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Enzymatic synthesis, purification and identification of bioactive trehalose ester derivatives for health applications

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ABSTRACT

In the present work, we attempted to produce novel trehalose ester derivatives catalyzed by lipase using trehalose and lipoic acid as substrates. Our aim was to select the lipase with the highest catalytic efficiency as a biocatalyst to efficiently produce trehalose ester derivatives with the lowest production cost for further industrial applications. The highest conversion yields of trehalose lipoate (75.9 ± 1.9%) were obtained at the following optimal conditions: reaction temperature 40 °C, reaction time 4 days, substrate molar ratio 1:4 (trehalose:lipoic acid), total enzyme activity 3000 PLU (propyl laurate units), four stepwise additions of lipoic acid (0.03 mmol/day) and a cosolvent ratio of 4:1 [DMSO:2-methyl-2-butanol(2M2B)]. After isolation and purification, the carboxyl group of lipoic acid was determined to be connected to the C6 hydroxyl group of trehalose using nuclear magnetic resonance. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity (%) of purified 6-o-trehalose lipoate was at least 2.5-fold higher than that of lipoic acid. This bioactive trehalose ester exhibits improved antioxidant activity, which may have higher health benefits than those of lipoic acid, in various functional product applications.

1. Introduction

Sugar fatty acid esters are non-ionic surfactants with good surface properties and are composed of mono-, di- or oligosaccharides esterified with fatty acids of various chain lengths (Chang and Shaw, 2009; Chaiyaso et al., 2006; Ferrer et al., 2005; Udomrati and Gohtani, 2014; Li et al., 2015). Because carbohydrates play a critical role in many biological processes, the exact number and positions of acyl substituents in a carbohydrate molecule significantly affect its bioactivity (Mendez and Salas, 2001). In recent years, esterified products of various carbohydrate have been shown to have antimicrobial activity (Marshall and Bullerman, 1994), antitumor activity (Okabe et al., 1999), plant growth inhibition (Woods and Swinton, 1991), insecticidal activity (Peterson et al., 1997), radical scavenging activity (Kim et al., 2015) and antibiotic activity (Kohya et al., 1986), indicating potential new markets for these products. Such ester derivatives can be synthesized by chemical and enzymatic methods. Chemical esterification reactions generally require high-temperature conditions

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and a non-specific alkaline catalyst, which often results in a broad product profile and generates unwanted colored byproducts (Chaiyaso et al., 2006; Cao et al., 1999). In contrast, the same esters prepared enzymatically from renewable and inexpensive substances under mild reaction conditions, can be minimized side reactions and labeled "natural" (Grillies et al., 1987). The amphiphilic nature of these esters as well as their non-toxic, non-allergenic, and biodegradable properties (Ducret et al., 1995) have resulted in increasing commercial attention as well as increased demand for these esters for food (Neta et al., 2012), detergent, cosmetic, pharmaceutical (Schiefelbein et al., 2010) and biomedical applications (Ferrer et al., 2005; Degn et al., 1999).

The use of biocatalysts having highly specificity, including lipases, esterases and proteases, might increase the possibility of obtaining specific isomers of a particular ester in high yield, since the exact number and locations of acyl substituents bound to acyl donor is very important (Ducret et al., 1995; Degn et al., 1999; Larios et al., 2004). However, in the case of sugar fatty acid esters, selecting an appropriate solvent with high sugar solubility as well as high enzyme activity and specificity is difficult. While the enzyme is usually more stable in more hydrophobic solvents with high log *P* values, most sugars (e.g., glucose, sucrose, and fructose) prefer hydrophilic organic solvents (Ducret et al., 1998; Van Tol et al., 1995; Li et al., 2015). Partially dissolved, solid-phase, co-solvent or metastable supersaturated solution strategies have therefore been developed to maintain high biocatalyst catalytic efficiency in less harmful organic solvents (e.g., acetonitrile, acetone, *tert*-butanol and 2-methyl-2-butanol) to avoid slow reaction rates and incomplete reactions (Degn et al., 1999; Chang et al., 2009).

