A Packed-Bed Enzyme Mini-Reactor for the Production of Structured Lipids Using Nonimmobilized Lipases

Sir:

Most research on enzymatic acidolysis for the production of structured lipids (SL) has focused on using either batch reactors or relatively large-scale continuous packed-bed reactors (1). In our previous studies for the production of SL, we developed batch enzyme reactors (2) as well as continuous packed-bed enzyme reactors from laboratory scale (3) to pilot plant (4). Batch reactors can be used from milligram flask level to ton production level; however, they often produce significant acyl migration during the lipase-catalyzed production (2). Therefore, it is preferable to use packed-bed reactors, in which acyl migration is reduced (4). The continuous reactors developed in our previous work had a dead volume from 50 mL to 1 L, indicating that the minimal requirement of substrate cannot be less than these amounts. However, in some applications, only small amounts of targeted SL are required, such as isotopically labeled SL. The production of such SL is often involved and requires rare or expensive starting materials. Accordingly, consumption of starting materials should be limited, and losses during processing should be minimized.

To minimize material consumption, improve product yields, and be able to produce small amounts of SL in packed-bed reactors, it is desirable to downsize the packed-bed reactors and also to simplify the processes. In this study, a mini-scale continuous packed-bed reactor was constructed using an HPLC column together with an HPLC pumping system. This system could allow the use of nonimmobilized lipases in such a continuous system, which is impossible in large-scale packed beds.

Refined fish oil (Aarhus Olie A/S, Aarhus, Denmark) and caprylic acid (Sigma, St. Louis, MO) were used in the acidolysis. The fatty acid composition of the oil (mol%) is C14:0 8.0, C14:1 0.4, C15:0 0.6, C15:1 0.2, C16:0 17.0, C16:1 6.6, C17:0 0.7, C17:1 0.7, C18:0 2.0, C18:1n-9 13.0, C18:1n-7 1.9, C18:2n-6 2.7, C18:3n-3 2.3, C18:4n-3 8.7, C20:1 4.2, C20:5n-3 7.4, C22:1n-11 9.6, C22:5n-3 1.3, and C22:6n-3 12.2. All solvents and reagents used were of analytical or chromatographic grade. Six nonimmobilized commercial lipase preparations donated by Amano (Nagoya, Japan) were directly used in this study, i.e., Lipase AP, *Aspergillus niger* (activity 12,000 units/g); Lipase P, *Pseudomonus* sp. (activity 30,000 units/g); Lipase AY, *Candida rugosa* (activity 30,000 units/g); Lipase AK, *P. fluorescens* (activity 25,000 units/g); Pancreatin F, pancreatic lipase (activity 1,000 units/g); and Lipase D, *Rhizopus delemar* (activity 1,650,000 units/g). The listed activities in parentheses are labeled hydrolytic activities and were provided by the producer. The water content of the lipase preparations was 4.6, 5.2, 3.8, 5.0, 6.3, and 7.9, respectively (wt%) by Karl Fischer determination (2). Lipozyme RM IM (Novozymes, Bagsvaerd, Denmark), a commercial immobilized lipase from a strain of *Rhizomucor miehei*, was also used in the mini-scale bed for comparison of overall performance (labeled activity from the producer 8.2 BIU/g, measured water content by Karl Fischer titration 3.5 wt%). The default mole ratio of fish oil to caprylic acid was 1:6. The water content of the mixture was 0.07% as determined by Karl Fischer titration.

The reactor was a stainless steel HPLC column (25 cm × 4.6 mm i.d.). Nonimmobilized lipases or Lipozyme RM IM (1.2–1.3 g) were packed in the column according to the previously reported method (4). The void fraction was also measured accordingly with both immobilized and nonimmobilized lipases (4). The substrate mixture was fed into the column reactor by the peristaltic HPLC pump. The reaction temperature was controlled by immersing the column in a water bath. The column was disconnected from the detector, and the eluate (product) was collected under a nitrogen blanket.

Incorporation of caprylic acid into the oil TAG was determined by GLC analysis after alkaline-catalyzed transmethylation to FAME as previously described (3). The gas chromatograph was equipped with an FID and a fused-silica capillary column (SP-2380, 60 m × 0.25 mm i.d.; Supelco Inc., Bellefonte, PA). Area percentages were recalculated into mole percentages based on the measured response factors and the molecular weights of the fatty acids. Acyl migration was determined by Grignard degradation of TAG in the samples, isolation of *sn*-2 MAG by TLC, transmethylation of the MAG by the KOH method, and determination of FAME composition by GC analysis (2).

