# Fatty Acid and Product Selectivities of Potato Tuber Lipid Acyl Hydrolase in Esterification Reactions with Glycerol in Organic Media

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**ABSTRACT:** Fatty acid [FA; butanoic ($C_4$); octanoic ($C_8$); tetradecanoic ($C_{14}$); and *cis*-9,12-octadecadienoic ($C_{18:2}$) acids] reaction selectivity and the corresponding acyl profiles in differentially accumulating acylglycerol (AG) products (mono-, di-, and triacylglycerols; MAG, DAG, TAG, respectively) were evaluated for Celite™-immobilized potato tuber lipid acyl hydrolase (LAH)-mediated esterification reactions in isooctane at 35°C and water activity of 0.19. The ordinal pattern of FA selectivities was $C_8 > C_{14} > C_{18:2} > C_4$, and the AG products accumulating were α-MAG > DAG > β-MAG > TAG. A dimensionless expression for fatty acid partitioning coefficient (FAPC) was contrived to represent the partitioning patterns of specific FA into specific AG pools on the basis of an equivalent extent of FA reaction. These FAPC values indicated that preferential partitioning of FA was as follows: $C_4$ was preferentially partitioned into TAG, DAG, and β-MAG; $C_8$ was preferentially partitioned into DAG; $C_{14}$ was preferentially partitioned into α,β-MAG; $C_{18:2}$ was preferentially partitioned into α,β-MAG and TAG. These findings infer that the tendency for LAH-mediated esterifications to accumulate MAG is based, in part, on a constraint in reactivity of α-MAG of ≥10 acyl carbon groups to serve as acceptors for further esterification events. The general approach taken in this study may assist in identifying the discrete steps in assembling structured glycerides where different biocatalysts exhibit the greatest degree or control of reaction selectivity.

Paper no. J9264 in *JAOCS 76,* 1119–1125 (October 1999).

**KEY WORDS:** Acylglycerols, esterification, fatty acid, lipid acyl hydrolase, microaqueous, potato tuber lipase, selectivity.

Interest in using biocatalysts to synthesize or modify fatty acyl esters has intensified over the last decade (1,2) as an extension of the maturing field of microaqueous enzymology (3–5). Because of the intrinsic selectivities of enzymes, biotransformations of oleochemicals and native lipid resources are projected to be effective for executing the strategic modifications required to prepare value-added derivatives. With particular reference to edible lipids, the prospect of preparing "structured glycerides," where control is exercised over the differential arrangement of fatty acids along the *sn*-glycerol backbone, is an especially intriguing and popular area of study. Although most of these efforts to date have focused on the use of positionally selective (most often *sn*-1,3-selective) lipases (6), future targets for structured lipid synthesis will likely impose increasing demand for, and sophistication in, control of reaction selectivity to yield specialty products of added value and tailored functionality. Furthermore, when studies start to fully address the efficacy of using lipases in reaction mixtures with multiple substrates, such as native triacylglycerols (TAG) instead of a pure fatty acid (FA) and/or TAG substrates, FA selectivity and product selectivity will be of increasing importance and relevance to transforming basal lipid resources.

Some of the important factors that govern the choice of lipases for mediating lipid transformations in microaqueous media revolve around the issues of catalytic activity (rate), unique selectivity or specificity, and safety (derived from a nontoxic or nonpathogenic host). The first two factors reflect intrinsic properties of a particular lipase, and these properties can sometimes be inversely related (7). The safety issue often relates to the use of "food-grade" enzymes, and is always of concern where transformed bioresources are to be used as comestibles. An enzyme that has been recently studied is a lipid acyl hydrolase (LAH) from potato tubers (8; Pinsirodom, P., and K.L. Parkin, unpublished data). LAH is unique in that it is broadly selective for lipid class and acts on acylglycerols (AG), glycoglycerolipids, and phosphoglycerolipids (9), and is selective for FA with 8–10 carbons in esterification reactions with glycerol and glycerol analogs (Pinsirodom, P., and K.L. Parkin, unpublished data). In terms of product selectivity in reactions with FA of ≥10 acyl carbons and glycerol, LAH is unique in that only monoacylglycerols (MAG) appear to accumulate (8), although preliminary results in our laboratory revealed that under certain conditions, diacylglycerols (DAG) also accumulate. Although LAH has long been recognized to possess wax ester synthesis and acyl transferase activities (9–13), the regenerated interest in its catalytic properties may be embedded in the desire to identify novel food-grade biocatalysts with unique selectivity. The unequivocal safety of potato tuber LAH stems from its being the major storage protein in potatoes, accounting for 20–40% potato tuber protein (14,15), or up to almost 1% fresh weight.

