Production of Alkyl Esters from Tallow and Grease Using Lipase Immobilized in a Phyllosilicate Sol-Gel

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ABSTRACT: The lipase-catalyzed synthesis of alkyl esters from tallow and grease using *Pseudomonas cepacia* lipase (PS-30) immobilized within a phyllosilicate sol-gel matrix was investigated. The effects of the presence of alcohol and of the amount of enzyme used were studied. The matrix-immobilized PS-30 lipase effectively converted grease and tallow to ethyl esters in greater than 95% yield when using ethanol. The final conversion of grease or tallow to alkyl esters was aided by the addition of molecular sieves (0.4 wt% of substrates) to the reaction mixture. The matrix-immobilized PS-30 enzyme was easily recovered and could be reused at least five times without losing its activity. Accordingly, the phyllosilicate sol-gel immobilized PS-30 lipase is potentially useful for the economic production of biodiesel fuel.

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KEY WORDS: Alkyl esters, biodiesel fuel, grease, immobilization, phyllosilicate, sol-gel, tallow, transesterification.

Biodiesel fuel is a term applied to monoalkyl ester derivatives of fats and oils when they are used as neat diesel fuel or diesel fuel extenders. These alternative fuels are receiving interest because of the concern over emissions generated by petroleum-based fuels. As a result, development of alternative fuels from agricultural fats and oils has become important (1,2). Because biodiesel fuel has a higher price than petrodiesel fuel, the use of lower-cost feedstocks, such as tallow or restaurant grease, instead of more costly refined vegetable oils would be advantageous.

Chemical approaches to the synthesis of alkyl esters from triglycerides have been used for decades (3,4), but there are several associated problems, including glycerol recovery, removal of inorganic salts, and the cost of refined feedstocks. In contrast, biocatalysis allows the synthesis of specific alkyl esters and facilitates the recovery of the glycerol co-product. Recently, interest has developed in the use of lipases, with or without immobilization in transesterification and interesterification reactions for the production of biodiesel (5–7). The research has concentrated on the use of lipases to transesterify higher molecular weight fatty acids to alkyl esters. Nelson *et al*. (5) demonstrated the lipase-catalyzed production of biodiesel from soybean oil, rapeseed oil, tallow, and recycled restaurant grease. They established conditions for converting tallow and recycled restaurant grease to alkyl esters in >95% yield using various commercial lipases. They also developed conditions effective for transesterifying feedstocks high in free fatty acid content to their respective alkyl esters. Although lipase-catalyzed esterification seems to be a viable method for the production of alkyl esters from tallow, grease, and vegetable oils, the extents of conversion and cost of their production have not been optimized. The search for efficient methods of immobilizing lipases also has emerged as an important field of interest because process economics demand repeated use of these biocatalysts. Recently, our laboratory developed a novel method of enzyme immobilization using phyllosilicate clay cross-linked by a sol-gel matrix (8,9). This new immobilization technique was applied to lipases, which enhanced their esterification activity and improved their stability and reusability. In this paper, we report the phyllosilicate sol-gel-immobilized, lipase-catalyzed transesterification of restaurant grease and tallow with normal and branched-chain alcohols to alkyl esters in high conversions. A potential application of this method is in the synthesis of biodiesel fuel from low cost feedstocks.

MATERIALS AND METHODS

Materials. Recycled restaurant grease was supplied by Kaluzany Bros., Inc. (Joliet, IL), and contained 8.5% free fatty acid (FFA). Beef tallow (0.75% FFA) was obtained from HRR Enterprise (Chicago, IL). Lipase from *Pseudomonas cepacia* (PS-30) was obtained from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). Phyllosilicate (montmorillonite Sy-1) was from Source Clay Minerals Repository (Columbia, MO). Cetyltrimethyl ammonium chloride (HDTMA) and tetramethylorthosilicate (TMOS) were obtained from Aldrich (Milwaukee, WI). Molecular sieve was obtained from J.T. Baker (Phillipsburg, NJ). All other reagents used were of the highest purity available from commercial suppliers.

