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Enzymatic synthesis of isomaltotriose palmitate and evaluation of its emulsifying property

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ABSTRACT

Enzymatic syntheses of oligosaccharide fatty acid esters are important owing to their wide range of industrial applications in the food, cosmetic, and pharmaceutical industries. Transesterification of isomaltotriose and palmitic acid vinyl ester, catalyzed by the metalloprotease thermolysin, was performed in organic solvents. The process parameters (reaction time and temperature) were optimized to achieve the highest yield of isomaltotriose palmitate (IP). The water content of the reaction system played a key role in the acylation of isomaltotriose. Dimethyl sulfoxide was thought to be the most suitable reaction medium by taking the degree of substitution of the modified isomaltotriose into account. The optimum reaction time, temperature, water content, and enzyme concentration were 24 h, 45 °C, 40%, and 0.05%, respectively, under which the product yield was as high as 89.7%. The enzyme operational stability study showed that thermolysin retained 51.5% of its initial activity for the synthesis of IP (even after repeated use for 72 h). Moreover, test results showed that the emulsifying capacity and emulsion stability of IP are 107.5 mL oil/g ester and 16.3%, respectively.

1. Introduction

Oligosaccharides are found on cell surfaces as glycoprotein or glycolipid conjugates and play important structural and functional roles in numerous biological recognition processes. Amphiphilic carbohydrate fatty acid esters are an important class of biodegradable and non-toxic surfactants with broad applications in the food, cosmetic, and pharmaceutical industries [1]. Their emulsifying and surfactant properties [2] may be modulated by the type of fatty acid, the sugar moiety, the degree of substitution, and the position of attachment to the fatty acid [3]. Moreover, these types of compounds also present interesting biological properties like the recently reported antibiotic, insecticidal properties, and antitumor activities (Hep-G2 and HeLa) of maltotriose fatty acid esters (6′-*O*-dodecanoylmaltotriose and 6′-*O*-palmitoylmaltotriose) [4,5].

Generally, transesterification reactions require high temperatures, which, in combination with the catalysts (tionyl chloride and K_2CO_3) used, might induce partial degradation of the oligosaccharide chains and discoloration. Moreover, region- selective chemical acylation of carbohydrates is complex due to the presence of multiple hydroxyl groups, which require protection and deprotection [6,7]. They may also lead to the formation of side products. When enzymes are used in organic media, they exhibit specificity [8], thermostability, molecular memory, and the capacity to catalyze synthetic reactions [9,10]. Therefore, enzymatic processes offer an attractive alternative route for the synthesis of oligosaccharide esters. Selective processes catalyzed by enzymes may be performed under mild conditions of temperature and pressure, thereby avoiding polymer degradation. The application of enzymes for modification of oligosaccharides will offer the advantage of high reaction specificity and region selectivity, which will generate products with controlled structure and functionality (Scheme 1).

Only a few reports are available on the esterification of carbohydrates with a degree of polymerization (DP) greater than two [11,12]. This may be due to their low solubility in most organic solvents and/or to the substrate specificity of the enzyme. *Bacillus thermoproteolyticus rokko* metalloprotease is able to catalyze the synthesis of raffinose, melezitose, and 1-kestose esters in dimethyl sulfoxide (DMSO); however, synthesis of isomaltotriose esters has not yet been studied [13]. A subtilisin-catalyzed synthesis of 6^{III}-*O*-butyryl maltotriose in pyridine has been reported [14]. Our current research explores the metalloprotease transesterification of isomaltotriose palmitate (IP), with the aim of developing a convenient synthetic method to obtain highly "hydrophobic" isomaltotriose derivatives and high overall conversion. In the present work, we found that IP can be efficiently synthesized from isomaltotriose

