Selectivity of Potato Tuber Lipid Acyl Hydrolase Toward Long-Chain Unsaturated FA in Esterification Reactions with Glycerol Analogs in Organic Media

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ABSTRACT: FA selectivity of a Celite-immobilized potato lipid acyl hydrolase (LAH) in esterification reactions with long-chain FA, including stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), α-linolenic acid (18:3), EPA (20:5), and DHA (22:6), and alcohol co-substrates (*n*-propanol, isopropanol, 1,3-propanediol, and glycerol) was studied in isooctane. Immobilized LAH was selective for FA of greater degrees of unsaturation (18:3 > 18:2 > 18:1 > 18:0) for all alcohol acceptors evaluated. Selectivity of LAH toward unsaturated C_{18} FA increased with an increase in water activity (a_w) from 0.19 to 0.90 for *n*-propanol, isopropanol, and 1,3-propanediol as alcohol co-substrates. In contrast, with glycerol as the alcohol co-substrate, selectivity of LAH toward these unsaturated C_{18} FA increased with a decrease in a_w from 0.90 to 0.19. In addition, immobilized LAH strongly discriminated against EPA and DHA for both 1,3-propanediol and glycerol as alcohol co-substrates.

Paper no. J10357 in *JAOCS 80*, 335–340 (April 2003).

KEY WORDS: Anhydrous, esterification, fatty acid selectivity, lipid acyl hydrolase, patatin, unsaturated fatty acid.

Because of their intrinsic reaction selectivities, lipolytic enzyme-catalyzed reactions in microaqueous media are viewed to have commercial potential for the preparation of structured lipids (1). Recent studies have concentrated on identifying and utilizing lipases with high selectivity for or against particular fatty acyl moieties for the purposes of FA fractionation and/or repositioning along the *sn*-glycerol backbone (2,3). Plant lipolytic enzymes are of great interest because they often display a unique acyl selectivity (4) and may often be considered safe for use in foods.

Patatin is the most abundant soluble protein fraction (20–40%) in potato tuber extracts (5) and has long been noted to possess lipid acyl hydrolase (LAH) activities (5,6). Similar to other lipolytic enzymes, patatin is capable of ester formation in aqueous co-solvent systems (7,8) as well as in microaqueous media (9–11). However, relative to lipases, potato LAH exhibits a rather narrow chain-length selectivity among saturated FA (C_4–C_{18}) substrates, particularly for C_8/C_{10} in both hydrolytic (12,13) and synthetic (11) modes. Water activity (a_w) and choice of alcohol co-substrate are important factors that influence both reactivity and FA selectivity of potato LAH in esterification reactions (11). In addition, with glycerol as the alcohol co-substrate, LAH is unique among lipolytic enzymes in that synthetic reactions are attenuated at the point where MAG accumulate as the principal end product (9,10). This feature appears to be related to a size restriction in activity toward MAG of $\geq C_{10}$, since DAG of chain lengths $<C_{10}$ can accumulate in reaction systems rich in FA $\leq C_{10}$ (10).

Phospholipids and glycolipids in plant tissues are rich in unsaturated FA. The rapid enzymic hydrolysis of endogenous phospholipids and galactolipids by LAH in potato tuber homogenates has long been noted (14). In addition, that potato LAH can catalyze the hydrolysis of these lipids *in vitro* has been well characterized (6,7). Since the preparation of structured lipids containing PUFA has received much attention (15), it is of interest to investigate the selectivity of LAH toward these FA in ester synthesis.

As an extension of earlier studies (9–11), the selectivity of potato LAH toward unsaturated 18- to 22-carbon FA in microaqueous media for esterification reactions with glycerol and glycerol analogs is reported in this paper.

EXPERIMENTAL PROCEDURES

Materials. Potato LAH was isolated as both crude and pure preparations from Russet Burbank potatoes and immobilized on Celite as described previously (10). HPLC grade *n*-propanol, isopropanol, and isooctane, and Celite®545 were obtained from Aldrich Inc. (Milwaukee, WI). FA (saturated), polyols, salt hydrates, protein isolation reagents, fish oil fractionation reagents, and lipoxygenase assay reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Oleic, linoleic, and α-linolenic acids were obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). Fish oil (menhaden) was obtained from Zapata Protein USA, Inc. (Reedville, VA). All other chemicals were obtained from Fisher Scientific (Chicago, IL).

