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Improved acylation of phytosterols catalyzed by *Candida antarctica* lipase A with superior catalytic activity

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ABSTRACT

This work reported a novel approach to synthesize phytosterol (β-sitosterol as a model) fatty acid esters by employing *Candida antarctica* lipase A (CAL A) which shows a superior catalytic activity to other lipases. A series of β-sitosteryl fatty acid esters (C2–C18) have been successfully prepared with structural identification of products by ^{1}H NMR and Fourier transform-infrared spectroscopy (FTIR). Compared to other immobilized lipases, CAL A achieves 6–14 times faster esterification of β-sitosterol with myristic acid. CAL A shows low activity toward short chain fatty acids (C2–C6), and remarkably high activity for medium and long chain ones ($\geq$C8). Reaction time, temperature, enzyme load, substrate ratio and concentration, and solvent property are found to profoundly influence reaction rates. A pronounced correlation between enzyme activities and log *P* values of solvents, among the solvents with a broad spectrum of log *P* values, was observed. 93–98% yield of β-sitosteryl esters could be achieved with hexane as solvent, fatty acid (C8–C18)/β-sitosterol (1:1, mol:mol), 5–10% CAL A load at 40–50 °C for 24 h. This work demonstrated the promising potential of CAL A in bioprocess of phytosterols for value-added application.

1. Introduction

Phytosterols are numbers of triterpene family, particularly C29- and C28-sterols consisting of a steroid skeleton with a hydroxyl group attached to the C-3 atom of the A-ring and an aliphatic side chain attached to the C-17 atom of the D-ring (see β-sitosterol as a representative structure in Fig. 1). Phytosterols have found a variety of applications in: (1) pharmaceuticals for production of therapeutic steroids [1]; (2) nutrition and functional foods by means of anti-cholesterol property [2], anti-cancer properties, anti-inflammatory, anti-artherogenicity activity, anti-oxidative activities [3–7] and anti-osteoarthritic properties [8] and (3) cosmetics as surfactants in creams and lipstick [9]. Recently, phytosterol products have been added into various commercial foods and drink products as a cholesterol-lowering agent [2,10] such as butter [11], low-fat yoghurt [12], low fat spreads, mayonnaise/salad dressing, cereal bars, chocolate, bakery products [13], milk [12,14], yoghurt minidrinks [15], orange juice [13] and beverage [16]. Abumweis et al. [13] suggested that the incorporation of phytosterols into spreads, mayonnaise/salad dressing, milk/yoghurt, was associated with a greater efficacy in LDL-cholesterol reduction than incorporation into other foods, suggesting the important role of the food matrix affecting the cholesterol-lowering property of phytosterols. The US Food and Drug Administration and the European Union Scientific Committee have thoroughly reviewed the safety of phytosterols before being used in functional foods [2].

Natural phytosterols have a low solubility in both water and fat, thus result in a poor intestine absorption [17]. For example, 50% of cholesterol entering the intestinal lumen can be absorbed whereas the absorptions of campesterol, β-sitosterol and β-sitostanol are in the ranges of 10–15%, 4–7% [18,19] and less than 1% [20], respectively. Hence, the effective dose of phytosterols is much high up to 25 g/day [21]. To improve the intestinal absorption and bioavailability of phytosterols, a feasible solution is to convert phytosterols into enzyme-liable lipophilic derivatives, such as fatty acid esters [22]. Miettinen and Vanhanen [22] have proved that that fat-soluble plant steryl and stanyl esters were able to lower plasma cholesterol level more efficiently than the corresponding homogenized crystalline plant sterol and stanol preparations.

Technical processes of chemical esterification or transesterification presently used for the preparation of steryl esters are generally performed at high temperature in the presence of chemical catalyst, accompanying with high energy consumption, browning of products and low selectivity [23]. As an alternative, enzymatic preparation of steryl esters has also been reported [24]. Lipases from *Candida rugosa* (lipase AYS), *Rhizomucor miehei* (Lipozyme TL IM), *Pseudomonas* sp. (lipase AK), *Aspergilus niger* (lipase AS), *Candida antarctica* B (CAL B) and *Alcaligenes* sp. (lipase QLM) have been used as biocatalysts [25–28]. In fact, the yields of phytosteryl esters catalyzed by those enzymes were generally low with longer reaction time. Vu et al. [27] examined lipase-catalyzed synthesis

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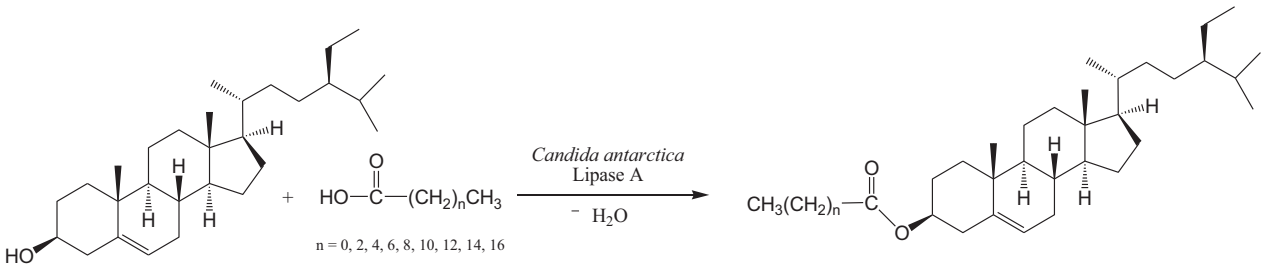

Fig. 1. *Candida antarctica* lipase A (CAL A) catalyzed esterification of β-sitosterol.

