 2 h reaction, and was maintained at ~60% after 4 h (Fig. 2). The maximum degree of esterification was 62.7% at 4 h and the initial velocity of PLE-catalyzed esterification was 2.69×10^{-2} units/mg protein/mL reactor for 2 h. With respect to time course of PLE-catalyzed esterification, the degree of esterification did not increase but gradually attenuated after 2 h. This is likely due to the reduced capric acid level, which causes an increase in the *G*/*F*-value, and because the deficiency of donor molecules relative to acceptor molecules decreased the esterification velocity.

3.2. Selection of surfactant and organic medium for the esterification

Because of the structural uniqueness of surfactants, they can locate the interfacial membrane between immiscible liquids, and stabilize two-phase systems. Therefore, the surfactant used to form the reverse micelles is one of the main factors to control the micelle size, percolation rate through materials of surfaces, and the interaction among nanodroplets [20]. With six kinds of surfactants, the relative activities were evaluated to select the suitable surfactant for the PLE-catalyzed esterification in reverse micelles (Table 1). The highest esterification activity of PLE could be found in the reverse micelles formed by AOT. The relative activities of the reverse micelles formed by DMPC, DPPC, and Triton X-100 were 12.0, 8.5, and 6.4%, respectively, compared to those formed with AOT. Esterification rarely occurred in the reverse micelles composed of polyethylene glycol 6000 or CTAB. It is well-known that the surfactants with around 1 of packing parameter are typically assembled to multilamellar- or cylinder-form vesicles in polar and nonpolar solvents [21]. The packing parameters of DMPC and DPPC are around 1, indicating that DMPC and DPPC could not form the stable spherical micellar structures enable to encapsulate PLE into the interior region. This would be the reason for the low relative activities of PLE in the vesicles formed by DMPC and DPPC.

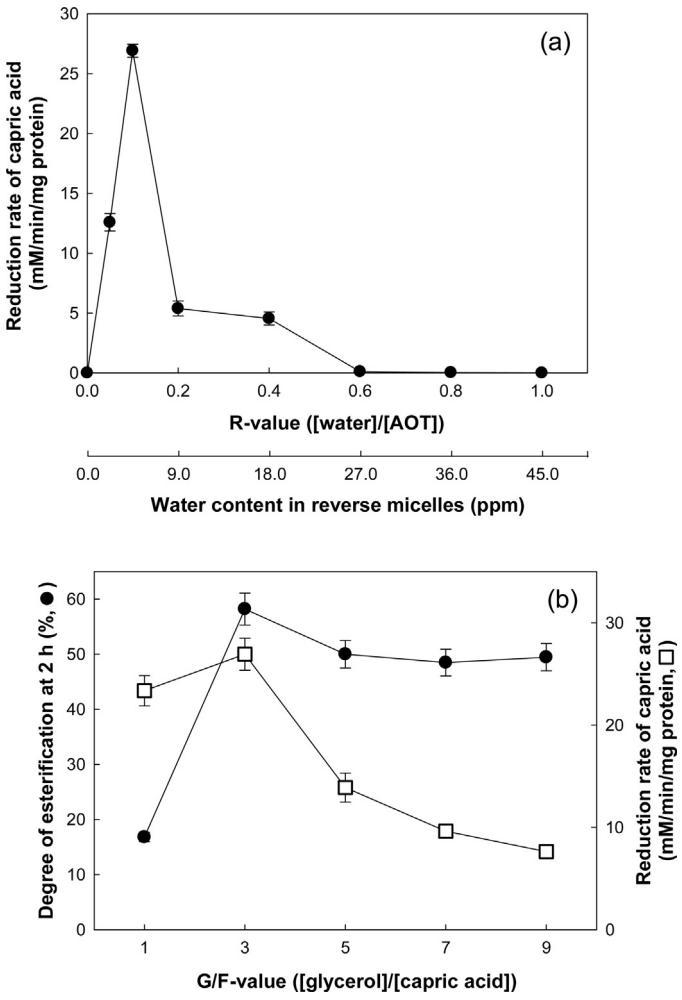

Fig. 3. Optimization of *R*-([water]/[AOT]) (a) and *G/F*-([glycerol]/[capric acid]) (b) values for the PLE-catalyzed esterification in 50 mM AOT/isooctane reverse micelles.