Concentration of EPA/DHA from menhaden oil. Menhaden oil was first saponified according to the method described by Medina *et al.* (16) with minor modifications. Fish oil (250 g) was mixed under nitrogen at 50°C with 500 g of an aqueous alcohol solution of NaOH (120 g NaOH and 1.25 g EDTA

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dissolved in 400 mL water and 400 mL 95% ethanol) until the mixture was completely saponified (about 1 h). The progress of saponification was monitored by taking a small amount of the reaction mixture, acidifying, extracting the lipid phase into hexane, and analyzing for the absence of esterified FA using GLC. Upon completion of saponification, the reaction mixture was acidified to pH 1 using HCl (HCl/water; 2:1,vol/vol) and the liberated FA fraction was recovered by extraction with 3 × 150 mL hexane. The combined hexane fractions were evaporated at 25°C using a vacuum rotary evaporator. The isolated FA fraction was supplemented with propyl gallate (0.01 wt%), flushed with nitrogen, and stored at −20°C until use.

EPA (20:5) and DHA (22:6) in the isolated FA fraction were concentrated by fractionation with urea as described by Medina *et al.* (16). While stirring constantly under nitrogen, the isolated FA fraction (25 g) was added to a hot (65–70°C) solution of 100 g of urea in 267 mL methanol. The reaction mixture was heated (65–70°C), stirred until clear, and then allowed to crystallize overnight at 4°C. After filtration under vacuum at 4°C, the liquid FA concentrate was reduced to about two-thirds of the original volume by vacuum rotary evaporation at 25°C. The FA concentrate was acidified by mixing with 125 mL of 0.1 N HCl and then extracted with 125 mL hexane. The hexane layer was collected and the lower layer was re-extracted with 50 mL hexane. The combined hexane phases were evaporated at 25°C using vacuum rotary evaporation. The n-3 FA concentrate with the addition of 0.01 wt% propyl gallate was flushed with nitrogen and stored at −20°C until use.

Analysis of FA composition. FA composition of menhaden oil, the liberated FA fraction, and the n-3 FA concentrate was analyzed by capillary GLC. Methylation of esterified FA in menhaden oil was done using sodium methoxide in methanol as described previously (11). Methylation of the saponified FA fraction and n-3 FA concentrate was done by direct transesterification with acetyl chloride/methanol (1:20) as described by Lepage and Roy (17). Methyl esters of FA were analyzed by GLC according to the method described below.

Lipoxygenase activity assay. The activity of lipoxygenase (LOX) was measured in both the crude and pure preparations of potato LAH in a 3-mL assay mixture containing of 50 mM sodium acetate buffer (pH 5.0), 0.5 mM sodium linoleate, 0.4% Tween-20, and 0.25 mg enzyme (protein). Assays were carried out at 20–23°C, and activity was monitored at 234 nm using an extinction coefficient of 2.5×10^4 $M^{-1}cm^{-1}$ for the conjugated diene. One unit of enzyme activity was defined as that generating 1 μmol of conjugated diene min^{-1} under the assay conditions.

Adsorption of polyols on silica gel. Owing to the solubility limitation of polyols (1,3-propanediol and glycerol) in isooctane, they were adsorbed onto silica gel (Merck, grade 9385, 230–400 mesh, 60Å average diameter, supplied by Aldrich) prior to introduction into the reaction mixture as described in our earlier report (10). Silica gel used in this manner furnishes a reservoir of polyol and prevents polar phase barriers to reactivity (18). If polyol interactions with silica gel and solubility are modulated by a_w, this may influence the reaction rates but not selectivity, as the rate is dependent on dissolved polyol but selectivity constants (competitive factors: α-values) are not (19). However, it is possible that some unanticipated interactions among a_w, polyol absorption, and enzyme reactivity may occur and influence the results obtained.

Esterification reaction mixtures. (i) C_{18} series of FA. A typical reaction mixture consisted of 20 mL isooctane solution containing 50 mM each of stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and α-linolenic acid (18:3) together with 50 mM of caprylic acid (C_8) as the reference substrate. Also included was one alcohol acceptor at 500 mM for *n*-propanol and isopropanol, or the molar equivalent level of polyol for silica gel-adsorbed polyols (1,3-propanediol and glycerol). a_w was controlled at 0.19, 0.69, and 0.90 by adding 1.2 g each member of the Na_2HPO_4-hydrate pairs, $0H_2O/2H_2O$, $2H_2O/7H_2O$, and $7H_2O/12H_2O$, respectively (20). The reaction mixture was pre-incubated by orbital shaking at 300 rpm and 35°C for 1 h to allow a_w and temperature to equilibrate. Reactions were initiated by adding 1.5 g Celite-immobilized potato LAH. Reaction subsamples were taken at predetermined intervals and centrifuged to remove solid matter prior to product analysis.

