Lipase Catalyzed Formation of Fatty Amides

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Certain lipase preparations were found to facilitate the preparation of fatty amides at 20°C in hexane. Lipase preparations investigated were from the fungi *Candida rugosa, Rhizomucor miehei* and porcine pancreas. Reactants were various primary alkylamines and fatty acid methyl esters or triglycerides. Moderate yields of fatty amides were obtained using a *R. miehei* lipase preparation which is immobilized on a solid support as catalyst, although all three lipase preparations showed some catalytic activity under these conditions and, in addition, showed different kinds of selectivity for fatty acid and alkylamine chain lengths. No reaction was observed in similar experiments using one fatty acid as the substrate or one secondary amine.

KEY WORDS: *Candida rugosa*, FAME, fatty acids, fatty triglycerides, primary amines, porcine pancreas, *Rhizomucor miehei*.

The activities of lipases as catalysts are being investigated to determine their potential application for the conversion of surplus fats and oils into higher value products for food and industrial uses. Lipases usually function at an oil-water interface to hydrolyze fats to fatty acids and glycerol. Recently Zaks and Klibanov (1) reported that some enzymes retain their activity even when the amount of water is greatly reduced and that the bulk of the water can be replaced by a number of organic solvents. They found that lipases, such as those of *C. rugosa, R. miehei* [previously *Mucor miehei* (2)]and porcine pancreas (P. pancreas), may serve as catalysts for reactions in organic solvents containing a minimal amount of water. More recently, Matos and co-workers (3) described the synthesis of peptides using the lipases from *C. rugosa* and P. pancreas as catalysts in organic solvents. Various uses of lipases in non-aqueous solvents have been the subject of a recent review by Gillis (4). Also, a few reports on the enzyme-catalyzed synthesis of fatty amides in organic solvents have appeared. Montet *et al.* (5) used the lipase from *R. miehei* to catalyze the formation of N-lauryloleyl-amides from N-laurylamine and oleic acid in organic solvents at 60°C. Similarly Chinsky, *et al.* (6) observed that the amidation of the bi-functional 6-amino-1-hexanol was catalyzed by a protease, Subtilisin, Carlsberg, in tert-amyl alcohol. V. Gotor and co-workers (7) observed that Yeast lipase (*C. rugosa*) catalyzed the reaction between ethyl (±)-2-chloropropionate and different aliphatic and aromatic amines yielding optically active amides.

Large quantities of fatty amides are produced in the United States each year (41 million pounds in 1984) (8); they comprise about 10% of all fatty nitrogen compounds produced. The fatty amides have high melting points and are quite stable physically and chemically; they also possess limited solubility in common organic solvents. Many applications in the textile, paper, wood, metal, rubber, plastics and coatings industries have been found for these compounds. The fatty amides are prepared commercially at temperatures ranging from 150–200°C and at varying pressures, up to 100 psig (9). Reaction times for these amide preparations can be as long as 10 hr and the processes are energy intensive. The application of new biotechnological techniques such as lipase catalysis may offer an improvement to the manufacture of fatty amides. Starting materials such as triglycerides or fatty acid methyl esters (FAMEs) from animal fats or vegetable oils could be reacted with a range of amines to produce amides. Since the lipase and the organic solvent can be recovered for reuse, this could further reduce the production cost. Moreover, the lipase catalyzed reactions would be carried out at mild temperatures and atmospheric pressure and the equipment required would be less costly. Therefore, it would appear that lipase catalysis for the production of fatty amides could have numerous advantages over conventional methods.

This report contains the results of a preliminary study of the application of three lipase preparations to the synthesis of fatty amides from fatty acid methyl esters and N-alkylamines in hexane. The three lipase sources selected for this initial investigation were *C. rugosa* (a yeast), *R. miehei* (a fungus) and P. pancrea. The experiments were designed to estimate the selectivity of these lipases with regard to the structure of the alkyl amine and the fatty acid moieties.

EXPERIMENTAL PROCEDURES

Materials. The enzymes used were *C. rugosa*, 792 + eq/hr/mg; and P. pancreatic, 25.9 + eq/hr/mg; lipases, from Sigma Chemical Co. (St. Louis, MO); *R. miehei*, 225 + eq/hr/mg; Lipozyme IM-20, immobilized on an anion exchange resin, from Novo Nordisk Bioindustrials, Inc. (Danbury, CT).

