Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.11 No.1, Issue of January 15, 2008

Received February 20, 2007 / Accepted July 9, 2007
DOI: 10.2225/vol10-issue4-fulltext-8
RESEARCH ARTICLE

Effect of methyl branching of C_8H_{18} alkanes and water activity on lipase-catalyzed enantioselective esterification of ibuprofen

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Financial support: The NIH supported this research through grant GM061565-01 A2.

Keywords: enantioselective, esterification, ibuprofen, lipase, solvent-effect, water activity.

Abbreviations: NSAIDs: non steroidal anti-inflammatory drugs

The purpose of this research was to study the effect of the methyl branching of a high log P alkane solvent and the water activity in the organic medium on the initial rate and the enantioselectivity of ibuprofen esterification catalyzed by *Candida rugosa* lipase. Resolution of ibuprofen is important because S-(+)-ibuprofen has the desired pharmacological activity, whereas the R-(-)-enantiomer causes much of the side effects. The *Candida rugosa* lipase-catalyzed reaction in isooctane at 40ºC and 0.73 water activity gave the best results, both in terms of the initial reaction rate and the enantioselectivity of the reaction. An increase in water activity allowed a higher reaction rate and enantiomeric excess in each of the four solvents. An increase in methyl branching did not necessarily increase the initial reaction rate, but it allowed a higher enantioselectivity, evidenced by an increase in the substrate enantiomeric excess.

There are many investigatory and marketed drugs which are racemic mixtures, consisting of equal quantities of two optical isomers called enantiomers. These racemic mixtures often have one enantiomer, the eutomer, which possesses most of the pharmacological activity while the other, the distomer, has relatively little pharmacological activity or

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may be responsible for the bulk of the toxicity associated with the drug (Page and Morley, 1999; Handley, 2001). It might be beneficial if the eutomer alone could be administered, thus reducing the dose and the toxicity associated with the therapy (Handley, 2001).

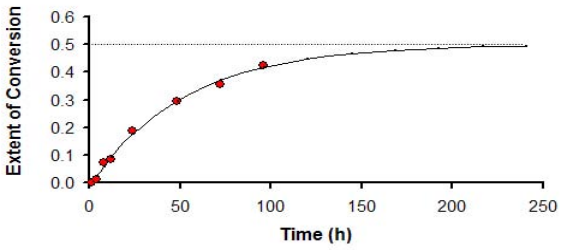

Figure 1. Extent of conversion as a function of time showing asymptotic approach to 0.5 (isooctane, 36 mM initial ibuprofen concentration, 0.73 a_w).

Synthesis of optically pure drugs has been performed by stereoselective crystallization (Sarasua et al. 2005), chemical asymmetric synthesis (Deshmukh, 2006), and preparative chiral chromatography (Ali et al. 2006; Wang et al. 2006). These processes are either time consuming or expensive. Since synthesis of individual enantiomers may be difficult or cost prohibitive, it is often more economical and convenient to prepare the racemic mixture and then resolve the active enantiomer at the completion of the synthetic sequence. Enzymes have become an important means to obtain optically pure compounds because they can enantioselectively catalyze esterification (Shang and Hsu, 2003; Won et al. 2006), transesterification (Kitano et al. 1999), hydrolysis (Li et al. 2004), and other chemical reactions involving chiral substrates. The advantages of biocatalytic reactions in organic solvents are (i) efficient catalysis of reactions with substrates that have poor aqueous solubilities, (ii) easier recovery of an insoluble enzyme from the organic medium for re-use, (iii) fewer undesirable side reactions that can occur with the participation of water, (iv) thermodynamic equilibria of processes such as esterification are unfavorable in water, and (v) the greater stability of some enzymes in anhydrous or nearly anhydrous media (Chen and Sih, 1989; Castro and Knubovets, 2003; Chenevert et al. 2006).

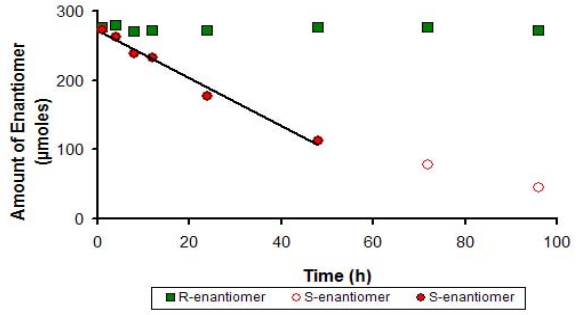

Figure 2. Enantioselectivity of the *C. rugosa* lipase-catalyzed reaction in isooctane at a 0.73 water activity and with an initial ibuprofen concentration of 36 mM. The initial reaction rate is calculated from the absolute value of the slope through the initial linear portion of the hyperbolic curve.

