# Application of Potato Lipid Acyl Hydrolase for the Synthesis of Monoacylglycerols

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**ABSTRACT:** Protein extracts from potato tubers contain a lipid acyl hydrolase (LAH) with an unusual selectivity. The component responsible for the enzyme activity is a group of closely related glycoproteins, known as patatin. Potato LAH catalyzes the rapid hydrolysis of monoacylglycerols (MAG), but in contrast expresses only low activity with di- and triacylglycerols. The selectivity of the LAH can be exploited for the synthesis of MAG from fatty acids and glycerol in microaqueous reaction systems. Oleic, linoleic, linolenic, capric, lauric, and myristic acids can be used as reactants, and in each case the reaction products contain >95 mol% MAG and <5 mol% diacylglycerol. By removing water from the reaction mixture by distillation under vacuum, excellent conversions of fatty acids into MAG are possible. Low conversions are observed with palmitic and stearic acids, because of the necessity to use a high temperature (70°C) to maintain a liquid reaction mixture. Potato LAH is rapidly inactivated at 70°C in the microaqueous reaction systems. Potato LAH also catalyzes the selective synthesis of monoesters from oleic acid and simple diols. With oleic acid and diglycerol, monoesters are again the main reaction products, but 17 mol% diesters are also formed.

*JAOCS 75*, 1489–1494 (1998).

**KEY WORDS:** Diglycerol, diol, fatty acid, glycerol, lipase, lipid acyl hydrolase, monoacylglycerol, patatin, potato.

Protein extracts from potato tubers contain a highly active lipid acyl hydrolase (LAH) (1,2). The component responsible for this enzyme activity is a family of closely related glycoproteins known as patatin (3,4). These glycoproteins constitute 20–40% of the total soluble protein or approximately 0.3% of the wet weight of the tubers (5,6). The substrate specificity of potato LAH is unusual. It catalyzes the rapid hydrolysis of monoacylglycerols (MAG), phospholipids, and galactolipids, while it has very low activity with diacylglycerols (DAG) and triacylglyerols (TAG) (4). Potato LAH can also express esterification and transesterification or acyl transferase activities. For example, synthesis of fatty acid alkyl esters was observed on incubation of potato protein extract with alcohols and either free fatty acids (FFA) or phospholipids (1,7). Because of its unusual specificity, potato LAH may be an effective catalyst for the synthesis of MAG. Under low water conditions reaction of glycerol with FFA to give MAG should be rapid in comparison with the subsequent conversion of MAG to DAG, leading to an accumulation of high levels of MAG in the reaction mixture.

MAG are an important group of emulsifiers used in food and personal-care products. They are usually produced from oils and fats by a high-temperature base-catalyzed glycerolysis reaction. The product from this reaction is a mixture of MAG, DAG, and TAG, and the >90% MAG required for many applications is isolated from the mixture by molecular distillation (8). There is an interest in developing alternative low-temperature enzyme-catalyzed processes for the production of "natural" MAG, particularly if high yields of pure MAG can be obtained without the necessity of using molecular distillation. Lipase-catalyzed syntheses of MAG have been reviewed by Bornscheuer (9). MAG can be synthesized from glycerol and FFA using microbial lipases, but the yields obtained are usually unacceptably low. High yields of MAG can be produced by lipase-catalyzed esterification of protected glycerol with FFA, but this route is complex, involving protection and deprotection of the glycerol moiety and the use of unnatural chemicals. High yields of MAG can also be obtained by lipase-catalyzed glycerolysis of fats and oils or fatty acid esters under controlled temperature conditions, the reactions being directed toward MAG formation by selective crystallization of MAG from the reaction mixtures (10–12). However, these glycerolysis reactions are slow, and high levels of lipase are required to achieve acceptable reaction rates.

In this paper we report the results of an investigation of the catalytic properties of potato LAH and show that the enzyme is an effective catalyst for the synthesis of MAG from glycerol and various fatty acids.

