Selectivity of Celite-Immobilized Patatin (Lipid Acyl Hydrolase) from Potato (*Solanum tuberosum* L.) Tubers in Esterification Reactions As Influenced by Water Activity and Glycerol Analogues as Alcohol Acceptors

Praphan Pinsirodom and Kirk L. Parkin*

Department of Food Science, 1605 Linden Drive, University of Wisconsin, Madison, Wisconsin 53706

Lipid acyl hydrolase (LAH; patatin) was purified from potato tubers by ammonium sulfate fractionation followed by anion-exchange and affinity chromatography. The major protein band of 40−43 kDa on SDS−PAGE appeared to be patatin, and it stained positive for lipase activity on native PAGE. Selectivity of a Celite-immobilized potato LAH in esterification reactions with *n*-acyl fatty acids (FA; C4, C6, C8, C10, C12, C14, C16, and C18) and alcohol acceptors (*n*-propanol, 2-propanol, 1,3-propanediol, and glycerol; 1,2-propanediol was not sufficiently reactive) was studied in isooctane. Immobilized LAH was highly selective for medium chain FAs (C8/C10) with a secondary optimum for chain lengths of C14/16. Water activity (a_w) influenced activity and FA selectivity of the enzyme. Initial rates of ester synthesis were greatest at a_w of 0.90 for all alcohol acceptors except for glycerol, where greatest initial rates were observed at a_w of 0.19. Immobilized LAH preparations exhibited a bell-shape pH profile with optimum activity at pH 6−7 for ester synthesis, and no effect of pH on FA selectivity was observed.

Keywords: *Patatin; potato lipid acyl hydrolase; fatty acid selectivity; esterification; microaqueous enzymology*

INTRODUCTION

Patatin is the common name for the major storage protein in potato (*Solanum tuberosum* L.) tuber, accounting for up to 40% of the total tuber protein (Racusen and Foote, 1980; Rosahl et al., 1986). It exists as up to 15 immunologically identical glycoprotein isoforms with slightly acidic p*I* and a monomeric molecular mass determined by SDS−PAGE to be about 40 kDa (Racusen, 1984; Sonnewald et al., 1989; Höfgen and Willmitzer, 1990; Bohac, 1991; Senda et al., 1996). In native form, patatin may exist as dimers or oligomers (Matsuda and Hirayama, 1979; Racusen and Weller, 1984; Racusen, 1986) and appears to be localized in vacuoles of parenchyma cells (Sonnewald et al., 1989). Patatin constitutes the major lipid carboxyl esterase activity in potato tissues, although other esterase enzymes also exist (Galliard and Dennis, 1974).

Patatin has long been noted to possess lipid acyl hydrolase (LAH) activity on both native and model lipid substrates with acyl chain lengths of 8−18 (Galliard, 1971; Racusen and Foote, 1980; Racusen, 1984; Andrews et al., 1988). Potato LAH has broad lipid class specificity and is hydrolytically active toward phospholipids, neutral lipids (mono- and diacylglycerols), glycolipids, and esters of long chain fatty acids (Galliard, 1971). Although its physiological function in the tuber is not clearly known, it was suggested to have a role in wound response (Dennis and Galliard, 1974) or to afford a defense by mediating phytoalexin production (Andrews et al., 1988) or to be involved in signal transduction, the latter by virtue of its phospholipase A_2-like activity (Senda et al., 1996).

A property that was noted for patatin, but received little sustained attention, was the capability to mediate wax ester synthesis (Galliard, 1971; Dennis and Galliard, 1974) and acyl-transfer reactions (Galliard, 1970, 1971; Galliard and Dennis, 1974; Andrews et al., 1988). In view of the advances in microaqueous enzymology over the last 15 years and the prospect of using lipolytic enzymes to prepare restructured glycerides and other value-added lipid derivatives (Zaks and Russell, 1988; Vulfson, 1993; Villeneuve and Foglia, 1997; Kim et al., 1998), it would seem appropriate to more fully evaluate the synthetic potential of potato tuber LAH. The fact that most industrial sources of lipases are derived from microorganisms (Godfrey, 1995; Gandhi, 1997) provides another compelling argument to explore the potential of novel food-grade enzymes.