Ibuprofen, a non-steroidal anti-inflammatory drug which is available as a racemic mixture, was selected as a model drug because there are several chemically related non steroidal anti-inflammatory drugs (NSAIDs) currently marketed or in clinical evaluation that are also racemic mixtures. Since the therapeutic properties of ibuprofen and most NSAIDs have been shown to reside in the S-(+)-enantiomer (Evans, 2001), it is beneficial to resolve the enantiomers. The kinetic resolution of ibuprofen enantiomers using lipase has been studied (Yu et al. 2004; Yu et al. 2005), and an increase in the hydrophobicity of the solvent can increase the reaction rate (Mustranta, 1992; Kim and Lee, 1996). Lipase from *Candida rugosa* is reported to show a greater preference for the esterification of the S-enantiomer of ibuprofen (Mustranta, 1992; Xie et al. 1998a) and of naproxen (Tsai et al. 1999; Shang and Hsu, 2003), whereas lipase from *Candida antarctica* prefers the R-enantiomer (Xie et al. 1998b; Park et al. 1999). The preference of *C. rugosa* lipase for the S-enantiomer of ibuprofen in the present study should be due to the presence of isoenzyme L1 in great excess over L2 (Alcántara et al. 2004). Isoenzyme L1 is apparently S-stereoselective and L2 is R-stereoselective (Alcántara et al. 2004), and the L1 isoenzyme is highly stereoselective (Lopez et al. 2004). Since lipase from *Candida antarctica* has been shown to possess low enantioselectivity (Roure et al. 1997), *Candida rugosa* lipase was selected as the enzyme for use in the present study. *Candida rugosa* lipase has also been shown to be more efficient and enantioselective in an organic solvent when compared to an aqueous or biphasic medium (Kim and Lee, 1996).

The enzyme will be allowed to catalyze the esterification reaction of enantiomers of ibuprofen with n-butanol. It is well known that a higher rate of esterification is obtained in a high log P organic solvent when using lipase as a biocatalyst, and isooctane has consistently outperformed other alkanes possessing the same or higher log P value in terms of esterification rate and enantioselectivity (Mustranta, 1992; Bhandarkar and Neau, 1997; Tsai et al. 1997; Ducret et al. 1998; Bhandarkar and Neau, 2000). For that reason, the effect of branching in an organic solvent on the initial reaction rate and the enantioselectivity of the reaction was studied. Several alkane solvents possessing eight carbons to yield a chemical formula of C_8H_{18}, but with different extents of methyl branching, were used. They are n-octane, 2-methylheptane, 2,5-dimethylhexane, and 2,2,4-trimethylpentane (also known as isooctane). (To extend this series further is problematic since 2,2,3,3-tetramethylbutane is a solid at room temperature). These alkanes have extremely similar densities of 0.6965 ± 0.0051 g/cm^3 (Lide, 2006) such that the density of the medium is not a determining factor in, for example, diffusion of the substrate or product. They differ in their vapor pressures, showing an increase in vapor pressure with an increase in the extent of methyl branching, as well as an increase in the boiling point with a decrease in methyl branching of these solvents (Yaws and Yang, 1992). These differences in

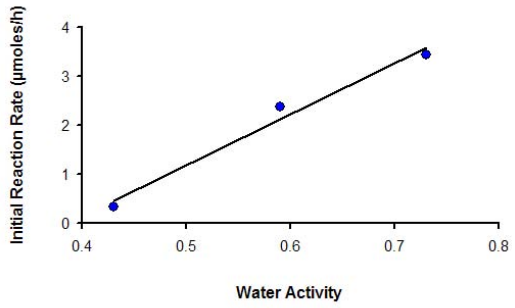

Figure 3. Initial reaction rate as a function of the water activity (isooctane, 36 mM initial ibuprofen concentration).

vapor pressure and boiling point reflect the differences in the interactive forces possible within the pure solvent that could also present the decreasing order of strength of the interactive forces possible with any solvated or suspended material. Thus, weakening of these intermolecular interactions increases with branching and may have an effect on the initial reaction rate and the enantioselectivity of the enzyme. These last two parameters indicate that their ability to relate to other molecules will differ in strength, and thus they prove to be a good set of solvents to study the effect of methyl branching on the reaction characteristics.