(ii) n-3 FA concentrate series (EPA/DHA). The n-3 FA concentrate prepared by urea fractionation was used for FA selectivity studies of potato LAH in esterification reactions, and only α-linolenic acid (18:3), EPA (20:5), and DHA (22:6) were analyzed. The addition of n-3 FA concentrate in the reaction mixture provided 50 mM of EPA, as well as 10.0 mM α-linolenic acid and 36.4 mM DHA (Table 1). α-Linolenic acid obtained from Doosan Serdary Research Laboratories was added to make the total amount of this FA equal to 50 mM. Therefore, the final reaction mixture consisted of 20 mL of isooctane solution containing 50 mM each of α-linolenic acid, EPA, and caprylic acid (as reference FA), and 36.4 mM DHA, together with one alcohol acceptor (1,3-propanediol or glycerol). All other reaction conditions were the same as described for the C_{18} series of FA except that a_w was controlled at the optima of 0.90 for 1,3-propanediol and at 0.19 for glycerol (11).

TABLE 1
FA Composition of Menhaden Oil, FA Liberated by Saponification, and n-3 FA Concentrate from Urea Fractionation

	% by weight		
FA	Menhaden oil	Saponified FA	n-3 FA concentrate
18:0	2.45	2.35	0.06
18:1	6.75	6.55	1.63
18:2	0.92	0.89	1.18
18:3	***2.80***	***2.75***	***4.49***
20:4	0.61	0.59	1.20
20:5	***10.4***	***10.3***	***23.1***
22:6	***7.45***	***7.35***	***18.3***
Others	68.6	69.2	50.0

[a]FA that were subject to analysis of lipid acyl hydrolase selectivity in this study appear in bold italics.

Determination of competitive factors (α-values). The FA selectivity of potato LAH was determined by a competitive factor (α), which is the ratio of the catalytic power (k_{cat}/K_m) of two substrates competing for the same enzyme active site (19). In practice, α-values are analyzed experimentally as differential reaction rates or progress of one substrate relative to a reference substrate (C_8 in this study) according to Equation 1:

$$\log([C_0]_A/[C_0 - C_x]_A = \alpha\log([C_0]_B/[C_0 - C_x]_B \quad [1]$$

where subscripts *A* and *B* represent two competing substrates (with *B* being the reference substrate), $[C_0]$ is the initial substrate concentration, $[C_x]$ is the product concentration, and $[C_0 - C_x]$ represents the substrate concentration remaining at a given sampling time interval, *x*.

A log–log plot of the above equation yields a linear plot with a slope equal to the α-value for any pair of substrates. The α-value of the reference substrate (C_8) was taken as 1.0, and for any other substrate, the greater the α-value, the more selective LAH is for that substrate by comparison (11). In practice, reactions were <20% complete for the determination of α-values, well within the 40–60% completion found to yield linear plots in previous studies (19).

Analysis of reaction progress. Reaction subsamples, which consisted of the ester products resulting from reactions, were subjected to derivatization to convert the esterified FA to their methyl esters using sodium methoxide in methanol as described previously (11). The methyl esters were analyzed using a Hewlett-Packard (HP) 6890 series gas–liquid chromatograph (Hewlett-Packard, Wilmington, DE) and an HP-INNOWAX cross-linked polyethylene glycol capillary column (30 m × 0.32 mm i.d. and 50-μm film thickness) and limonene as an internal standard. The temperature program for the C_{18} series of FA 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 another 4 min. The temperature program for the n-3 series of FA was holding at 100°C for 1 min, ramping to 145°C at 20°C/min, then to 200°C at 30°C/min, and finally to 250°C at 5°C/min, and holding for another 4 min.

RESULTS AND DISCUSSION

Preparation of n-3 FA concentrate enriched in EPA/DHA. The FA profiles of original menhaden oil, liberated FA fraction obtained after saponification, and the n-3 FA concentrate from urea fractionation are given in Table 1. Most of the PUFA were enriched about two- to threefold after fractionation with urea, particularly EPA and DHA, the weight percentage of which increased from 10.3 to 23.1% and 7.4 to 18.3%, respectively.

