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Immobilization of *Rhizopus japonicus* lipase on celite and its application for enrichment of docosahexaenoic acid in soybean oil

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Abstract

Rhizopus japonicus lipase was immobilized on Celite, the immobilized enzyme acquired 6.34 units of transesterification activity over the native enzyme which does not show any significant transesterification activity in *n*-hexane. However, there was no improvement in the thermal stability of Celite-immobilized lipase. The preparation is shown to be promising for its end application in producing structured lipid. An incorporation of 25% docosahexaenoic acid (DHA) into soybean oil was observed in *n*-hexane media in 24 h with the immobilized lipase.

1. Introduction

The commercial value of fats and oils depends on their physico-chemical and nutritional properties which, in turn, are dependent on their triglycerides and fatty acid composition. Thus, improvement in the fatty acid composition of naturally-occurring fats and oils from the viewpoint of their intended application has been an area of extensive research (Kaufman & Ruebusch, 1990; Macrae, 1986). Transesterification is one of methods for modification of oils and fats. Lipase-catalyzed transesterification, due to enzyme specificity, provides a precise control over incorporation of desired fatty acid at a specific glycerol position, because operations under mild reaction conditions are preferred over harsh and non-specific chemical catalysis or random physical blending (Bosley, 1997; Villeneuve & Foglia, 1997; Vulfson, 1994). Several industrial applications of lipase transesterification, such as production of cocoa butter equivalents (Coleman & Macrae, 1980), polyunsaturated fatty acid rich structured lipids (Huang & Akoh, 1994), milk fat substitutes (King & Padley, 1990), medium chain and low calorie trigylcerides (Huang & Akoh, 1996) from low value oil, have been reported. However, the high costs of enzyme and scale-up limit application of lipases. For this reason, chemical catalysts are preferred over lipases but an additional step for purification of desired end-product is needed in such cases because of the random mixture of end-products obtained (Haumann, 1997). Similar problems, in the case of other industrial enzymes, have been overcome by immobilization, imparting reusability and stability. However, for lipases the immobilization method and support should be such that immobilized lipase can work well in an organic solvent. This is because the primary reaction of lipase is hydrolysis and, for interesterification to take place, the water content in the medium should be low so that the equilibrium can be shifted toward reverse direction or synthesis (Macrae, 1983). Transesterification, thus typically, is performed in an organic solvent to control water content of medium to the desired level (Valivety, Halling, Peilow & Macrae, 1994). For this reason choice becomes restricted to a method of immobilization and suitable matrix which can work well in organic solvents. Although a number of useful immobilized lipases have been reported and some of these have been found very useful for application of lipase-catalyzed transesterification in lipid processing and modification (Balcao, Paiva & Malcata, 1996; Bosley, 1997), search still continues for better preparation in terms of cost, enhanced activity, stability and reusability. In the present study, relatively inexpensive *Rhizopus japonicus* lipase was immobilized on a Celite support, which is

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considered suitable for working in solvents because of its porosity and low cost. We observed good transesterification activity for Celite-immobilized lipase in *n*-hexane and it was found to be reasonably useful for enrichment of docosahexaenoic acid (DHA) into soybean oil.

2. Materials and methods

2.1. Materials

R. japonicus lipase having sn-1,3 specificity (Saiken 100) was supplied by Nagase Biochemicals, Osaka, Japan. The enzyme preparation contained 11% protein, 75% lactose as stabilizer and the rest was non-nitrogenous protein. Mono, di- and tripalmitin, mono-, di- and tristearin, 1-palmitoyl 3-stearoyl glycerol, and docosahexaenoic acid ethyl ester having 99% purity (Cat no. D-2661) were procured from Sigma Chemical Co., USA; 1,2- dipalmitoyl-3-stearoyl glycerol and 1,3-distearoyl-2-palmitoyl glycerol were gifts from Unilever Colworth Lab, UK. Celite 535 from Wako Chemical Co. was used as the support for the immobilization. All other reagents were of analytical grade.

2.2. Immobilization of lipase on Celite

R. japonicus lipase was immobilized on Celite by simple adsorption following the method described by Triantafyllou, Adlercreutz and Mattiasson (1993). Lipase (1.0 g) is dissolved in 20 ml double-distilled water then mixed with 5 g of Celite and kept stirring for one hour followed by freeze-drying. The freeze-dried preparation was stored at 4°C for further use.

