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Synthesis of monoglyceride containing omega-3 fatty acids by microbial lipase in organic solvent

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SUMMARY

An enzymatic method for synthesis of monoglyceride from 1,2-isopropylidene glycerol and n-3 polyunsaturated fatty acid concentrate was investigated in organic solvent. Optimal reaction conditions for monoglyceride synthesis by lipase were established. Lipase IM-60 from *Mucor miehei* produced yields of monoglyceride of up to 80% in this system. The resultant monoglyceride contained 76.2% n-3 polyunsaturated fatty acid (eicosapentaenoic acid, 43.3%; docosahexaenoic acid, 32.7%). Isooctane and hexane were suitable organic solvents for monoglyceride synthesis and optimal initial water content was 2.5%. Lipase IM-60 was relatively stable in organic solvent and is easily recovered for reuse.

INTRODUCTION

Monoglycerides are widely used in food preparations to promote the formation and stability of emulsions. They may be produced chemically by promotion of transesterification reactions between triglycerides and glycerol at elevated temperatures (200 °C). However, the extreme conditions produce unwanted side reactions leading to formation of dark-colored products [10]. Different enzymatic routes for production of monoglycerides have been reported [1,12]. Lipase-catalyzed transesterification reactions between triglyceride and glycerol produce mixtures of mono-, di-, triglycerides as well as free fatty acids and reaction rates tend to be low. Lipase-mediated monoglyceride production from isopropylidene glycerol and fatty acid has been proposed as an alternative approach as illustrated in Fig. 1 [4, 9]. The product of the enzymatic reaction, acetone glycerol acyl ester is converted to monoglyceride by acid hydrolysis.

Concerns regarding the amount and nature of lipids consumed in the human diet and the consequences in terms of health and disease have stimulated the development of n-3 fatty acid enriched products [3]. Diets rich in n-3 polyunsaturated fatty acid (PUFA), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to reduce the risk of coronary heart disease [2]. In this paper an enzyme-based process for efficient production of monoglyceride containing a high percentage of omega-3 fatty acids is described.

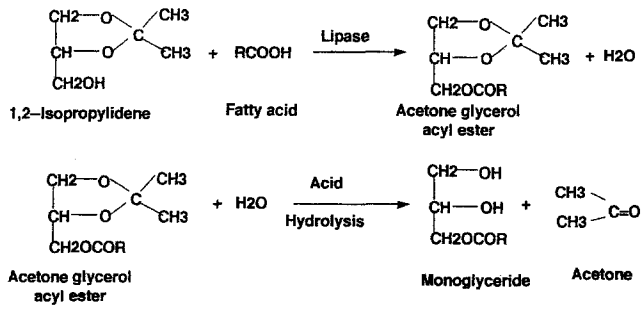

Fig. 1. Lipase-mediated synthesis of monoglyceride from isopropylidene glycerol.

MATERIALS AND METHODS

Chemicals and materials

Cod liver oil was obtained from R.P. Scherer (Windsor, Ont., Canada). Six lipase preparations were obtained from different sources, as shown in Table 1. Lipase IM-60 from *M. miehei*, immobilized on a microporous anion exchange resin was a gift of Novo Industri, Bagsvaerd, Denmark. Lauric, myristic, palmitic, stearic and oleic acids and 1,2-isopropylidene glycerol were obtained from Sigma Chemical Co. (St Louis, MO, USA). All solvents used were analytical grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Molecular sieve material was obtained from BDH (Toronto, Ontario, Canada). Highly polyunsaturated n-3 fatty acids concentrate from cod liver oil was prepared by saponification and extraction of fatty acids according to the method of Haagsma et al. [5]. The product contained 81.4% EPA and DHA.

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TABLE 1

Lipases examined

Enzyme origin	Abbreviations	Source	Specificity
1. *Candida cylindracea*	CCL	Sigma Chemical Co.	Random
2. Porcine pancreas	PPL	Sigma Chemical Co.	1,3-Specific
3. *Rhizopus* sp.	FAP-15	Amano International Enzyme Co.	Random
4. *Penicillium* sp.	G	Amano International Enzyme Co.	1,3-Specific
5. *Pseudomonas* sp.	PS-30	Amano International Enzyme Co.	Random
6. *Mucor miehei*	IM-60	Novo Nordisk Inc.	1,3-Specific

Esterification reaction

Reaction mixtures for monoglyceride synthesis from 1,2-isopropylidene glycerol and free fatty acids consisted of: 1,2-propylidene glycerol, 1 g (about 7.5 mmol); fatty acid (about 7.5 mmol); n-hexane, 3 ml; and water, 2% v/v unless otherwise stated. Reaction mixtures were placed in 50-ml Erlenmeyer flasks with silicone-capped stoppers to prevent evaporation of reactants. The reaction was initiated by addition of 6000 U lipase (dry powder). The resulting suspension was agitated on an orbital shaker at 200 r.p.m. at 37 °C.