Lipase immobilization. The procedure used to entrap lipase PS-30 within a phyllosilicate sol-gel matrix was as described by Hsu *et al*. (8,9). Briefly, in the standard method lipase PS-30 powder was suspended in water (150 mg/mL). The suspension was mixed and then centrifuged at $2,000 \times g$ to remove particulate material. Protein content in the supernatant (1.25 mg/mL) was determined by a modified Lowry assay using serum albumin as the standard (10). An aliquot of the supernatant (2.4 mL) was added to an aqueous suspension of phyllosilicate clay (5.4 mL, 3.3% wt/vol), which pre-

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viously had been saturated with sodium ions and then exchanged with alkylammonium ions by the addition of 600 µL of 1 M HDTMA. The mixture was mixed for a few seconds, and 1 mL of 1 M sodium fluoride, TMOS (1.05 mL), and water (1.05 mL) were added. This mixture was mixed briefly, kept in an ice bath for 3 h, and then set at room temperature overnight to complete the TMOS polymerization process. The mixture was washed with distilled water to remove the untrapped enzyme and then was air-dried. The final protein concentration in the matrix (approximately 1 mg protein per 100 mg immobilized lipase) was calculated by subtracting protein content in the water wash from the original protein supernatant concentration. The dried residue was used as the immobilized lipase preparation for this study.

Enzymatic transesterification. Grease or tallow [174 mg, 0.2 mmol: molecular weight of grease was determined by fatty acid composition in the grease (11)] was weighed into a 10-mL Teflon-stoppered Erlenmeyer flask, and then 4 mol equiv of alcohol (e.g., methanol, ethanol, 95% ethanol, *n*-propanol, isopropanol, *n*-butanol, or isobutanol) was added. Immobilized lipase PS-30 (100 mg) or free lipase PS-30 (10 mg) was then added, and reaction was started by placing the flask into an orbital water bath shaker at 50°C, 500 × *g*. For the time course studies, the reaction was scaled up fivefold and the reaction followed by taking 10-µL aliquots at selected time intervals. Each aliquot was transferred into a 16 × 125 mm screw-capped culture tube containing 3 mL of hexane/ether (1:1, vol/vol) and 1 mL of 10 wt% sodium chloride solution added. After mixing, the organic phase was removed by a pipette and passed through a column of anhydrous sodium sulfate (2 g packed in 10 × 100 mm jumbo Pasteur pipette). The eluate from the column was collected, the solvent removed under a stream of nitrogen, and the residue dissolved in hexane (1 mg/mL) for compositional analysis (12). All results are an average of two trials with triplicate determinations for each data point.

Analysis of reaction products. The percentages of alkyl esters (AE), free fatty acids (FFA), monoacylglycerols (MAG), diacylglycerols (DAG), and triacylglycerols (TAG) in reaction mixtures were determined using a high-performance liquid chromatography (HPLC) method developed in our laboratory (12). Reaction mixtures were separated on a cyanopropyl (CN) column (Phenomenex, Torrance, CA) using a binary mobile phase of hexane and methyl *t*-butyl ether, both containing acetic acid (0.4%, vol/vol), with detection by an evaporative light-scattering detector (MK III; Varex, Burtonsville, MD).

RESULTS AND DISCUSSION

Effect of lipase PS-30 concentration on transesterification of grease to alkyl ester. Various amounts of lipase PS-30 powder were dispersed in water (from 50 to 200 mg/mL), clarified by centrifugation, and the resulting supernatants used to prepare a series of phyllosilicate sol-gel immobilized lipase PS-30 preparations. The prepared matrix-immobilized enzymes were subsequently used to catalyze the transesterification of restaurant grease with 95% ethanol at 50°C for 24 h (Fig. 1). Under these conditions, the percentage conversion of grease to ethyl esters increased as the amount of free lipase used to prepare the immobilized lipase preparation approached 150 mg/mL. Increasing the amount of lipase PS-30 above 150 mg/mL did not significantly increase the transesterification activity of the immobilized lipase preparation (Fig. 1). The same immobilized lipase preparations and reaction conditions (50°C, 24 h reaction) were used to catalyze the transesterification of tallow with 95% ethanol. The most effective conversions of tallow to ethyl esters (>95% conversion) also were achieved when the amount of free lipase used to prepare the immobilized lipase preparation was between 120 and 150 mg/mL (data not shown). Thus, in both instances the highest conversion of restaurant grease or tallow to ethyl esters was obtained when the immobilized lipase preparation was prepared with the supernatant from the 150 mg/mL free lipase PS-30 dispersion.