LOX activity in potato LAH preparations. It was judged important to confirm that any contaminating LOX activity in the potato LAH preparations would not affect an evaluation of selectivity for unsaturated FA. Therefore, both crude (ammonium sulfate precipitation) and pure (additional steps of DEAE-Sephacel and Con A-Sepharose column chromatography) potato LAH preparations were assayed for LOX activity. Only the crude preparation contained LOX activity at 1.22 units mg^{-1} enzyme, and no LOX activity was detected in the pure preparation of potato LAH.

Both crude and pure preparations of potato LAH showed no difference in selectivity patterns for the C_{18} series of FA (with *n*-propanol; data not shown), confirming that LOX activity in crude potato LAH was insufficient to degrade and decrease 18:2/18:3 levels (by oxygenation) to affect the pattern of selectivity observed for LAH in these studies.

FA selectivity of potato LAH. (i) C_{18} series of FA. Competitive factors calculated from the progress curves for esterification of the C_{18} series of FA with glycerol and glycerol analogs using C_8 as a reference FA are shown in Figures 1–4. Although less selectivity was observed for unsaturated FA

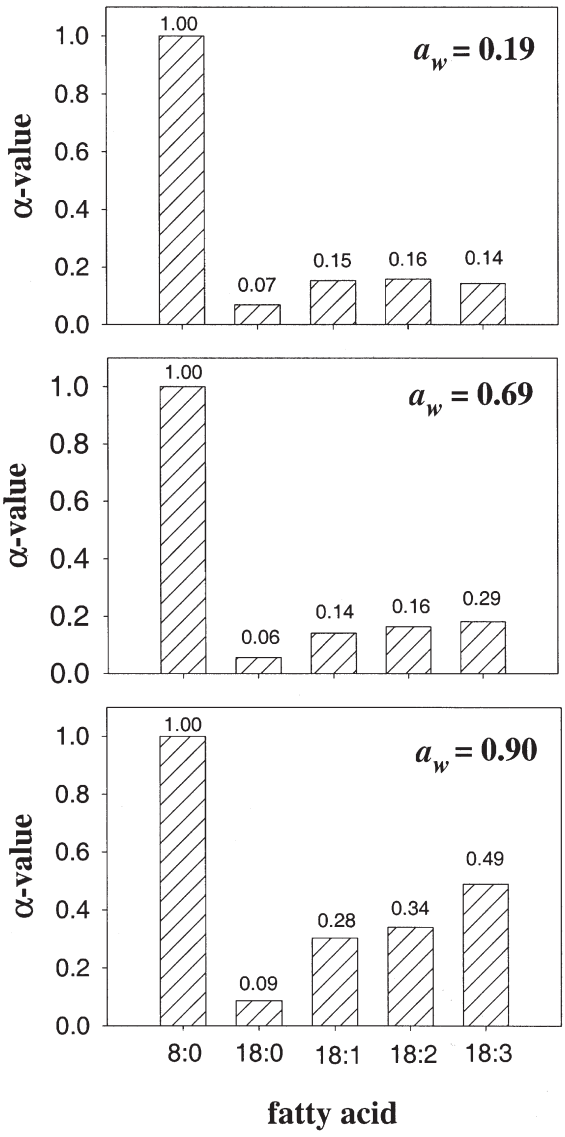

FIG. 1. Selectivity profiles (competitive factors: α-values) for the esterification of FA and *n*-propanol in isooctane by potato lipid acyl hydrolase (LAH) at a water activity (a_w) of 0.19, 0.69, and 0.90. Results are from two experiments with a CV of about 5%.