Estimation of degree of synthesis

The reaction progress was followed by free fatty acid titration with 0.05 N NaOH. The degree of synthesis (%) is defined as the percentage of initial fatty acids consumed in the reaction mixture.

Identification of reaction products

At the end of the reaction 3 ml water was added and the enzyme was filtered off. The aqueous phase was extracted twice with 3 ml volumes of diethyl ether. The combined organic layers were subsequently washed with 3 ml of 0.5 N NaOH. The resulting 1,2-isopropylidene glycerol-3-acyl ester was hydrolyzed by addition of 1 ml of concentrated hydrochloric acid to obtain monoacyl glycerol. The monoglyceride was analyzed by TLC, developed in a solvent containing 80:20:1.5 petroleum:ethyl ester:acetic acid. Lipid spots were identified using a 12.5% solution of phosphomolybdic acid. Fractions corresponding to monoglyceride were scraped from the plate and derivatized for GC analysis.

Gas chromatography

Each lipid class separated by TLC was methylated according to the method of Holub and Skeaff [7]. The methyl esters of fatty acids were dissolved in n-hexane for analysis. Analysis was performed using a Gas Chromatograph GC-14A (Shimadzu Inc., Kyoto, Japan) with CR 601 data integrator equipped with a megabore column DB-225 and a flame ionization detector (FID). Helium was the carrier gas, at a flow rate of 38 ml min^{-1}. The injection port and FID temperature were both 250 °C and the column temperature was 210 °C. Peaks of fatty acid esters were identified and calibrated using standard fatty acids. Pentadecaenoic acid (15:0) was used as an internal standard.

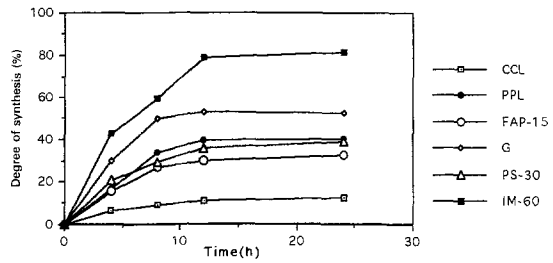

Fig. 2. Conversion versus reaction time for the synthesis of monoglyceride by various lipases. (The reaction mixture contained 1 g 1,2-isopropylidene glycerol, 2.5 g n-3 PUFA concentrate, 50 μl H_2O, 6000 U lipase and 3 ml n-hexane.)

RESULTS AND DISCUSSION

The esterification of 1,2-isopropylidene glycerol and n-3 polyunsaturated fatty acid concentrate in organic solvent by six lipases at equivalent enzyme units are compared in Fig. 2. Degree of synthesis catalyzed by the 1,3-specific lipases, IM-60, G and PPL, was higher than that catalyzed by the random lipases, CCL, FAP-15 and PS-30. Immobilized lipase IM-60 synthesized monoglyceride at the highest rate. We have also found this enzyme to be very stable in organic solvent and to be easily recovered for reuse. It was, therefore, used in all further studies.

The relative rates of esterification of 1,2-isopropylidene glycerol by various C_{12}–C_{18} and omega-3 fatty acids in organic solvent by lipase IM-60 were compared (Fig. 3).

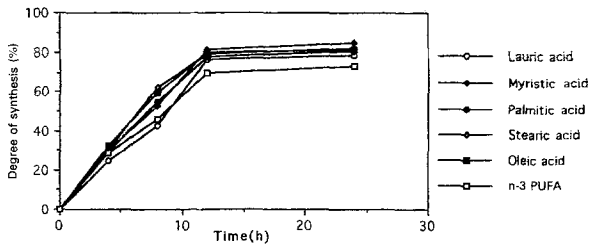

Fig. 3. Progress curve of synthesis of monoglyceride from 1,2-isopropylidene glycerol and various kinds of fatty acid by lipase IM-60. (The reaction mixture contained 1 g 1,2-isopropylidene glycerol, 2 g fatty acid, 50 μl H_2O, 6000 U lipase IM-60 and 3 ml n-hexane.)

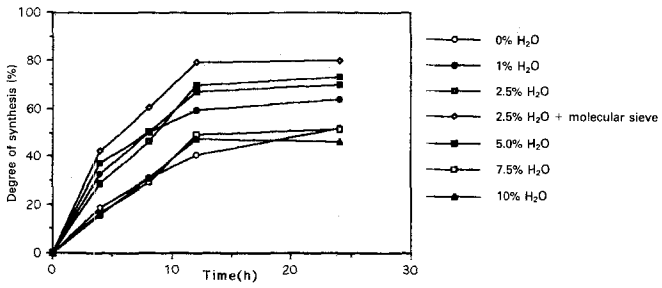

Fig. 4. Effects of initial water content on monoglyceride synthesis by lipase IM-60. (The reaction mixture contained 1 g 1,2-isopropylidene glycerol, 2.6 g n-3 PUFA concentrate, 3 ml n-hexane, 6000 U lipase IM-60 and various amounts of water.)