Lipase assay. Lipase activity was measured at room temperature, according to the method of Baillargeon and Sonnet (10), at pH 7.7 instead of pH 7.3. The rate of free fatty acid release was followed by addition of 0.100 N NaOH under nitrogen using the Radiometer titration system in pH stat mode. Enzyme free blanks showed no uptake of base.

Supplies and instruments. Thin-layer chromatography (TLC) silica Gel G plates, scored 2.5 × 20 cm, 250 microns, was from Analtech, Inc. (Newark, DE). C_4-C_{16} n-acyl chlorides, ethyl, diethyl, n-butyl, n-methyl butyl, n-nonyl, n-dodecyl and n-tetradecylamines, octanoic acid, methyl myristate and docosane were from Aldrich Chemical Co. (Milwaukee, WI); while the n-hexylamine, trioctanoin, methyl butyrate and methyl octanoate were purchased from Eastman Kodak Co. (Rochester, NY). Shaker-water Bath Incubator, Precision-Dubnoff, was purchased from Thomas Scientific (Swedesboro, NJ); Fisher-Johns melting point apparatus, from Fisher Scientific Co. (Pittsburgh, PA); gas

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chromatography, Hewlett Packard Model 5890 Gas Chromatograph with automatic sampler and a Hewlett Packard integrator Model No. 3396, from Hewlett Packard Co. (Palo Alto, CA); shaker-incubator, from New Brunswick Scientific Co., Inc. (Edison, NJ); and Radiometer Titration System from Radiometer, Inc. (Copenhagen, Denmark).

Methods of synthesis. Trioctanoin was prepared as described in the literature (11). Standard fatty amides were prepared using the following general procedure. N-Nonylamine, 6.6 g (0.05 moles) was dissolved in 100 mL of methylene chloride, containing 10 mL of pyridine, and chilled to 10°C. Octanoyl chloride, 7 g (0.04 moles) was added dropwise to the amine solution over a 5 min period and stirred for 1 hr. Excess pyridine was neutralized with 4N hydrochloric acid and the organic layer washed with distilled water until neutral. The organic phase was passed through a Florisil (adsorbent magnesium silicate; Floridin Co., Berkley Springs, WV) column to remove impurities. The product was crystallized from methylene chloride solution at 0°C. Residual amounts of free fatty acid were removed by titrating an alcoholic amide solution with 0.1 N NaOH, drying and repeating the above purification procedure. N-Nonyloctanamide, 7.5 g (m.p. 49.0–49.5°C) was obtained in 70% yield, infrared: NH absorption at 3300 cm^{-1}, amide absorption at 1638 cm^{-1} and 1548 cm^{-1}. Purity was ascertained by TLC. Eighteen amide standards were prepared according to this procedure.

Small scale lipase catalyzed reactions were carried out by mixing 0.5 mL of a 0.1 M solution of fatty acid ester (in hexane containing 0.02% water by volume) and 0.5 mL of a 0.1 M solution of amine (same solvent) with 100 mg of lipase preparation suspended in 0.5 mL of the same solvent in a 15 × 100 mm test tube which was then sealed and shaken at 100 rpm in an incubator at 20°C for 72 hr. Amide formation was monitored by TLC and quantitated by comparison with standards. N-Nonylamine was reacted with methyl octanoate under these conditions both in the presence and absence of lipase and no amide formation was observed in the latter case.

Large scale preparation of n-butylpalmitamide was carried out as follows: Methyl palmitate, 5.0 g (0.018 moles) and n-butylamine, 1.5 g (0.019 moles) were dissolved in 75 mL of the hexane and 25 g of *R. miehei* lipase preparation, was added. The mixture was shaken at 100 rpm and room temperature for 5 days. The amide appeared as a white coating on the surface of the catalyst. After filtration, the amide was removed from the catalyst by washing with warm hexane washing. n-Butylpalmitamide (4.3 g) was obtained, 80% yield (m.p. 72–72.5°C). I.R. was NH absorption at 3300 cm^{-1}, amide absorption at 1640 cm^{-1} and 1548 cm^{-1} cm^{-1}. Anal. calc. for $C_{20}H_{41}$ NO: C = 77.10, H = 13,27, N = 4.50. Found C = 76.80, H = 13.33, N = 4.36. The reaction was repeated with the recovered lipase and produced 4.8 g of n-butylpalmitamide, 85% yield, (m.p. 72–72.5°C).