The objective of this study was to evaluate the selectivity patterns of LAH in microaqueous media for reactions suitable for preparing structured glycerides from FA substrate mixtures.

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Both FA substrate and AG product selectivity were assessed in an effort to obtain a comprehensive account of the combined reactivity of FA and FA partitioning into various AG pools.

## EXPERIMENTAL PROCEDURES

*Materials.* LAH was isolated as a crude preparation from Russet Burbank potatoes and immobilized on Celite™, preconditioned at pH 7.0 with buffer (Pinsirodom, P., and K.L. Parkin, manuscript in preparation). FA, glycerol, salt hydrates, and protein isolation reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Celite™ 545 and solvents (all high-performance liquid chromatographic grade) were obtained from Aldrich Inc. (Milwaukee, WI). Thin-layer chromatography (TLC) plates (Whatman PK6F silica gel, 60 Å, 500-μm thickness) and all other chemicals were obtained from Fisher Scientific (Chicago, IL).

*Adsorption of glycerol on silica gel.* To avoid enzyme agglomeration and diffusion limitations resulting from undissolved glycerol in isooctane, glycerol was adsorbed onto silica gel prior to introduction into the reaction mixture (16). Equal weights of glycerol and silica gel (Merck, grade 9385, 230–400 mesh, 60 Å average pore diameter, supplied by Aldrich) were mixed with an Omni Mixer homogenizer (Warrenton, VA) until a homogeneous, friable powder was obtained (Lee, C.-H., and K.L. Parkin, unpublished data). The resulting powder was stored in a desiccator prior to use.

*Reaction parameters.* FA selectivity was evaluated in a multicompetitive substrate reaction system containing four *n*-FA [butanoic ($C_4$), octanoic ($C_8$), tetradecanoic ($C_{14}$), and *cis*-9,12-octadecadienoic ($C_{18:2}$) acids] as acyl donors, each at 100 mM with an equivalent of 600 mM immobilized glycerol in 50 mL of isooctane at 35°C. Water activity ($a_w$) was controlled at 0.19 by adding 2.5 g each of $Na_2HPO_4 \cdot (H_2O)_0$ and $Na_2HPO_4 \cdot (H_2O)_2$ (17). Reaction mixtures were preincubated at 35°C for 30 min in an orbital shaker at 300 rpm, and Celite™-bound enzyme (5.0 g; preconditioned with isooctane and salt hydrates at $a_w$ 0.19) was added to initiate the reaction. Subsamples were periodically withdrawn from the reaction mixtures, centrifuged to remove particulate enzyme, and then analyzed as described below.

*Analysis of reaction progress.* Reaction subsamples were often combined with 1(3)-MAG (α-MAG), 2-MAG (β-MAG), DAG, and TAG standards of either hexadecanoic or dodecanoic acid to facilitate locating the AG bands by TLC, especially when specific AG species were present in limited amounts. Preliminary experiments with various levels of standards indicated that recoveries from TLC plates were >95%. Samples were spotted on duplicate boric acid-impregnated (coated with 3.0% boric acid in ethanol) TLC plates and one plate each was developed by petroleum ether/diethyl ether/acetic acid (65:35:1, vol/vol/vol) and chloroform/acetone/methanol/acetic acid (157:40:2:1, by vol). These two systems were used to resolve and isolate TAG and DAG fractions and α-MAG and β-MAG fractions, respectively. AG bands were visualized by 0.1% 2,7-dichlorofluorescein spray and ultraviolet light, and analyzed by a general procedure (18). Zones corresponding to the different AG pools were scraped and combined with 2.0 mL 0.5 M methanolic sodium methoxide and 100 μL methyl decanoate (internal standard), and then incubated at 50°C for 10 min prior to adding 400 μL hexane and 4 mL saturated NaCl. The mixture was vortexed for 2 min and then centrifuged at about 2,000 × *g* for 3–5 min, and the supernatant was analyzed for fatty acid methyl esters (FAME) with a Hewlett-Packard (HP) 6890 series gas–liquid chromatograph (Hewlett-Packard, Wilmington, DE) equipped with a flame-ionization detector and an HP-INNOWAX cross-linked polyethylene glycol capillary column (30 m × 0.32 mm i.d. and 0.50 μm film thickness; Hewlett-Packard). Injector and detector temperatures were 220 and 230°C, respectively. The temperature program involved holding at 50°C for 2 min, ramping to 220°C at 18°C/min, then to 250°C at 10°C/min and holding for another 4 min.