Recently, the use of potato LAH in mediating esterification reactions between glycerol and fatty acids (FA) to prepare monoacylglycerols has been reported (Macrae et al., 1998). Although this study demonstrated the synthetic potential of LAH, there is a need to fully understand the factors that impact LAH reactivity and selectivity before one can make an informed decision as to how LAH could be used in a practical setting. In the present study, potato tuber LAH-mediated esterification reactions between FAs and alcohols modeling the reactivity of the glycerol backbone were studied to gain insight as to the reaction selectivity of LAH in organic media. In addition, the influence of selected environmental parameters (a_w, pH) on the progress of LAH-mediated reactions was evaluated.

* Corresponding author [tel, 608-263-2011; fax, 608-262-6872; e-mail, klparkin@facstaff.wisc.edu].

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MATERIALS AND METHODS

Materials. Potato tubers used were of the Russet Burbank cultivar, distributed by Circle Valley Produce, Inc. (Idaho Falls, ID). Tubers were purchased at local retailers and held at 4 °C for up to 10 days prior to use. DEAE-Sephacel and Con A-Sepharose were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). *p*-Nitrophenyl (PNP-laurate), insoluble polyvinylpolypyrrolidone (PVPP), methyl α-D-glucoside, gel-staining chemicals, salt hydrates, polyols, and FAs were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC grade *n*-propanol, 2-propanol, and isooctane and Celite 545 were obtained from Aldrich Inc. (Milwaukee, WI). All other chemicals were obtained from Fisher Scientific (Chicago, IL).

Purification of Patatin. Patatin was purified by the modified method of Bohac (1991) except that the size-exclusion chromatography step was omitted and all steps were done at ~4 °C. Potato tuber tissue (250 g) was homogenized in 250 mL of 25 mM sodium phosphate (pH 7.0) containing 2 mM cysteine and 0.05 g of PVPP g^{-1} fresh weight of tissue. The homogenate was filtered, centrifuged, and then subjected to precipitation by 40−70% saturated ammonium sulfate and subsequent dialysis to provide a "crude" patatin preparation. Subsequent steps of DEAE-Sephacel and Con A-Sepharose column chromatography yielded a "pure" patatin.

Enzyme Assays and Protein Determination. LAH activity of patatin preparations was assayed with the synthetic substrate, PNP-laurate, according to the method of Bohac (1991). One unit of activity was defined as 1 μmol of *p*-nitrophenol produced min^{-1}. Protein contents were determined by the Coomassie blue dye-binding assay (Bradford, 1976) using bovine serum albumin as a standard. Where reported, initial reaction velocities were derived from the initial, linear portion of the reaction progress curves based on analysis for fatty acid ester formation.

Electrophoresis. SDS−PAGE and native PAGE were performed with Bio-Rad Mini-Protein II electrophoresis system using Bio-Rad precast ready gels (12% resolving gel, 4% stacking gel; Bio-Rad Laboratories, Hercules, CA). Proteins were visualized by Coomassie Brilliant Blue R-250 and lipase activity staining.

Lipase activity staining for the native PAGE gels was accomplished by simultaneous capture method (Gabrifl, 1971) using α-naphthyl caprate as the staining solution.

Immobilization of Patatin. Crude and pure patatin preparations were immobilized onto Celite. Celite was pretreated by washing with 50 mM sodium phosphate buffer (pH 7.0) on a Buchner funnel until the pH of the filtrate was 7.0. The residue was dried at 130 °C for about 8 h and stored in a desiccator until use.

The pretreated Celite (10 g) was added to 25 mL of patatin solution in 50 mM sodium phosphate buffer (pH 7.0) that contained 550 units of LAH activity. After the mixture was mixed and incubated at 4 °C for 30 min, 100 mL of chilled acetone (−70 °C) was added, and the suspension was filtered through a Buchner funnel using Whatman No. 1 filter paper. The immobilized patatin was washed on the filter paper with another 100 mL of chilled acetone and dried in a vacuum desiccator for about 6 h at 25 °C.

Immobilized patatin preparations, adjusted to different pH values, were prepared in the same manner as described above, but with different buffer solutions. Buffers used for the pretreatment steps were 0.2 M sodium citrate phosphate buffer (pH 4.0, 5.0, 6.0), 0.25 M sodium phosphate buffer (pH 7.0, 8.0), and 0.1 M glycine NaOH buffer (pH 9.0, 10.0).