With C_{12}–C_{18} fatty acids the degree of conversion ranged from 78.9–84.9%. The degree of conversion to monoglyceride was slightly less (76.15%) when n-3 PUFA concentrate was used as substrate and the resultant monoglyceride was found to contain 43.3% EPA and 32.7% DHA.

The effect of initial water content on monoglyceride synthesis by lipase IM-60 from 1,2-isopropylidene glycerol and n-3 PUFA concentrate in hexane is illustrated in Fig. 4. Maximum monoglyceride synthesis was observed with a moisture content of 2.5% (v/v). Reduction of water content in the reaction mixture was attempted by the addition of molecular sieve pellets (4A) as dehydrating agents in order to obtain a higher conversion rate. Addition of 1.0 g of molecular sieve to the reaction medium containing 2.5% moisture resulted in a degree of synthesis of 80.2%.

The effect of organic solvent on the catalytic activity of lipase in the esterification of 1,2-isopropylidene glycerol with omega-3 concentrate and with myristic acid was examined (Table 2). With both fatty acid substrates highest degrees of synthesis were observed with isooctane and hexane.

Optimum n-3 PUFA concentration to 1,2-isopropylidene glycerol ratio for monoglyceride synthesis by lipase IM-60 was also investigated. Ratios of 0.5, 1.0, 2.0, 4.0 of fatty

TABLE 2

Effects of organic solvent on the monoglyceride synthetic reaction by lipase IM-60

Organic solvent	Degree of synthesis (%)	
	myristic acid	n-3 PUFA concentrate
Pentane	81.5	69.8
Hexane	84.9	73.4
Heptane	79.8	70.4
Isooctane	86.7	76.4
Acetone	24.6	11.2
Chloroform	34.6	22.4
Benzene	14.2	6.70

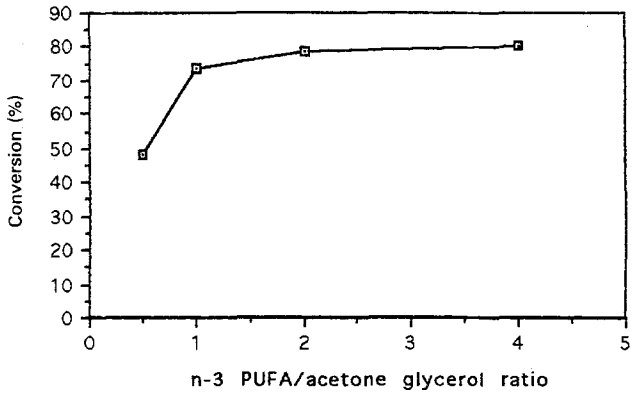

Fig. 5. Effect of fatty acid to 1,2-isopropylidene glycerol ratio on monoglyceride synthesis by lipase IM-60. (The reaction contained 3 ml of substrate mixture of various ratios, 3 ml n-hexane, 6000 U lipase IM-60 and 2.5% H_2O.)

acid to 1,2-isopropylidene were tested (Fig. 5). When this ratio was greater than 1, a maximum conversion of about 80% was obtained.

Use of 1,2-isopropylidene glycerol as a substrate for esterification by omega-3 fatty acids is advantageous from a processing perspective. The substrate is easily formed from glycerol and acetone by mild acid catalysis. The product of lipase esterification is easily converted to free monoglyceride by mild acid hydrolysis. The observation that higher rates of esterification were obtained with 1,3-specific lipases rather than random attacking lipases may reflect the positional preference of these enzymes for the available sn-1 hydroxyl group. Only one hydroxyl is available for esterification resulting in production of one glyceride form (monoglyceride). On this basis, the procedure has advantages over triacylglycerol hydrolysis [11] and glycerol esterification reactions [6,8] where monoglyceride is substantially contaminated with di- and triglycerides.

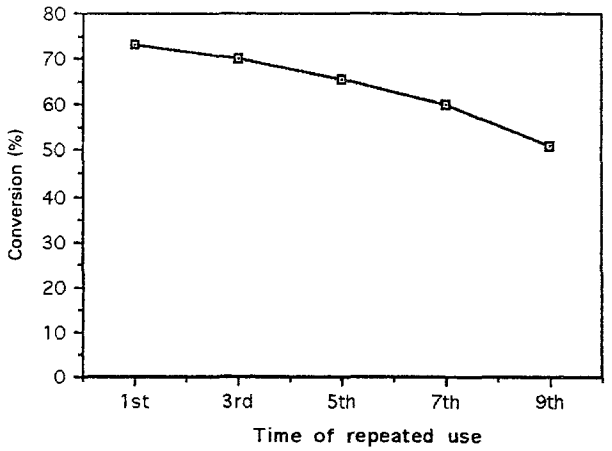

Fig. 6. Operational stability of lipase IM-60 for synthesis of monoglyceride. (The reaction mixture contained 1 g 1,2-isopropylidene glycerol, 2.5 g n-3 PUFA concentrate, 2.5% H_2O, 6000 U lipase IM-60 and 3 ml n-hexane.)

ACKNOWLEDGEMENTS

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