*Experimental replication.* The results provided in this report are presented as means from at least two experimental trials, each done with duplicate samples contributing to each datum point, and where the coefficient of variation (CV) was estimated to be on the order of 8%.

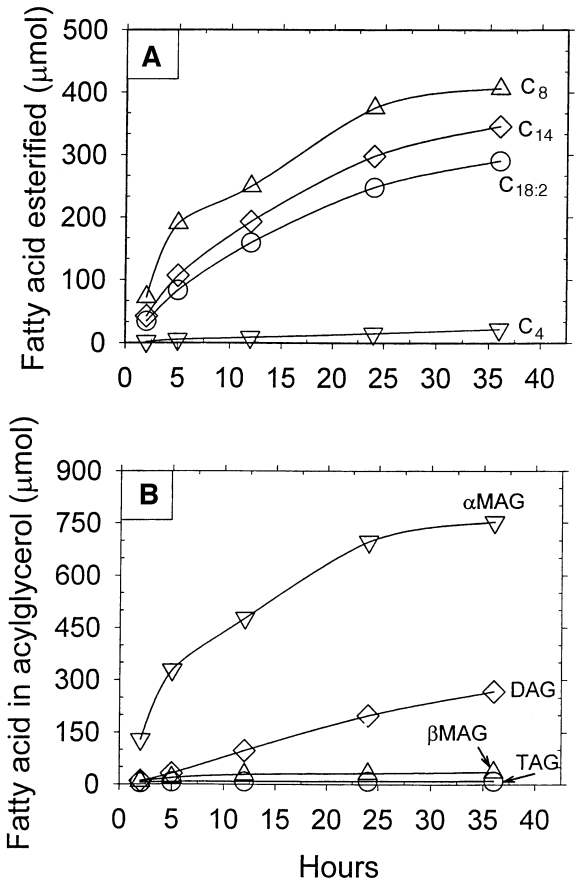


**FIG. 1.** Progress of esterification reactions between competitive fatty acid substrates and glycerol mediated by lipid acyl hydrolase (LAH). (A) Time course of differential incorporation of fatty acids into acylglycerols; (B) time course of differential accumulation of acylglycerols. $C_4$, butanoic; $C_8$, octanoic; $C_{14}$, tetradecanoic; $C_{18:2}$, *cis*-9,12-octadecadienoic; αMAG, 1(3)-monoacylglycerol; βMAG, 2-MAG; DAG, diacylglycerol; TAG, triacylglycerol.

## RESULTS AND DISCUSSION

*Reaction progress*. LAH exhibited selectivity toward FA substrates on the order of $C_8 > C_{14} > C_{18:2} > C_4$ in reactions with glycerol in isooctane (Fig. 1A). This pattern of selectivity was reported earlier for the saturated FA substrates with several glycerol analogs (Pinsirodom, P., and K.L. Parker, unpublished data), and $C_{14}$ was a better FA reactant than $C_{18:2}$ in esterification reactions with glycerol in the absence of organic solvent (8).

During the course of reaction, most of the esterified FA accumulated in the α-MAG pool, with lesser amounts accumulating in the DAG pool and the lowest levels accumulating in the β-MAG and TAG pools (Fig. 1B). By the end of the reaction period, the molar ratio of AG products, α-MAG/DAG/β-MAG/TAG, was 228:41:11:1. TAG were not synthesized in LAH-mediated esterification reactions with glycerol in the absence of organic solvent (8), and this is not surprising, given the lack of hydrolytic activity of LAH observed on TAG (8,9,19). It stands to reason that if LAH cannot recognize TAG as a substrate for hydrolysis, then it may not easily recognize TAG as a product of synthesis, presumably based on a lack of compatibility (i.e., binding) between TAG and the active site of LAH. What was surprising was the molar ratio of α-MAG/DAG products of 5.6:1 observed at the end of the reaction period (Fig. 1B), in light of the previous report of product molar ratios of MAG/DAG of >50:1 under similar reaction conditions (8). The relative lack of DAG formation in the latter case cannot be attributed solely to a lesser ability of LAH to recognize or synthesize DAG compared to MAG, since hydrolytic activity of LAH on DAG is as much as 20% that of activity on MAG (9). Thus, some other factor(s) may contribute to the AG product selectivity of LAH, and we sought to identify this factor through subsequent analyses.

