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Control of water activity in lipase catalysed esterification of chiral alkanoic acids

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ABSTRACT

Candida rugosa lipase (CRL) catalysed enantioselective esterification of racemic 2-methylhexanoic acid was performed with 1-decanol in organic solvent at constant water activity (a_w). The a_w was maintained either by a salt hydrate pair added in the reaction mixture or by performing the reaction in an air tight desiccator over an aqueous saturated salt solution. It was found that the enatiomeric ratio and average reaction rate profiles were similar when controlling the water activity for the esterification reaction with these two different methods. But, in some cases the average rate of the reaction and the enantioselectivity of CRL was affected when ions from the salt hydrate pairs were present in the reaction mixture.

1. Introduction

The effect of water activity (a_w) on enzyme-catalysed reactions in non-aqueous media is well studied [1–5] and it is widely accepted that each enzyme has specific a_w requirements [2,6]. There are some examples in the literature on the effect of a_w on the enantioselectivity of enzymes [7–10] but also some research groups have found that a_w does not influence the enantioselectivity of an enzyme [11,12].

Different methods have been used to control the water activity in enzyme-catalysed reactions in non-aqueous media. Several research groups have pre-equilibrated the reactants, reaction medium, and the enzyme with an aqueous saturated salt solution of known a_w before starting the reaction [1,4,6,9,11,13–17]. With this method it is possible to obtain the required a_w at the start of the reaction but if it is an esterification that is studied, water is released as the reaction progresses and the a_w of the system changes. If the a_w is too high reversal reactions like hydrolysis of the formed ester might occur [3,18]. On the other hand if the a_w is too low then the reaction rate might be greatly reduced [3]. To maintain a constant a_w throughout the reaction, salt hydrate pairs have been widely used [3,5,7,19,20]. However, some papers have been published lately presenting alternative methods to the addition of salt hydrates directly in to the reaction mixture to maintain constant water activity throughout the reaction. These include, circulating a saturated salt solution of known a_w through silicone tubing immersed in the reaction mixture [21,22], pumping a saturated salt solution of known a_w through a microporous hollow fibre polypropylene membrane in a packed bed membrane reactor [23] or performing the reaction in open eppendorf tubes in an air tight plexiglass chamber with saturated aqueous solutions of various salts [10]. More recently some research groups have used even more sophisticated methods as computer aided a_w control system to maintain a constant a_w in enzyme-catalysed reactions [24,25].

Many of the salt hydrates have not been tested for the compatibility with biocatalysts and that adverse effects of the ions on the biocatalyst are a potential concern, but evidence so far shows that this is not a serious problem [3–6]. But, Zacharis et al. reported that ions from the salt hydrate pairs might associate with the enzyme and modify its microenvironment and if ions such as Cu(II) are transferred to the enzyme, inactivation of the enzyme may be the result [20]. Some researchers have observed an impact on the reaction rate and the lipase enantioselectivity when the a_w was maintained using salt hydrates or when metal salts were employed in the reaction [7,26–28]. Kvitingen et al. reported that some salt hydrates showed anomalous behavior in the CRL catalysed esterification of butanoic acid [19]. Can it be that the ions of these salt hydrates not only adjust the water activity but also affect both the enantiomeric ratio and the rate of esterification? This prompted

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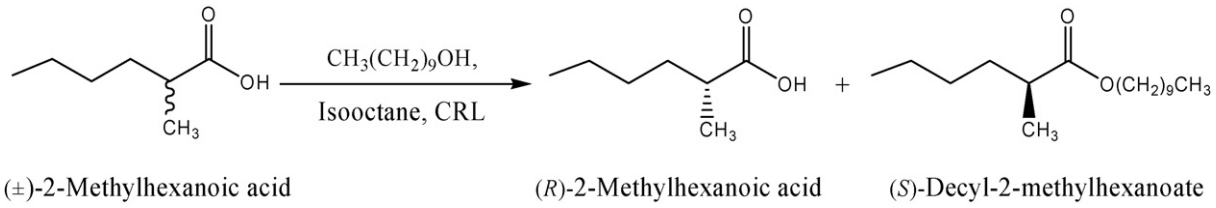

