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Short communication

Enzymatic synthesis of structured lipids using a novel cold-active lipase from *Pichia lynferdii* NRRL Y-7723

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ABSTRACT

Structured lipids (SL) were synthesized by the acidolysis of borage oil with caprylic acid using lipases. Six commercial lipases from different sources and a novel lipase from *Pichia lynferdii* NRRL Y-7723 were screened for their acidolysis activities and Lipozyme RM IM and NRRL Y-7723 lipase were selected to synthesize symmetrical SL since recently NRRL Y-7723 lipase was identified as a novel cold-active lipase. Both lipases showed 1,3-regiospecifity toward the glycerol backbone of borage oil. The effects of enzyme loading and temperature on caprylic acid incorporation into the borage oil were investigated. For Lipozyme RM IM and NRRL Y-7723 lipase, the incorporation of caprylic acid increased as enzyme loading increased up to 4% of total weight of the substrate, but significant increases were not observed when enzyme loading was further increased. The activity of NRRL Y-7723 lipase was higher than that of Lipozyme RM IM in the temperature range between 10 and 20 °C.

1. Introduction

Lipase-catalysed modifications have been used to alter the fatty acid composition and physicochemical, nutritional, and functional properties of fats and oils to meet particular applications. Lipases can catalyse a variety of reactions including hydrolysis, esterification, and transesterification with a wide range of substrates (Gunstone, 1999). By using enzymatic transesterification, it is possible to incorporate an acyl group at a specific position in the triacylglycerol (TAG). By contrast, chemical transesterification does not produce this regiospecificity because of the random nature of the reaction (Willis, Lencki, & Marangoni, 1998; Xavier Malcata, Reyes, Garcia, Hill, & Amundson, 1990). Hence, industries have interests in exploring new lipases with unique characteristics and high activities that are being derived from newly examined microorganisms. Of those lipases, cold-active lipases are particularly attractive for the consideration of product stability and energy savings, rendering broad applications in such as detergent formulations, fine chemistry catalysis, and specifically food processing like SL synthesis requiring mild reaction conditions to avoid oxidative deterioration (Marshall, 1997). Recent our study (Kim, Hou, Kwon, & Shin, 2010) reported that a novel lipase from a mesophilic yeast strain *Pichia lynferdii* Y-7723 was highly active at low temperature. Relative hydrolysis activity of lipase Y-7723 at 10 °C compared to 35 °C was twice higher than that of Lipozyme RM IM. These results augmented us to address quick application of this enzyme for SL synthesis using acidolysis reaction.

Borage oil is one of the most important and commercially available sources of γ-linolenic acid (GLA, 18:3*n*−6), an essential fatty acid (Gunstone, 1992; Horrobin, 1992). GLA belongs to the *n*−6 family of polyunsaturated fatty acids (PUFA). In humans and other mammals, GLA is the first metabolite formed during the bioconversion of linoleic acid (18:2*n*−6) to prostaglandins by Δ-6 desaturase (Brenner, De Tomas, & Peluffo, 1965; Marcel, Christiansen, & Holman, 1968). GLA has physiological functions in modulating immune and inflammatory responses (Kunkel, Ogawa, Ward, & Zurier, 1981), and is effective for treating atopic eczema (Bamford, Gibson, & Renier, 1985; Manku, Horrobin, Morse, Wright, & Burton, 1984) and rheumatoid arthritis (Jantti, Seppala, Vapaatalo, & Isomaki, 1989). Thus, GLA-containing oil, especially borage oil, is used for pharmaceutical and dietary purposes (Barre, 2001). Medium-chain triacylglycerols (MCT) also offer numerous health benefits and have been widely studied for medical, nutritional, and food

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applications. MCT have been used to treat fat absorption abnormalities that occur in premature infants and in patients with cystic fibrosis (Gupta, Rathi, & Bradoo, 2003). MCT are burned quickly for energy and are not deposited in the adipose tissue (Chan, McCowen, & Bistrian, 1998). However, physical mixtures of MCT and long-chain triacylglycerols retain their original individual absorption rates. Structured lipids (SL) containing medium-chain fatty acids (MCFA) at the *sn*-1, 3 positions and long-chain fatty acids at the *sn*-2 position are more readily absorbed and oxidised for energy as compared to long-chain triacylglycerols (Jandacek, Whiteside, Holcombe, Volpenhein, & Taulbee, 1987). These products can be efficiently synthesized by exchanging fatty acids at the primary positions and/or *sn*-2 position of TAG with desired fatty acids using a variety of lipases.

In this paper, we present the production of SL containing GLA and MCFA by acidolysis of borage oil with caprylic acid using a novel cold-active lipase (NRRL Y-7723 lipase) from *P. lynferdii* NRRL Y-7723. The effects of enzyme loading and temperature on the lipase-catalysed acidolysis reaction using the NRRL Y-7723 lipase were examined, and the activity of the lipase was also compared to that of a frequently used immobilised lipase namely, Lipozyme RM IM. In addition, in order to discover the regiospecifity of the NRRL Y-7723 lipase, fatty acid profiles at the *sn*-2 position of modified TAG were also investigated.

2. Materials and methods

2.1. Materials

Borage oil, caprylic acid (99%), fatty acid standards, and pancreatic lipase were purchased from Sigma Chemical Company (St. Louis, MO, USA). Immobilised lipases from *Rhizomucor miehei* (Lipozyme RM IM) and *Burkholderia cepacia* (Lipase PS-C) were provided by Novo Nordisk BioChem North America Inc. (Franklinton, NC, USA) and Amano Enzymes (Troy, VA, USA), respectively. Other free lipases from *Candida rugosa* (Lipase AY), *Pseudomonas fluorescens* (Lipase AK), *B. cepacia* (Lipase PS), and *Mucor javanicus* (Lipase M) were obtained from Amano Enzymes.