## EXPERIMENTAL PROCEDURES

*Materials.* Potato tubers (variety: Santé) were donated by the National Institute of Agricultural Botany, Cambridge, United Kingdom.

Oleic acid (90%), ethane diol, 1,2-propane diol, and 1,3-propane diol were obtained from Aldrich Chemical Company (Gillingham, United Kingdom). Capric acid, palmitic acid, tributyrin, and gum arabic were purchased from Fluka Chemicals (Gillingham, United Kingdom). Myristic acid (Prifrac

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2942), glycerol (Pricerine 9098), diglycerol and 6-*O*-lauroyl ethylglucoside were donated by Unichema International (Gouda, The Netherlands). Other fatty acids, fatty acid esters, acylglycerols, insoluble polyvinyl polypyrrolidone, bovine serum albumin, trimethyl-chlorosilane, and bis-trimethylsilylacetamide were obtained from Sigma Chemical (Poole, United Kingdom). Octyl-sepharose CL-4B was purchased from Pharmacia (St. Albans, United Kingdom). Other chemicals were supplied by Fisher Scientific International (Loughborough, United Kingdom).

*Preparation of potato protein extract.* Potato tubers were washed, peeled, and diced into pieces, which were immersed immediately into water containing 0.01% sodium metabisulfite and a suspension of 10% insoluble polyvinyl polypyrrolidone (PVPP). After draining off the aqueous mixture, the potato pieces were put into plastic bags and stored frozen at –18°C.

An aliquot of the potato pieces (1.6 kg) was allowed to thaw and homogenized in a Waring blender at 4°C for 1 min with 1.5 L of 100 mM sodium phosphate buffer (pH 7.0) containing 0.02% sodium metabisulfite and 1% insoluble PVPP. The homogenate was filtered through three layers of cheesecloth, and the filtrate centrifuged at 8,000 × *g* for 30 min. The supernatant (2.7 L) was dialyzed overnight at 4°C against 10 L of deionized water. The resulting dialyzed mixture was centrifuged at 18,000 × *g* for 1 h, and the supernatant was shell frozen and reduced to a volume of approximately 100 mL by freeze-drying. After thawing, the concentrated solution was centrifuged at 18,000 × *g* for 1 h, and the supernatant was shell frozen and freeze-dried to give the potato protein extract as a powder (7.2 g) containing approximately 50% protein.

*Purification of LAH.* Protein extraction and purification were carried out at 4°C. Potato tubers (400 g) were washed, diced, and immediately immersed in 400 mL of 25 mM potassium phosphate buffer, pH 7.0, containing 0.038% sodium metabisulfite and 1% insoluble PVPP. The mixture was then homogenized for 1 min using a Waring blender, and the homogenate was filtered through three layers of cheesecloth. The filtrate was centrifuged at 10,000 × *g* for 30 min. Ammonium sulfate was added to the resulting supernatant to give 40% saturation, and the mixture was centrifuged at 10,000 × *g* for 45 min. The pellet was discarded, and further ammonium sulfate was added to the supernatant to give 70% saturation. The mixture was centrifuged at 10,000 × *g* for 45 min and the supernatant discarded. The pellet was suspended in 70 mL of 35 mM potassium phosphate buffer containing 10% ammonium sulfate, and the suspension was centrifuged at 20,000 × *g* for 10 min. The resulting pellet was discarded and the supernatant used for further purification of the LAH by hydrophobic interaction chromatography.

An octyl-sepharose CL-4B column (2.5 × 10 cm) was equilibrated with 5 bed vol of 25 mM potassium phosphate buffer, pH 7.0, containing 10% ammonium sulfate at a flow rate of 3 mL/min. The supernatant (65 mL) was applied to the column, which was then washed with a further 3 bed vol of the same buffer again at 2 mL/min. LAH was eluted from the column with distilled water using the same flow rate.