The unique characteristics (e.g., resists freezing and moisturizing) of trehalose have greatly enhanced its market demand. In 2000, trehalose production was 20,000 tons/year with a market value of approximately 50 million US dollars (Paku et al., 2003). However, due to the highly hydrophilic nature of trehalose, the applications of this sugar have been restricted to the food and cosmetic industries. The development of the biocatalytic synthesis of trehalose derivatives with novel physiological activity and higher lipophilicity is important for expanding its range of applications (Paku et al., 2003). Although many researchers have investigated enzymatic esterification of carbohydrate fatty acid ester by different strategies, few studies have focused on using low-cost direct esterification of trehalose to meet the needs of industry. For example, Cao et al. (1999) reported lipase-catalyzed direct esterification of 6-o-glucose palmitate in a mainly solid-phase system. The highest conversion (84%, 24 h) and productivity (0.69 mmol product/gram lipase/h) of 6-o-glucose were achieved with CAL-B lipase immobilized on polypropylene (CAL-B EP-100). The enzyme's activity on most disaccharides such as sucrose, lactose, trehalose and maltose was very low; however, complete conversion was possible with glucose. Chen et al. (2005) investigated the enzymatic synthesis of monolinoleoyl trehalose, maltose, and cellobiose by *Candida antarctica* lipase-catalyzed condensation using linoleic acid as the acyl donor in an organic solvent. Using a mixture of pyridine and *tert*-butanol as the reaction medium resulted in a high product concentration of linoleoyl trehalose and maltose. Additionally, linoleoyl trehalose showed the strongest surface activity. Li et al. (2015) demonstrated the conformation of commercial immobilized *C. antarctica* lipase B (CALB) binding mono-ester affected by organic solvents essentially determined degrees of esterification (DE). For direct esterification of fructose laurate case, the CALB conformation was preferred to binding mono-ester in methyl ethyl ketone (MEK). Kim et al. (2015) reported the biosynthesis of cinnamate derivatives with sugar alcohols can overcome the poor properties of solubility and stability of cinnamic acid. The final product 6-o-cinnamoyl-sorbitol was obtained and was found to have radical scavenging activity, using immobilized *C. antarctica* lipase as biocatalyst.

In the present work, we attempted to produce novel trehalose ester derivatives catalyzed by lipase using trehalose and lipoic acid as substrates. Our aim was to select the lipase with the highest catalytic efficiency as a biocatalyst to efficiently produce trehalose ester derivatives with the lowest reaction time and cost for further industrial applications. All reaction parameters, including reaction time (1–6 days), reaction temperature (30–50 °C), substrate molar ratio (trehalose:lipoic acid = 1:1–1:5), enzyme amount (0.01–0.05 U) and ratios of co-solvent combinations 1:1 and 8:1 (dimethyl sulfoxide (DMSO):*tert*-butanol, respectively), were evaluated to better understand their effects on the molar conversion of trehalose lipoate. Nuclear magnetic resonance (NMR) was used to determine the chemical structure of the purified product, and the biological activity of the product was analyzed in this study.

2. Materials and methods

2.1. Reagents and chemicals

Commercial lipase AY (type VII; 807 U/mg) from *Candida rugosa*, Trypsin (EC 3.4.21.4; 1564 BAEE units/mg solid), a pancreatic protease, D-(+)-trehalose dehydrate (>99%) and D,L-lipoic acid (≧99%) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Immobilized granulated lipase from *C. antarctica* B (Novozym 435 immobilized on acrylic resin) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Molecular sieve 4 Å was purchased from Davison Chemical (Baltimore, MD, USA). All other chemicals were of analytical reagent grade. All reagents and chemicals were dehydrated with molecular sieves for 24 h.

2.2. Biosynthesis of trehalose ester derivatives

In a preliminary test for enzyme screening, we attempted to use lipase AY and Novozym 435 as green biocatalysts to directly esterify trehalose and lipoic acid in the presence of different organic solvents (DMSO and/or *tert*-butanol). Each reaction contained 0.01 M trehalose, 0.02 M lipoic acid, and 0.2 g enzyme and was carried out at 40 °C for 72 h.

In a typical reaction, trehalose (0.01 M; 0.03 mmol) and lipoic acid (0.04 M; 0.12 mmol) were mixed with the organic solvent (DMSO), followed by the addition of 0.1 g/mL enzyme [e.g., Novozym 435; 10,000 PLU (propyl laurate units)/g] in 15 mL sealed glass test tubes. Reaction mixtures were mixed in an orbital shaking water bath (200 rpm) at 40 °C for 4 days. All samples were centrifuged at 13,000 rpm for 15 min to remove the immobilized enzyme, and the upper layer was filtered with 0.45 μm nylon membrane filters (Millipore Millex-HN 13 mm, Billerica, MA, USA) and stored at −20 °C for further quantification and purification. Overall changes in chemical structures are diagrammed as shown in Fig. 1.

To examine the effect of different reaction parameters, all reactions were performed in 15 mL of sealed glass test tubes. Trehalose lipoate synthesis was catalyzed by Novozym 435