Scheme 1. CRL catalysed enantioselective esterification of (±)-2-methylhexanoic acid.

us to investigate an alternative method for controlling the water activity where the impact of such salt hydrate pairs is avoided. Thus, as a model reaction we studied the enantioselective esterification of a 2-methylalkanoic acid in an organic solvent at controlled water activity using a method which avoided the addition of the salt hydrate pairs into the reaction (Scheme 1). We report in this paper the differences and similarities in the enantiomeric ratio and the esterification rate observed when a_w was maintained either by employing the traditional method with salt hydrate pairs in the reaction mixture or when performing the reaction in an air tight desiccator over an aqueous saturated salt solution of known water activity.

2. Experimental

2.1. Materials and methods

Candida rugosa lipase-type VII (Lot: 074K0685, activity: 1410 units/mg solid) was purchased from Sigma–Aldrich and stored in a desiccator over silica gel at 6 °C. Racemic 2-mehtylhexanoic acid and 1-decanol was purchased from Sigma–Aldrich. Accurel 200–350 μm (EP 100) was purchased from Accurel systems, AKZO Faser AG, Obernburg, Germany and stored in a desiccator over silica gel at 6 °C. Iso-octane was purchased from Fluka and used without further purification.

2.2. Analytical methods

Gas chromatography was used to monitor the progress of enzymatic reactions (Varian 3400 C_X, equipped with an Alltech ECONO-CAP EC-1 column (30 m × 0.32 mm ID × 0.25 μm), carrier gas: N_2, 13 psi). Enantiomeric excesses of the remaining substrate acid with 2*R*-configuration (ee_s) and the 2*S*-configurated product ester (ee_p) were determined after LC-separation and reduction ($LiAlH_4$) to the corresponding alcohols and then analysed by GC using a chiral column. (Varian 3300 equipped with a β-dex 225 column, 30 m × 0.25 mm × 0.25 μm; He, 18 psi.) Retention time for (*R*)-2-methylhexanol was 16.5 min and for (*S*)-2-methylhexanol was 17.0 min. The enantiomeric ratio (*E*) was calculated according to the equation $E = \ln[1 - c\ (1 + ee_p)]/\ln[1 - c\ (1 - ee_p)]$ where $c = ee_s/(ee_s + ee_p)$ [29].

2.3. Immobilisation of CRL on Accurel 200–350 μm (EP 100)

CRL was immobilised on Accurel 200–350 μm (EP 100) following a procedure similar to that was published previously [30–32]. A solution/suspension of CRL in sodium phosphate buffer (20 mM, pH 7.0) was prepared by stirring 16 g CRL in 400 ml phosphate buffer and centrifuged at 2700 rpm for 2 min to remove the insoluble impurities. The resulting supernatant was separated and used in the immobilisation procedure. 2.0 g Accurel was wetted with 24 ml ethanol. To the ethanol wetted Accurel sample supernatant from the centrifuged fraction was added and stirred at room temperature for 15 h and then filtered and washed with several fractions of ethanol. The sample was then left on the filter under suction for 1 h to remove the majority of the solvent before complete drying at room temperature and at low pressure under suction for 3 days over silica gel in a desiccator. At that time no further weight loss was recorded [32].

2.4. General procedure for CRL catalysed enantioselective esterification of 2-methylhexanoic acid at constant water activity (a_w) in presence of added salt hydrate pairs

A 0.15 M standard solution of 2-methylhexanoic acid and 1-decanol in iso-octane was prepared and used in all reactions.