*Distribution of esterified FA in specific AG pools*. The profile of FA distribution among the AG fractions obtained by TLC also was evaluated during the course of reactions (Fig. 2). $C_4$ was largely confined to the DAG pool, with lesser portions similarly distributed between the other AG pools (Fig. 2A). In contrast, $C_8$ was partitioned into the α-MAG pool, with moderate proportions found in the DAG pool and low levels in the other AG pools (Fig. 2B). Both $C_{14}$ and $C_{18:2}$ were distributed similarly among the AG pools as was $C_8$, except that there was a greater preference for the former FA to

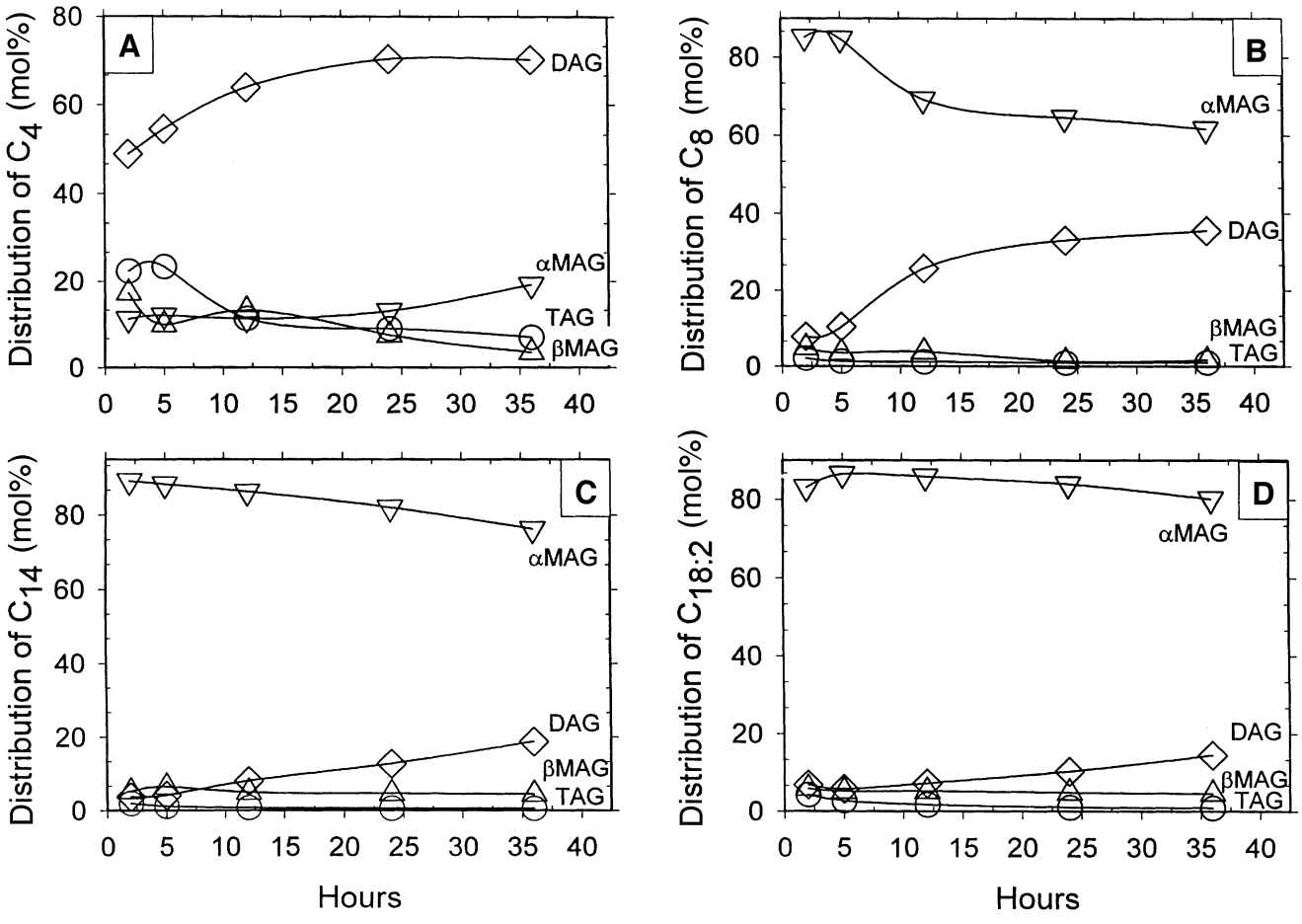


**FIG. 2.** Mol% distribution of esterified fatty acids among acylglycerol pools. Distribution of (A) $C_4$, (B) $C_8$, (C) $C_{14}$, (D) $C_{18:2}$. See Figure 1 for abbreviations.

be found in the α-MAG rather than DAG pool (Fig. 2C,D).