Time course of alkyl ester production from grease. At least 4 mol equiv of 95% ethanol was required to maximize the conversion of grease to its ethyl esters using either free or immobilized lipase PS-30 (data not shown). Typical time course plots for this reaction are shown in Figure 2. For the reaction catalyzed by free lipase PS-30, the conversion approached 80% after 3 h, but after 22 h only 85% of the grease was converted to esters. Increasing the reaction time did not increase the conversion. For the immobilized lipase PS-30 reaction, the initial conversion rate was slower in that after 5 h only 70% conversion to esters had been obtained. The conversion, however, continuously increased as the reaction time increased. After 22 h reaction, >95% of the grease had been converted to ethyl esters. This suggested that the immobilized

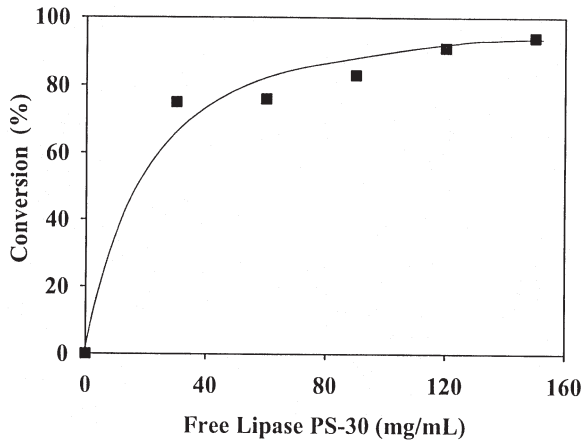

FIG. 1. Transesterification activity of phyllosilicate sol-gel immobilized lipase PS-30 as a function of the amount of free lipase PS-30 used to prepare the immobilized lipase. A mixture of grease (0.2 mmol), 95% ethanol (0.8 mmol), and immobilized lipase PS-30 (150 mg) was shaken (500 × *g*) for 24 h at 50°C. Conversion to ethyl esters determined by high-performance liquid chromatography. Percent conversion is area percent of ethyl esters peaks of total detector area. Lipase PS-30 (from *Pseudomonas cepacia*) supplied by Amano Pharmaceutical Co. (Nagoya, Japan).

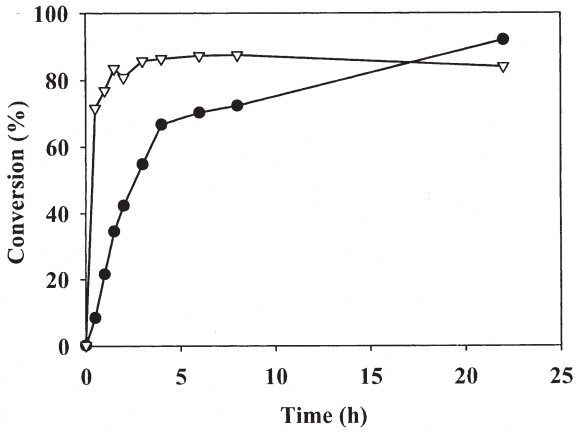

FIG. 2. Time course of reaction for lipase PS-30-catalyzed transesterification of grease to ethyl esters. Grease (0.2 mmol), 95% ethanol (0.8 mmol), and either free lipase PS-30 powder (▽, 10 mg) or immobilized lipase PS-30 (●, 100 mg) was shaken (500 × *g*) at 50°C. At selected time intervals each reaction mixture was analyzed for ester production by high-performance liquid chromatography. For manufacturer see Figure 1.

lipase PS-30 was a more effective catalyst for alkyl ester synthesis than the free lipase.