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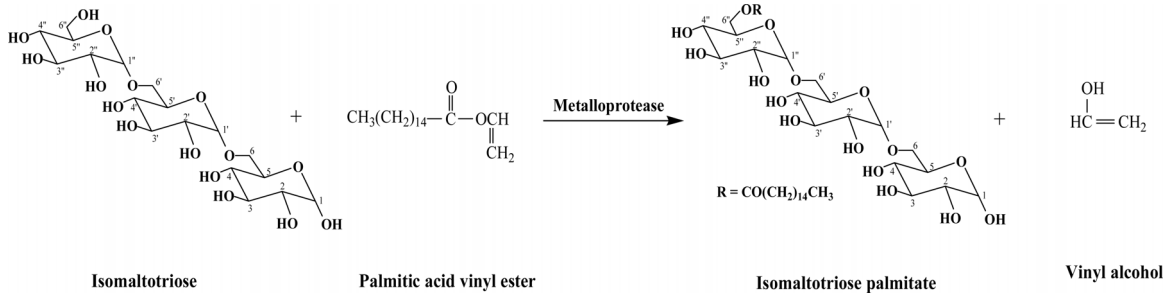

Scheme 1. Metalloprotease catalyzed transesterification of isomaltotriose with palmitic acid vinyl ester.

and palmitic acid vinyl ester using thermolysin in DMSO. To optimize the production of IP, various parameters influencing enzyme activity, such as reaction temperature, organic solvent, acyl donor, isomaltotriose/palmitic acid vinyl ester molar ratio, and water content, were investigated. Moreover, the moisture absorption rate, moisture retention rate, and emulsifying capacity of IP were investigated. Finally, the residual enzyme activity after repeated operations was assayed to evaluate the practicality of the developed method.

2. Materials and methods

2.1. Materials

Thermolysin from *B. thermoproteolyticus rokko* (Type X, lyophilized powder, 67 units/mg protein), palmitic anhydride, methyl palmitate, tripalmitin, and palmitic acid vinyl ester were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Isomaltotriose was a product of Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMSO (>99.5%), cyclohexane, *n*-hexane, and *tert*-butanol (>99%) were obtained from Merck (Darmstadt, Germany). All other chemicals used were of reagent grade.

2.2. Preparation of the immobilized metalloprotease

Metalloprotease was immobilized on support by the following procedure. Metalloprotease (0.4 g) was dissolved in 8 ml of 10 mM phosphate buffer (pH 7.0) and mixed with 1.6 g support (nano-magnetite (Fe_3O_4)) which was prewashed in 10 mM phosphate buffer (pH 7.0). Then 48 ml of cold acetone (−20 °C) was added and the mixture was stirred at 4 °C with a magnetic stirrer in an ice bath for 30 min. The immobilized lipase was filtered and dried first in air, and then in a vacuum at room temperature. The immobilized catalyst was stored at 4 °C.

2.3. Optimization of isomaltotriose palmitate synthesis in organic solvents

IP was synthesized using a 15-mL reaction vessel via the transesterification of isomaltotriose (10 mM) with palmitic acid vinyl ester (10 mM) in DMSO [13]. The reaction mixture (1 mL) contained 0.5 mg of the biocatalyst. The reactions were performed at 45 °C with orbital shaking (200 rpm). The reaction was quenched by removing the enzyme by filtration. The transformation products were analyzed by thin layer chromatography (TLC) after removing the reaction solvent by vacuum evaporation. Several experiments were conducted to optimize the reaction conditions, such as enzyme source, reaction time (6–48 h), reaction temperature (25–60 °C), acyl donor type, molar ratio (0.3–3.0) of isomaltotriose to palmitic acid vinyl ester, water content (20–80%) of the reaction mixture, and reaction solvent, to obtain the best yield of IP. The effects of several parameters on IP yield were studied while keeping all other conditions constant. To study the effect of the metal ions on analog synthesis, metalloprotease was dissolved in 2 mM metal ion solution and lyophilization was performed by a Savant Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY, USA) under 50 mTorr for 24 h before use [15]. The residual enzyme activity was assayed using the optimized experimental conditions after 1–4 cycles of operations to evaluate the reaction conditions developed. The metalloprotease was previously lyophilized using a Savant Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY, USA) under 50 mTorr for 24 h before use.