There is a common belief that an enzyme requires the presence of some water to perform properly in an organic medium (Chen and Sih, 1989). Since the non-covalent bonding of water to the enzyme maintains the catalytically active conformation of the enzyme, the organic solvent must still be able to provide a minimal amount of water. Although addition of water to a reaction medium is common, this technique falls short in that catalytic activity drops when the water level in the organic media is increased beyond an optimum level, which would occur with esterification reactions since water is a product of the reaction. The activity of water experienced by the lipase can be known, as the activity of water in the organic reaction medium can be controlled using appropriate pairs of hydrates of inorganic salts (Halling, 1992).

There are several advantages to using salt hydrates as water activity buffers, most importantly: (i) a broad list of pairs of salt hydrates and the water activity they maintain is available in the literature, (ii) simplicity of the experimental set up, and (iii) since water will be generated in the reaction being studied, and this water may change the moisture in the enzyme microenvironment, the presence of a material that will pick up excess water or distribute required water would help in maintaining the uniformity of conditions throughout the length of the experiment. To control water activity in the range of 0.43 to 0.73 at 40ºC, appropriate pairs of salt hydrates (Halling, 1992) will be added to the reaction medium.

The first objective of the study was to add knowledge about how a broad set of conditions, namely *Candida rugosa* lipase, the organic solvent, and the water activity, influence the progress of lipase-catalyzed enantioselective esterification reactions. The second objective was to explore the effect of water activity on the initial reaction rate and enantioselectivity of those reactions. The third objective was to determine whether an increase in methyl branching of the alkane solvent has an effect on the initial reaction rate or enantioselectivity of the *Candida rugosa* lipase-catalyzed reactions.

MATERIALS AND METHODS

Candida rugosa lipase (1140 Units/mg solid, substantially free of amylase and protease) was purchased from Sigma Chemical Company (St. Louis, MO). Sigma Chemical Company states that one unit will hydrolyze 1.0 micro equivalent of fatty acid from olive oil in one hr at pH 7.2 at 37ºC.

2,2,4-Trimethylpentane and n-octane were purchased from Aldrich (St. Louis, MO), and 2-methylheptane and 2,5-dimethylhexane from Pfaltz & Bauer (Waterbury, CT) and Frinton Laboratories (Vineland, NJ), respectively. Racemic ibuprofen was available from Alfa Aesar (Ward Hill, MA) and S-(+)-ibuprofen was purchased from Fluka Chemical Corporation (St. Louis, MO). Water activities in the range of 0.43 to 0.73 at 40ºC are controlled using the following salt hydrate pairs (Halling, 1992):

$Na_2HPO_4 \cdot 7H_2O/2H_2O$ $a_w = 0.73$
$Na_4P_2O_7 \cdot 10H_2O/0H_2O$ $a_w = 0.59$
$NaBr \cdot 2H_2O/0H_2O$ $a_w = 0.43$

$Na_2HPO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot 2H_2O$ and $Na_4P_2O_7 \cdot 10H_2O$ were purchased from Fluka Chemical Corporation, whereas $Na_4P_2O_7 \cdot 0H_2O$ was available from Sigma Chemical Company. Anhydrous sodium bromide salt was available from Alfa Aesar, and the dihydrate form was prepared using a literature method (Halling, 1992). Briefly, the required hydrate form is obtained by incubating the salt in an atmosphere of controlled water activity which can be produced by a saturated salt solution. For example, the

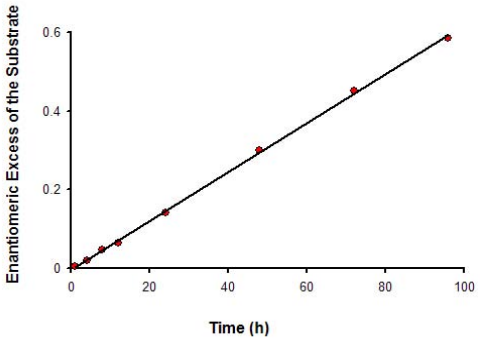

Figure 4. Representative plot of data for reactions at 0.59 and 0.73 water activities (n-octane, 60 mM initial ibuprofen concentration, 0.73 water activity). All of the 96 hrs data were used to calculate the rate of improvement in the enantiomeric excess of the substrate in these cases.

