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Candida antarctica lipase A effectively concentrates DHA from fish and thraustochytrid oils

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ABSTRACT

The fatty acid selectivity of *Candida antarctica* lipase A (CAL-A) was applied to produce DHA concentrate by controlling the rate and extent of hydrolysis. Calcium was utilized to achieve a higher degree of hydrolysis. CAL-A was not regioselective but rather fatty acid selective, showing sequential selectivity for saturated, monounsaturated and polyunsaturated fatty acids in the order of increasing double bonds. Based on its strong initial preference for saturates, CAL-A was used to concentrate 82% docosahexaenoic acid (DHA) and 11% omega-6 docosapentaenoic acid (DPA-n6) after partial hydrolysis of algal oil. *Thermomyces lanuginosus* (TL 100L) lipase was used to partially remove DPA-n6, further concentrating DHA to 89%. CAL-A was immobilized on octadecyl-activated resin without altering its fatty acid selectivity.

1. Introduction

Lipases are an important group of biocatalysts with high versatility. Lipases catalyse the hydrolysis of fats into fatty acids and glycerol at the water-lipid interface (Olusesan et al., 2011) and because of their compatibility with a broad range of solvents, they are able to catalyse a range of reactions (Akai et al., 2006; Houde, Kademi, & Leblanc, 2004; Mathesh et al., 2016; Park & Kazlauskas, 2003). One important application of lipases is the concentration of omega-3 fatty acids from fish oil. Omega-3 fatty acid consumption, particularly that of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been associated with a decreased risk of cardiovascular, neurological and inflammatory mediated diseases, as well as with the regulation of cellular activity (De Caterina & Massaro, 2005; Mazza, Pomponi, Janiri, Bria, & Mazza, 2007). Recent mild methods have been developed for the concentration of EPA and DHA from fish oil using lipases, providing high quality omega-3 concentrates useful for food fortification and pharmaceutical applications (Aarthy, Saravanan, Ayyadurai, Gowthaman, & Kamini, 2016; Akanbi, Adcock, & Barrow, 2013). Thraustochytrid oil is a potential replacement for fish oil in food and pharmaceutical products, particularly those that require high DHA levels, such as infant formula. However, this oil is high in saturated fats and omega-6 DPA, which limits its application. Concentration of DHA from this oil, through removal of saturated fat and omega-6 DPA would be useful for some nutritional applications.

Candida antarctica, a basidiomycetous yeast, produces two distinct lipases, known as CAL-A and CAL-B (Høegh, Patkar, Halkier, & Hansen, 1995; Ishii, 1988). CAL-B is the most well studied of these two lipases and has been shown to catalyse a wide range of reactions. The crystal structure of CAL-B was reported in 1994 (Uppenberg, Patkar, Bergfors, & Jones, 1994), while that for CAL-A was reported more recently (Ericsson et al., 2008). CAL-A has some useful properties, including high thermostability and an ability to tolerate a broad range of pHs (Patkar et al., 1993; Zamost, Nielsen, & Starnes, 1991). However, the regiospecificity and fatty acid selectivity of CAL-A remain undetermined, with respect to omega-3 long chain polyunsaturated fatty acids (LCPUFA). Calcium ions (Ca^{2+}) influence the interfacial activation and activity of some lipases, by stabilizing the lipases against denaturation (El Khattabi, Van Gelder, Bitter, & Tommassen, 2003). Both CAL-A and CAL-B have some calcium dependence (Anderson, Larsson, & Kirk, 1998; Dimitrijevic et al., 2012; Nielsen, Ishii, & Kirk, 1999), although this is highly dependent on the type of substrate (Cheng, Angkawidjaja, Koga, & Kanaya, 2012). The impact of Ca^{2+} on the rate and extent of fish oil hydrolysis by CAL-A remains undetermined.

In the current study, we investigate the fatty acid and positional selectivity of CAL-A for a broad range of fatty acids including EPA and DHA, using a combination of Iatroscan-FID, GC-FID and ^{13}C NMR spectroscopy. Subsequently we show that CAL-A be can immobilized for multiple reuse. This immobilised lipase was then

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applied to the concentration of DHA from alga oil. DHA was further concentrated by applying TL 100 lipase in a subsequent concentration step.

2. Materials and methods

2.1. Materials

Anchovy oil was provided by Lipa Pharmaceuticals Pty. Ltd. (Minto, NSW Australia) while tuna and alga oils were provided by Ocean Nutrition Canada (now DSM). *Candida antarctica* lipase A, CAL-A with a declared activity of 6000 LU/g and TL100 lipase were purchased from Novozymes Pty. Ltd. (North Rocks, NSW Australia). Enzyme immobilization carriers, Octadecyl (ECR8806), Epoxy (ECR8209) and Amino (ECR8309) resins were purchased from Purolite Ltd. (South Wales, UK). Their pore sizes were: 500–700 Å (Octadecyl resin, ECR8806), 600–1200 Å (Epoxy resin, ECR8209) and 600–1200 Å (Amino resin, ECR8309). Gas and thin layer chromatography standards were purchased from Sigma-Aldrich (Castle Hill, Australia) and Nu-Chek Prep (Elysian, MN, USA). All other chemicals used were of analytical grade.