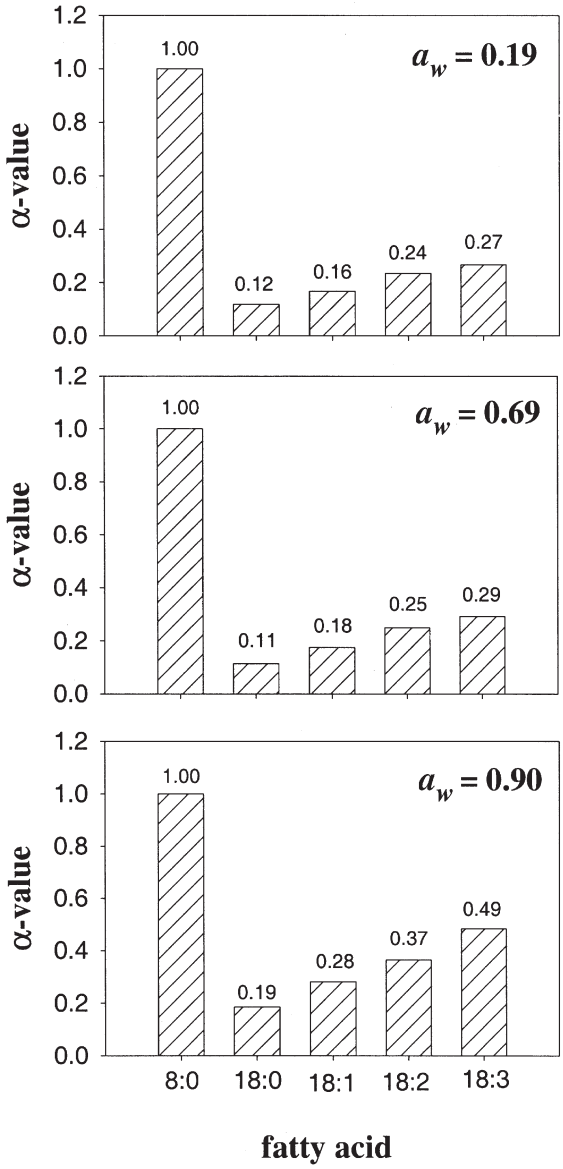

FIG. 2. Selectivity profiles (competitive factors: α-values) for the esterification of FA and isopropanol in isooctane catalyzed by potato LAH at a_w 0.19, 0.69, and 0.90. Results are from three experiments with a CV of about 8%. For abbreviations see Figure 1.

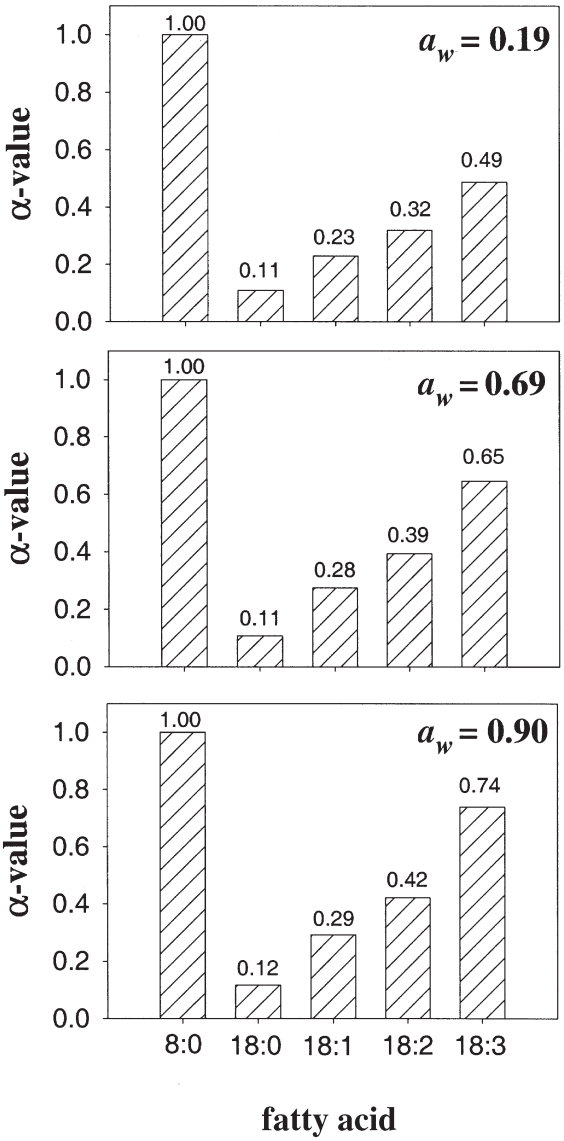

FIG. 3. Selectivity profiles (competitive factors: α-values) for the esterification of FA and 1,3-propanediol in isooctane catalyzed by potato LAH at a_w 0.19, 0.69, and 0.90. Results are from two experiments with a CV of about 3%. For abbreviations see Figure 1.

compared to C_8, potato LAH exhibited a general trend of greater selectivity for FA of greater degree of unsaturation (18:3 > 18:2 > 18:1 > 18:0) regardless of a_w and the identity of the alcohol co-substrate. Interestingly, the similar pattern of unsaturated FA selectivity of LAH was observed for hydrolysis reactions of methyl esters (6) or 1(3)-MAG (13), with 18:3 > 18:2 > 18:1 > 18:0. The greater flexibility of fatty acyl chains with higher degrees of unsaturation and/or lower molar volumes may facilitate reactivity with LAH and the alcohol co-substrate.