Although these analyses were informative in terms of documenting the partitioning of esterified FA into different AG pools, any trends may be obscured by the differences in extent of FA reactivity and the sizes of the individual AG pools. Thus, it was judged necessary to evaluate the partitioning of esterified FA into AG pools by a means that would account or normalize for the differences in reactivity just stated. To do this, the following relationship was developed:

$$\frac{\text{mol FA}_X \text{ in AG}_Y \text{ species}}{\text{mol FA}_{\text{Tot}} \text{ in AG}_Y \text{ species}} \div \frac{\text{mol FA}_X \text{ in AG}_{\text{Tot}}}{\text{mol FA}_{\text{Tot}} \text{ in AG}_{\text{Tot}}} = \text{FAPC} \quad [1]$$

where the subscripts *X* and *Y* refer to a *specific* esterified FA or AG product pool, the subscript Tot refers to the levels of all esterified FA or AG product pools, and FAPC is an abbreviation for fatty acid partitioning coefficient. This dimensionless relationship represents the relative proportioning of a specific FA into a specific AG pool compared to the relative reactivity of that specific FA among competing FA. This ratio also represents the biopartitioning of a specific FA into a specific AG pool for an equivalent degree of reaction. If there is no selectivity in terms of biopartitioning of FA into AG pools, then the value of this ratio will be 1.0. Values >1.0 indicate a preference for a specific FA for that AG pool and values <1.0 indicate an exclusion of a specific FA for that AG pool. The validity of this approach was confirmed by calculating [Σ mol fraction $FA_i \cdot FAPC_i$] for the four FA for each AG pool and arriving at unity in each case, indicating a conservation of mass.

*FAPC.* The data in Figures 1 and 2 were transformed to yield FAPC values for each AG pool as defined by Equation 1 (Fig. 3). Because of the dynamic changes in FAPC during reaction progress, an integration of FAPC values over the entire reaction period was done to provide for an overall and quantitative assessment of partitioning behavior of FA into specific AG pools (Table 1). It should be emphasized that the conclusions drawn from these analyses are within the context of an equivalent or normalized degree of FA incorporation into AG products in an attempt to analyze for biopartitioning behavior (Fig. 1A shows that FA incorporation was not equivalent).

$C_4$ was largely excluded and $C_8$ was slightly excluded from the α-MAG pool, whereas the two longest chain-length FA were selectively enriched in this pool (Fig. 3 and Table 1). In contrast, $C_4$ was preferentially partitioned into all other AG pools relative to other FA reactants. Thus, it appeared that $C_4$ was most easily incorporated into AG structures where greater steric constraints for enzyme–substrate interaction would be predicted for product formation relative to α-MAG as a prod-

