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Synthesis of fatty acid sterol esters using cholesterol esterase from *Trichoderma* sp. AS59

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ABSTRACT

We recently reported the characterization of novel cholesterol esterase (EC. 3.1.1.13) from *Trichoderma* sp. and preliminary work on sterol ester synthesis. In the present study, we further examined the enzyme ability to synthesize cholesterol esters from cholesterol and free fatty acids of various chain lengths, and compared the fatty acid specificity in synthesis with that in hydrolysis. The enzyme catalyzed the synthesis of medium- and long-chain fatty acid cholesterol esters, but failed to synthesize short-chain fatty acid esters. The fatty acid specificities in the synthesis and hydrolysis of cholesterol esters were entirely different from each other. Unlike other lipolytic enzymes, the enzyme was largely independent of water content in the synthesis of cholesterol oleate, and it achieved near-complete esterification in the presence of an equimolar excess of oleic acid. Of additional interest is the finding that the addition of *n*-hexane markedly enhanced the esterification activities on all the medium- and long-chain saturated fatty acids used. Based on these findings, we attempted to synthesize stigmasterol stearate as a food additive to lower cholesterol levels in blood plasma, and found that the enzyme catalyzed effective synthesis of the ester without the need of dehydration during the reaction, indicating the potential utility of the enzyme in the food industry.

1. Introduction

Since the 1950s, it has been well established that ingestion of foods enriched with plant sterols or stanols (hydrogenated form of sterols) can significantly reduce serum and LDL cholesterol concentrations [1,2]. The most common plant sterols are β-sitosterol, campesterol, and stigmasterol, which are structurally very similar to cholesterol, and classified as 4-desmethylsterols of the cholestane series [3]. The primary cholesterol-lowering mechanism of plant sterols/stanols is their ability to reduce intestinal cholesterol absorption. An inverse correlation between plant sterol ingestion and cholesterol absorption was revealed earliest by Sugano et al. [4–6], and positive correlations between cholesterol absorption and plasma LDL cholesterol concentration have been found in humans [7,8]. Early studies examined the cholesterol-reducing ability of plant sterols/stanols using up to 2.5 g/day ingested in solid crystalline form. Later, it was established that the sterols/stanols in edible fat products are more effective in lowering blood cholesterol levels than those in crystalline form. These findings encouraged attempts to incorporate these substances into margarine, mayonnaise, and salad dressing for use to reduce blood cholesterol levels.

Despite their potential abilities, the use of plant sterols/stanols has been limited by their high melting point and low solubility in both water and oil phases. As a food additive to be used widely, they should also be conveniently incorporated into food, without organoleptic effects. Free sterols/stanols, however, have solubility as low as 2% in fats and oils, and are difficult to be dissolved in butter or margarine. In the 1970s, Mattson et al. discovered that esterifying plant sterols with long-chain fatty acids increased their solubility in edible oils 10-fold, and that esterification did not impair their ability to inhibit cholesterol absorption [9–11]. Moreover, results from the studies conducted to date indicate that plant sterol/stanol ester-containing foods are safe and effective for reducing blood cholesterol levels [12–14]. Therefore, commercial plant sterols/stanols extracted from soybean oil or tall oil are currently esterified with long-chain fatty acids, allowing maximal incorporation into a limited amount of fats and oils. On the other hand, cholesterol as well as other intercellular lipids including ceramides and fatty acids is known to contribute the barrier function and water-holding capacity in the stratum cornea, and is used as an ingredient of skin and hair cosmetics [15]. As with plant sterols/stanols, cholesterol is used as long-chain fatty acid esters to enhance the handling quality and miscibility.

Usually, fatty acid esters of sterols/stanols are prepared by chemical esterification [16–19]. It generally requires higher temperature, extreme pH, or poisonous inorganic catalysts, thus favoring the formation of side products such as dehydrated or