To 2-methylhexanoic acid (97.7 mg, 0.75 mmol) and 1-decanol (119 mg, 0.75 mmol) in iso-octane (5 ml) 1.0 mmol of lower hydrate and 0.5 mmol of higher hydrate of the salt hydrates respectively were added to maintain the desired water activity (a_w) [$Na_2HPO_4/Na_2HPO_4{\cdot}2H_2O$ ($a_w = 0.15$), $Na_2CO_3/Na_2CO_3{\cdot}1H_2O$ ($a_w = 0.22$), $Na_4P_2O_7/Na_4P_2O_7{\cdot}10H_2O$ ($a_w = 0.46$), $Na_2HPO_4{\cdot}2H_2O/Na_2HPO_4{\cdot}7H_2O$ ($a_w = 0.57$), $Na_2HPO_4{\cdot}7H_2O/Na_2HPO_4{\cdot}12H_2O$ ($a_w = 0.74$), $Na_2SO_4/Na_2SO_4{\cdot}10H_2O$ ($a_w = 0.76$)] [3,5,7,19]. The enantioselective esterification was started by addition of 86.2 mg of immobilised CRL. After stirring for an appropriate time at 20 °C the reaction was stopped below 40% conversion by filtering off the enzyme. The exact conversion was obtained from the equation $c = ee_s/(ee_s + ee_p)$ [29] at the point when the reaction was interrupted and the average reaction rate was calculated from this conversion and the total reaction time (see Table 1 and Fig. 3). The remaining substrate was completely separated, judged by TLC and GC, from the product ester via liquid chromatography (using an increasing gradient of distilled diethyl ether (0–100%) in distilled n-pentane as eluent). The acid and the ester were separately reduced with $LiAlH_4$ in Et_2O and the enantiomeric excesses (ee_s and ee_p) of the obtained enantiomerically enriched *R*- and *S*-alcohols were determined using a GC with a β-dex 225 column. The *S*-(+)-enantiomer is the faster reacting enantiomer in the resolution and this was confirmed by the sign of optical rotation of the 2-methylhexanol obtained from the product ester [33]. The enantiomeric ratio (*E*) was calculated according to the equation $E = \ln[1 - c\ (1 + ee_p)]/\ln\ [1 - c\ (1 - ee_p)]$ where $c = ee_s/(ee_s + ee_p)$ [29].

2.5. General procedure for CRL catalysed enantioselective esterification of 2-methylhexanoic acid at constant water activity (a_w) over an aqueous saturated salt solution

The standard solution from above (Section 2.4) and 172 mg immobilised CRL were separately equilibrated with aqueous saturated salt solution of known a_w for 16 h in two air tight desiccators (fitted with a rubber septum on the lid) [LiCl ($a_w = 0.12$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.52$), $CuCl_2$ ($a_w = 0.68$), KBr ($a_w = 0.84$), $BaCl_2$ ($a_w = 0.91$)] [1,6,9,13–17,21,23]. The esterification reaction was started by transferring 10 ml of the equilibrated standard solution with a syringe into the beaker containing CRL via the rubber septum as shown in the Fig. 1 without opening the lid. The reac-

Table 1
CRL catalysed esterification of 2-methylhexanoic acid with 1-decanol in iso-octane at 20 °C in the presence of added salt pairs that gives different water activities or in a desiccator over saturated salt solutions with different water activities.

Entry	a_w	Added salt pair	Aq. saturated salt solution	Average reaction rate[a] (%/h)	Conversion c[b] (%)	ee_s (%)	ee_p (%)	E[c]
1	0.12	–	LiCl	2.5	17.6	15.0	70.3	6.6
2	0.15	Na_2HPO_4(2/0)	–	3.7	18.3	17.3	77.4	9.4
3	0.22	Na_2CO_3(1/0)	–	0.8[d]	6.2	5.1	77.0	8.3
4	0.33	–	$MgCl_2$	3.4	23.5	22.6	73.4	8.1
5	0.46	$Na_4P_2O_7$(10/0)	–	3.9	29.5	31.8	76.0	9.9
6	0.52	–	$Mg(NO_3)_2$	3.1	21.6	20.8	75.7	8.9
7	0.57	Na_2HPO_4(2/7)	–	4.0	29.0	33.5	83.0	8.0
8	0.68	–	$CuCl_2$	3.0	21.2	20.6	76.7	9.3
9	0.74	Na_2HPO_4(12/7)	–	4.3	25.5	27.3	79.9	10.4
10	0.75	–	NaCl	3.3	22.8	23.0	78.0	10.1
11	0.76	Na_2SO_4(10/0)	–	4.0	24.0	25.4	81.0	12.2
12	0.84	–	KBr	3.0	21.3	21.3	78.6	10.3
13	0.91	–	$BaCl_2$	2.6	18.1	17.4	78.6	9.9