*Protein determination.* Protein concentration was measured using BioRad (Hemel Hempstead, United Kingdom) protein assay reagent according to the manufacturer's instructions, with bovine serum albumin as a protein standard.

*LAH activity assays.* Emulsions of 0.5% acylglycerol or fatty acid ester in water containing 2% gum arabic and 0.5% calcium chloride were prepared by sonication for 3 min using a Bandolin (Berlin, Germany) HD200 Sonopuls homogenizer. To determine LAH activity with the various substrates, 20 mL of emulsion was stirred in the reaction vessel of an Autotitrator (VIT90; Radiometer, Copenhagen, Denmark) maintained at 40°C, and the pH of the emulsion was raised to 8.0 by addition of sodium hydroxide solution. Enzyme was added and release of fatty acid measured at constant pH 8.0 by autotitration with 0.1 N sodium hydroxide. For each substrate, a control was run using LAH inactivated by heating at 100°C for 5 min. One unit of LAH released 1 μmol of fatty acid in 1 min.

*LAH-catalyzed acylglycerol synthesis.* In a typical reaction a mixture of oleic acid (1.5 g; 5.32 mmol), glycerol (0.57 g; 6.2 mmol), water (70 mg), and potato protein extract (100 mg) was stirred at 50°C in a stoppered tube. Samples were taken periodically from the reaction mixture for analysis by gas–liquid chromatography (GC) and thin-layer chromatography (TLC). For GC analysis the weighed samples (approximately 50 mg) were dissolved in 2 mL of a 1:1 mixture of acetone and pyridine containing 5 mg/mL of tridecanoic acid internal standard. Aliquots (100 μL) the resulting solutions were derivatized by addition of pyridine (300 μL) and 100 μL of a 9:1 mixture of bis-trimethylsilylacetamide and trimethylchlorosilane. The silylated lipids were analyzed by GC using a 10 m × 0.53 mm capillary column coated with a 0.25 μm film of 5% phenyl polysiloxane. A temperature program of 100 to 300°C at 15°C/min was used. The FFA, MAG, DAG, and glycerol components of the samples were quantified against the internal standard using response factors calculated from GC of derivatized pure samples of the individual components. For TLC analysis, samples (approximately 5 mg) were dissolved in dichloromethane (0.3 mL). Small aliquots of the resulting solutions were applied to silica gel TLC plates which were then developed using a mixture of petroleum ether (84%), diethyl ether (15%), and formic acid (1%). The developed plates were sprayed with a 1% solution of phosphomolybdic acid in 1:1 ethanol/water mixture, then heated for 5 min at 120°C to visualize the acylglycerols. Initial reaction rates were calculated from the amount of acylglycerols formed during the first 2 h of reaction. When required, water was removed from the water mixture by application of a vacuum (<50 mbar) to the headspace of the reaction tube using an oil pump.

*LAH-catalyzed diol ester synthesis.* Mixtures of oleic acid (2.82 g; 10 mmol), diol (10 mmol), and water (50 mg) were stirred in stoppered tubes at 30°C with potato protein extract (200 mg). After 6 h the reaction mixtures were analyzed by GC using the method for acylglycerols described above.

*LAH-catalyzed diglycerol ester synthesis.* A mixture of diglycerol (0.9 g; 5.42 mmol), oleic acid (1.45 g; 5.14 mmol),

and water (70 mg) was stirred at 50°C with potato protein extract (100 mg). A vacuum (<50 mbar) was applied to the headspace of the reaction tube using an oil pump. After 48 h the reaction mixture was analyzed by GC using the method described above.

## RESULTS

*Protein extraction from potato tubers.* Protein was extracted by homogenizing diced tubers in buffer containing sodium metabisulfite and PVPP. To obtain crude protein extracts for use as catalysts for MAG synthesis, the homogenates were filtered, dialyzed, and freeze-dried. More than 50% of the total soluble protein in the tubers was recovered in the freeze-dried powders, which had an LAH activity of approximately 50 U/mg assayed with monoolein.