Preparation of Immobilized Substrates. To compensate for the limitation of solubility of hydrophilic substrates (1,2- and 1,3-propanediols and glycerol) in isooctane and prevent multi-liquid-phase behavior, polyol substrates were adsorbed onto silica gel according to a method described by Castillo et al. (1997). Ten grams of polyol and 10 g of silica gel were mixed until homogeneous. Thus, 1 g of silica gel-adsorbed (immobilized) substrate contained 0.5 g of polyol.

Competitive Assays for Selectivity Determination. FA selectivity of patatin in ester synthesis was investigated using a competitive assay method described by Deleuze et al. (1987) and Rangheard et al. (1989). A typical reaction mixture consisted of 20 mL of isooctane solution containing 50 mM each of multiple *n*-FAs (designated as C4, C6, C8, C10, C12, C14, C16, and C18) and one alcohol acceptor at 600 mM for *n*-propanol and 2-propanol, or an equivalent level of addition for immobilized polyols (1,2- and 1,3-propanediol and glycerol). Salt hydrate pairs were used to control the water activity (a_w) of the reaction system at 0.19, 0.69, and 0.90 by adding 1.2 g of each member of the Na_2HPO_4 hydrate pairs: $0H_2O/2H_2O$, $2H_2O/7H_2O$, and $7H_2O/12H_2O$, respectively (Halling, 1992). The reaction mixture was then preincubated for 30 min by orbital shaking at 300 rpm and 35 °C to allow a_w and temperature to equilibrate. Reactions were initiated by adding 2 g of immobilized patatin (routinely, immobilized crude patatin was used, unless otherwise stated). Reaction subsamples were removed at predetermined intervals and centrifuged to remove solid matter prior to product analysis.

Determination of Competitive Factors (α-Values). The FA selectivity of patatin 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. The theory is described in Rangheard et al. (1989, 1992) and Deleuze et al. (1987). In practice, α-values are analyzed experimentally as differential reaction rates/progress of one substrate relative to a reference substrate (C8 in this study) according to the following equation:

$$\log([C_o]_A/[C_o - C_x]_A) = \alpha \log([C_o]_B/[C_o - C_x]_B)$$

where subscripts A and B represent two competing substrates, with B being the reference substrate (cf. Berger and Schneider, 1991; Chang et al., 1999), $[C_o]$ is the initial substrate concentration, $[C_x]$ is the product concentration, and $[C_o - C_x]$ 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 is taken as 1.0 and for any other substrate, the greater the α-value, the more selective (reactive) that substrate is by comparison. We obtained progress curves typical for this type of experimental approach (cf. Figures 1 and 2 in Rangheard et al., 1989).

Sample Preparation and Gas Chromatography Analysis. In a screw-cap test tube, a reaction subsample (0.15 mL) was derivatized by adding 1.5 mL of hexane containing limonene as an internal standard and 0.1 mL of 1 M sodium methoxide in methanol (Christie, 1992). The mixture was vortexed for 3 min and then orbitally shaken at 300 rpm for 15 min. The derivatized sample was centrifuged to remove precipitated material. Methyl esters of fatty acids (FAME) were analyzed by gas−liquid chromatography (GC) using a model 6890 gas chromatograph (Hewlett-Packard, Wilmington, DE) equipped with a HP-5 cross-linked 5% phenyl methyl siloxane capillary column (30.0 m × 0.32 mm × 0.25 μm) and flame ionization detector (FID). The samples were auto-injected, and the injector and detector temperatures were set at 220 and 230 °C, respectively. The column temperature program was ramped from 40 to 100 °C at 17 °C min^{-1} and then from 100 to 220 °C at 10 °C min^{-1}. Quantification of FAME was done relative to external and internal standards.

RESULTS AND DISCUSSION

Purification of Patatin from Potato Tuber. Only a modest purification of patatin was obtained (Table 1). This was expected, since at least 20% of the soluble protein of potato tuber is patatin (Racusen and Foote, 1980) and 4−5-fold purifications are realistic. SDS−PAGE (gel not shown) confirmed that the major protein band originally existing in the crude extract and that being enriched during purification was 40−43 kDa, consistent with previous reports on the size of this enzyme (Racusen and Foote, 1980; Racusen, 1984).