In reverse micelles, organic solvents affect the stability and initial velocity of encapsulated biocatalysts and solubilization of substrate(s), droplet size, and inter-droplet interaction [10]. Isooctane, acetone, benzene, cyclohexane, *n*-hexane, and heptane were evaluated for their effect on enzyme-catalyzed esterification, as they are frequently used as the organic media for various enzyme-catalyzed reactions. Water-miscible solvents such as ethanol and methanol which cannot form reverse micelles with an aqueous phase were not considered [8]. AOT which displayed the greatest PLE activity in reverse micelles was used for formation of reverse micelles with the various organic solvents. The effect of the organic solvent on PLE-catalyzed esterification in the reverse micelles is summarized in Table 1 (the bottom). Isooctane was the most effective organic solvent for PLE-catalyzed esterification of glycerol and capric acid in the reverse micelles. Acetone, benzene, and cyclohexane showed very low relative activities, 18.0, 8.4, and 1.3%, respectively. However, no enzyme activity was detected in the reverse micelles prepared with *n*-hexane and heptane. Therefore, AOT and isooctane were selected as the surfactant and organic reaction medium, respectively, to characterize PLE-catalyzed esterification in the reverse micelles. This combination was consistent with previous reported results [22].

3.3. Optimization of R- and G/F values for the esterification in reverse micelles

The *R*-value dominantly regulates the size of aqueous microdomain entrapping the enzyme and affects the stability of the entrapped enzyme, which indicates the degree of hydration of the reverse micelles [23]. For this reason, it has been utilized to evaluate the dependence of enzyme activity on the water content. In order to determine the effect of *R*-value on PLE-catalyzed esterification in the reverse micelles, *R*-values were varied from 0 to 1.0 by changing the ratio of water concentration to 50 mM AOT in isooctane solution to minimize the possible denaturing effect of the surfactant on the enzyme [24]. The maximum reduction rate of capric acid was obtained from the optimal amount for activation of PLE for an esterification, but not for hydrolysis reaction (Fig. 3-a). At low *R*-values (0 to 1.0), the conversion rate increased dramatically with the increment of the *R*-value. However, at an *R*-value of >0.1, the data showed a low reduction rate of capric acid compared with that at an *R*-value of 0.1. In particular, PLE-catalyzed esterification was not found at an *R*-value of >0.8.

The water in reverse micelles can be classified as immobilized (below 2.0), hydrated (4.0 to 10.0), or free (above 10.0) state, depending on the *R*-value [25]. In this study, the optimized *R*-value for PLE-catalyzed esterification was 0.1, much lower than 2.0. As described above, in reverse micelles with a very low *R*-value, the AOT exists in a distorted state because of water deficiency in the point of rotational isomerism of surfactant molecules, and it is difficult to form a micelle sphere including a water-pool to hydrate enzymes. Thus, PLE is suspected to be solubilized inside of glycerol-AOT micelle spheres in AOT/isooctane-reverse micelles following the induced-fit model, one of three models of solubilization of protein molecules suggested by Martinek et al. [26]. In this model, the size of the inner cavity of glycerol-AOT micelles can increase in order to solubilize the enzyme. The other two models are the water-shell and fixed size models, which require enough water at an *R*-value of >2.0 to form a water-surfactant micelle sphere. At low *R*-values, the micro-viscosity of the micellar water is higher than that of the bulk solution [27,28]. Therefore, the maximum activity of PLE in esterification of capric acid and glycerol in the low *R*-value state may be explained by a reduction in the competition between glycerol and water molecules and by the elimination of spontaneous fluctuations of protein structure, which disturb the catalytic conformation in aqueous solution [26].

During enzyme-catalyzed synthesis, the ratio of donor to acceptor is an important factor to achieve high productivity and determine product composition. In esterification of glycerol with fatty acids catalyzed by PLE, the fatty acid acts as a donor and glycerol acts as an acceptor. The *G/F*-value can usually be controlled by varying the concentration of glycerol to a fixed concentration of fatty acid. To maximize the synthetic efficiency in synthesis reactions such as esterification, a higher concentration of acceptor than of donor is generally required due to competition of acceptor molecules with solvent molecules, such as water, at an intermediate state. However, the amount of water was limited to an *R*-value of 0.1 in this study. Simultaneously, the concentration of glycerol in the aqueous phase of the reverse micelles was fixed to minimize the possibility of unexpected and undesirable structural changes in the reverse micelles. The concentration of capric acid was thus varied as a constant concentration of glycerol. The effect of a *G/F*-value between 1.0 and 9.0 was estimated by decreasing the concentration of capric acid to 100 mM glycerol. As shown in Fig. 3-b, degree of esterification at 2 h of reaction time was proportionally increased with the increase in the *G/F*-value from 1.0 to 3.0. However, increasing the *G/F*-value to >3.0 did not further increase the degree of esterification and decreased drastically reduction rate of capric acid. Finally, the maximum levels