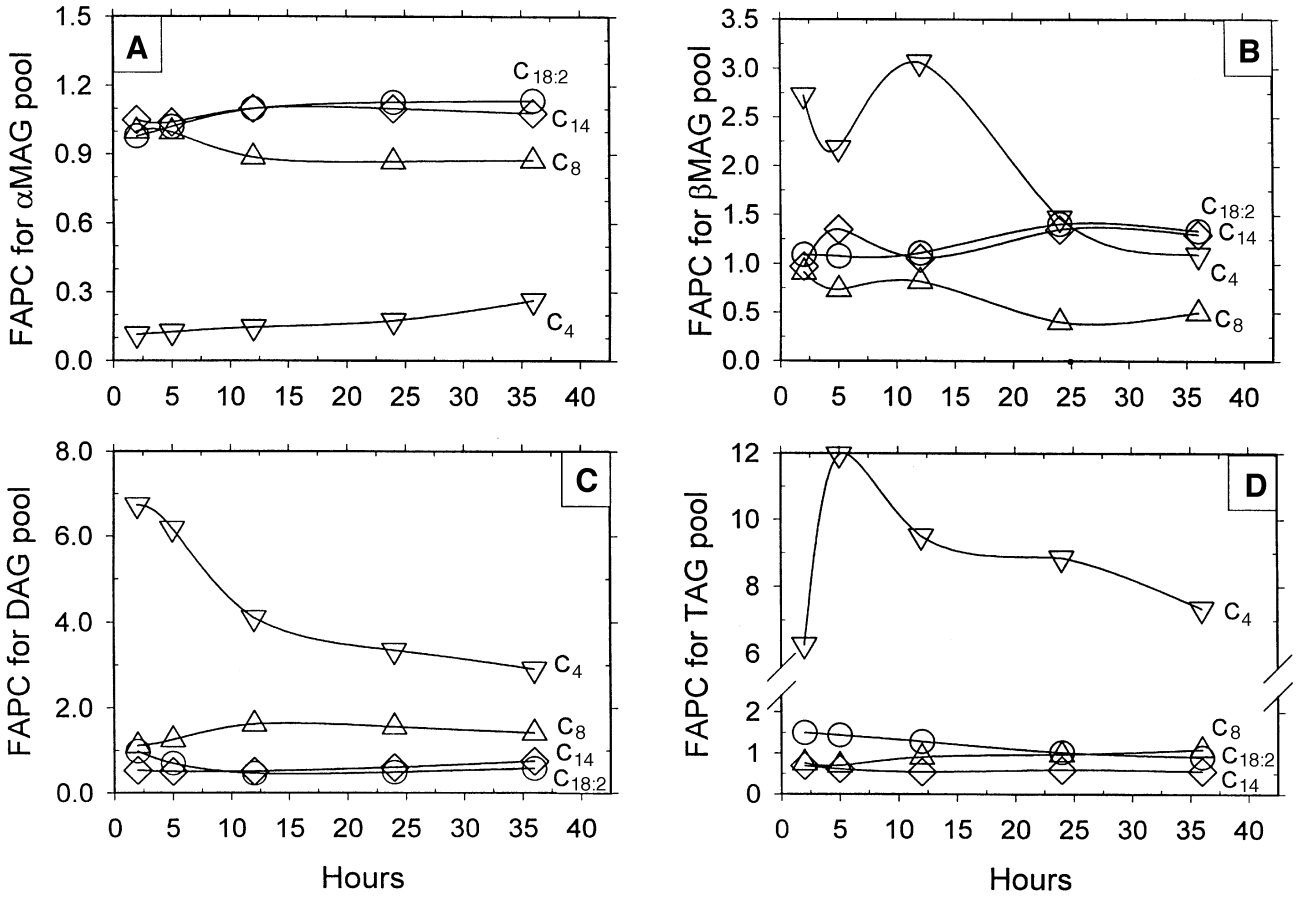


**FIG. 3.** Fatty acid partitioning coefficient (FAPC) values for fatty acids among acylglycerol pools. FAPC for (A) $C_4$, (B) $C_8$, (C) $C_{14}$, (D) $C_{18:2}$. See Figure 1 for abbreviations.

**TABLE 1**
**Integrated FAPC Values for Entire Reaction Progress[a]**

| Esterified FA species | Integrated FAPC value | | | |
|---|---|---|---|---|
| | αMAG | βMAG | DAG | TAG |
| $C_4$ | 0.17 | 2.03 | 3.84 | 8.77 |
| $C_8$ | 0.90 | 0.58 | 1.33 | 0.92 |
| $C_{14}$ | 1.07 | 1.23 | 0.58 | 0.61 |
| $C_{18:2}$ | 1.07 | 1.19 | 0.55 | 1.11 |

[a]The relationship between fatty acid partitioning coefficient (FAPC) and reaction progress curves was integrated as areas below straight line plots (using data from Fig. 3) for the period of 2–36 h, relative to the area represented by a nonselective reaction (FAPC value = 1.00) integrated over the same time entire period and assigned a value of 1.00; FA, fatty acid; $C_4$, butanoic; $C_8$, octanoic; $C_{14}$, tetradecanoic; $C_{18:2}$, *cis*-9,12-octadecadienoic; αMAG, 1(3)-monoacylglycerol; βMAG, 2-MAG; DAG, diacylglycerol; TAG, triacylglycerol.

uct. Other trends observed were that $C_8$ was most excluded from the β-MAG pool and was preferentially partitioned into the DAG pool (albeit less favorably than was $C_4$). FAPC trends for $C_{14}$ and $C_{18:2}$ were similar for most AG pools, being slightly favored for α,β-MAG and slightly excluded from DAG pools; a difference was observed for partitioning into TAG, and perhaps the flexibility of the 18:2 acyl chain is conducive to its greater preference for, or ease of, incorporation into TAG.