2.2. Enzymatic hydrolysis

Two grams of oil and 0.5 mL CAL-A neat lipase solution were placed in a glass bottle containing 6000 μL buffer solution (20 mM sodium phosphate buffer pH 7.0, a known concentration of $CaCl_2$, and 0.03% w/v gum Arabic) and the mixture was flushed with nitrogen. Hydrolysis was carried out at 45 °C under nitrogen with magnetic stirring at 200 rpm and aliquots of sample were withdrawn at different time intervals. Buffer solution was added periodically in order to maintain the pH at 7.0. The acylglycerol and free fatty (FFA) portions of each sample were separated by extracting acylglycerols from the mixture with *n*-hexane, after adjusting the pH to 9 by adding 0.5 M KOH (30% ethanol solution). FFA in the water layer were then extracted with *n*-hexane after adjusting the pH to 1 with 4 M HCl, as described previously (Gamez-Meza et al., 2003). The glycerol fraction was further concentrated in DHA using TL100 lipase, as previously described (Akanbi et al., 2013) with some modifications. Briefly, the CAL-A concentrated acylglycerol fraction (750 mg) was mixed with 0.2 mL TL 100 lipase in a glass bottle containing 2.5 mL buffer solution (20 mM sodium phosphate buffer pH 7.0, 20 mM $CaCl_2$, and 0.03% w/v gum Arabic) and incubated at 40 °C under nitrogen for 60 min, while maintaining the buffer at pH 7.0 throughout the reaction. Workup of the acylglycerol fraction was carried out as described above.

2.2.1. Hydrolysis degree and lipid class analysis by capillary chromatography (Iatroscan)

Lipid classes were determined before and after hydrolysis using capillary chromatography with flame ionisation detection (Iatroscan MK5, Iatron Laboratories Inc., Tokyo, Japan). The Iatroscan settings were: air flow rate; 200 mL/min, hydrogen flow rate; 160 mL/min and scan speed of 30 s/scan. The chromarods were cleaned by scanning twice using the above conditions before applying sample. One microlitre of each lipid fraction in hexane was spotted onto the rods with the aid of an auto pipette along the line of origin on the rod holder and developed for 22 min in a solvent tank containing hexane/diethyl ether/acetic acid (60:17:0.2, v/v/v). Percent hydrolysis was determined using SIC-480 II software for multiple chromatogram processing, by comparing the percentage peak areas of the triacylglycerol (TAG) before and after hydrolysis (Akanbi, Sinclair, & Barrow, 2014). TLC standards purchased from Nu-Chek Prep were used to identify each lipid class.

2.2.2. Effect of calcium concentration on lipid hydrolysis by CAL-A

The effect of calcium concentration on lipid hydrolysis by CAL-A was determined using substrate mixtures with different calcium concentrations, ranging between 0 and 20 mM. A hundred microlitres (100 μL) of lipase was added to 10 mL emulsion (2% v/v anchovy oil, 20 mM sodium phosphate buffer pH 7.0, 0–20 mM $CaCl_2$, and 0.03% w/v gum arabic) in a 50 mL flask and incubated under nitrogen at 45 °C for 180 min with magnetic stirring at 200 rpm. Aliquots of samples were withdrawn at different time intervals and hydrolysis degrees were determined as described earlier.

2.3. Fatty acid analysis by gas chromatography

Fatty acids in oils before and after hydrolysis were methylated and the resulting fatty acid methyl esters (FAMEs) were analysed using an Agilent 6890 gas chromatograph with flame ionisation detection (FID) equipped with a BPX70 SGE column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The oven was programmed from 140 °C (5 min hold) to 220 °C (5 min hold) at a rate of 4 °C/min for a total run time of 30 min. A volume of 1 μL of solution was injected with a split ratio of 50:1 (injector temperature, 250 °C). Helium was used as the carrier gas (1.5 mL/min, constant flow). Detector gases were 30 mL/min hydrogen, 300 mL/min air and 30 mL/min nitrogen. Peak areas were integrated by ChemStation software and corrected using theoretical relative FID response factors (Ackman, 2002).

2.4. ^{13}C NMR spectroscopy

The quantitative ^{13}C NMR spectra of oils were recorded under continuous ^{1}H decoupling at 24 °C in a Brucker Avance 500 MHz spectrometer. Scan conditions were: spectral width (SWH); 21306.8 Hz, acquisition time (AQ); 1.55 s, receiver gain (RG); 2050, dwell time (DW); 23.47 μs, line broadening (LB); 0.8 Hz, number of scans (NS); 15,000. For each analysis, 0.5 g of oil in 700 μL $CDCl_3$ (99.8% pure) was used and the residue of each fatty acid at different positions was quantified by integration of peak area ratios obtained from three spectra fittings.

2.5. Infrared-based protein quantitation

Protein contents were determined using a Direct Detect Spectrometer (Millipore, Australia), an IR-based assay-free protein quantitation method. This method involves the use of a sample card with four hydrophilic polytetrafluoroethylene membrane positions sized for easy sample application and surrounded by a hydrophobic ring to retain analysed sample within the IR beam. By design, the positions on the card are numbered from 1 to 4, starting from the bottom. Position 1, by software default, is reserved for the blank measurement while the three remaining card positions are used for the protein samples. Prior to analysis, lipase samples were diluted to 2 mg/mL using sodium phosphate buffer and (2 μL) was placed on each membrane position and protein content was determined. Buffer solution was used as a reference blank. The results of each assay were an average of three replicates with standard deviation and coefficient of variation calculated directly with the Direct Detect Spectrometer.

2.6. Lipase immobilization

CAL-A lipase was immobilized on three resins (octadecyl ECR8806, amino ECR8309 and epoxy ECR8209 resins) following the protocols supplied by Purolite Ltd. (South Wales, UK) with some modifications. For each immobilization protocol, 2 g of resin and 12 mL of lipase solution were used. Lipase solution was prepared by mixing 4 mL liquid lipase (containing 60.8 ± 3.2 mg protein) with 8 mL buffer.