Table 1. Purification of Potato LAH

purification step	total act (units)[a]	recov of act (%)	total prot (mg)	specific act (units/mg)	purif (fold)
crude extract	593	100	769	0.77	1.0
40−70% $(NH_4)_2SO_4$	335	56.5	339	0.99	1.3
DEAE-Sephacel	234	39.5	176	1.33	1.7
Con A-Sepharose	124	20.8	38.1	3.25	4.2

[a] One unit of activity is equivalent to 1 μmol of *p*-nitrophenol produced min^{-1} from PNP-laurate at 20−22 °C.

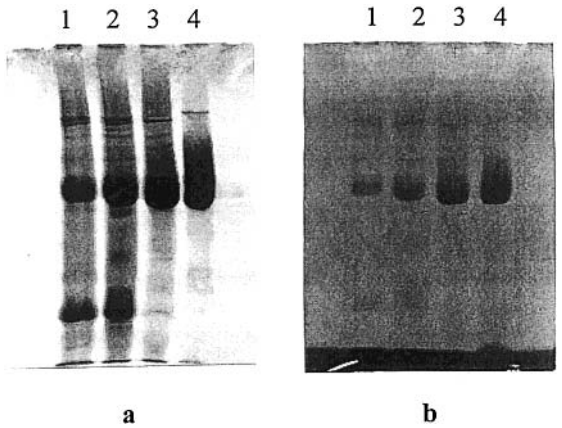

Figure 1. Native PAGE gels of patatin purification, visualized with Coomassie blue (a) and lipase activity (b) stains: crude extract (lane 1); ammonium sulfate-precipitated fraction (lane 2); DEAE-Sephacel-eluted fraction (lane 3); Con A-Sepharose-eluted fraction (lane 4).

Although a few contaminating bands of lower molecular weight were observed in the sample eluted from Con A-Sepharose, the purity of the preparation based on the 40−43-kDa band (presumptively patatin) was estimated at >97% by densitometry.

Results from native PAGE with Coomassie blue and lipase activity staining also supported the premise that LAH activity of the preparations at various stages of purification was inextricably linked to the major 40−43-kDa band (Figure 1). For the crude extract and ammonium sulfate fraction, the major (patatin) band and both lower and higher molecular weight bands showed lipase/esterase activity (Figure 1b, lanes 1, 2). However, only the major (patatin) band in the DEAE-Sephacel and Con A-Sepharose samples showed this same activity (Figure 1b, lanes 3, 4). For the studies presented in the balance of this paper, routinely the ammonium sulfate-precipitated ("crude" patatin) preparation was used. In some cases, the Con A-Sepharose-eluted ("pure" patatin) sample was evaluated to confirm that the characteristics of the active agent were similar between these preparations.

Effect of a_w on Ester Synthesis. Preliminary studies on the optimization of LAH activity revealed that a_w was a critical parameter. The initial rate of esterification of alcohols (*n*-propanol, 2-propanol, 1,3-propanediol) with the best FA substrate (defined by later experiments as C10) increased with increasing a_w between 0.19 and 0.90 (Table 2). In contrast, glycerol showed a greater initial rate of esterification with the best FA substrate (C8) at the lowest a_w in this range. It is unlikely that the difference of using C8 and C10 is responsible for this trend, and this same trend of reactivity of alcohols was found with other lipases (from *Rhizomucor miehei*, *Candida antarctica* B, and *Pseudomonas cepacia*) during the course of parallel studies in our laboratory (Lee and Parkin, unpublished observations).

Table 2. Initial Rates of Esterification Catalyzed by Immobilized Crude Potato LAH in Isooctane as a Function of a_w[a]

	initial rate of esterification (μmol h^{-1} g^{-1} of enzyme) at a_w		
alcohol acceptor	0.19	0.69	0.90
n-propanol	4.18	10.1	20.9
2-propanol	0.48	1.94	4.09
1,3-propanediol	20.0	28.1	29.8
glycerol	12.8	9.63	4.24

[a] C8 and C10 were used for reactions with glycerol and all other alcohol acceptors, respectively. Results are from 3 experiments with a CV of 5%.

The greater initial rate of esterification at greater a_w observed for the alcohols other than glycerol may represent an intrinsic dependence of enzyme activity, and different lipases have different a_w optima for reactivity (Valivety et al., 1994). This effect of a_w may be on overall enzyme conformational plasticity (Klibanov, 1997) or confined to a more localized region of the enzyme, such as the 'hinges and lid' structure in lipases (Valivety et al., 1992). If a_w only exerts an intrinsic effect on enzyme activity, then it would be reasonable to expect the same a_w dependence of reaction rates regardless of choice of alcohol acceptor substrate. The fact that reactions with glycerol exhibited anomalous behavior indicates multiple effects of a_w.