The dynamics of changes in FAPC for some FA and AG pools over the progress of reactions are likely attributable to several phenomena. One is that the profile and acyl composition of AG species that are subject to further reaction (esterification) are in a constant state of flux. It would appear certain that each AG species influences FA reaction selectivity in a characteristic and differential manner, such that dynamic changes in AG levels will change the pattern of FA reactivity and partitioning into different AG pools. A second reason is that some AG pools are relatively small (β-MAG and TAG) compared to others (α-MAG and DAG), such that even modest changes in the levels and acyl composition of these smaller AG pools will have a large impact on FAPC values over the course of the reaction. Evidence of this phenomenon comes from the observations that FAPC values in the dominant AG pools (α-MAG and DAG; Fig. 3A,C) were subject to gradual and smooth transitions, whereas FAPC values in smaller AG pools (β-MAG and TAG; Fig. 3B,D) were subject to more abrupt and oscillatory transitions.

This work is an extension of our previous efforts to determine "intrinsic" FA selectivity of enzyme-mediated esterification reactions in organic media, and how selectivity is modulated by the nature of the alcohol acceptor (Pinsirodom, P., and K.L. Parkin, unpublished data; Lee, C.H., and K.L. Parkin, unpublished data; 20). The present study was designed to infer factors that control product selectivity in these reactions, one of which is intrinsic FA selectivity exhibited by the biocatalyst. The case study of potato tuber LAH provides a good example, since both FA ($C_{8/10}$ favored) (Pinsirodom, P., and K.L. Parkin, unpublished data) and product (MAG favored) (8) selectivity are established, and it would seem important to know why the enzyme behaves as it does in the latter case.

The specific question we wished to address was, "Why do LAH-mediated esterification reactions in organic media yield mostly MAG?" (8). Based on prior observations, the tendency of such reactions in such apolar media as used by Macrae *et al.* (8) [an oleic acid, glycerol, and water mixture with a calculated log partitioning (*P*) coefficient between 1-octanol/water of >5, using the fragmental constant approach (21) or reported log *P* values (22)] would favor formation of as many fully acylated AG products (DAG and TAG) as the specific enzyme will permit (23–26).

It would seem implausible to accept that the preference of LAH for MAG over DAG by a factor of at least 5 in hydrolysis reactions (8,9) (assuming the same degree of selectivity in synthetic reactions) is solely responsible for the 50:1 ratio of MAG/DAG products observed (8). Our results (Figs. 1–3; Table 1) suggest that another important factor is simply an acyl chain-length restriction (*viz.*, substrate selectivity) of LAH reactivity toward α-MAG, such that α-MAG with acyl carbon chain lengths of >10 are recalcitrant reactants for further esterification. This was examined further by evaluating the time course of esterification reactions with individual FA (Fig. 4). Clearly, as FA acyl chain length increased, so did the tendency to accumulate α-MAG and restrict accumulation of DAG. The relative levels of FA in β-MAG and TAG were generally <2–5% those of α-MAG and DAG (data not shown), similar to the data shown in Figure 1. (The relative rates and progress of reactions in Fig. 4 should not necessarily be expected to be quantitatively similar to those in Figures 1,2, because reactant concentrations and composition are quite different.)

The approach of applying FAPC analyses to reaction progress curves may provide for a diagnostic analysis and prediction of which step(s) of acylation by an enzyme reaction are most subject to selectivity and/or are most important to controlling product profile. However, to be of greatest value, FAPC analysis must be taken in the context of relative reactivity of FA and the pool size of different AG species. For example, in the case of LAH, selectivity of the first glycerol acylation step would appear to be most important, as it yields the pool of alcohol acceptors (MAG) that show even greater discrimination among FA for subsequent reaction (range of integrated FAPC values increases from MAG to DAG, *cf.* Table 1). Even though $C_4$ would be expected to become more reactive with MAG acceptors than with glycerol, the fact that $C_4$ is a poor FA substrate for LAH (Fig. 1) (Pinsirodom, P., and K.L. Parkin, unpublished data) dictates that only a limited amount of DAG accumulates, and it is enriched primarily in $C_8$. In addition, trends in FAPC values in the context of established patterns of FA reaction selectivity may help explain the presence of large or small AG pools (the small pools are caused by either a lack of formation or from their relative reactivity, and so do not attain elevated steady-state levels). With reference to the preparation of structured lipids, the type of approach used in this report may provide clear indications of which enzymes would exhibit greatest selectivity at various steps in assembling structured glycerides (TAG).