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oxysterols leading to low yields and complicated purification procedures. Accordingly, interest in lipase-catalyzed sterol/stanol ester synthesis has recently claimed increasing attention mainly due to the potential of mild conditions and high yields [20–22]. These reactions generally need only a small amount of water or addition of water-immiscible organic solvents to prevent hydrolysis of ester once formed during the reaction. Although several reports of this kind have been published [23–35], those on sterol ester synthesis catalyzed by cholesterol esterase are limited. Recently we isolated a fungal strain *Trichoderma* sp. AS59 producing novel cholesterol esterase extracellularly [36]. The enzyme exhibited not only wide-ranging hydrolytic activities on cholesterol esters of short-, medium- and long-chain fatty acids, but also high ability to synthesize cholesterol and stigmasterol oleates in high yields. Of special interest in connection with the synthetic ability of the enzyme is the recent finding that both plant sterols/stanols and stearic acid have cholesterol-lowering properties, which are significantly enhanced when they are esterified. The conclusion was derived from the observation that dietary consumption of plant sterols enriched in stearate significantly reduced serum concentrations of low-density lipoprotein cholesterol in adults with normal lipid profiles and those with hypercholesterolemia [37,38]. These reports prompted us to further examine the ability of the enzyme to synthesize a variety of fatty acid sterol esters including the stearate. This will serve to assess the potential of the enzyme as a biocatalyst for industrial production of useful plant sterol esters, and to check if there is any correlation between the fatty acid specificities in the synthesis and hydrolysis of the esters. Enzymatic esterification between sterol and fatty acid that are solid at room temperature is still challenging work.

2. Materials and methods

2.1. Enzyme and chemicals

Cholesterol esterase from *Trichoderma* sp. AS59 was purified from a 3-day liquid culture according to the method already reported [36]. The purified enzyme was dialyzed against distilled water, and lyophilized before use. One unit of hydrolytic activity (U) was defined as the amount of the enzyme producing 1 μmol of free cholesterol per minute using cholesterol linoleate as substrate. Cholesterol (purity >99%), long-chain unsaturated fatty acids (oleic, linoleic, and arachidonic acids; >99%), cholesterol esters of the unsaturated fatty acids (99%), stigmasterol (95%), methyl oleate (>99%), triolein (>99%), tristearin (>99%), and 5α-cholestane (>97%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methyl oleate and triolein contained 90–100 ppm of water as measured by Carl–Fisher method. Saturated fatty acids of different chain lengths (C2–C18; >97%), and their cholesterol esters (>95%) were from Tokyo Chemical Industry (Tokyo, Japan).

2.2. Optimization of factors affecting direct esterification

Before esterification of cholesterol with free fatty acids (FFAs) of different chain lengths, several factors affecting the reaction including the enzyme amount, pH, temperature, molar ratio of FFA to cholesterol were investigated using cholesterol and oleic acid as substrates. Unless otherwise noted, the enzyme was used as dissolved in a certain amount of a buffer in such a way that the final water content in the reaction mixture was 30 wt%. The effect of pH was studied using 2 M phosphate buffer (pH 5.0, 6.0, 7.0) or 2 M Tris–HCl buffer (pH 8.0), but the effect of water content was examined using the phosphate buffer (pH 5.0). A mixture comprising 200 μmol of cholesterol, 200 μmol, 400 μmol, or 600 μmol of oleic acid, and 100 μmol of 5α-cholestane (an internal standard) was combined with 25 U, 50 U, or 100 U of the enzyme in a hermetically sealed vial. The whole mixture was mixed up for 1 min with a glass rod, and statically incubated for a certain period at 20 °C. In addition, we used *n*-hexane as an organic solvent to see how it affects the esterification. In the beginning the enzyme stability to the solvent was checked in the following way: to 1 mL of the enzyme solution (0.44 U/mL of the phosphate buffer, pH 5.0) was carefully added 2 mL of the organic solvent along the inner wall of the reaction vial. As a control, the same amount of the buffer was added to the original enzyme solution. Each of the mixtures was statically incubated for 19 h at 20 °C, and was assayed for hydrolytic activity. The enzyme stability was further examined in the presence of both cholesterol and a FFA according to the following procedures: a mixture comprising 200 μmol of cholesterol, 400 μmol of oleic or stearic acid, and 100 μmol of 5α-cholestane was combined with 50 U of the enzyme dissolved in 100 μL of the phosphate buffer, and was mixed up for 1 min with a small glass rod. The final water content was 30 wt%. To each of the reaction vials except those for control was carefully added 2 mL of the organic solvent as described above. Half the vials were statically incubated for 19 h at 20 °C. The remaining vials were vortexed for 10 s, and then incubated likewise. After the incubation, 4 mL of chloroform was added to each of the mixtures to extract the products and the remaining reactants. A portion of the chloroform layer was taken out, diluted exactly six times with chloroform, and analyzed by thin-layer chromatography and gas-liquid chromatography. The products were determined using 5α-cholestane and commercially available cholesterol esters as standards.