The specific trend observed with glycerol may be attributable to the reduced tendency for aggregation of solids (enzyme, immobilized glycerol, and salt hydrates) in the reaction mixture as a_w is reduced. In keeping with this rationale, reactions with 1,3-propanediol exhibited an a_w dependence that was intermediate to (iso)propanol and glycerol alcohol acceptor systems and also had a lesser tendency to aggregate than did glycerol. The net effect of aggregation can be a reduction in the surface area and creation of diffusional barriers between enzyme and substrate. Interestingly, when similar reactions with glycerol were run in a solvent-free, biphasic reaction medium, no aggregation of the reaction mixture was apparent and the initial rate of ester synthesis was greater at a_w 0.90 than at 0.19 and 0.69 (data not shown).

When 1,2-propanediol was used as alcohol substrate, the rate of ester synthesis was too low to provide for accurate analysis. Less than 1% conversion was observed after 19-h reaction time at each a_w evaluated. Therefore, FA selectivity using 1,2-propanediol as an alcohol acceptor was not evaluated further. However, it has been reported that esterification of 1,2-propanediol and oleic acid in a solvent-free medium catalyzed by patatin showed reactivity, in fact, greater than that with 1,3-propanediol (Macrae et al., 1998). Taken collectively, the influence of the nature of the continuous phase and the interdependence of this parameter with a_w dependence and alcohol reactivity merit further study. Knowing the interdependence of these factors may assist in the design of processes to exhibit the reaction selectivity desired in a specific lipid modification.

FA Selectivity of Patatin. Regardless of a_w and the identity of the alcohol acceptor species used in esterification reactions, patatin was highly selective for medium chain length FAs (C8 and C10) (Figures 2−5).

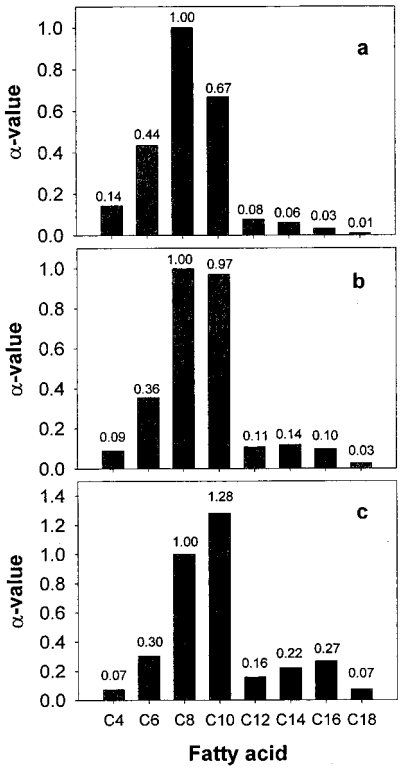

Figure 2. FA selectivity profiles for esterification reactions with *n*-propanol in isooctane catalyzed by potato LAH at a_w 0.19 (a), 0.69 (b), and 0.90 (c). Results are from 2−3 experiments with a CV of about 4%.

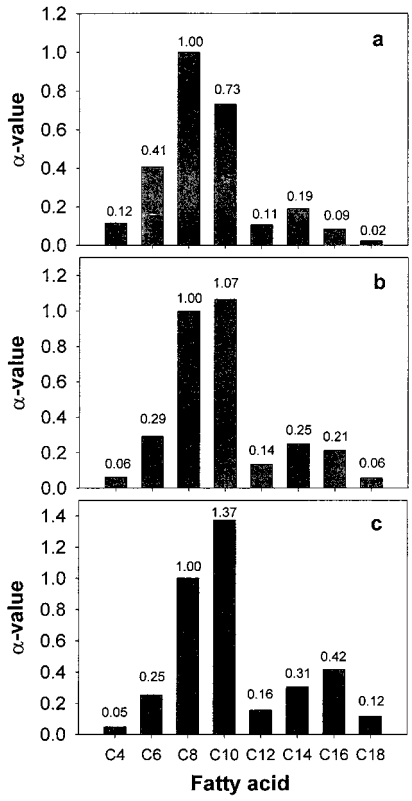

Figure 3. FA selectivity profiles for esterification reactions with 2-propanol in isooctane catalyzed by potato LAH at a_w 0.19 (a), 0.69 (b), and 0.90 (c). Results are from 2−3 experiments with a CV of about 5%.