Another feature of enzyme reaction selectivity demonstrated in these studies was that FA reaction selectivity is modulated

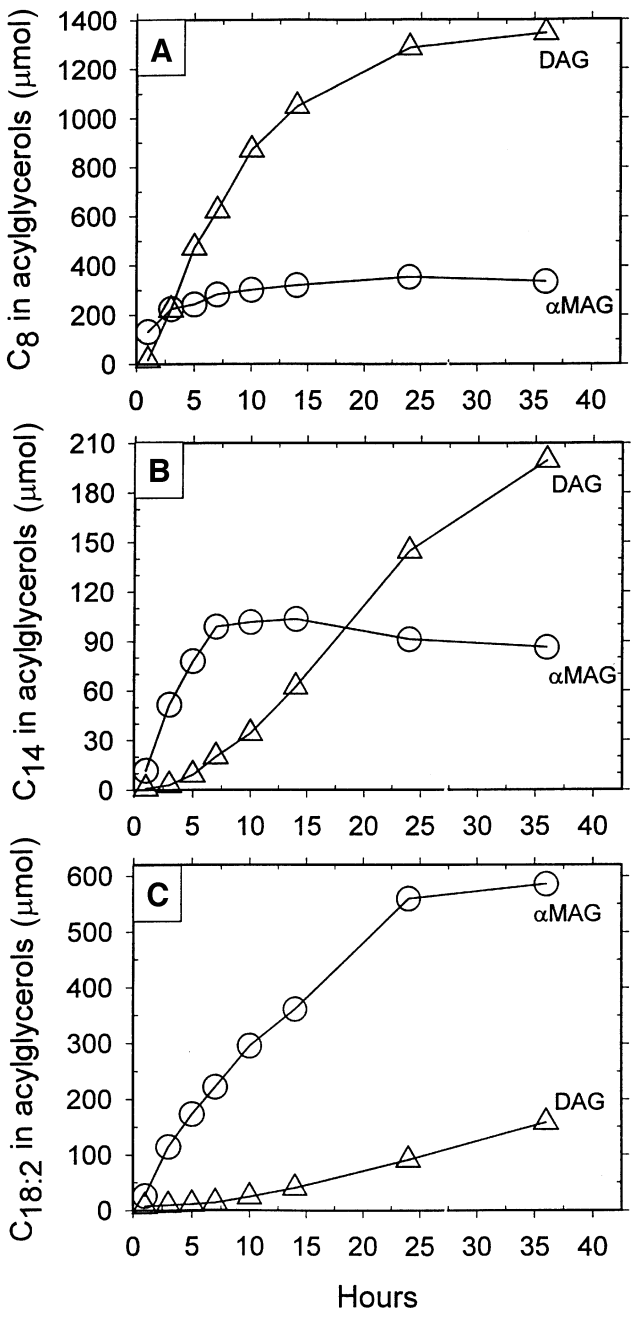


**FIG. 4.** Progress of esterification reactions between single fatty acid substrates and glycerol mediated by LAH. Time course of differential incorporation into αMAG and DAG for (A) $C_8$, (B) $C_{14}$, (C) $C_{18:2}$. See Figure 1 for abbreviations.

for the remaining -OH sites for reaction as the glycerol backbone becomes increasingly acylated. We predicted this in an earlier report (20), and this suggests that overall FA selectivity for higher glyceride synthesis cannot be predicted on the basis of reaction selectivity with FA and glycerol alone. Rather, the influence of the nature and location of the *sn*-acyl group already present along the glycerol backbone needs to be determined before reasonable predictions of reaction outcomes yielding DAG and TAG can be made.

The results in this report also suggest that $C_4$ becomes preferentially esterified at *sn*-2-glycerol relative to the other FA reactants (Table 1). This would need to be confirmed by positional analysis of the AG products, but this conclusion is in keeping with the previous suggestions that steric constraints about the *sn*-2-glycerol are important in conferring reaction selectivity of lipases (20,27,28).

It should be stressed that interpretation of the results obtained in this study are embedded within the reaction parameters evaluated. The nature of the solvent and the water activity used may influence reaction selectivity for any enzyme (29), although how much of an influence these parameters have remains to be determined.

## ACKNOWLEDGMENTS

The authors thank the U.S. Department of Agriculture (grant no. 95-37500-2023), the College of Agricultural and Life Sciences at the University of Wisconsin-Madison through Hatch funds WIS03878 and WIS04032, and the Royal Thai Government Ministry of University Affairs (for providing P. Pinisirodom with a graduate fellowship) for supporting this work.

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