2.4. Purification and thin-layer chromatography

When the enzymatic reaction reached equilibrium, usually after 24 h of reaction time, the solvent was evaporated using a rotatory evaporator. Then, distilled water was added (3 mL) and the mixture vigorously stirred. The mixture was extracted with *n*-butanol-cyclohexane (25 mL; 3/1, v/v). The organic phases were pooled, washed four times with 17 mL of distilled water (4 × 17 mL) to remove residual isomaltotriose, and centrifuged at 10,000 rpm for 5 min to precipitate the enzyme. The crude ester phase was washed four times with *n*-hexane at room temperature in a separatory funnel, and the ester phase was then dried by using anhydrous Na_2SO_4. After removing the solvent with a rotary evaporator, qualitative analysis of ester products was carried out by TLC. TLC analysis was performed on silica gel Plates 60 F_{254} (Merck, Germany) using chloroform: methanol: water (1:0.61:0.12, v/v/v) as the developing system [16]. The plates were sprayed with diphenylamine–aniline–phosphoric acid reagent (acetone containing 10% (v/v) 85% phosphoric acid, 1% (v/v) aniline, and 1% (w/v) diphenylamine) and heated at 110 °C for 5 min to develop isomaltotriose and isomaltotriose ester spots. R_f values of different substrates and products were as follows: 0.81 (isomaltotriose), 0.40 (palmitic acid vinyl ester), and 0.80 (IP), respectively. The yield of IP was calculated according to the following equation:

$$\text{Yield of IP}(\%) = \frac{\text{Actual yield of IP}}{\text{Theorectical yield of IP}} \times 100\%$$

Triplicate samples were each analyzed twice.

2.5. Mass analysis

Electrospray ionisation mass spectra were obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan) using the DA-6000 data analysis system. Argon was used as bombarding gas at 6 kV and 10 mA. The accelerating voltage was maintained at 10 kV. *m*-Nitrobenzyl alcohol (NBA) was used as a matrix.

2.6. Emulsion capacity

Emulsions were formed inside a 200 mL beaker by use of a continuous stirring apparatus; this apparatus consisted of a regulated/stabilized 9-V power supply, a burette, a stirrer, a beaker with the emulsion, and a digital milliameter. The stirrer consisted of a stainless steel rod holding a perspex bridge fixed to a 9-V DC motor spindle by means of a plastic adaptor. A milliameter monitored the current drawn by the stirrer motor to maintain a constant speed; the greater the viscosity of the emulsion, the greater the current drawn. IP (0.2 g) was made into a solution in 40 mL of methanol in a conical flask. Olive oil was then added at a rate of 0.5 mL s^{-1} from a burette until the emulsion collapsed, as indicated by a sharp fall in the motor current resulting from the sudden breakdown in viscosity. The volume of olive oil added up to this inversion point was noted and the emulsifying capacity expressed as mL of oil per g of ester.

2.7. Emulsion stability

The emulsion stability was determined by following the procedure used for the emulsifying capacity, except that 17.5 mL of olive oil was added rather than adding olive oil until the emulsion breakpoint. IP (0.16 g) solution in methanol was made and used for emulsifying capacity. A 50-mL aliquot of the resultant emulsion was measured into a 25-mL graduated cylinder and allowed to stand at room temperature (25 °C). The amount of methanol separated was noted after 0 and 30 min, respectively. The emulsion stability (%) was determined using the following equation:

$$\text{Emulsion stability } (\%) = \frac{100 - M_t}{100 - M_o} \times 100$$

where M_o is the volume of the methanol layer for 0 min in the resultant emulsion, and M_t is the volume of the methanol layer for 30 min in the resultant emulsion.

2.8. Statistical analysis

A variance analysis of the results was carried out using the General Linear Model Procedure from SAS Statistical Software, Version 6.11 [17]. The enzyme source, reaction time, temperature, acyl donor, organic solvent, and water content were each tested in triplicate.