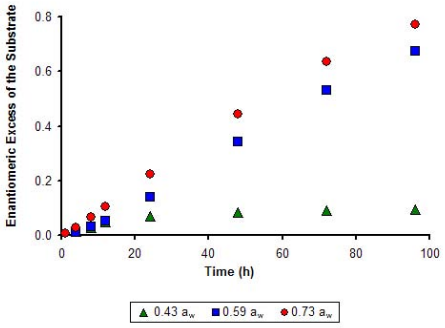

Figure 5. Enantiomeric excess of the substrate as a function of time, showing the increase in enantiomeric excess at each time point with an increase in the water activity (isooctane, 60 mM initial ibuprofen concentration).

anhydrous salt of disodium hydrogen phosphate can be obtained by equilibrating the salt at any water activity comfortably within the range 0 to 0.163 a_w. The advantage of saturated salt solutions is that they can gain or lose water without changing the vapor pressure. As the salt required to be generated is sodium bromide dihydrate, it is essential to equilibrate the salt at any water activity comfortably within the range of 0.163 to 0.610 a_w, as described by Halling (1992). In this study, a saturated salt solution of sodium bromide was used to control the water activity. Sodium bromide at 25ºC is known to give a saturated salt solution water activity of 0.576 (Greenspan, 1977). The sodium bromide dihydrate product obtained was characterized by comparing the following ratios:

$$\frac{\text{molecular weight of sodium bromide dihydrate}}{\text{molecular weight of sodium bromide}} = \frac{\text{target mass}}{\text{initial mass}}$$

to determine the target mass. The dihydrate formed by this technique came to 96% of its target mass.

Experimental set-up

Twenty milliliter screw-capped vials with caps lined with foamed polyethylene were used as reaction vessels. This capped vial was found to adequately resist the loss of the volatile solvents for up to 96 hrs. Stock solutions of ibuprofen and 720 mM n-butanol in the solvent being studied were prepared in advance. The 720 mM concentration for n-butanol was chosen so that the n-butanol concentration in the reaction mixture remains essentially unchanged during the course of the reaction.

To prepare a reaction mixture, 10 ml of ibuprofen solution was first added to the reaction vessel. To this, 196 mg of powdered *Candida rugosa* lipase was added. To maintain the desired water activity, 100 and 900 mg of the higher and lower hydrates, respectively, from the appropriate pair of salt hydrates were also added. The lower hydrate was in excess since water would be generated as the reaction progressed and uptake of water would generate the higher hydrate. At least four vials were used for each concentration of ibuprofen at a desired water activity in each organic solvent. The vials and their contents were shaken in a Polyscience microprocessor controlled shaker bath at 40ºC at 165 cycles/min for 24 hrs to allow equilibration of the enzyme and solvent system at the target water activity provided by the salt hydrates. Five milliliters of 720 mM n-butanol previously equilibrated for 24 hrs at the target water activity were added to the reaction mixture resulting in an n-butanol concentration of 240 mM. Shaking was then begun and 40 µl samples were taken at 4, 8, 12, 24, 48, 72, and 96 hrs. Each sample was filtered through a hydrophobic Millipore 0.45 µm pore size PTFE filter (Millipore Corporation, Bedford, MA). The organic solvent was evaporated immediately after sampling in a Savant Speed Vac SPD101B (Global Medical Instrumentation, Ramsey, MN) and the samples were analyzed using a chiral HPLC method.

HPLC analysis

The concentration of the unreacted enantiomers of ibuprofen was determined by an enantioselective HPLC method using a chiral α_1-acid glycoprotein column, based on modification of a literature method (Geisslinger et al. 1994). The α_1-acid glycoprotein column was purchased from Chrom Tech Inc. The chromatographic system consisted of a Series 1050 pump, injector and a UV detector set at 225 nm (Hewlett Packard, Palo Alto, CA). The mobile phase consisted of 15 mM potassium phosphate buffer (pH 7.0) with 0.25 mM N,N-dimethyloctylamine as a charge modifier. The flow rate was maintained at 0.9 ml/min and the injection volume was 20 µl.

Data analysis

The initial reaction rate and the enantiomeric excess of the remaining substrate molecules are two parameters that have been used as the basis to compare the performance of different organic solvents at different water activities (Bhandarkar and Neau, 2000). The initial reaction rate is obtained from the absolute value of the slope of the linear portion of the hyperbolic curve obtained by plotting substrate mass present as a function of time, and it is reported in units of µmoles/hrs. The enantiomeric excess of the remaining substrate molecules was calculated using the following formula (Chen et al. 1982):

$$ee_s = ([R] - [S])/([R] + [S]) \quad [1]$$

where [R] is the concentration of the slower reacting R-enantiomer and [S] is the concentration of the faster reacting S-enantiomer at the time at which the enantiomeric excess is calculated. The enantiomeric excess of the substrate will be calculated for all time points up to 96 hrs and the ee_s provided by each solvent at a particular water activity at a particular point of time will be compared. The extent of conversion, c, is the fraction of product formed at

any given time. It is calculated using the formula (Chen et al. 1982):

$$c = 1 - (([R] + [S])/([R]_o + [S]_o)) \quad [2]$$

where $[R]_o$ is the initial concentration of the R-enantiomer, and $[S]_o$ is the initial concentration of the S-enantiomer.