2.3. Transesterification reaction using tripalmitin and stearic acid as substrates

The substrate tripalmitin (PPP) and stearic acid (S) 250 mg were each dissolved in 50 ml *n*-hexane (dehydrated similarly as described above to decrease the water concentration to 10 mg/l). Modified lipase (30 mg) was added into this reaction mixture and the reaction was carried out with constant stirring at 500 rpm and 40°C for 5 h. The samples were withdrawn periodically, filtered with a 0.5 μm filter (LCR 13 LH, Millipore Co., USA) and dried at 60°C under nitrogen. The dried samples were dissolved in 1 ml of hexane containing internal standard (1 mg hexadecane/ml) and analyzed by a gas chromatograph equipped with a capillary column (0.25 mm internal diameter, 25-m column length, 0.1 μm film thickness) under the operating temperature 80 to 360°C programmed at the rate of 10°C/min, injector 370°C and flame ionization detector (FID) 400°C. The transesterification reaction products, 1,2-dipalmitoyl-3-stearoyl glycerol (PPS) and 1,3-distearoyl-2-palmitoyl glycerol (SPS), have quantified against the standard provided by Unilever Colworth Lab., UK. One unit of enzyme activity is defined as the mmols of PPS formed per h per mg of protein.

2.4. Preparation of DHA rich soybean oil

Soybean oil (100 mg) and DHA (50 mg) in 5 ml *n*-hexane were used as substrates and transesterification was monitored after adding 50 mg enzyme. The amount of DHA incorporated into triglycerides (TG) was estimated in periodically withdrawn aliquots in first subjecting samples to thin-layer chromatography for TG separation (Li & Ward, 1993) and then methylating TG by 6% HCl/methanol at 80°C for 2 h (Huang & Akoh, 1994). The methylated TG were analyzed by GC as described above after dissolving in 1 ml hexane containing hexadecane as internal standard.

2.5. Hydrolysis activity using p-nitrophenyl palmitate (PNP) as substrate

The hydrolysis activity was assayed following the method as described by Furutani, Ooshima and Kato (1995). The liberation of *p*-nitrophenol from PNP by lipase reaction was monitored spectrophotometrically for increase in the absorbance at 410 nm as a function of time. The unit of activity is defined as the μmoles of *p*-nitrophenol released per min per mg of protein.

2.6. Protein estimation

The protein content was determined by dye binding assay (Bradford, 1976) Water concentration in the reaction system was analyzed by Karl Fisher Coulometer.

3. Results and discussion

3.1. Immobilization of R. japonicus lipase on Celite

Lipases have been immobilized by various immobilization methods for their use in industrial application in fat and oils processing or their restructuring. It is established that a nearly ideal support for lipase immobilization should be suitably porous to overcome the mass transfer constraints to work well for a water partition effect between, enzyme, support and organic solvent (Bosley & Clayton, 1994). Celite, being inexpensive, is preferred over other ideally useful porous support such as controlled porous glass. Besides these, synthetic polymers such as polyethylene and polypropylene being hydrophobic, are yet another good choice but are too costly (Wisdom, Dunnill & Lilly, 1984). In present work, immobilization of *R. japonicus* lipase (a less expensive enzyme) on a Celite matrix was studied. The

lipase (1 g/20 ml water) was found to adsorb well in Celite (5 g) in one hour. In total 360 mg enzyme was adsorbed per g of Celite under the above immobilization conditions and 64% protein was recovered in subsequent washings as unabsorbed enzyme (Table 1). Interestingly, as can be seen in Table 1, although native enzyme showed no/or very little transesterification activity, the immobilized preparation acquired a good transesterification specific activity of 6.23 units for transesterification between tripalmitin and stearic acid. This may possibly be attributed to a catalytic conformation favorable to transesterification induced by immobilization. Triantafyllou et al. (1993) reported 10.6 units of activity on immobilization of *Candida rugosa* lipase on Celite for transesterification between tributyrin and pentan-2-ol in iso-octane medium. Mustranta, Forssell and Poutanen (1993) also found a similar increase from native *Candida cylindracea* (920 nkat/mg) to Celite-immobilized (11,000 nkat/g support). Thus the transesterification activity observed for Celite-immobilized *R. japonicum* is quite comparable to other preparations. However, the yield of 36% is rather low but it is compensated by the fact that, whereas native *R. japonicum* lipase showed no transesterification activity towards tripalmitin and stearic acid as substrate in *n*-hexane media, immobilized lipase acquired a high transesterification activity of 6.23 units. The yield in the case of Celite-immobilization has often been low because binding is due to adsorption, which is a weak interaction and leaches into washings. Attempts to use glutaraldehyde following the adsorption led to further loss in activity in our case.