Most lipases have been shown to exhibit greater selectivity toward C_{18} FA with greater degrees of unsaturation in esterification and interesterification reactions. These lipases include those from *Penicillium* sp., *Candida cylindracea*, *Rhizopus arrhizus*, *C. rugosa*, *C. lipolytica*, and porcine pancreas (2,21). However, lipases that depart from this behavior include those from *Chromobacterium viscosum* (2), *Mucor javanicus* (21), and *Rhizomucor miehei* (22). In addition, Macrae *et al.* (9) reported a greater amount of MAG produced from esterification reactions with glycerol catalyzed by potato LAH in a solvent-free system when FA with greater degrees of unsaturation were used as acyl donors (18:3 > 18:2 > 18:1 > 18:0).

a_w was a factor that affected FA selectivity of potato LAH, particularly toward 18:3. A general trend was observed for increasing selectivity toward unsaturated FA as a_w increased from 0.19 to 0.90 for *n*-propanol, isopropanol, and 1,3-propanediol as alcohol co-substrates (Figs. 1–3). Reaction systems with glycerol as alcohol cosubstrate behaved in the opposite fashion in that selectivity toward unsaturated FA was

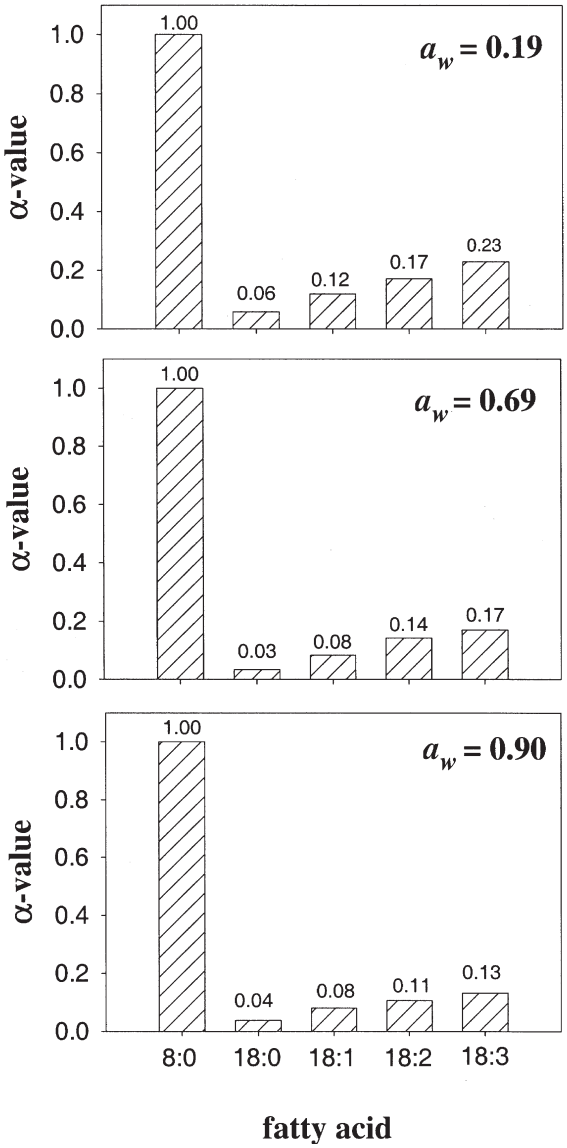

FIG. 4. Selectivity profiles (competitive factors: α-values) for the esterification of FA and glycerol in isooctane catalyzed by potato LAH at a_w 0.19, 0.69, and 0.90. Results are from three experiments with a CV of about 6%. For abbreviations see Figure 1.