2.3. Reactions between cholesterol and FFAs of different chain lengths

Direct esterification of cholesterol with each of FFAs of different chain lengths was conducted to examine the activity in ester synthesis with due regard to the optimized reaction conditions established above. A mixture comprising 200 μmol of cholesterol, 400 μmol of a FFA, 100 μmol of 5α-cholestane (hereinafter referred to as a standard substrate mixture) was combined with 50 U of the enzyme in the phosphate buffer (pH 5.0), and was mixed up for 1 min with a glass rod. The final water content was adjusted to 30 wt%. The reaction vial was statically incubated at 20 °C with and without the addition of 2 mL of *n*-hexane. For the reactions with short-chain FFAs, namely, acetic, butyric, and caproic acids, the experimental scale was decupled to permit direct determination of pH of the substrate mixtures with a Horiba 9669-10D microelectrode (Horiba, Kyoto, Japan). The pH was adjusted to 5.0 and 3.5 with M NaOH or M HCl before the addition of the enzyme, and the final water content was brought to 5 wt% and 30 wt%. Then 50 U of the powdered enzyme were added to the substrate mixture, followed by mixing up for 1 min with a glass rod. For a biphasic reaction, *n*-hexane was carefully added to the reaction mixture as described. All the vials were statically incubated for various lengths of time at 20 °C. The products and the remaining reactants were determined in duplicate by gas–liquid chromatography described below.

2.4. Reactions between cholesterol and methyl oleate/triolein

A mixture comprising 200 μmol of cholesterol, 400 μmol of methyl oleate or 133 μmol of triolein, and 100 μmol of 5α-cholestane was combined with 50 U of the enzyme dissolved in 12 μL (5 wt% water content) and 100 μL (30 wt% water content) of the phosphate buffer (pH 5.0), and was mixed for 1 min with a glass rod to start the reaction. The whole mixture was statically incubated for 5 h and 19 h at 20 °C.

2.5. Synthesis of stigmasterol stearate by direct esterification

A mixture comprising 200 μmol of stigmasterol, 400 μmol of stearic acid, 100 μmol of 5α-cholestane was combined with 50 U, 100 U, and 200 U of the enzyme dissolved in 100 μL of the phosphate buffer (pH 5.0), and was mixed up for 1 min with a glass rod. The whole mixture was statically incubated for 1–19 h at 20 °C after the addition of 2 mL of *n*-hexane. During the course of the reaction, samples were withdrawn and analyzed by gas–liquid chromatography, and the remaining portion of the reaction mixture was chromatographed over silica gel for identification.

2.6. Silica gel column chromatography

After the reaction of stigmasterol with stearic acid, 4 mL of $CHCl_3$ was added to the reaction mixture to extract the products. The organic layer was transferred to a vial, dried in a vacuum, and the residue was redissolved in 10 mL of *n*-hexane. Coexisting free stearic acid was ionized by the addition of 1 mL of 0.5 N KOH in 30% ethanol solution, and transferred to the alkaline layer. Then the organic layer was put on a column (1 cm × 10 cm) packed with silica gel 60 (Merck, Darmstadt, Germany) as a slurry in *n*-hexane, and the product was eluted with 50 mL of a mixture of *n*-hexane/ethyl acetate (98:2 (v/v)). Fractions were checked by TLC, and those showing one spot at an R_f value of 0.7 were collected. After the eluate was evaporated in a vacuum, the product was identified by infrared spectroscopy and mass spectrometry.

2.7. Thin-layer chromatography (TLC)

Reaction products were detected qualitatively by TLC using a silica gel 60 plate (Merck, Darmstadt, Germany). An aliquot of the enzyme reaction mixture was put on the silica gel plate, which was then developed in a mixture of *n*-hexane/diethyl ether/acetic acid (80/20/1 (v/v)), sprayed with 50% sulfuric acid in ethanol, and heated at 120 °C for 5 min. R_f values of sterols and sterol esters were near 0.1 and 0.7, respectively.

2.8. Gas–liquid chromatography (GLC)

Quantitative analyses of sterols, FFAs, and their esters in the reaction mixture were conducted with a Shimadzu GC1700 gas chromatograph (Kyoto, Japan) connected to a DB-1ht capillary column (0.25 mm × 5 m; J&W Scientific, Folson, CA). Argon at a flow rate of 80 mL/min was used as a carrier gas, and the splitting ratio was 1/40. In the course of analysis, the column temperature was raised from 120 to 345 °C at 15 °C/min, and then maintained at 345 °C for 2 min. Both the injector and