2.2. Preparation of crude NRRL Y-7723 lipase

P. lynferdii NRRL Y-7723 was obtained from the culture collection of National Center for Agricultural Utilization Research (Peoria, IL, USA). The stock culture was maintained in a cryogenic vial containing 0.4 ml of glycerol and 0.6 ml of YM medium (1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone w/v) in a deep freezer at −70 °C prior to use. For the production of lipase, the culture was incubated in 250 ml conical flask containing 100 ml of production medium (YM medium plus 1% (v/v) soybean oil) for 72 h with reciprocal shaking at 150 rpm and 25 °C. After incubation, the culture was centrifuged at 4 °C and the supernatant without cell was collected and lyophilised at −50 °C. The dried crude enzyme sample was stored at −20 °C prior to use.

2.3. Lipase-catalysed acidolysis reaction

Acidolysis reactions were performed in a 25-ml screw-capped Erlenmeyer flask to synthesize SL. Borage oil (240 mg; average MW 872) was mixed with 160 mg caprylic acid (MW 144) at a mole ratio of 1:4 (borage oil/caprylic acid) in 3 ml *n*-hexane. The lipase of interest (1.25–20% of the weight of reactant) was then added. The mixture was stirred in an orbital shaker water bath (Model G76; New Brunswick Scientific Co. Inc., New Brunswick, NJ, USA) at 300 rpm and temperatures ranging from 10 to 70 °C.

2.4. Analysis of products

Once the reaction was complete, the enzymes were removed by filtration. The modified TAG were isolated using TLC, developed with petroleum ether/ethyl ether/acetic acid (80:20:0.5, by vol.), and detected with 0.2% 2,7-dichlorofluorescein in methanol solution under UV light. The band corresponding to the TAG was scraped off the TLC plate and methylated according to AOCS official method Ce 2-66 (AOCS, 1990). The fatty acid methyl esters (FAME) were extracted with 3 ml *n*-hexane, dried over sodium sulphate, and concentrated under nitrogen. A gas chromatograph (Varian 3800; Varian Inc., Walnut Creek, CA, USA) equipped with a Supelcowax 10 fused-silica capillary column (30 m × 0.25 mm i.d.; Supelco, Bellefonte, PA, USA) and flame-ionisation detector was used. The column was held at 190 °C for 1 min and programmed to rise to 240 °C at the rate of 1.5 °C/min and held for 10 min. The carrier gas was helium, and the total gas flow rate was 50 ml/min. The injector and detector temperatures were 240 and 280 °C, respectively. The FAME were identified by comparing the retention times with standards.

2.5. Hydrolysis by pancreatic lipase

Pancreatic hydrolysis was used to determine the positional distribution of the fatty acid in TAG (Luddy, Barford, Herb, Magidman, & Riemenschneider, 1964). Five milligrams of TAG were mixed with 2 ml of 1 M Tris–HCl buffer (pH 7.6), 0.5 ml of 0.05% bile salts, 0.2 ml of 2.2% $CaCl_2$, and 3 mg of pancreatic lipase. The mixture was incubated in a water bath at 37 °C for 2 min, vortexed vigorously, extracted with diethyl ether, and dried using anhydrous sodium sulphate. The mixture was then placed on a silica gel G TLC plate (Alltech Associates Inc., Deerfield, IL, USA) and developed

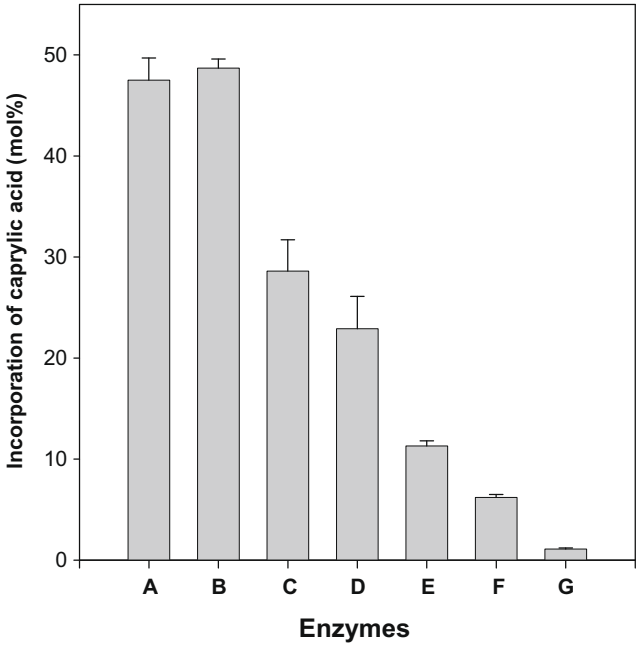

Fig. 1. Extent of incorporation of caprylic acid in borage oil by various enzymes. Enzymes: (A) NRRL Y-7723 lipase (from *Pichia lynferdii*); (B) Lipozyme RM IM (an immobilised lipase from *Rhizomucor miehei*); (C) lipase AK (from *Pseudomonas fluorescens*); (D) lipase PS-C (an immobilised lipase from *Burkholderia cepacia*); (E) lipase PS (from *Burkholderia cepacia*); (F) lipase AY (from *Candida rugosa*); (G) lipase M (from *Mucor javanicus*). Reaction conditions: a mixture consisting of 240 mg borage oil, 160 mg caprylic acid, 40 mg enzyme (10% of weight of total substrate), and 3 ml *n*-hexane was incubated for 24 h in an orbital shaker operating at 300 rpm and 40 °C.