For protein purification, homogenate was filtered, centrifuged, and subjected to ammonium sulfate fractionation. Approximately 50% of the LAH activity was recovered in the 40–70% ammonium sulfate fraction (Table 1). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed that both the crude homogenate and the ammonium sulfate fraction contained proteins with molecular weights (MW) of approximately 40 kDa as major components. Patatin proteins are known to have MW of approximately 40 kDa (13).

*LAH purification.* A recent publication reported that patatin could be separated from other enzymes in potato protein extracts by hydrophobic interaction chromatography (14). Therefore, this technique was used to purify LAH from potato tubers. A peak of protein with LAH activity was eluted from octyl-sepharose with distilled water (Fig. 1). The active fractions from this peak were collected, and SDS–PAGE of protein from the combined fractions showed two major bands with MW of approximately 40 kDa contaminated by much smaller amounts of lower-MW proteins. The two major bands from an SDS-PAGE gel were electroblotted onto paper and sequenced, the first 24 amino acids of the stronger band and the first 10 amino acids of the weaker band being determined. The *N*-terminal sequences were identical to each other and to published patatin sequences (15). This confirms that the two 40 kDa proteins are patatin-like, and supports previous work showing that patatin expresses LAH activity (3,4).

The specific LAH activity of the partially purified protein was 308 U/mg (Table 1). Because at least 20% of the soluble protein of potato tubers is known to be patatin, the 11-fold increase in specific activity observed on partial purification of the protein is larger than expected. Possibly the protein is activated during purification by removal of an LAH inhibitor. The high specific activity of freeze-dried potato protein extract (approximately 120 U/mg protein) in comparison with the crude homogenate suggests that the inhibitor is a low-MW substance which can be separated from LAH by dialysis.

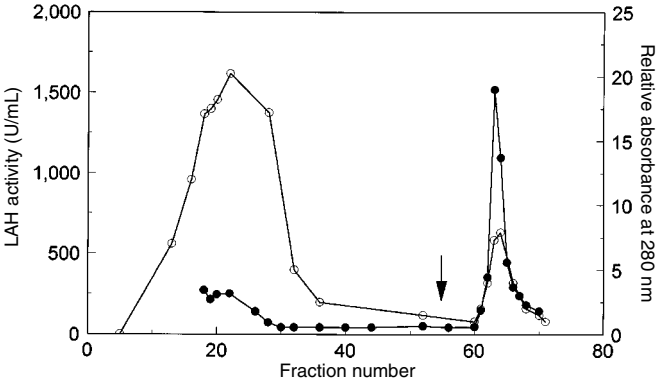


**FIG. 1.** Octyl-sepharose chromatography of potato protein. Chromatographic conditions are described in the Experimental Procedures section. Lipid acyl hydrolase (LAH) activity was assayed using monoolein emulsions. Fraction size = 4 mL. The arrow indicates the switch to elution with water. (●), LAH activity; (○), absorbance at 280 nm.

*Substrate specificity of LAH.* The hydrolytic activity of freeze-dried potato protein extract was examined using various substrates. Under the assay conditions used, activities >0.5 U/mg extract could be detected. High hydrolytic activity was observed with MAG and the glycolipid, 6-*O*-lauroyl ethylglycoside, but very low activity was obtained with diolein, TAG, and fatty acid alkyl esters (Table 2). These results confirm that potato LAH is selective for the hydrolysis of MAG and other polar fatty acid esters.