Of equal interest was that FAs other than C8/C10 were markedly less reactive, as indicated by the reduced α-values. This narrow FA selectivity of patatin appears

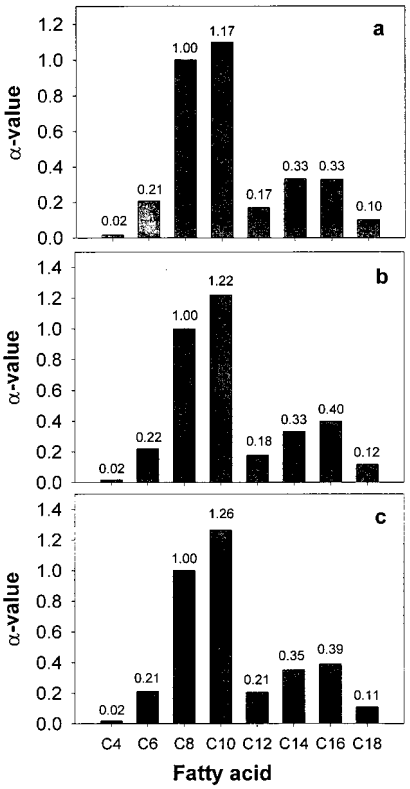

Figure 4. FA selectivity profiles for esterification reactions with 1,3-propanediol in isooctane catalyzed by potato LAH at a_w 0.19 (a), 0.69 (b), and 0.90 (c). Results are from 2−3 experiments with a CV of about 2%.

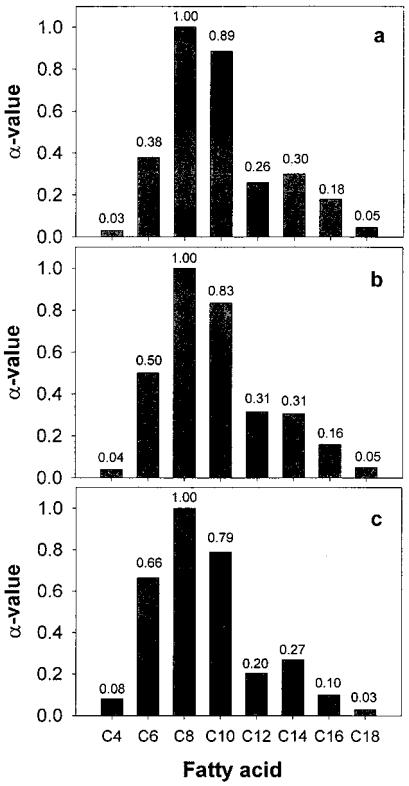

Figure 5. FA selectivity profiles for esterification reactions with glycerol in isooctane catalyzed by potato LAH at a_w 0.19 (a), 0.69 (b), and 0.90 (c). Results are from 2−3 experiments with a CV of about 3%.

to be unique compared to other acyl hydrolases in similar reaction configurations. For example, *R. miehei*

and *Geotrichum candidum* lipases show a broad FA selectivity for C8 to C18 in esterification reactions with (iso)propanol (Rangheard et al., 1989). *C. antarctica* B and *P. cepacia* lipases also have broad FA selectivity in esterification reactions with the same alcohols used in the present study (Chang et al., 1999). Macrae et al. (1998) observed a preference of patatin for C10 and C14 in reactions with FAs and glycerol in solvent-free media, but the use of different reaction temperatures for different FA substrates renders equivocal any comparisons between FA reactivity.

Results from the competitive assays in the present study also indicated a general trend of double optima for FA chain lengths of C8/C10 and C14/C16 with all alcohol substrates. Interestingly, hydrolysis of *p*-nitrophenyl FA esters by patatin also showed double optima for FAs C10 and C16 (Andrews et al., 1988). Multiple FA optima in esterification reaction in microaqueous media have been observed for lipases from *C. rugosa*, *G. candidum*, and *R. miehei* (Sonnet, 1988; Rangheard et al., 1989, 1992; Gandhi et al., 1995; Ader et al., 1997), but this apparent anomaly has received little attention except in a couple of studies (Parida and Dordick, 1993; Chang et al., 1999). It has been proposed that lipases contain multiple subsites within the binding pockets hosting the reactive acyl moiety, with greater catalytic efficiency manifest for substrates of specific sizes (Parida and Dordick, 1993; Kazlauskas, 1994). Other reasons offered for multiple FA chain length optima include multiple acyl binding sites and thermodynamic optimization of the scissile acyl binding site for more than a single acyl chain length substrate (Chang et al., 1999). It is likely that patatin also possesses multiple substrate (FA) binding regions and subsites, given its broad selectivity toward lipid classes, and that multiple FA optima are common among lipid acyl-modifying enzymes.

