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Production of cinnamoyl lipids using immobilized *Proteus vulgaris* K80 lipase and an evaluation of their antioxidant activity

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ABSTRACT

Natural phenolic acids, such as cinnamic acid, have antioxidant and other biological activities. However, their hydrophilic groups reduce antioxidant efficiency in fat and oil systems. To overcome this restriction in their usage, natural phenolic acids have been modified via an enzymatic transesterification reaction. The lipase-catalyzed transesterification reaction has received attention due to advantages, such as stereo and regioselectivity and substrate specificity. In this study, ethyl cinnmate (40 mM) and triolein (240 mM) were used as substrates and immobilized *Proteus vulgaris* K80 lipase (1 U) was used as enzyme catalyst. When the interesterification reaction was performed in 5 mL *n*-hexane/toluene (85:15, v/v) at 35 °C for 72 h, cinnamoyl monooleyl glycerol (CMOG) and cinnamoyl dioleyl glycerol (CDOG) were produced and the conversion yield was 70%. These cinnamoylated lipid products were collected by preparative liquid chromatography and identified using high resonance mass spectrometry. CMOG and CDOG showed radical scavenging activities of 45% and 69%, respectively, in *n*-hexane system using DPPH assay and 56% and 20%, respectively, in dichloromethane/DMSO (8:2, v/v) system using ABTS assay.

1. Introduction

Antioxidants are molecules that inhibit oxidation of other molecules, which is a chemical reaction that produces free radicals, leading to chain reactions that can damage cells. Antioxidants are used in the food, cosmetic, and pharmaceutical industries. For example, synthetic antioxidants, such as butylated hydroxyltoluene (BHT) and butylated hydroxyanisole (BHA), are frequently used due to their high antioxidant activity in non-polar media [1]. However, some properties of BHT and BHA, such as high volatility and instability at high temperature, have restricted their industrial application. In addition, side effects, such as neuronal damage and colon cancer, have been reported in a mice model, indicating potential carcinogenicity [2,3]. Consumers have started to prefer natural antioxidants rather than synthetic antioxidants [4].

Phenolic acids in fruits, vegetables, and aromatic herbs [5] have strong antioxidant activities. In addition, phenolic acids extracted from natural sources have anti-inflammatory and anti-carcinogenic properties [6,7]. However, their hydrophilic characteristics reduce solubility in lipophilic media. As a result, the antioxidant activity of phenolic acids decreases in lipophilic media, such as oils, fats, and emulsions [8]. To overcome this drawback, lipase catalyzed-esterification or transesterification of phenolic acids with a lipophilic moiety, such as oleyl alcohol, has been studied to increase their solubility in lipophilic media [9,10]. Lipase-catalyzed bioconversion reactions, such as trans, inter, or esterification, have been employed widely by virtue of their mild reaction conditions, high substrate specificity, and high regio and enantioselectivity [11]. Moreover, the stability of lipase improves when high concentrations of lipid substrates are used in organic solvent systems [12].

Proteus vulgaris K80 lipase is a stable enzyme in various organic solvents, suggesting that it could be used in trans, inter, or esterification reactions [13]. Lipase K80 was recently immobilized on MA-DVB resin using an adsorption and cross-linking method with glutaraldehyde. The enzyme has been characterized biochemically and has been used to synthesize an omega 3-fatty acid methyl ester [14].

Lipases immobilized onto beads are useful catalysts for transesterification in non-aqueous solvent system. They are mixed well with non-polar reactants and hence provide minimal steric hindrance to reactants for accessing enzyme active sites. In addition, they can be recovered easily after transesterification reaction [15,16].

In this study, we performed an immobilized lipase K80-mediated interesterification reaction with ethyl cinnamate and

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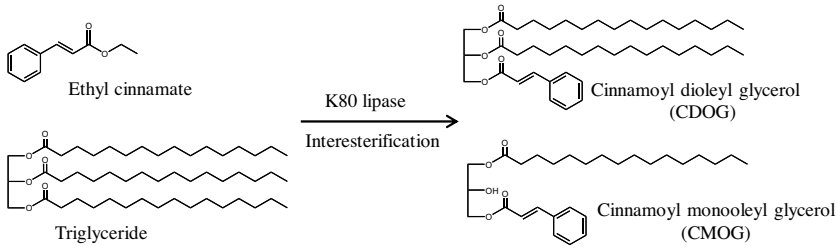

Scheme 1. Interesterification reaction using ImmK80 lipase. ImmK80 performs interesterification reaction using ethy cinnamate and triolein to produce cinnamoyl dioleoyl glycerol (CDOG) and cinnamoyl monooleoyl glycerol (CMOG).

triolein to produce cinnamoyl lipids (Scheme 1). Cinnamic acid is a phenolic acid with the basic backbone structure of phenyl propanoids and has antioxidant capacity [17]. We optimized the organic solvent system, substrate concentration, and mole ratio of substrates using immobilized lipase K80. We confirmed that the cinnamoyl lipid products were cinnamoyl monooleyl glycerol (CMOG) and cinnamoyl dioleyl glycerol (CDOG). Finally, we evaluated their radical scavenging activities using diphenylpicrylhydrazyl (DPPH) assay and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay in various solvent systems.