According to the previous studies, the average void fraction varied between 0.44 and 0.47 for Lipozyme RM IM in large beds (4). The measured values for the present mini-bed were 0.46–0.48, slightly higher than the last measured, probably due to the smaller size of the bed. Thus, the void volume of the mini-scale continuous reactor was 1.9–2.0 mL (the full bed volume was 4.15 mL for Lipozyme RM IM). The average void fraction for nonimmobilized lipases was in the range of 0.35–0.38 as measured by the same method. Different nonimmobilized lipases used in this study only had slight differences in void volume. Therefore, the void volume for nonimmobilized lipases in a packed-column format was less than 1.5 mL since both full-bed volume and void fraction were smaller. This indicates that as little as a few milliliters of substrates can be processed successfully in this mini-scale packed-bed

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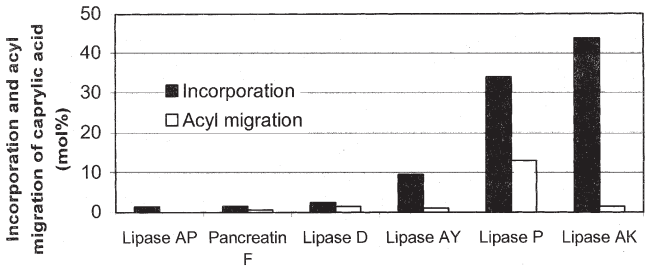

FIG. 1. Screening of nonimmobilized lipases with respect to the overall incorporation and acyl migration of caprylic acid into fish oil in the mini-scale packed-bed reactor. Conditions: substrate mole ratio 1:6, flow rate 0.01 mL/min, temperature 50°C, and no water addition. Lipase preparations donated by Amano (Nagoya, Japan).

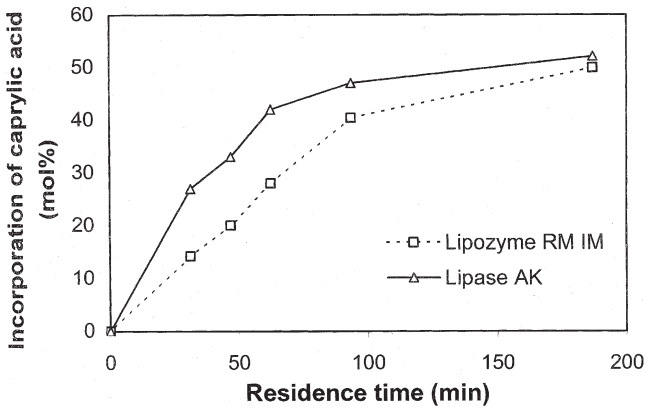

FIG. 2. Time courses of Lipase AK (Amano, Nagoya, Japan) and Lipozyme RM IM (Novozymes, Bagsvaerd, Denmark) for the enzymatic acidolysis of fish oil with caprylic acid in the mini-scale packed-bed reactor. Conditions: substrate mole ratio 1:6, reaction temperature 50°C, and no water addition.

reactor using either immobilized or nonimmobilized lipases. This allows for production of very small quantities of SL with packed-bed reactors or the use of a small amount of substrates.

Six nonimmobilized lipases were screened in the same packed-bed mini-reactor for their overall performance in the production of SL using fish oil and caprylic acid. The reaction conditions were: substrate mole ratio 1:6 (fish oil/caprylic acid), flow rate 0.01 mL/min, and temperature 50°C. Based on the overall product output of the reactions, Lipase AK and Lipase P showed higher incorporation of caprylic acid than other lipases examined (Fig. 1). However, Lipase P produced higher acyl migration than Lipase AK. Therefore, Lipase AK was selected as the best nonimmobilized lipase for the production of SL in the mini-scale reactor.

A time-course study was made to examine the overall performance of Lipase AK (nonimmobilized) in comparison with Lipozyme RM IM (immobilized) in the same reactor and under the same conditions. The applications of Lipozyme RM IM have been well received, and its performance is well understood. Therefore, the time courses will visualize the level of overall performance of Lipase AK in this special mini-bed reactor. The same amounts (weight) of the two lipase preparations (in powder/particle, used as obtained from the producers) were used for the packing of the beds. Lipase AK, the nonimmobilized lipase, showed a slightly higher incorporation than Lipozyme RM IM in the time course (Fig. 2). This indicates that production of SL in the mini-scale packed-bed reactor is feasible with nonimmobilized lipases because they can provide a reaction performance similar to the immobilized enzyme. The possibility of using nonimmobilized enzymes in packed-bed reactors provides convenience, but the physical stability of packed beds using nonimmobilized lipases is low as observed in this study. In general, lipase activity declines faster with nonimmobilized lipases than with immobilized ones (5). Since the objective of the mini-scale packed-bed reactor was to produce small amounts of special SL, these phenomena were not crucial to the applications.

Therefore, a mini-scale packed-bed reactor using an HPLC column was demonstrated to have an operational performance similar to large-scale packed-bed reactors for the production of SL. In some cases, a nonimmobilized lipase, like immobilized lipases, can be used in the mini-scale packed-bed reactor. Among the nonimmobilized lipases examined, Lipase AK exhibited slightly better overall acidolysis performance than Lipozyme RM IM under similar reaction conditions.

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