In addition, these results show an effect of a_w on FA selectivity of patatin (Figures 2−4). The optimum FA chain length shifted from C8 to C10 and from C14 to C16 as a_w increased from 0.19 to 0.90 for reactions with *n*-propanol, 2-propanol, and 1,3-propanediol as alcohol acceptor. This may be attributable to a greater plasticity/flexibility of the enzyme active site at greater a_w and, in turn, an increasing ease of accommodation of substrates that may otherwise pose steric constraints. Again, the effect of a_w on FA selectivity in reactions with glycerol as alcohol acceptor (Figure 5) did not conform to the trend observed for the other alcohols, as a preference for C8 and C14 was maintained throughout the range of a_w evaluated. In fact, increasing a_w in reaction with glycerol led to enhanced reactivity with short chain FAs (C4/C6), a trend opposite of that seen with the other alcohol acceptors.

FA selectivity for ester synthesis was also determined using immobilized "pure" patatin, for reactions with both *n*-propanol and 2-propanol as alcohol acceptors. The same results as observed for the immobilized "crude" patatin were observed in terms of FA selectivity and influence of a_w (data not shown). These results indicated that patatin was responsible for the patterns of FA selectivity in the esterification reactions evaluated with the crude preparation.

Effect of pH. It is known that enzymes are rather rigid in microaqueous media and this confers their ability to structurally "remember" the pH of the last aqueous environment that they encountered (Zaks and Klibanov, 1985). To investigate an influence of pH on the reactivity of patatin in microaqueous media, immobilized patatin preparations were pretreated over a pH range of 4−10, prior to being used in simple esterification reactions (C8 for glycerol and C10 for *n*-propanol, 2-propanol, and 1,3-propanediol). The pH profiles for initial rates of ester synthesis by patatin for all alcohol acceptors were typically bell-shaped with an optimum activity at pH 6−7. The same optimum pH has been reported for the hydrolysis of monoolein in an aqueous medium (Galliard, 1971). Thus, unlike pancreatic lipase, which exhibits optimum activity at pH 8.4 in microaqueous media with no distinct pH optimum observed for hydrolytic activity in an aqueous medium (Zaks and Klibanov, 1985), patatin appears to retain its general pH dependence regardless of the nature of the reaction medium.

In addition, FA selectivity of immobilized patatin preparations at pH 5, 7, and 9 for esterification with 1,3-propanediol was also investigated at a_w 0.19, 0.69, and 0.90. No influence of the pH of enzyme on FA selectivity was observed (data not shown). Collectively, these results indicate that pH of the enzyme preparation influenced reactivity, but not FA selectivity, in esterification reactions.

CONCLUSION

Potato patatin/LAH has synthetic capabilities similar to those of other lipases and can catalyze ester modification reactions in organic media. However, relative to other lipolytic enzymes, patatin appears to have a rather narrow chain length selectivity among FA substrates, and specifically for C8/10, FAs that have long been recognized as important constituents for medium chain glycerides (Babayan, 1968), generally considered a health-promoting value-added product (Merolli et al., 1997). A secondary optimum for FA chain length was noted, and dual FA optima appear to be a common property of lipases in general (Chang et al., 1999). Environmental factors that influence both reactivity and FA selectivity of patatin are a_w and choice of alcohol acceptor. As for the latter factor, the observation that marked differences in activity and selectivity were observed for reactions between 1,2- and 1,3-propanediols and glycerol implicates the nature of the *sn*-2 site along the glycerol backbone as an important control factor for reactivity, as has been noted earlier for lipases (Rogalska et al., 1993; Stadler et al., 1995; Chang et al., 1999). Reaching a full understanding of how selectivity of patatin can be controlled for the range of ester modification reactions leading to the preparation of structured glycerides will be essential to identifying suitable technological applications of this novel food-grade enzyme.

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