The effect of LAH purification on the relative rates of hydrolysis of monoolein, diolein, triolein, and methyl oleate was investigated. In this case, when appropriate, higher concentrations of enzyme were used in the assays, and activities >0.05 U/mg protein could be detected. The results given in Table 3 show that, on purification, the enzyme became more selective for hydrolysis of monoolein, and the relative rates of hydrolysis of diolein, triolein, and methyl oleate observed

**TABLE 1**
**Purification of Lipid Acyl Hydrolase (LAH) from Potato Tubers**

| | Total protein (mg) | Total activity[a] (U) | Specific activity (U/mg) | Recovered activity (%) |
|---|---|---|---|---|
| Crude homogenate | 2045 | 55,800 | 27.3 | — |
| 40–70% Ammonium sulfate fraction | 444 | 27,001 | 60.3 | 48 |
| Octyl-sepharose peak | 24 | 7,400 | 308.3 | 13 |

[a]Activities were determined using monoolein emulsion as substrate.

**TABLE 2**
**Substrate Specificity of LAH in Potato Protein Extract[a]**

| Substrate | Activity (U/mg powder) | Relative activity |
|---|---|---|
| Monoolein | 65.0 | 100 |
| Diolein | <0.5 | <1 |
| Triolein | <0.5 | <1 |
| Monolaurin | 6.5 | 10 |
| Monocaprin | 40.8 | 62.8 |
| Tributyrin | <0.5 | <1 |
| Methyl oleate | <0.5 | <1 |
| Butyl oleate | <0.5 | <1 |
| 6-*O*-Lauroyl ethylglucoside | 28.0 | 43.1 |

[a]For abbreviation see Table 1.

**TABLE 3**
**Effect of Purification on Substrate Specificity of Potato LAH**

| Substrate | Relative activity[a] | | |
|---|---|---|---|
| | Crude homogenate | 40–70% Ammonium sulfate fraction | Octyl-sepharose peak |
| Monoolein | 100 | 100 | 100 |
| Diolein | 1.0 | 0.5 | 0.1 |
| Triolein | 0.4 | 0.3 | <0.1 |
| Methyl oleate | 0.6 | 0.4 | <0.1 |

[a]In each case activities are expressed relative to monoolein (=100). The specific activities observed with monoolein are given in Table 1. For abbreviation see Table 1.

with partially purified enzyme were extremely low. Possibly a nonselective lipase/esterase, which was present at low levels in the crude homogenate, was removed during LAH purification.

*LAH-catalyzed acylglycerol synthesis.* Freeze-dried potato protein extract was stirred at 50°C in a sealed vessel with a mixture of oleic acid and glycerol (molar ratio 1:1.17) containing 3.4% added water. The progress curve for the reaction shows that the major product was MAG, with only a low level of DAG synthesized even on prolonged incubation (Fig. 2). Examination of the final reaction product by TLC showed that TAG were not formed. Because LAH-catalyzed MAG synthesis is a reversible reaction, removal of water generated by the reaction should increase the product yield. Therefore, a second reaction was run in which water was removed from the reaction mixture by application of a vacuum to the headspace of the reaction vessel. As expected, an improved yield of MAG was obtained (Fig. 2). Only a low level of DAG was formed, and >90% of the oleic acid was converted into acylglycerols.

The effects of reaction temperature and added water on LAH-catalyzed MAG synthesis were investigated. The results given in Table 4 show that 50°C is the preferred reaction temperature. The initial reaction rate increased with increasing temperature up to 60°C, but the greatest final MAG yield was obtained at 50°C, suggesting that significant enzyme inactivation occurred during reaction at 60°C. At 70°C enzyme inactivation was rapid, and only a small amount of MAG was produced. At all temperatures only low levels of DAG were synthesized. Addition of water to the reaction mixture had little effect on the reaction rate. The reactants and protein extract were not rigorously dried before use, and the small amounts of water present in these materials were sufficient for expression of catalytic activity by the potato protein extract.

