LIPASE-CATALYSED SYNTHESIS OF FATTY ACID MODIFIED CHITOBIOSE IN A LOW-WATER ORGANIC SOLVENT Effect of Water Distribution in a Multiphase Reaction System

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Abstract: Lipase-catalysed condensation of chitobiose and myristic acid in low-water acetone media was investigated. A product identified as monomyristoyl chitobiose was obtained using immobilized lipase B from *Candida antarctica* (Novozym 435). The product yield was significantly affected by reaction conditions, such as the initial water concentration in acetone, the initial water content of immobilized lipases, and the amount of added molecular sieves. The product yield varied in the range 0–10%. The effects of the reaction conditions are discussed in relation to the quantitative distribution of water in the reaction system, that is, water adsorbed onto immobilized enzymes, water adsorbed onto molecular sieves, and free water dissolved in the organic solvent.

Keywords: lipase; sugar-based surfactant; condensation; organic solvent; water distribution.

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INTRODUCTION

Sugar-based surfactants with mono-, di- or trisaccharide hydrophilic groups and fatty acid or higher alcohol hydrophobic groups are widely utilized in the food, pharmaceutical, cosmetic and detergent industries. For their intended uses, such surfactants should be highly biocompatible, and therefore it is desirable to synthesize them enzymatically.

The synthesis of sugar-based surfactants by lipase-catalysed condensation of sugars and fatty acids in low-water organic solvents has been widely studied as reviewed in the literatures (Plou *et al.*, 2002; Adachi and Kobayashi, 2005). In these systems, water is a reaction product in the condensation reaction and thus affects the reaction equilibrium (Kobayashi and Adachi, 2004). Furthermore, the small amount of water bound to enzyme molecules is so-called essential water, which is critical for enzymatic catalytic activity in low-water media (Rupley *et al.*, 1983; Zaks and Klibanov, 1988).

Synthetic reactions catalysed by enzymes are often carried out in a multiphase system consisting of solid enzymes, organic solvent and desiccant. Water in such systems must be distributed among the components. Therefore, to reveal the effects of water on the synthetic reaction, the water distribution among the enzymes, solvent and desiccant in the reaction system should be considered quantitatively.

In this study, we synthesized fatty acid–modified chitobiose in low-water acetone media using immobilized lipase B from *Candida antarctica*. This enzyme has been used for acylation of various sugars including di- and trisaccharides (Woudenberg *et al.*, 1996; Ferrer *et al.*, 2000; Plou *et al.*, 2002; Zhang *et al.*, 2003b; Chen *et al.*, 2005). Acetone was used as a reaction medium because it can be easily separated from products by evaporation due to its low boiling point and is recognized as safe for use in food processing in many countries and areas. We studied the effects of the water concentration in acetone, the water content of the catalysts, and the amount of added molecular sieves on the condensation of chitobiose and myristic acid. The effects of these factors are

discussed in relation to the quantitative distribution of water among the components in the reaction system. A comprehensive method for evaluating the effects of water on the product yield is presented.

MATERIALS AND METHODS

Materials

An immobilized preparation of lipase from *C. antarctica* fraction B (CALB), designated Novozym 435 (a product of Novo Nordisk A/S, Bagsvaerd, Denmark), was purchased from Sigma Co. (St Louis, MO, USA). The support material of Novozym 435 is a porous acrylic resin with a diameter of 0.3–0.9 mm. Myristic acid, acetone, and molecular sieves 3A were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Chitosan HD (98% deacetylated chitosan) was kindly supplied by Yaegaki Bio-industry Inc. (Himeji, Japan) and used as a starting material for preparation of chitobiose. All other chemicals were obtained from commercial sources.

Preparation of Chitobiose

Chitosan HD was hydrolysed using immobilized chitosanases (Ichikawa *et al.*, 2002; Kuroiwa *et al.*, 2002; Kuroiwa *et al.*, 2003). The hydrolysates were purified by cation-exchange column chromatography (Uchida *et al.*, 1989), and chitobiose-HCl salt was obtained. The salt was applied to an anion-exchange column packed with Amberlite IRA-410 resin (Organo Co., Tokyo, Japan) and eluted with deionized water to remove HCl. Water was removed by evaporation, the residue was dissolved in 3 mL of methanol, and 30 mL of acetone was added to precipitate chitobiose. The precipitate was recovered by centrifugation and dried under reduced pressure. The resulting white powder was used without further treatment.