Table 1
Activities of *R. japonicus* lipase immobilized into Celite

Properties	Native	Celite-immobilized
Yield (%)[a]	100	36
Hydrolysis activity (%)[b]	100	18.6
Transesterification activity (mM PPS/ mg/h)[b]	0	6.23

[a] Yield is defined as the amount of protein bound to Celite considering the total protein used for immobilization as 100%.

[b] Hydrolysis and transesterification were determined towards *p*-nitrophenyl palmitate, stearic acid and tripalmitin, respectively, as described and defined in Materials and methods.

Hydrolysis activity of immobilized lipase decreased to 18% of that of the native form. Basri, Ampon, Yunus, Razak and Salleh (1992) also reported 70–98% decrease in hydrolytic activity in their bisimido ester-modified lipase, which gained transesterification activity. They have suggested that increased hydrophobicity, altering the microenvironment near the active site, may have caused this effect.

3.2. *Thermal stability of immobilized* R. japonicus *lipase*

In order to asses the thermal stability, the immobilized enzyme preparation was incubated at 45°C and residual activity determined in the periodically withdrawn samples. No significant improvement was observed in the thermal stability of immobilized enzyme

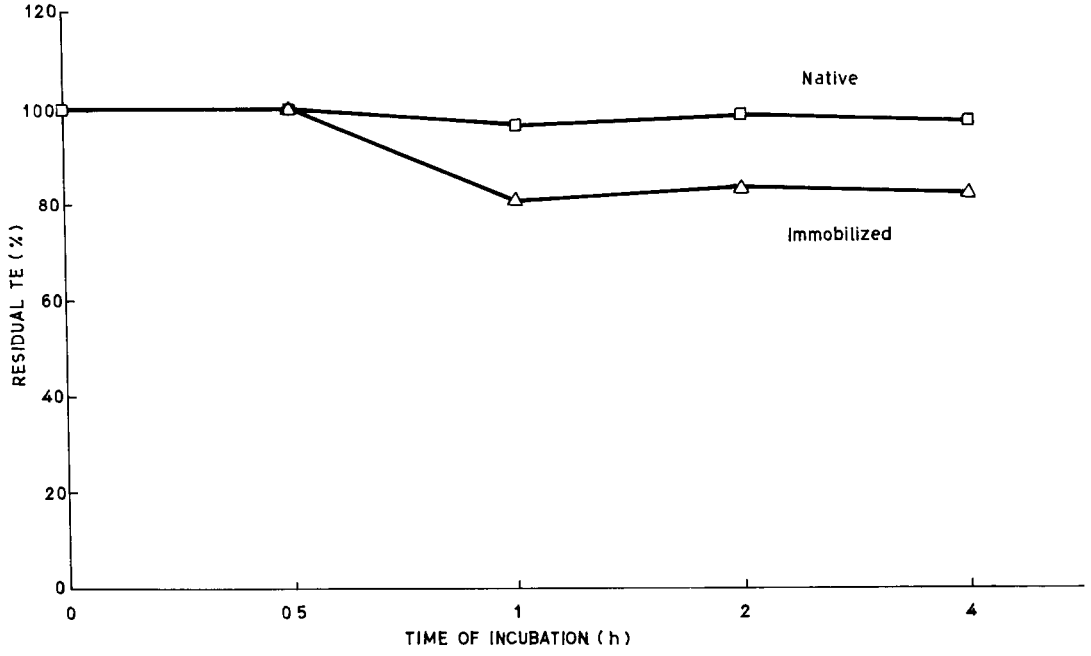

Fig. 1. Thermal stability of native and Celite-immobilized *R. japonicus* lipase. Enzymes (500 mg) in 5 ml distilled water were incubated at 45°C. Samples were drawn at various interval of time and freeze-dried. The residual activity of lipase was determined for the hydrolysis of *p*-nitrophenyl palmitate following the standard method. Only hydrolysis activity was determined to compare the thermal stability of native and immobilized forms, not the transesterification as native has no/or very little intersesterification activity: —◇— native; —▽— Celite-immobilized.