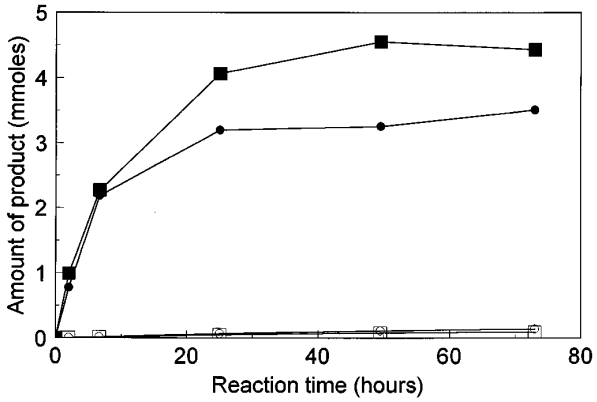


**FIG. 2.** Acylglycerol synthesis catalyzed by potato protein extract. Oleic acid (5.32 mmol) and glycerol (6.2 mmol) were reacted as described in the Experimental Procedures section. Products formed by reaction in a stoppered tube: (●), MAG; (○), DAG. Products formed with water removal under vacuum: (■), MAG; (□) DAG. MAG, monoacylglycerol; DAG, diacylglycerol; for other abbreviation see Figure 1.

**TABLE 4**
**Effect of Temperature and Added Water on LAH-Catalyzed MAG Synthesis[a]**

| Temperature (°C) | Added water (mg) | MAG formed | | |
|---|---|---|---|---|
| | | 6.5 h (mmol) | 24 h (mmol) | 48 h (mmol) |
| 40 | 0 | 1.00 | — | — |
| 40 | 70 | 1.05 | 2.55 | 2.76 |
| 50 | 0 | 1.57 | 3.52 | — |
| 50 | 70 | 2.18 | 3.20 | 3.51 |
| 60 | 70 | 2.89 | 3.10 | 2.92 |
| 70 | 70 | 0.48 | 0.52 | 0.48 |

[a]Mixtures of oleic acid (5.32 mmol), glycerol (6.2 mmol), and various amounts of water were stirred with potato extract (100 mg) at various temperatures. MAG, monoacylglycerol; for other abbreviation see Table 1.

Various saturated and unsaturated fatty acids were tested as reactants for LAH-catalyzed acylglyerol synthesis. Where possible a reaction temperature of 50°C was used, but, because of their melting points, higher temperatures had to be used for myristic, palmitic, and stearic acids to give a liquid reactant mixture. The results given in Table 5 show that the reactions run at 50°C with capric, lauric, and $C_{18}$ unsaturated fatty acids had similar initial rates and produced substantial amounts of MAG after 6 h of reaction. The reaction run at 60°C with myristic acid had a higher initial rate and produced more MAG after 6 h. At 70°C with palmitic and stearic acids, measurement of initial reaction rates was not possible because of rapid catalyst inactivation, and much lower amounts of

**TABLE 5**
**Acylglycerol Synthesis from Various Fatty Acids[a]**

| Fatty acid | Temperature (°C) | Initial reaction rate (μmol/min/g/extract) | Products formed after 6 h | |
|---|---|---|---|---|
| | | | MAG (mmol) | DAG (mmol) |
| Capric | 50 | 310 | 1.40 | 0.02 |
| Lauric | 50 | 241 | 1.65 | 0.05 |
| Myristic | 60 | 445 | 3.13 | 0.04 |
| Palmitic | 70 | — | 0.91 | 0.02 |
| Stearic | 70 | — | 0.17 | <0.01 |
| Oleic | 50 | 214 | 1.50 | 0.04 |
| Linoleic | 50 | 217 | 1.59 | 0.03 |
| Linolenic | 50 | 254 | 2.53 | 0.03 |

[a]Mixtures of fatty acid (5.0 mmol), glycerol (6.1 mmol), and water (70 mg) were stirred with potato protein extract (50 mg) at various temperatures. DAG, diacylglycerol; for other abbreviation see Table 4.

**TABLE 6**
**LAH-Catalyzed Diol Ester Synthesis[a]**

| Diol | Monoester formed (mmol) | Diester formed (mmol) |
|---|---|---|
| Ethane diol | 2.78 | 0.13 |
| 1,2-Propane diol | 4.49 | <0.02 |
| 1,3-Propane diol | 3.90 | 0.04 |

[a]Mixtures of oleic acid (10 mmol), diol (10 mmol), and water (50 mg) were stirred with potato extract (200 mg) for 6 h. For abbreviation see Table 1.