[a] Gas chromatography was used to monitor the amount of produced ester but the average reaction rate was calculated from the conversion obtained from the equation $c = ee_s/(ee_s + ee_p)$ [29] at the point when the reaction was interrupted and the total reaction time.
[b] The conversion was obtained from the equation $c = ee_s/(ee_s + ee_p)$ [29] at the point when the reaction was interrupted.
[c] Calculated according to the equation $E = \ln[1 - c\,(1 + ee_p)]/\ln[1 - c\,(1 - ee_p)]$ where $c = ee_s/(ee_s + ee_p)$ [29].
[d] 2-Methylhexanoic acid forms a sodium salt together with the salt pair Na_2CO_3 0/1 and the concentration of protonated acid is therefore low in the solution which explains for the low reaction rate [20,21].

tion mixture was continuously stirred for an appropriate time and quenched below 40% conversion. The exact conversion was obtained from the equation $c = ee_s/(ee_s + ee_p)$ [29] at the point when the reaction was interrupted and the average reaction rate was calculated from this conversion and the total reaction time (see Table 1 and Fig. 3). The enantiomeric ratio (E) was calculated according to the equation $E = \ln[1 - c\,(1 + ee_p)]/\ln[1 - c\,(1 - ee_p)]$ where $c = ee_s/(ee_s + ee_p)$ [29].

3. Results and discussion

When we performed the enantioselective esterifications in an air tight desiccators the humidity changes just above the reaction mixture were constantly monitored using Center® 313 Humidity Temperature Meter with datalogger. As the esterification progressed, the humidity just above the reaction mixture increased which might indicate that the salt solution removed the water as it was formed from the reaction. Saturated salt solutions are known to give a constant a_w depending on the type of salt used [1,4,6,9,13–17].

When using salt hydrate pairs with different crystal water that give different water activities (see entries 2, 7 and 9 in Table 1 when different combinations of hydrated Na_2HPO_4 were used), the E-value (E = 9.4, 8.0 and 10.4 respectively) is affected by the change in water activity. It should also be noted that the E-values from Table 1 for entries 9 and 11 are different even though the reactions were performed at similar water activities. When performing the reactions at similar water activities using the two different methods for the control of a_w (compare entries 2 and 11 with entries 1 and 10), it can be seen that the addition of phosphate ions in entry 2 and sulphate ions in entry 11, Table 1 increased the E-values.

It can be seen from Figs. 2 and 3 that the enantiomeric ratio and average esterification rate are somewhat different for reactions in the presence of salt hydrates and for the ones performed in a desiccator over a saturated salt solution. In Fig. 2, the curve presenting the enantiomeric ratio for the esterifications in a desiccator over a saturated salt solution was smooth and the curve for the esterifications in presence of salt hydrate pairs was somewhat crooked. This indicates that the ions have an influence on the enantiomeric ratio. In Fig. 3 the average esterification rate curve in the presence of salt hydrates shows that the average rate in all entries, except for entry 3 (Table 1), is somewhat higher when the ions are present in the reaction mixture. For entry 3 when Na_2CO_3 is added most of the substrate acid is probably removed from the organic phase as a sodium salt which results in a very slow average production of the ester [19].

