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Preparation of phytosteryl ester and simultaneous enrichment of stearidonic acid via lipase-catalyzed esterification

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ABSTRACT

The simultaneous synthesis of a phytosteryl ester and enrichment of stearidonic acid (SDA) were performed via a one-step lipase-catalyzed esterification of fatty acids from Ahiflower™ seed oil with phytosterol. A commercial lipase (Lipase OF) from *Candida rugosa* was employed as a biocatalyst. Three solvents were screened and cyclohexane was selected as a suitable reaction medium. The effects of enzyme loading, temperature, and solvent amount were investigated. The conversion as well as the SDA content were significantly improved by adding molecular sieves after 1 h of reaction. The optimum conditions were the enzyme loading of 10% (based on the total substrate weight), the temperature of 30 °C, and the solvent amount of 4 mL (based on a substrate weight of 2 g), respectively. The maximum conversion and SDA content in the residual fatty acid were 81 mol% and 58 mol%, respectively, under the optimum conditions. The SDA content increased a 3.4-fold from 17 mol% in Ahiflower™ seed oil to 58 mol%.

1. Introduction

Phytosterols are widely present in plants, especially fruits, vegetables, nuts, grains, and legumes [1]. The most common phytosterols are β-sitosterol, stigmasterol, and campesterol. The chemical structures of phytosterols are very similar to that of cholesterol, and thus these compounds can have pharmacological properties, such as inhibiting the absorption of cholesterol and reducing low density lipoprotein (LDL) and total cholesterol levels in blood [2]. The hydrogenation of phytosterols leads to the formation of their saturated analogues, phytostanols, which are also known to efficiently lower cholesterol. Despite their health benefits, free phytosterols are not readily commercialized because of their high melting points and low solubilities, both in water and oils [3,4]. In addition, owing to their low physical reactivities and poor solubilities, phytosterols must be ingested in very high doses [5]. In contrast, phytosteryl esters have significantly lower melting points and much better solubilities in fats, potentially allowing their practical application in fat-based foods. Moreover, they also exhibit significantly higher cholesterol-lowering activity compared with the free phytosterols [6].

Ahiflower™ seed oil is a dietary oil rich in stearidonic acid (SDA, 18:4 *n*-3) that is extracted from *Buglossoides arvensis* seeds [7]. It is worth noting that this plant is a non-genetically modified organism that can contain up to 20% SDA [8]. SDA is an *n*-3 polyunsaturated fatty acid (PUFA) and the first metabolite in the bioconversion of α-linolenic acid (ALA, 18:3 *n*-3) to longer chain, highly unsaturated fatty acids [9]. In human bodies, ALA is converted to SDA by Δ6-desaturase, and consequently, SDA is turned into EPA and DHA via desaturation and elongation. However, due to the fact that Δ6-desaturase is limited in humans, only 2–5% of ALA can be turned into EPA whereas 20–30% of SDA can be converted to EPA. Therefore, the level of interest in SDA is increasing because it provides efficient means of increasing EPA content. SDA possesses similar biological properties to eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3), both of which have been recognized as having health-promoting effects in human. These effects include the prevention of cardiovascular, inflammatory and autoimmune diseases [10,11].

Numerous papers have reported the synthesis of phytosteryl esters from free phytosterols and fatty acids originating from vegetable oils via enzymatic esterification [3,12]. Phytosteryl ester containing pinolenic acid was also synthesized successfully using an immobilized lipase from our previous study [13]. Meanwhile, many studies, which were to enrich PUFAs with physiological benefits from various vegetable or fish oil using lipase-catalyzed selective esterification, also have been

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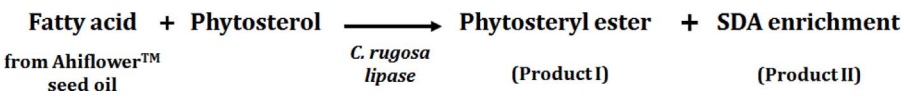

Scheme 1. Preparation scheme of synthesis of phytosteryl esters and SDA enrichment using *C. rugosa* lipase as the biocatalyst.

attempted [14–17]. Over the last decades, the studies on the synthesis of phytosteryl ester as well as enrichment of the PUFAs by lipase-catalyzed esterification have attracted great attention. However, both studies have been attempted separately via lipase-catalyzed reaction. To the best of our knowledge, there have been no attempts to simultaneously synthesize phytosteryl esters and enrich the PUFAs.

In this study, the synthesis of phytosteryl esters and the enrichment of SDA were carried out simultaneously in a solvent system via a one-step lipase-catalyzed esterification (Scheme 1). Phytosterols from soybeans and fatty acids from Ahiflower™ seed oils were employed as substrates and a lipase from *Candida rugosa* was used as a biocatalyst. The effects of the type of solvent, enzyme loading, temperature, and the amount of solvent were investigated by monitoring the time course of the reaction under different conditions. The effects of molecular sieves were also studied.

2. Materials and methods

2.1. Materials

Ahiflower™ seed oil was donated by the Cheminex Corporation (Gyeonggi-do, Republic of Korea). Lipase OF from *Candida rugosa* was also donated by the Meito Sangyo Co., Ltd. (Nagoya, Japan). β-Sitosterol (from soybean, ≥70%) was purchased from Sigma Aldrich Co. (Seoul, Republic of Korea). All of the other chemicals used in this study were analytical grade unless otherwise noted.