The reversibility of the reaction could be a problem when the water activity is controlled (Wu and Liu, 2000). For reversible systems, the enantiomeric excess of the substrate is dependent on not only the extent of conversion and the enantiomeric ratio, but also on an equilibrium constant, K. Chen et al. (1987) presented modifications to the calculation of the enantiomeric ratio, E, that reflect the effect of reversibility by inclusion of an equilibrium constant, K:

$$E = \frac{\ln[1- (1 + K)(c + ee_s\{1 - c\})]}{\ln[1- (1 + K)(c - ee_s\{1 - c\})]} \quad [3]$$

and Wu and Liu (2000) provided a means to estimate the value of K:

$$K = \frac{1 - X^e_s}{X^e_s} \quad [4]$$

where X^e_s is the extent of conversion of the faster reacting S-enantiomer of ibuprofen when the equilibrium is established.

RESULTS AND DISCUSSION

Effects of the solvent and water activity on the extent of conversion

The extents of conversion at 48 hrs are reported in Table 1. The extent of conversion at 48 hrs is reported because one can distinguish at this time between two reactions that might have similar extents of conversion at 96 h. When the sum of the concentration of the R- and S-enantiomers of ibuprofen at the 96 h time point were compared to the initial ibuprofen concentration, it was found that less than 2% of the total was esterified in the reactions involving 2,5-dimethylhexane, 2-methylheptane, or n-octane at a water activity of 0.43. The values for reactions at 0.43 water activity in these cases are not reported in Table 1. Isooctane proved to be the exception, with 7.5% of the ibuprofen esterified at 96 hrs at a water activity of 0.43. Alcántara et al. (2004) also reported low yields of esters of ketoprofen using *Candida rugosa* lipase at 30ºC when the water activity was less than 0.5. At 0.73 and 0.59 water activities, each of the reaction conditions studied provided an extent of conversion that approached 0.5 with time. For example, see Figure 1 for the data for the reaction in isooctane with an initial 36 mM ibuprofen concentration at 0.73 a_w. That the extent of conversion approaches a value of 0.5 is in keeping with the observation that the R-enantiomer of ibuprofen essentially does not react, and therefore the conversion cannot exceed 50% of the initial racemic ibuprofen until the slower reacting R-enantiomer no longer provides a negligible reaction. Note that a higher water activity resulted in a higher extent of conversion. There is no evidence of a trend with respect to the influence of methyl branching of the solvent on the extent of conversion. Apparently when these solvents are equilibrated to a particular water activity they might lose their differences in their ability to relate to the enzyme. The data indicate that control of the water activity can essentially eliminate a solvent effect, with the exception of 2,5-dimethylhexane results that are substantially lower than found with the other reactions. Among the reaction conditions studied, isooctane with a water activity of 0.73 provides the conditions for a maximum extent of conversion at 48 hrs.

Effects of the solvent and water activity on the initial reaction rate

Initial reaction rates were obtained from the absolute value of the slope of the initial linear portion of the hyperbolic curve obtained by plotting S-(+)-enantiomer mass present in the reaction as a function of time (Figure 2), and it is reported in micromoles/h. The R-(-)-enantiomer of ibuprofen showed an insignificant esterification of less than 5% at 96 hrs for each reaction. Initial reaction rates, reported in Table 2, were calculated for each reaction except those at 0.43 water activity in n-octane, 2-methylheptane, and 2,5-dimethylhexane, where the reaction rates were negligible. Data up to 48 hrs were used for calculation of initial reaction rates in each case. In isooctane, a minimum four-fold increase in the initial reaction rate was observed with an increase in the water activity in the medium from 0.43 to 0.73 at each initial ibuprofen concentration.

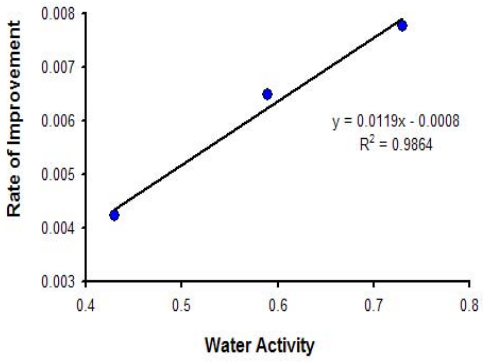

Figure 6. The rate of improvement (h^{-1}) in the enantiomeric excess of the substrate as a function of the water activity. Isooctane, 36 mM initial ibuprofen concentration.