2. Materials and methods

2.1. Materials

Commercially available *Candida antarctica* lipase (CalB) immobilized on acrylic resin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemical reagents, including ethyl cinnmate (99.5%), ABTS, DPPH, potassium persulfate, *p*-nitrophenyl palmitate, 2-butanone, and a molecular sieve (4 Å) were also purchased from Sigma-Aldrich. Methanol, ethanol, isopropanol, *n*-hexane, cyclohexane, toluene, and dichloromethane were purchased from Junsei (Tokyo, Japan). All solvents for the high-performance liquid chromatography (HPLC) analysis were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of immobilized lipase K80

The recombinant plasmid pKLE [18] containing the *P. vulgaris* K80 lipase gene was transformed into *Escherichia coli* BL21 (DE3). The transformants were cultured in 1 L of Luria–Bertani medium containing 100 μg/mL ampicillin at 37 °C and 210 rpm. The bacteria were cultured until the optical density at 600 nm reached 0.5. Then, recombinant protein production was induced with 1 mM isoprophy-β-D-thiogalactopyranoside for 18 h at 20 °C and 210 rpm. The cultured cells were harvested via centrifugation (6000*g*, 10 min) and resuspended in 20 mL Tris-HCl buffer (100 mM, pH 8.0). The cells were disrupted by sonication (Vibra Cell; Sonics and Materials, Newtown, CT, USA), and the cell-free extract obtained by centrifugation (10,000*g*, 20 min) was used for enzyme immobilization.

For immobilization, 1 g of metacrylate-divinyl benzene (MA-DVB) resin and 10 mL of methanol were mixed and treated for 1 h at 25 °C with shaking at 160 rpm. After washing the MA-DVB resin with potassium phosphate buffer (50 mM, pH 6.5), 10 mL of the cell-free extract (400 mg protein) containing 0.5 M ammonium sulfate was added and incubated for 4 h at 25 °C with shaking (160 rpm). Then, 25 mM glutaraldehyde was added and incubated for 20 h at 4 °C with stirring. The immobilized lipase K80 (ImmK80) was stored at 4 °C.

2.3. Measurement of lipase activity

ImmK80 transesterification activity was measured by a method described previously [19]. Briefly, *p*-nitrophenyl palmitate (pNPP) was used as a substrate for the spectrometric assay. The reaction mixture containing 500 μL of 20 mM substrate in *n*-hexane, 35 μL ethanol, and 5 mg ImmK80 was incubated for 3 min at 35 °C with shaking at 210 rpm. After incubation, ImmK80 was centrifuged to the bottom of tube and 25 μL of the supernatant was mixed with 1 mL 0.1 M sodium hydroxide. The mixture was measured at 415 nm using a spectrophotometer. One unit of enzyme activity was defined as the quantity of enzyme required to generate 1 μmol of *p*-nitrophenol via the transesterification of pNPP substrate in 1 min.

2.4. Interesterification reaction of ethyl cinnamate with triolein

The interesterification reaction of ethyl cinnamate with triolein was performed in a 20 mL screw capped vial according to the method of Karboune et al. [20]. The standard reaction mixture consisted of 40 mM ethyl cinnamate, 40 mM triolein, and 200 mg of molecular sieve (4 Å) in 5 mL of a *n*-hexane/toluene mixture (85:15, v/v). Enzymatic reactions were initiated by the adding 1 U (transesterification assay) ImmK80 to the reaction mixture. The reaction mixture was incubated at 35 °C, with continuous shaking at 210 rpm for 72 h. The interesterification reactions were terminated by removing ImmK80 by decanting off the organic solvent media.

To test the effect of substrate amount on the ImmK80-mediated interesterification reaction, the reaction was conducted with various concentrations (4–160 mM) of both substrates. The molar ratio of ethyl cinnamate and triolein was 1:1 (v/v), and the other reaction conditions were the same as described above.

To test the effect of the amount of triolein on the ImmK80-mediated interesterification reaction, the reaction was performed using increasing concentrations of triolein (40–240 mM) and a fixed concentration of ethyl cinnamate (40 mM). The other reaction conditions were the same as described above.

A CalB-mediated reaction was performed for comparison using increasing concentrations of triolein (4–24 mM) and a fixed concentration of ethyl cinnamate (4 mM) using 150 mg CalB and a reaction temperature of 55 °C. The other reaction conditions are the same with the standard condition described above.

The effect of the reaction media, consisting of a mixture of *n*-hexane and a selected organic solvent (85:15, v/v), on the interesterification reaction was investigated. Organic solvents, including *n*-hexane, cyclohexane, toluene, dichloromethane, and 2-butanone, with log P values of 4.0, 2.7, 2.31, 1.0, and 0.29, respectively, were used [21].

Finally, the time course for the interesterification reaction was measured under optimized conditions; 40 mM ethyl cinnamate, 240 mM triolein, 1 U ImmK80, 200 mg molecular sieve, 5 mL *n*-hexane/toluene (85:15, v/v).