2.2. Preparation of fatty acids from Ahiflower™ seed oil

Ahiflower™ seed oil (150 g) was added to a solution of sodium hydroxide (60 g) in distilled water (150 mL) and ethanol (99%, 450 mL). The mixture was refluxed with stirring at 500 rpm for 1 h, then transferred into a separatory funnel. Water (300 mL) was added to the reaction mixture and the aqueous layer containing the saponifiable matter was acidified by adding 6 N HCl (to a pH of 1) to generate the free fatty acids. The upper layer containing the fatty acids was subsequently extracted with *n*-hexane (300 mL) and this extract phase was washed twice with distilled water (150 mL). The *n*-hexane layer containing the fatty acids was then dried over anhydrous sodium sulphate and the solvent was removed using a rotary evaporator at 40 °C. Residual *n*-hexane in the fatty acid was removed completely by nitrogen flushing at 40 °C. The fatty acids in Ahiflower™ seed oil consist of 5.8 mol% palmitic acid, 1.8 mol% stearic acid, 10.4 mol% oleic acid, 12.3 mol% linoleic acid, 5.1 mol% γ-linolenic acid, 47.4 mol% α-linolenic acid, and 17.2 mol% stearidonic acid.

2.3. Esterification of the phytosterol with fatty acids from Ahiflower™ seed oil

The enzymatic esterification of fatty acids from the Ahiflower™ seed oil with phytosterol was carried out in a batch reactor system. Initially, phytosterol (1.2 g) and the fatty acid mixture (0.8 g) (equivalent to a molar ratio of 1:1) were placed in a 50 mL water-jacketed glass vessel and solvent was added. The reaction mixture was then preheated to the desired temperature using a water circulator and stirred at 300 rpm. The reaction was initiated by adding *Candida rugosa* lipase. During the reaction, sample aliquots (70 mg) of the reaction mixture were withdrawn at th appropriate time intervals and diluted with chloroform, then filtered through a 0.45 μm nylon microfilter (Pall Corporation, Port Washington, NY, USA) to remove the lipase.

2.4. Analytical methods

Conversions were determined using a gas chromatograph (Model 3800; Varian, Palo Alto, CA, USA) equipped with a fused silica capillary column (DB-1ht, 15 m × 0.25 mm i.d. × 0.15 μm film thickness, J & W Scientific, Folsom, CA, USA) and a flame ionization detector (FID). The column was held at 120 °C for 3 min and programmed to rise to 370 °C at a rate of 25 °C/ min. The column was then held at 370 °C for 5 min. The column flow was 1.5 mL/min and the split ratio was 1/50. The injector and detector temperatures were maintained at 370 °C. The conversion (mol%) was defined as the degree of the synthesis of phytosteryl ester and was calculated as following Eq. (1):

$$\text{Conversion (mol\%)} = a/(a + b) \times 100 \quad (1)$$

Where a is the moles of the phytosteryl ester in the reaction mixture and b is the moles of fatty acids in the reaction mixture.

To determine the SDA content in the residual fatty acid, 30 mg of sample dissolved in chloroform was loaded onto a thin layer chromatography (TLC) silica gel 60 F_{254} plate (Merck KGaA, Darmstadt, Germany) and developed using petroleum ether/diethyl ether/acetic acid (80:20:0.5, by vol) as the eluent. The free fatty acids, phytosterol, and phytosteryl ester were detected by spraying the TLC plate with a 2,7-dichlorofluoroscein solution (0.2% in 95% methanol). The band corresponding to the free fatty acids was subsequently scraped off and methylated with 14% BF_3 in methanol. The resulting fatty acid methyl ester (FAME) was analysed by gas chromatography using a Supelcowax 10 fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm; Supelco, Bellefonte, PA, USA) and an FID. The column was held at 180 °C for 1 min and then heated to 215 °C at a rate of 1.5 °C/min and held at that temperature for 10 min. Helium was used as the carrier gas at a flow rate of 1 mL/min and split ratio was 1/50. The injector and detector temperatures were set at 240 and 250 °C, respectively. The FAMEs were identified by comparison with the retention time of a standard. Heptadecanoic acid was used as an internal standard.

3. Results and discussion

3.1. Solvent screening for the optimum reaction medium

In general, the catalytic activity displayed by enzymes in organic solvents is different from that in water [18]. Because proteins are insoluble in most organic solvents, enzyme powder suspensions in solvents must be stirred or shaken vigorously to eliminate barriers for mass-transfer in substrates. In addition, hydrophobic solvents are usually superior to hydrophilic solvents as enzymatic reaction medium because the latter have a greater tendency to strip the tightly bound water that is essential for catalytic activity from the enzyme molecules [19]. The proper choice of solvent can also help to make the reaction mixture sufficiently fluid, because the high viscosity of the oil can hinder mobility in the reaction solution, thereby reducing reactivity [20]. Organic solvents have various physicochemical effects on enzyme molecules and it is widely believed that the nature and polarity of the organic medium in which enzymatic reactions take place affect the activity of lipases [21]. This occurs because the solvent alters the native conformation of the enzyme by disrupting hydrogen bonding and hydrophobic interactions, potentially changing both activity and stability [22].

The hydrophobicity of a solvent can be expressed by its log *P* value, which is defined as the logarithm of the partition coefficient of a given compound in the standard two-phase octanol/water system [23]. A higher log *P* indicates a more hydrophobic solvent. In this work, three