To predict the enantioselectivity in this type of lipase catalysed reaction when changing the water activity, it is preferable to not add ions into the reaction. On the other hand if an optimal E-value is the goal the best strategy is to test both methods for control of a_w for the

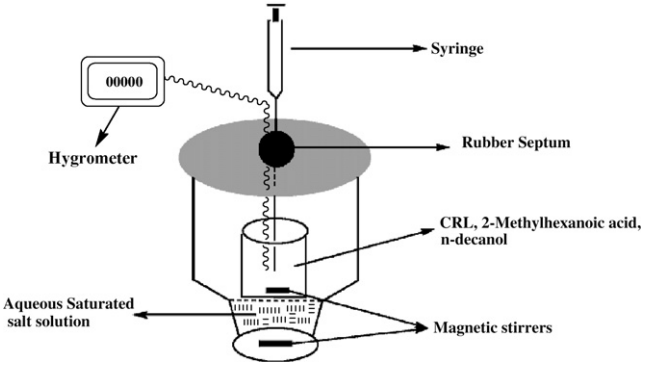

Fig. 1. CRL catalysed esterification of 2-mehtylhexanoic acid with 1-decanol in iso-octane at 20 °C in an air tight desiccator over an aqueous saturated salt solution.

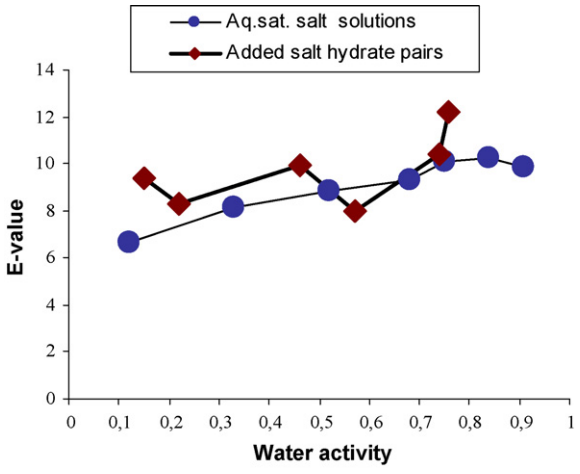

Fig. 2. E-values in the enantioselective esterification of 2-mehtylhexanoic acid with 1-decanol in iso-octane at 20 °C in the presence and absence of salt hydrate pairs.

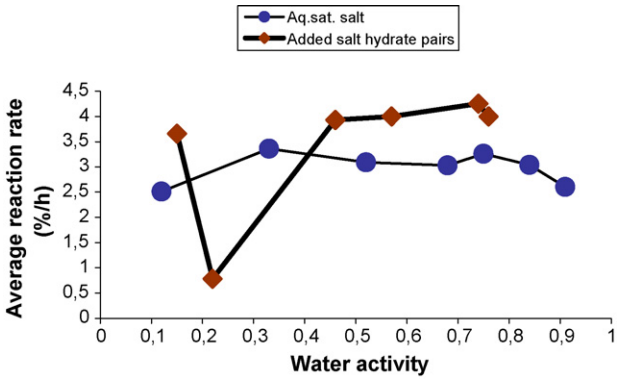

Fig. 3. Average reaction rate of the enantioselective esterification of 2-mehtylhexanoic acid with 1-decanol in iso-octane at 20 °C in the presence and absence of salt hydrate pairs.

best enantiomeric ratio. The enantiomeric ratio is only somewhat higher for reactions performed in the presence of salt hydrate pairs compared to the esterifications performed in the desiccator over a saturated salt solution (Fig. 2). Thus, due to the similarity between the curves, the effects of various additives on the lipase enantioselectivity can preferably be studied at different water activities by using the desiccator method in which the effects of salt hydrates is avoided.

4. Conclusions

We conclude that the enantioselectivity of the lipase may be modified in presence of salt hydrate pair ions. The water activity profiles for a particular lipase is complex and while using salt hydrates or other metal salts one should pay attention to that the ions from the salt might influence the enantiomeric ratio and the average reaction rates. Thus, to avoid the direct contact between the salt hydrates and the lipase one can study the lipase catalysed reaction at a specific a_w using the desiccator method described in this paper.

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