Enzyme and Microbial Technology 82 (2016) 51–57

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

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

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ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form 4 August 2015
Accepted 21 August 2015
Available online 28 August 2015

Keywords:
Porcine liver carboxylesterase
Esterification
Reverse micelles
1-monocaprin
Enzyme kinetics

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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http://dx.doi.org/10.1016/j.enzmictec.2015.08.014
0141-0229/

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