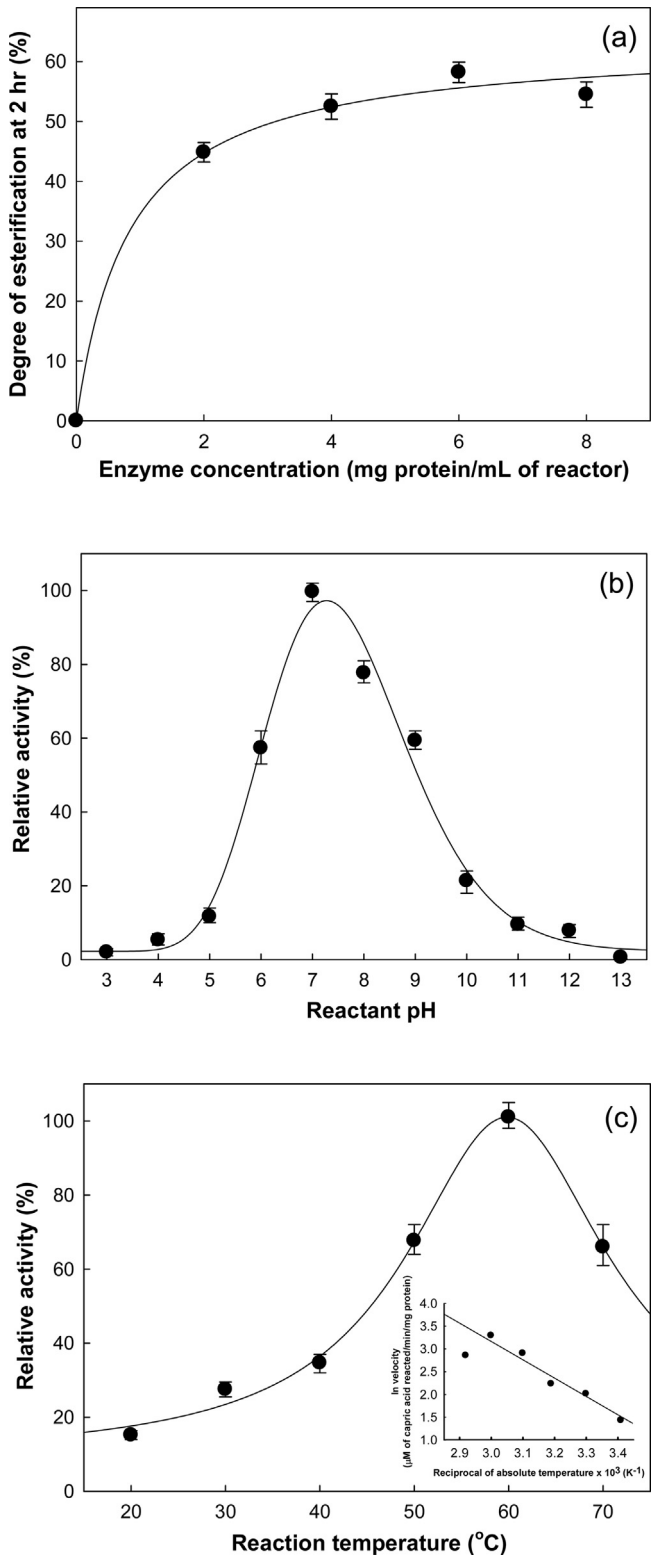

Fig. 4. Effects of enzyme concentration (a), reactant pH (b), and reaction temperature (c) on the PLE-catalyzed esterification.

of esterification degree (58.2%) and capric acid reduction velocity (26.92 μM/min/mg protein) could be obtained from 3.0 of *G*/*F*-value. Therefore, the optimum *G*/*F*-value was determined as 3.0 and identical to that of carboxylesterase from *Calotropis procera* R. Br. [8].

3.4. Characteristics of PLE-catalyzed esterification in AOT/isooctane reverse micelles

General characteristics of PLE-catalyzed esterification were investigated by determining the effects of the enzyme concentration (2.0-8.0 mg/mL), pH (3–13), and temperature (20–70 °C) on the esterification in reverse micelles. The degree of esterification at 2 h was measured and gradually increased from 44.9 to 58.2% at enzyme concentration of 2.0 and 6.0 mg/mL, respectively. However, increasing the enzyme concentration to >6.0 mg/mL could not increase the degree of esterification any more (Fig. 4-a). Thereafter, 6.0 mg protein/mL reactor was employed as the optimum concentration of enzyme for the further study. The dependence of PLE activity on pH was shown as a bell-shaped curve in Fig. 4-b, and the highest value was observed at pH 7.0 with 60 °C. PLE showed <20% activity at a pH of <5.0 and >10.0 compared with that at an optimum pH. The optimum pH 7.0 was slightly closer to a neutral pH compared with pH 8.0, the optimum pH for hydrolysis of *p*-nitrophenyl acetate in an aqueous system [29]. It is thought that a neutral pH is advantageous for solubilizing PLE in the reverse micelles, including the anionic surfactant AOT, as determined by the induced-fit model. The effect of temperature on PLE-catalyzed esterification was investigated at temperature from 20 to 70 °C. As illustrated in Fig. 4-c, PLE activity increased proportionally with function from 20 to 60 °C, but dropped at 70 °C. The highest degree of esterification was achieved at 60 °C after 2 h reaction. Therefore, 60 °C was determined to be the optimum temperature for PLE-catalyzed esterification. Temperature plays an important role in enzyme-catalyzed reaction. Increases of reaction temperature make a low viscosity of the reaction mixture. The low viscosity has a potential to increase the rate of interaction between substrates and enzyme molecules, thereby enhancing enzymatic activity [30]. The Arrhenius equation has been widely used as a model to determine the temperature dependence of the reaction rate [31]. The activation energy (E_a) of the esterification reaction in AOT/isooctane reverse micelles calculated from the Arrhenius plot [Eq. (1)] was relatively low, 7.75 kcal/mol (inserted in Fig. 4-c), when compared with 8.40 kcal/mol reported in a two-phase system for a similar temperature range [32]. The difference might be caused by the increased interfacial area between PLE molecules solubilized in the glycerol pool containing a very small amount of water (core in the reverse micelles) and capric acid (substrate) solubilized in isooctane (continuous phase in the reversed micellar system). Therefore, we reconfirmed that the AOT/isooctane reversed micellar system could be efficient and suitable for the enzyme-catalyzed esterification because it enhanced the collision factor between the enzyme and substrate molecule.