Lipase PS-30-catalyzed transesterification of grease and tallow with primary and secondary alcohols. In using solventless conditions and in the presence of molecular sieves to eliminate water as an important factor, both the free and immobilized lipase PS-30 were tested for their ability to transesterify grease and tallow to their corresponding alkyl esters with primary or secondary alcohols (Table 1). The alcohols studied included methanol, ethanol, 95% ethanol, *n*-propanol, isopropanol, *n*-butanol, and isobutanol. In general, Table 1 shows that better conversions to esters were obtained when immobilized lipase PS-30 was used as the catalyst. For example, the transesterification of grease with either primary or secondary alcohols was effective using immobilized PS-30 lipase (conversions from 84–94%), whereas under the same conditions the free lipase gave lower conversions (47–89%) and the poorest yields for methyl esters. Previous studies by Nelson *et al.* (5) indicated that the lipase-catalyzed methanolysis of oils and fats was extremely sensitive to the presence of water. In these previous experiments, we did not control the water content of the reactions, which may account for the low methyl ester yield. However, fair yields of ethyl esters were obtained with free lipase when using 95% ethanol, which indicates that methanol may inhibit the activity of the free enzyme but not the immobilized enzyme. For tallow, the free lipase PS-30-catalyzed reactions gave ester yields comparable to those obtained when grease was the substrate (Table 1). Again, the poorest conversion was for the methanolysis reaction. Previous studies on the transesterification of tallow with methanol and ethanol using free lipase PS-30 also reported low ester yields with these alcohols (6,13). On the other hand, immobilized lipase PS-30 gave good ester yields from tallow using primary alcohols (82–94% conversions) but poor conversions (32–68%) with the secondary alcohols.

TABLE 1
Free and Immobilized Lipase PS-30-Catalyzed Transesterification of Tallow and Grease with Alcohols[a]

	Alkyl esters[b,c] (%)			
	Grease		Tallow	
Alcohol	Free PS-30	Immobilized PS-30	Free PS-30	Immobilized PS-30
Methanol	47 ± 2	94 ± 3	45 ± 2	92 ± 4
Ethanol	81 ± 2	88 ± 2	76 ± 2	91 ± 3
95% Ethanol	81 ± 2	94 ± 3	85 ± 2	82 ± 2
n-Propanol	87 ± 1	87 ± 1	87 ± 2	85 ± 3
n-Butanol	89 ± 3	94 ± 2	93 ± 3	92 ± 4
Isopropanol	75 ± 2	90 ± 2	65 ± 3	32 ± 2
Isobutanol	87 ± 2	84 ± 2	75 ± 3	68 ± 3

[a]Reactions contained 2 mmol of grease or tallow, 8 mmol of alcohol, and 100 mg of immobilized lipase (PS-30, from *Pseudomonas cepacia*; Amano Pharmaceutical Co., Nagoya, Japan) or 10 mg of free lipase powder. Reactions were carried out at 50°C for 18 h in an orbital shaking bath (500 × *g*) in the presence of 0.4 wt% of molecular sieves.
[b]Percentage of alkyl esters determined by high-performance liquid chromatography as described in the Materials and Methods section.
[c]Each data point is the average of two trials with triplicate determinations.

Effect of molecular sieves on the conversion of grease to alkyl esters. Grease was transesterified with 95% ethanol at 50°C using both free and immobilized lipase PS-30 in the presence and absence of molecular sieves (Fig. 3). Molecular sieves (0.4 wt% of substrate) were added to the reaction mixtures at 0, 1, 2, 3, 4, and 6 h, and the reaction was continued for a total of 18 h. Figure 3 shows that for both the free and immobilized lipase PS-30-catalyzed reactions, conversions were higher and occurred faster in the presence of molecular sieves. For example, in reactions catalyzed by immobilized lipase PS-30, in the presence of molecular sieves added at 3 h and continued overnight, the conversion to esters reached