Analysis by thin-layer chromatography. The lipase was removed from the reaction mixtures by filtration through Whatman No. 2 filter paper, and the residue was rinsed with three 5 mL washings of hot acetone. The samples were concentrated at room temperature under N_2 to 1/3 volume, and then diluted with 1 mL hexane. The silica plates were prewashed with the developing solvent mixtures. The plates were spotted with 5–10 μL of sample and developed sequentially with solvent A containing benzene, diethyl ether, ethyl acetate and acetic acid (80:10:10:1, v/v/v/v) for 8 cm, dried under nitrogen and then solvent B containing hexane, diethyl ether and formic acid (80:20:2, v/v/v) for 13 cm, followed by drying. Visualization of sample spots was accomplished by spraying the plates with 60% sulfuric acid and charring on a hot plate. R_f values of fatty amides were then compared to those of known standards for identification.

The amide spots were quantitated by preparative TLC on 500 μm thick silica plates. The weighed sample was dissolved in ethyl acetate/methanol (80:20, v/v) and applied to the plate by means of a streaker Applied Science Laboratories, Inc. (State College, PA). The plates were then developed in solvent A and solvent B according to the above procedure. Upon drying, the plate was sprayed with a 0.1% ethanolic solution of 2,7-dichlorofluorescein which permitted detection of the amide band under UV light. The band was scraped from the plate and extracted with warm ethyl acetate/methanol (80:20, v/v) into preweighed vials. The solvent was removed under N_2 and the vials were reweighed to determine amide yield. All analyses were run in duplicate and averages are reported.

To determine the limits of the TLC procedure, the relatively volatile, low mol. wt. amide standards (i.e., ethylbutyramide, n-butylhexanamide and n-butyloctanamide) were examined and minimal losses (ca. 2–4%) were found. Complete recovery of silica gel-free products were obtained with higher molecular weight, standard, fatty amides.

RESULTS AND DISCUSSION

The lipases from *R. miehei, C. rugosa (Cylindracea)* and P. pancreas, due to their ready availability, were selected for a study of lipase catalyzed amidation. In order to determine the effect of different fatty chemical structures, octanoic acid, methyl octanoate and trioctanoin were reacted with n-nonylamine in the presence of lipase and the yield of n-nonyloctanamide determined (see Table 1). Conditions similar to those of Zaks and Klibanov (1) were used in these experiments, except that the shaking speed was reduced to 100 rpm. We found that no amidation occurred in the experiments in which octanoic acid was the fatty substrate. However, amidations took place in reactions involving the other two fatty substrates. Under these experimental conditions, a larger amide yield was obtained from methyl octonoate than from trioctanoin when the lipases from *R. miehei* and P. pancreas were used as catalysts. The *R. miehei* lipase was mounted on a support which facilitated its recovery and reuse. Since the reactions, catalyzed by *R. miehei* lipase, produced higher amide yields than those from *C. rugosa* and P. pancreas, we restricted further experimentation to the *R. miehei* lipase.

Table 2 gives the results of the preparation of a series of amides of increasing molecular weight. Long

TABLE 1

Lipase Catalyzed Production of N-Nonyloctanamide

Lipase	Reaction	% Amide yield[c]
Rhizomucor miehei	*a*	46.4
	b	22.2
Candida rugosa	*a*	18.8
	b	18.8
Porcine pancreas	*a*	27.3
	b	13.1

[a]Methyloctonoate + n-Nonylamine + *lipase* → N-Nonyloctanamide.
[b]Trioctanoin + n-Nonylamine + *lipase* → N-Nonyloctanamide.
[c]By preparative TLC.

TABLE 2

***R. miehei* Catalyzed Formation of Amides**

Amide	Yield (%)
Nonylbutyramide	4
Tetradecylbutyramide	9
Butylhexanamide	1
Dodecylhexanamide	8
Butyloctanamide	51
Ethyldecanamide[a]	21
Butyldecanamide	51
Nonyldecanamide	13
Tetradecyldecanamide	18
Butyllauramide	50
Butylmyristamide	51
Dodecylmyristamide	18
Ethylpalmitamide[a]	27
Butylpalmitamide	61
Hexylpalmitamide	45
Nonylpalmitamide	17
Dodecylpalmitamide	10
Tetradecylpalmitamide	15

[a]The ethylamine used contained 25% by volume of water.