of β-sitosteryl esters of medium chain fatty acids (C6:0–C12:0), where AYS (from *C. rugosa*) was found to be the most effective to achieve 40.3% yield of β-sitosteryl laurate in 48 h. Villeneuve et al. [25] obtained 85% yield of canola phytosterols of oleic acid in 72 h employing *C. rugosa* lipase as a biocatalyst. Albeit these progresses, developing more efficient reaction approach is still of academic and practical interests. *C. antarctica* lipase A (CAL A) is reported to have specific activity to accept highly branched acyl groups and sterically hindered alcohols as substrates [29]. This specificity of CAL A is of great interest for acylation of phytosterols which have a secondary alcohol and a bulky structure; however, to date employing CAL A as biocatalyst for acylation of phytosterols has not been investigated systemically. Thus, this work attempts to systemically examine the ability of CAL A in catalyzing acylation of phytosterols using β-sitosterol as a model substrate. The effects of enzyme concentration, substrate molar ratio, reaction temperature and time, and polar/non-polar organic solvents were intensively investigated. Primary results from this study indicate that the CAL A is able to achieve 6–14 times efficiency for synthesis of β-sitosteryl fatty acid esters higher than most commercial immobilized lipases. The formation of β-sitosteryl esters with different fatty acids (C2–C18) was confirmed by ^{1}H NMR and Fourier transform-infrared spectroscopy (FTIR).

2. Materials and methods

2.1. Lipases and chemicals

β-Sitosterol, acetic acid (C2:0), butyric acid (C4:0), hexanoic acid (C6:0), octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Immobilized lipase *C. antarctica* lipase A, NZL-101 (CAL A) was purchased from Codexis, Inc., (Pasadena, CA, USA). Immobilized *C. antarctica* lipase B (CAL B, Lipozyme 435), immobilized *Thermomyces lanuginosus* (Lipozyme NS-40044 TLL) and immobilized *T. lanuginosus* (Lipozyme TL IM) were obtained from Novozymes A/S (Bagsvaerd, Denmark). All solvents such as acetone, acetonitrile, butanone, chloroform, ethyl acetate, *n*-propanol, *n*-hexane and *n*-heptane were of HPLC grade and obtained from Sigma–Aldrich (St. Louis, MO, USA). All the enzymes were used as received; no further treatment has been done.

2.2. Lipase catalyzed synthesis of β-sitosterol-saturated fatty acid esters

In a typical reaction, β-sitosterol was mixed with myristic acid (C14:0) at a mole ratio of 1.0:1.0 (mol/mol) at the concentration of 0.2 M in 3 mL hexane in the presence of 10% lipase (wt% of β-sitosterol). Myristic acid was selected as a model saturated fatty acid because of its preferable chain length (>12, belonging to long chain fatty acids), lower melting point (54.4 °C) and better solubility (than palmitic and stearic acids) in many solvents. The reaction was conducted in a 10 mL capped vial at 50 °C for 72 h with agitation at 500 rpm. The reaction was monitored with periodically sample withdrawing. The aliquot was dissolved in 1 mL hexane for HPLC analysis. To assess catalytic efficiency of the lipases from other sources, Lipozyme 435 (*C. antarctica* lipase B, CAL B), Lipozyme NS 40044 TLL (*T. lanuginosus* lipase) and Lipozyme TL IM (*T. lanuginosus*), which have been proven to the most effective enzymes in catalyzing transesterification of steryl esters [30], were employed as biocatalyst to carry out esterification of β-sitosterol with myristic acid.

2.3. Parameterization of CAL A catalyzed synthesis of β-sitosteryl myristate

Parameters considered important for enzymatic synthesis of β-sitosteryl ester in this work are reaction time, temperature, enzyme concentration, substrate ratio, substrate concentration and solvent property.

Effect of enzyme concentration on synthesis of β-sitosteryl myristerate was examined by varying CAL A concentrations from 3% to 30% (wt% of β-sitosterol). The concentrations of both β-sitosterol and myristic acid were 0.2 M with hexane as reaction solvent. The reaction was performed at 50 °C with agitation at 500 rpm for up to 72 h. The reaction was monitored with periodically sample withdrawing and HPLC analysis.

Examination of the effect of mole ratio of myristic acid/β-sitosterol was carried out similarly as for enzyme concentration, but the concentration of CAL A was fixed at 5%, and mole ratio of myristic acid/β-sitosterol varied from 5/1 to 1/1. The reaction was monitored with sample withdrawing at the set time and HPLC analysis.

Likewise, examination of the effect of reaction temperature was performed with varied temperature from 30, 40, 50 to 60 °C, while other conditions were 0.2 M β-sitosterol and myristic acid in hexane, 5% enzyme load, and agitation at 500 rpm. The reaction progress was monitored with periodically sample withdrawing at the set time; and the aliquots were properly diluted in hexane for HPLC analysis.

Acetone, acetonitrile, butanone, *n*-hexane and *n*-heptane, chloroform, methyl *tert*-butyl ether (MTBE) and toluene with varied log *P* values were selected for evaluation of the effects of solvents. The reaction was conducted at 40 °C, 0.2 M β-sitosterol and myristic acid, 5% CAL A (wt% of β-sitosterol), agitation at 500 rpm. The reaction progress was monitored by sampling at desired time.

Effect of substrate concentrations on synthesis of β-sitosteryl myristerate was studied by varying concentrations of β-sitosterol and myristic acid from 0.05 to 0.5 M (the mole ratio of myristic acid/β-sitosterol fixed at 1:1) in the presence of 31.1 mg CAL A (5%, wt% of β-sitosterol at 0.5 M), with hexane as reaction solvent. The reaction was carried out at 50 °C with agitation at 500 rpm for