The highest initial reaction rate was observed in isooctane at a water activity of 0.73. Figure 3 shows the increase in the initial reaction rate of 36 mM ibuprofen in isooctane with an increase in water activity from 0.43 to 0.73. It should be noted that water activity might show a different effect on lipase-catalyzed reactions when lipase is obtained from different sources. In the case of lipase from *Rhizomucor miehei*, high esterification rates were reported at a water activity of 0.12, and lipase from *Rhizopus niveus* was also fairly active at low water activity (Valivety et al. 1992). The highest water activity, 0.73, provided the fastest initial reaction rates in each reaction in the present study. Under those conditions where the active site of the enzyme has an affinity for the substrate, maintaining the optimum water activity can still fine tune the activity to provide further improvements in the reaction.

Although isooctane, the most highly branched of the solvents, provided the maximal initial reaction rate, there was no trend with an increase in methyl branching. The unbranched alkane, n-octane, yielded higher initial reaction rates than 2,5-dimethylhexane and 2-methylheptane allowed. 2,5-Dimethylhexane provided the lowest initial reaction rates.

Effects of the solvent and water activity on the enantiomeric excess of the substrate

The enantiomeric excess of the substrate was found to increase as the reaction proceeded, even to the last time point, 96 hrs. See Figure 4 for a plot of the enantiomeric excess of the substrate as a function of time for the esterification of 60 mM ibuprofen in n-octane at a water activity of 0.73. The enantiomeric excess of the substrate at 48 hrs for each of the reactions where a reaction was observed is reported in Table 3. A decrease in the enantiomeric excess of the substrate at any time point is observed with a decrease in the water activity of the system (Figure 5 and Table 3). It is well documented that a certain amount of water is necessary for catalytic activity in organic media, but that an excessive amount of water can result in hydrolysis of the product. Therefore, control of the water activity is important for esterification reactions in organic media. In the case of *Candida rugosa* lipase-catalyzed esterification of flurbiprofen with n-butanol in isooctane at 30ºC, the maximum enantiomeric excess at each time point was obtained with a water activity of 0.65, but a lower enantiomeric excess was observed with an increase in the water activity to 0.85 (Bhandarkar and Neau, 2000). Under the conditions of the present study, a decrease in enantiomeric excess of the substrate was therefore expected in going from 0.59 to 0.73 water activity. The fact that this was not observed suggests that the critical water activity at 40ºC, above which the enantiomeric excess would begin to decline, is higher than 0.73. The increase in temperature to 40ºC likely shifts the maximum water activity to a higher value. Since the water activity in the present study was maintained using the salt hydrate pairs suggested by Halling (1992), the highest water activity that can be achieved at 40ºC is only 0.75, and the water activity that provides the maximum enantiomeric excess of the substrate could not be confirmed.

The slope of a plot of enantiomeric excess of the substrate as a function of time (Figure 4) is designated here as the rate of improvement in the enantiomeric excess of the substrate (RI) to allow comparison across water activities and solvents. For those reactions at 0.43 water activity, there was a linear relationship between enantiomeric excess of the substrate and time only in the initial portion of the plot and the slope of this initial linear portion of the plot is reported as the rate of improvement in the enantiomeric excess of the substrate. A plot showing the rate of improvement in the enantiomeric excess of the substrate as a function of the water activity for the esterification of 60 mM ibuprofen in n-octane is shown in Figure 6. Similar plots were observed for each of the solvents at each ibuprofen initial concentration. The RI for the esterification reactions under various conditions are reported in Table 4.

It was suspected that the plateau observed in the plots of the concentration of the S-enantiomer as a function of time and in the plots of enantiomeric excess of the substrate as a function of time for reactions at the lowest water activity might be due to establishment of an equilibrium. The equations allow calculation of the equilibrium constant, K, and the enantiomeric ratio, E, for this equilibrium situation. The E and the corresponding value of K (if it needed to be calculated) for the various reactions are presented in Table 5. Since the enantiomeric ratio is not dependent on the initial substrate concentration, there is only one E value reported for each solvent at each water activity. Note that only the lowest water activity resulted in a situation where

the S-enantiomer was obviously not undergoing a reaction to completion. E values of 5-10 are considered modest (Chen et al. 1982), and these modest values in Table 5 could reflect the reported detrimental effect of n-butanol on the esterification of non-steroidal anti-inflammatory drugs catalyzed by *Candida cylindracea* (now *Candida rugosa*) lipase (Wu and Liu, 2000). Nevertheless, the effect of methyl branching is observed in the values of E, which is seen to increase with an increase in methyl branching. The values of E for 2-methylheptane and 2,5-dimethylhexane are not statistically different from each other at each of the water activities, although the mean values indicate that the greater methyl branching of 2,5-dimethylhexane results in a greater enantiomeric ratio.