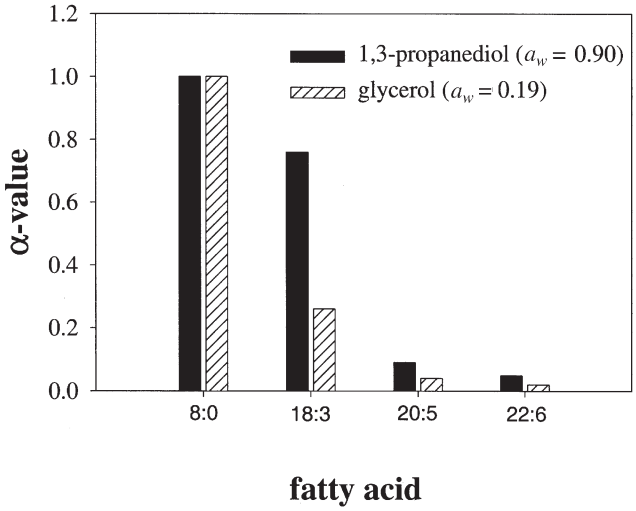

FIG. 5. PUFA selectivity (competitive factors: α-values) of potato LAH in esterification reactions with 1,3-propanediol (at a_w 0.90) and glycerol (at a_w 0.19) in isooctane and α-linolenic acid. Results are from three experiments with a CV of about 5%. For abbreviations see Figure 1.

suppressed with increasing a_w (Fig. 4). The initial rate of esterification reaction with glycerol catalyzed by potato LAH is greatest at a_w 0.19, while it is greatest at a_w 0.90 for all other alcohol co-substrates evaluated (11). The opposite effect of a_w on initial rates of ester synthesis between glycerol and other alcohol cosubstrates may be related to the opposite effect of a_w on FA selectivity of LAH observed between these same alcohol groups in this study.

(ii) EPA/DHA series. Competitive factors for the esterification reactions using the n-3 FA concentrate with 1,3-propanediol and glycerol are shown in Figure 5. Potato LAH strongly discriminated against EPA and DHA for both alcohol acceptors evaluated. Although the greater numbers of double bonds would create greater flexibility in the acyl chains of 20:5 and 22:6 compared to 18:3, the longer chain length of the former acyl groups may pose steric constraints on reactivity and reduce selectivity in reactions with LAH. Molecular modeling of eight other lipases indicated that only acyl chain lengths of $\leq C_{18}$ can fit completely within the binding pocket (23).

The determination of competitive factors using multicompetitive assay systems is theoretically independent of substrate concentration (19). Using substrates at different concentrations, such as when using the n-3 FA concentrate in this study, will cause individual reaction rates to vary but should still yield the same rate constants (α) for each FA substrate. This was confirmed by comparing the competitive factors analyzed from two esterification reactions with 1,3-propanediol at different FA concentrations (5–100 mM) (data not shown). The relative FA selectivity constants of other lipases also have been demonstrated to be independent of substrate concentration (21).

The poor selectivity that potato LAH exhibited toward EPA and DHA is similar to the discrimination against EPA and DHA observed for many lipases, such as those from *R. arrhizus*, *C. cylindracea*, *C. viscosum*, *R. miehei*, and *Brassica napus* (2,3). It has been proposed that the discrimination against unsaturated FA having the first double bond from the carboxyl end as a *cis*-4 (e.g., 22:6n-3), *cis*-6 (e.g., 18:1n-12, 18:3n-6, 18:4n-3), or a *cis*-8 (e.g., 20:3n-6) is a common feature among lipases (2).

In conclusion, potato LAH exhibited greater selectivity toward C_{18} FA as the degree of unsaturation increased in esterification reactions, consistent with previous observations on the hydrolytic selectivity of this enzyme (6,12). LAH discriminated against EPA and DHA in esterification reactions regardless of the type of alcohol cosubstrates, similar to most other lipolytic enzymes characterized to date. This finding,

along with previous observations (10,11), indicates that potato LAH is a suitable catalyst for preparing partial glycerides enriched in medium- and long-chain PUFA as intermediates in preparing structured lipids.

ACKNOWLEDGMENTS

The authors are grateful for support from the U.S. Department of Agriculture (grants 95-37500-2023 and 99-35503-8166), the College of Agricultural and Life Sciences of the University of Wisconsin–Madison through Hatch Funds (WIS03878 and WIS04032), and The Royal Thai Government, Ministry of University Affairs, to conduct this work.

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[Received June 13, 2002; accepted December 11, 2002]