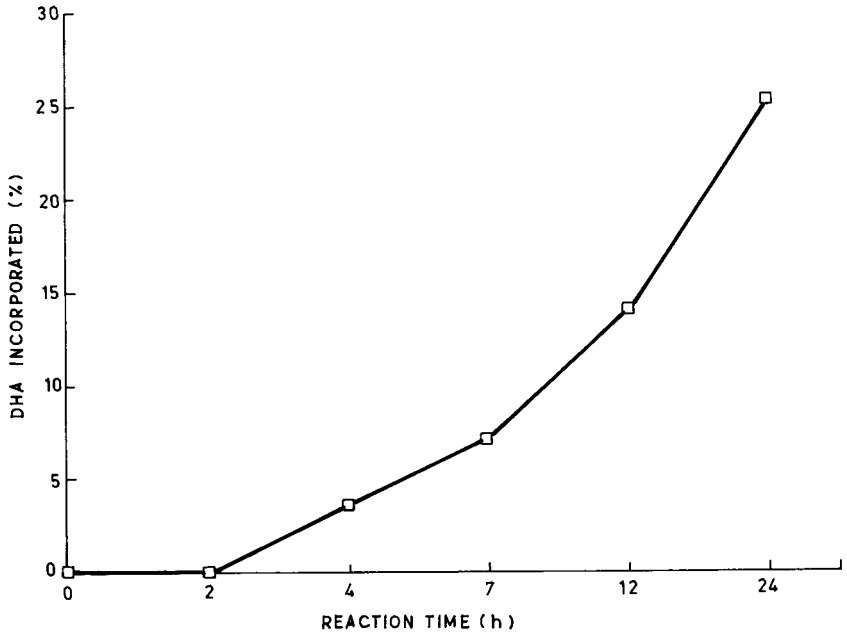

Fig. 2. Time course of DHA incorporation into soybean oil. Soybean oil (100 mg) and DHA ethyl ester (50 mg) in 5 ml dehydrated *n*-hexane were incubated with immobilized lipase (50 mg) at 40°C with 500 rpm stirring. The aliquots were periodically withdrawn and DHA incorporated was monitored in samples.

over the native form (Fig. 1). Nevertheless, as the immobilized lipase was found to be stable at 40°C for 12 h, it can well be used at this temperature for intended application.

3.3. Enrichment of DHA in soybean oil

Polyunsaturated fatty acids (PUFA), especially n-3 type docosahexaenoic acids (DHA) and eicosapentaenoic acids (EPA), have been proved to be essential for brain function and growth (Newton, 1997). Fish or fish oil is a rich source of these fatty acids but their use in the daily diet is not universal. Because of universal use of vegetable oil and fats in food preparation worldwide these are considered as alternative vehicles for carrying n-3 PUFA, DHA and EPA. Thus, one of the main applications of the lipase-catalyzed transesterification reaction is incorporation of DHA/EPA into vegetable oils. Considering that the Celite-immobilized *R. japonicus* lipase was reusable and working well in organic solvents with high transesterification activity, it was studied for assessing its efficiency to incorporate DHA into soybean oil triglyceride.

Fig. 2 shows the incorporation of DHA into soybean oil over the period of time. Both native and immobilized lipase preparations gave 18–25% DHA enrichment in 24 h, immobilized lipase being the more efficient catalyst. Immobilized lipase could be reused without any significant loss in activity. A similar PUFA enrichment, using immobilized *Candida antarctica* lipase to give 32.9% DHA in soybean oil, has been reported by Huang and Akoh (1994). Also, 43% EPA in the case of primrose oil in 24 h by *C. antarctica* lipase (1/3 ratio substrate, 50 mg oil, and 10 mg enzyme in hexane) has been reported by Akoh, Jennings and Lillard (1996). Thus, preliminary results giving 25% enrichment by using Celite-immobilized lipase in our case are promising enough. Further studies are required to scale up the process for final application of immobilized enzyme. Nevertheless Celite-immobilized *R. japonicus* lipase seems to work well as a less expensive and suitable preparation for lipase applications.

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