$$\ln k = \ln A - \frac{Ea}{R}\left(\frac{1}{T}\right) \quad (1)$$

where k is the rate constant, A the pre-exponential factor, E_a the activation energy, R the gas constant, and T the absolute temperature.

3.5. Determination of kinetic parameters

PLE-catalyzed esterification in reversed micellar system followed Michaelis–Menten kinetics. And Hanes-Woolf plot of ([capric acid]/initial velocity) versus [capric acid] gave a straight line as shown in Fig. 5. Kinetic parameters of Michaelis-Menten equation could be calculated from Hanes-Woolf equation [Eq. (2)] of the esterification activities. To determine the kinetic parameters, PLE-catalyzed esterification was conducted in the reverse micelles at *R*-value of 0.1, *G*/*F*-value of 3.0, pH 7.0, and 60 °C with 6 mg/mL PLE. The apparent K_m and V_{max} obtained from least square analysis were 16.44 mM and 38.91 μM/min/mg protein, respectively. And

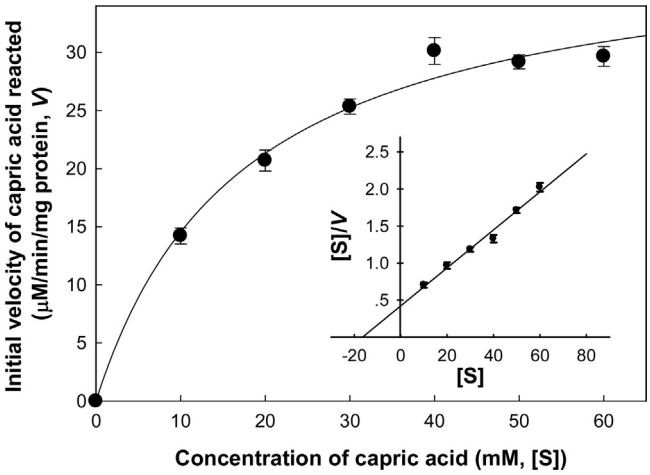

Fig. 5. Michaelis–Menten curve and Hanes–Woolf plot (inserted) for the determination of kinetic parameters in the PLE-catalyzed esterification.

the specific activity of the enzyme for the esterification in reversed micellar system, which means the initial velocity exerted by 1 mg protein of the enzyme, was determined as 2.69×10^{-2} units/mg protein/mL reactor.

$$\frac{[S]}{V} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}} \quad (2)$$

where [*S*] is the concentration of a substrate, *V* the initial velocity, V_{max} the maximum velocity, and K_m the substrate concentration at which half of V_{max}.

4. Conclusion

The reversed micellar system for PLE-catalyzed esterification was established with AOT (surfactant) and isooctane (reaction medium). Among surfactants applied, the surfactants having the ability to form the stable spherical micellar structures showed the high catalytic efficiency of the PLE-catalyzed esterification, indicating that these surfactants were enabled to encapsulate PLE into the interior region. PLE was most suitable enzyme to selectively produce 1-monocaprin from capric acid and glycerol in the reverse micelles. It was revealed that *R*-value was a significant factor to make PLE esterification reaction specific and *G*/*F*-value also an important factor to reach the high degree of esterification. Although the significant factors affecting PLE activity were revealed, the way how these factors affected PLE activity is unclear. Therefore, to account for how glycerol behaviors in the reverse micelles, not as a substance, and what makes esterification specific in the reverse micelles under the established condition, not hydrolysis specific, the experiment should be conducted to provide the concrete evidence for above questions.

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