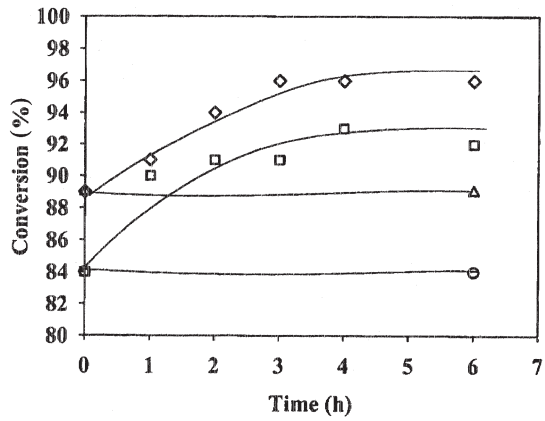

FIG. 3. Transesterification of grease catalyzed by lipase PS-30 with ethanol without and with molecular sieves. Grease (0.2 mmol) and 95% ethanol (0.8 mmol) were shaken (500 × *g*) with either free (10 mg) or immobilized lipase PS-30 (100 mg) at 50°C. After 1, 2, 3, 4, and 6 h reaction (times shown on abscissa), 0.4 wt% of molecular sieves was added and the reaction continued for a total of 18 h (□, free lipase PS-30 with molecular sieve; ○, free lipase PS-30 without molecular sieve; ◇, matrix-immobilized lipase PS-30 with molecular sieve; △, matrix-immobilized lipase PS-30 without molecular sieve). For manufacturer see Figure 1.

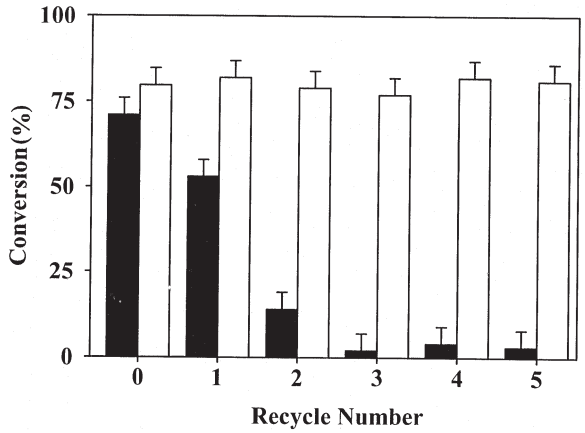

FIG. 4. Reusability of free and matrix-immobilized lipase PS-30 in the synthesis of alkyl esters of grease. Reactions were conducted with grease (0.2 mmol), 95% ethanol (0.8 mmol), and matrix-immobilized lipase PS-30 (150 mg) or free lipase (10 mg) at 50°C for 18 h. At the end of each cycle, the enzyme was recovered, washed with hexane, and dried. Fresh substrates were added to start the next reaction: free lipase PS-30 (■), immobilized lipase PS-30 (□). For manufacturer see Figure 1.

96%. In preliminary experiments, we confirmed that addition of exogenous water to these reactions was not necessary to activate the lipase. In fact, the addition of water slightly decreased the alkyl ester yields (data not shown). Thus, the addition of molecular sieves shifts the reaction equilibrium toward ester formation instead of hydrolysis. Previously, Nelson *et al*. (5) showed that an immobilized *Candida antarctica* lipase preparation did not require pretreatment with water for alcoholysis to occur. In subsequent work, Shimada *et al.* (13) showed that conversion of vegetable oil to alkyl esters using the same immobilized *C. antarctica* lipase decreased when water was added to the transesterification mixture.

Recyclability of immobilized lipase PS-30 for the transesterification of grease. The reusability of matrix-immobilized lipase PS-30 compared to the free lipase is shown in Figure 4. Grease was transesterified with 95% ethanol at 50°C in the solvent-free system for 18 h, and conversion to alkyl esters was determined by HPLC. Ester yields for each transesterification cycle are shown in Figure 4. After each reaction, both the free and immobilized lipase PS-30 catalysts were recovered, washed with hexane, and dried under nitrogen before reuse. The next cycle of transesterification was conducted with fresh reagents (grease and 95% ethanol) using the recovered enzymes. The data (Fig. 4) show that immobilized lipase PS-30 could be used at least six times without significant loss of activity, whereas free lipase PS-30 lost 50% of its initial activity when it was reused in the second cycle and after the third cycle lost most of its activity. Additional recycling studies after five were not performed, but the data from this study suggest that the immobilized lipase PS-30 prepared for this study can be reused repeatedly. Accordingly, this new immobilized lipase preparation may have potential for improving the economics of biodiesel production from alternative feedstocks, namely, recycled restaurant grease and tallow.

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