Lipase-Catalysed Synthesis of Acylated Chitobiose

Chitobiose (0.05 mmol), myristic acid (0.05 mmol), Novozym 435 (100 mg), and molecular sieves 3A (0–250 mg) were placed in a 30-mL Erlenmeyer flask. Five millilitres of acetone containing the prescribed amount of water was added to the flask. The flask was tightly sealed with a screw cap and incubated in a water bath at 40°C. After 72 h, acetone was evaporated from the reaction mixture, 15 mL of chloroform/methanol (2 : 1, v : v) was added to the solid residue, and Novozym 435 and molecular sieves were removed by filtration. After evaporation of the solvent, the solid residue was dried under reduced pressure and used for analysis.

Isotherms for Adsorption of Water onto Immobilized Enzymes and Molecular Sieves

A specified amount of Novozym 435 or molecular sieves 3A and acetone containing a prescribed amount of water were incubated in a screw-capped glass vial at 40°C. After a 100-h incubation, the water concentration in acetone was measured by Karl Fischer titration. The amount of water adsorbed was calculated with the following equation based on the mass balance of water:

$$q_e = q_i + V(C_i - C_e)/m \quad (1)$$

Analytical Methods

Reaction products were detected by thin-layer chromatography (TLC). TLC developing conditions were obtained from the literature (Gorbach *et al.*, 1994). Spots were visualized with ninhydrin reagent (0.25% ninhydrin in 2-propanol) or 50% sulphuric acid. The colour intensity of the spots visualized with 50% sulphuric acid was quantified by using NIH Image analysis software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Chemically synthesized *N*-monomyristoyl chitobiose (Gorbach *et al.*, 1994) was used as the standard. The yield of the reaction product was calculated based on the colour intensity of the spot of the standard.

Reaction products were purified by high-performance liquid chromatography on a Capcell pak NH_2 column (4.6 × 250 mm; Shiseido Co., Ltd, Tokyo, Japan). Acetonitrile/water (85 : 15, v : v) was used as the mobile phase at a flow rate of 1.0 mL min^{-1}. The column temperature was kept at 45°C. The MALDI-TOF-MS spectra of the purified products were obtained with a BIFLEX III spectrometer (Bruker Daltonics, Bremen, Germany) at an acceleration voltage of 19 kV, using 2,5-dihydroxybenzoic acid as the matrix.

RESULTS AND DISCUSSION

Lipase-Catalysed Acylation of Chitobiose

Lipase-catalysed acylation of chitobiose was carried out in the presence of myristic acid. Figure 1 shows TLC chromatograms of the reaction mixture after a 72-h incubation at 40°C. A product having an R_f value near that of the standard (lane 1, $R_f = 0.3$) was detected in lane 2. This product was identified as monomyristoyl chitobiose on the basis of the following

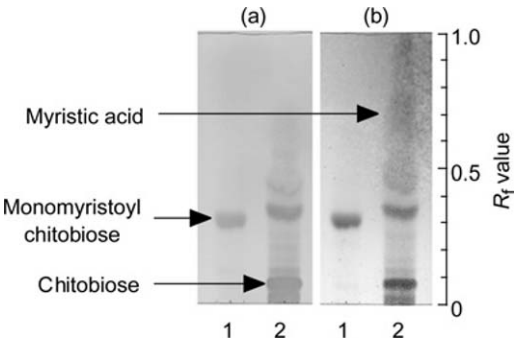

Figure 1. TLC chromatograms of the reaction mixture after a 72-h incubation at 40°C. Spots were visualized with ninhydrin reagent (a) and with 50% sulphuric acid (b). Lane 1: chemically synthesized standard; lane 2: reaction mixture for enzymatic synthesis. The solid residues remaining after removal of solvent were dissolved in 1 mL of methanol and applied to a silica gel 60 plate (Merck). 1-Butanol/ethanol/water/25% ammonia solution (40 : 40 : 15 : 5, by volume) was used as a developing solvent.