MAG were produced. With all the fatty acids only small amounts of DAG were formed in the reaction mixtures.

*Synthesis of esters from diols and diglycerol.* Equimolar mixtures of oleic acid and ethane diol, 1,2-propane diol, or 1,3-propane diol containing 1.4% added water were stirred at 50°C with 5.5% potato protein extract. Some monoester formation was observed in the early stages of the reactions, but the rates of ester production rapidly decreased on more prolonged incubation, suggesting that LAH was inactivated at 50°C in the presence of the diols. Therefore, a second series of reactions was run at 30°C with oleic acid and the diols. The results given in Table 6 show a 30–45% conversion of oleic acid into diol monoesters after 6 h of reaction. Only small amounts of diesters were formed in the reaction mixtures.

A mixture of oleic acid and a 5% molar excess of diglycerol was stirred with 4.3% potato protein extract at 50°C. A vacuum was applied to the headspace of the reactor and the reaction was run for 48 h. Extensive esterification occurred with the formation of diglycerol monoesters (2.60 mmol), some diglycerol diesters (0.56 mmol), and a small amount of MAG (0.18 mmol). The overall conversion of oleic acid into esters was 76%. MAG was produced from the low level of glycerol (4%) present in the diglycerol reactant.

## DISCUSSION

The data presented in this paper show that potato tuber LAH is an effective catalyst for the synthesis of MAG from FFA and glycerol in a microaqueous reaction system. A range of common fatty acids can be used as reactants, although problems arise with the longer-chain ($>C_{14}$) saturated fatty acids because of the necessity to use a high reaction temperature (70°C) to melt the fatty acids. At 70°C potato LAH is rapidly inactivated in the microaqueous reaction mixtures. By using potato protein extract as catalyst with approximately equimolar amounts of fatty acid and glycerol, the reaction products are >90% MAG. Similar selectivity has never previously been reported for MAG synthesis catalyzed by lipolytic enzymes. By removing water from the reaction mixture, excellent conversion of fatty acid into pure MAG can be achieved.

Immobilization of lipolytic enzymes can improve their activity and stability in microaquoeus reaction systems (16). Immobilization of potato LAH onto a porous support material might increase its stability, allowing efficient synthesis of MAG from the high-melting-point saturated fatty acids. Successful immobilization of the protein will also enable the catalyst to be recovered and recycled for preparation of multiple batches of MAG.

LAH is present in potato tubers at a high concentration (approximately $250 \times 10^3$ U/kg) and is potentially available in large quantities, for example as a by-product of potato starch manufacture. Production of the enzyme by fermentation using a genetically engineered yeast or mold should also be possible. LAH produced by this route would not be contaminated by other lipases/esterases, which may be present in potato protein extracts. Consequently, the cloned LAH might express improved selectivity in MAG synthesis giving higher yields of purer MAG products.

Potato LAH catalyzed the synthesis of esters of simple diols such as ethane and propane diols, and the enzyme was highly selective, giving reaction products containing >90% monoesters. With the tetraol diglycerol, some selectivity for monoester synthesis was observed, but approximately 25% diesters were present in the reaction products. Possibly the enzyme loses selectivity for monoester synthesis if, as in diglycerol, some of the polyol hydroxyl groups are separated by more than two carbon atoms. Potato LAH catalyzed rapid hydrolysis of the glycolipid, 6-*O*-lauroylethylglucoside. It will be of interest to examine the effectiveness of the enzyme as a catalyst for the synthesis of this ester and other glycolipid surfactants.

## ACKNOWLEDGMENTS

To Roy Dobb for help with analysis of reaction products, and to Amanda Hellyer for advice on protein purification and characterization techniques.

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[Received June 1, 1998; accepted August 19, 1998]