CONCLUDING REMARKS

The *Candida rugosa* lipase-catalyzed reaction in isooctane at 40ºC and a water activity of 0.73 gave the best results, both in terms of the initial reaction rate and the enantioselectivity of the reaction. In each of the four solvents, the activity of the enzyme increased with an increase in the water activity of the system, supporting the claim that a minimal quantity of water is required for an

enzyme to perform its function in organic media. An increase in water activity allowed a faster initial reaction rate in each of the four solvents. An increase in methyl branching did not necessarily increase the initial reaction rate. However, an increase in methyl branching allowed a higher enantioselectivity, evidenced by the increase in the substrate enantiomeric excess at each time point and by the improvement in the enantiomeric ratio.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Ana Iriarte and Joseph Mattingly from the School of Biological Sciences at UMKC for helpful discussion.

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APPENDIX.

Table 1. Extent of conversion of *Candida rugosa* lipase-catalyzed esterification of ibuprofen at 48 hrs under various conditions.

Ibuprofen Concentration		60 mM	48 mM	36 mM	24 mM
Isooctane	a_w = 0.73	0.29 ± 0.01	0.23 ± 0.01	0.29 ± 0.01	0.27 ± 0.02
	a_w = 0.59	0.20 ± 0.01	0.21 ± 0.01	0.23 ± 0.01	0.24 ± 0.01
	a_w = 0.43	0.06 ± 0.01	0.06 ± 0.01	0.03 ± 0.02	0.03 ± 0.01
2,5-Dimethylhexane	a_w = 0.73	0.17 ± 0.04	0.12 ± 0.01	0.07 ± 0.01	ND
	a_w = 0.59	0.11 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	ND
	a_w = 0.43	NR	NR	NR	ND
2-Methylheptane	a_w = 0.73	0.28 ± 0.01	0.25 ± 0.01	0.24 ± 0.01	0.24 ± 0.01
	a_w = 0.59	0.24 ± 0.01	0.21 ± 0.01	0.18 ± 0.01	0.23 ± 0.01
	a_w = 0.43	0.13 ± 0.002	0.056 ± 0.002	0.028 ± 0.002	NR
n-Octane	a_w = 0.73	0.26 ± 0.01	0.22 ± 0.05	0.21 ± 0.01	0.22 ± 0.01
	a_w = 0.59	0.24 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.25 ± 0.01
	a_w = 0.43	0.074 ± 0.005	0.066 ± 0.008	0.086 ± 0.015	0.189 ± 0.001

ND: indicates the reaction was not performed.
NR: indicates no reaction was observed.

Table 2. Initial reaction rates of *Candida rugosa* lipase-catalyzed esterification of ibuprofen under various conditions.

Ibuprofen Concentration		60 mM	48 mM	36 mM	24 mM
Isooctane	a_w = 0.73	5.02 ± 0.13	3.76 ± 0.04	3.44 ± 0.14	1.91 ± 0.08
	a_w = 0.59	3.89 ± 0.07	3.25 ± 0.12	2.37 ± 0.09	1.55 ± 0.14
	a_w = 0.43	1.12 ± 0.04	0.77 ± 0.02	0.33 ± 0.04	0.22 ± 0.06
2,5-Dimethylhexane	a_w = 0.73	2.62 ± 0.28	1.46 ± 0.03	0.85 ± 0.03	ND
	a_w = 0.59	1.57 ± 0.13	0.94 ± 0.02	0.58 ± 0.08	ND
	a_w = 0.43	NR	NR	NR	ND
2-Methylheptane	a_w = 0.73	3.64 ± 0.10	2.63 ± 0.20	2.02 ± 0.05	1.48 ± 0.06
	a_w = 0.59	3.12 ± 0.24	2.31 ± 0.16	1.94 ± 0.06	1.20 ± 0.12
	a_w = 0.43	0.71 ± 0.14	1.03 ± 0.18	1.01 ± 0.09	NR
n-Octane	a_w = 0.73	4.00 ± 0.06	2.91 ± 0.08	2.54 ± 0.17	1.68 ± 0.08
	a_w = 0.59	3.82 ± 0.07	2.45 ± 0.06	2.18 ± 0.08	1.51 ± 0.06
	a_w = 0.43	0.27 ± 0.05	0.46 ± 0.04	0.28 ± 0.01	0.05 ± 0.01

ND: indicates the reaction was not performed.
NR: indicates no reaction was observed.

Table 3. Enantiomeric excess of the substrate at 48 hrs revealing little effect from initial ibuprofen concentration, but influences of both water activity and solvent.