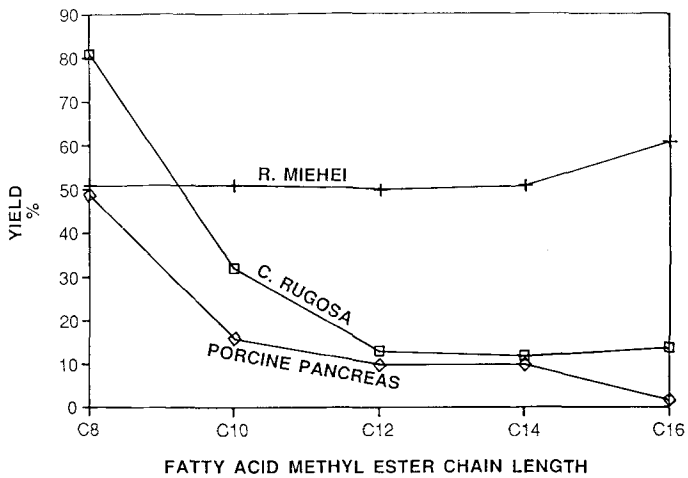

FIG. 1. Reaction of butylamine with fatty acid esters of varying chain length catalyzed by lipase preparations of *C. rugosa*, *R. miehei* and P. pancreas.

chain amides > 275 molecular weight were difficult to measure by GLC, so TLC was used to detect and quantify these amides. About 97% of the measured amounts of known fatty amides were recovered by this method.

It can be seen from the amide yields reported in Table 2 that reaction occurred in all of the combinations of amines and fatty acid methyl esters, and that up to 50% of certain amides were formed. These small scale reactions did not provide optimum conditions for amide formation; however, some trends are apparent. The apparent lack of selectivity by the *R. miehei* lipase preparation for fatty acid chain length from octanoic to palmitic was compared with that of the *C. rugosa* and P. pancreas lipase preparations using n-butylamine (Fig. 1). The same weights (100 mg) of the three lipase preparations were used here even though all three had quite different relative activites (see Experimental Procedures section) and the results have not been corrected for the different relative activities. Obviously, as the chain length of the fatty acid methyl ester increased, the corresponding amide yields dropped dramatically in the reactions catalyzed by the *C. rugosa* and P. pancreatic lipase preparations. As noted above, the *R. miehei* lipase preparation gave moderate yields of amide throughout, and apparently was not affected by the chain length of the fatty substrate.

We attempted to determine the preference of the *R. miehei* lipase preparation for primary *vs.* secondary amines by reacting methyl palmitate with ethylamine and diethylamine. However, the ethyl amine contained 25% water by volume and the yield of amide was suppressed by competition of the water with the amine for the acyl lipase complex. TLC of the reaction mixture indicated substantial conversion of the methyl palmitate to palmitic acid. Nevertheless, the measured yield of ethylpalmitamide was about 35%, indicating that the lipase preparation is quite active for this amine, too. The lipase preparation did not catalyze any amide formation with diethylamine and methyl palmitate; however, due to a small amount of water present here (the diethylamine contained about 2% water by volume) some hydrolysis of the ester occurred. Similar results were observed with N-methylbutylamine and triethylamine.

n-Butylpalmitamide was selected for a larger scale synthesis catalyzed by the *R. miehei* lipase preparation because we wished to determine the isolatable yield of a representative amide and to obtain a sufficient quantity for complete characterization. N-butylamide has poor solubility in hexane and rapidly drops out of solution as it forms. It can be readily purified by crystallization. An 80% isolated yield of essentially pure amide was obtained with relatively less lipase preparation than used in the small scale experiments, although the reaction was run for 5 days instead of the usual 3. It is important to note in that experiment the *R. miehei* lipase preparation could be recovered, washed and reused to give an 85% yield of n-butylpalmitamide.

It would appear that these lipases may be used to produce a wide variety of fatty amides. The three lipase preparations investigated showed different degrees of activity and selectivity. Only one solvent was

investigated; others may show different results. The experience with the ethylamine containing 25% by volume water indicates that nucleophilic reagents compete for the acyl-enzyme complex, and that a selectivity may be controlled. A great advantage is expected in thermally-sensitive reactions since high amide yields were obtained at 20°C. The lightly colored fatty amides were readily separated by filtration from the enzymes which, at least in one case, were recovered for reuse. Further investigation is required to determine the full potential of these lipase catalyzed amide reactions.

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