Ibuprofen Concentration		60 mM	48 mM	36 mM	24 mM
Isooctane	a_w = 0.73	0.442 ± 0.0127	0.402 ± 0.0126	0.419 ± 0.0053	0.462 ± 0.0074
	a_w = 0.59	0.343 ± 0.0101	0.330 ± 0.0088	0.313 ± 0.0019	0.303 ± 0.0093
	a_w = 0.43	0.0852 ± 0.0001	0.0783 ± 0.0061	0.0818 ± 0.0001	0.0907 ± 0.0045
2,5-Dimethylhexane	a_w = 0.73	0.247 ± 0.0078	0.201 ± 0.0029	0.161 ± 0.0067	ND
	a_w = 0.59	0.118 ± 0.0056	0.104 ± 0.0011	0.0836 ± 0.0046	ND
	a_w = 0.43	NR	NR	NR	ND
2-Methylheptane	a_w = 0.73	0.396 ± 0.0023	0.385 ± 0.0045	0.380 ± 0.0056	0.378 ± 0.0011
	a_w = 0.59	0.361 ± 0.0010	0.318 ± 0.0026	0.303 ± 0.0070	0.286 ± 0.0064
	a_w = 0.43	0.042 ± 0.0022	0.068 ± 0.0077	0.028 ± 0.0017	NR
n-Octane	a_w = 0.73	0.300 ± 0.0065	0.279 ± 0.0001	0.250 ± 0.0065	0.300 ± 0.0040
	a_w = 0.59	0.215 ± 0.0022	0.224 ± 0.0071	0.182 ± 0.0011	0.185 ± 0.0042
	a_w = 0.43	0.014 ± 0.0003	0.026 ± 0.0067	0.037 ± 0.0093	0.037 ± 0.0007

ND: indicates the reaction was not performed.
NR: indicates no reaction was observed.

Table 4. The rate of improvement (RI in h^{-1}) in the enantiomeric excess of the substrate and the fit of the estimating line expressed as the coefficient of determination, r^2. The value of RI precedes the value of r^2.

Ibuprofen Concentration		60 mM	48 mM	36 mM	24 mM
Isooctane	a_w = 0.73	0.0085 0.9933	0.0077 0.9919	0.0078 0.9929	0.0086 0.9880
	a_w = 0.59	0.0071 0.9915	0.0068 0.9968	0.0065 0.9972	0.0063 0.9973
	a_w = 0.43	0.0038 0.9528	0.0039 0.9590	0.0042 0.9731	0.0045 0.9402
2,5-Dimethylhexane	a_w = 0.73	0.0051 0.9989	0.0041 0.9977	0.0033 0.9941	ND
	a_w = 0.59	0.0032 0.9514	0.0028 0.9603	0.0021 0.9790	ND
	a_w = 0.43	NR	NR	NR	ND
2-Methylheptane	a_w = 0.73	0.0075 0.9921	0.0074 0.9935	0.0073 0.9929	0.0073 0.9951
	a_w = 0.59	0.0073 0.9966	0.0067 0.9967	0.0064 0.9962	0.0059 0.9946
	a_w = 0.43	0.0024 0.9652	0.0037 0.9958	0.0033 0.9858	NR
n-Octane	a_w = 0.73	0.0062 0.9992	0.0057 0.9788	0.0051 0.9758	0.0060 0.9980
	a_w = 0.59	0.0048 0.9901	0.0051 0.9729	0.0041 0.9856	0.0042 0.9909
	a_w = 0.43	0.0017 0.9439	0.0020 0.9754	0.0021 0.9303	0.0025 0.9868

ND: indicates the reaction was not performed.
NR: indicates no reaction was observed.

Table 5. The substrate-concentration independent enantiomeric ratio for the various batches, revealing evidence of the effect of methyl branching and confirming the effect of water activity on the esterification reactions. The value of the equilibrium constant, K, is found in parentheses after the enantiomeric ratio if K was not zero.

Ibuprofen Concentration		**Enatiomeric Ratio**
Isooctane	a_w = 0.73	14.9 ± 2.7
	a_w = 0.59	9.7 ± 1.9
	a_w = 0.43	4.0 ± 0.8
2,5-Dimethylhexane	a_w = 0.73	11.0 ± 1.6
	a_w = 0.59	6.5 ± 1.4
	a_w = 0.43	NR
2-Methylheptane	a_w = 0.73	10.1 ± 1.4
	a_w = 0.59	6.1 ± 1.3
	a_w = 0.43	NR
n-Octane	a_w = 0.73	6.6 ± 1.1
	a_w = 0.59	3.4 ± 0.3
	a_w = 0.43	2.0 ± 0.4 (7.3 ± 1.3)

NR: indicates no reaction was observed.