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USE OF SALT HYDRATES TO BUFFER OPTIMAL WATER LEVEL DURING LIPASE CATALYSED SYNTHESIS IN ORGANIC MEDIA: A PRACTICAL PROCEDURE FOR ORGANIC CHEMISTS

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Abstract: Enzyme catalysed reactions in mainly organic media depend very much on the amount of water in the system. We have shown that addition of appropriate solid salt hydrates to the reaction mixture is a simple and convenient method to obtain optimal water level conditions throughout the reaction. As a model reaction the esterification of butanoic acid with butanol catalysed by lipase from *Candida rugosa* was chosen. Variations in the amount of enzyme, in the solvent and in the concentration of reactants were made.

INTRODUCTION

There is at present considerable interest in the use of enzymes as catalysts in organic chemical synthesis.[1,2] Among the reactions that are studied are hydrolysis, esterifications and transesterifications catalysed by hydrolytic enzymes such as lipases, esterases and proteinases. Organic chemists using enzymes as catalysts tend to focus on the starting materials and the end products without paying much attention to the intermediate "biochemical machinery" necessary for catalysis. No matter how useful this approach may be, it is necessary to pay attention to the reaction conditions such as the nature of the catalyst, the solvent and, above all, the amount of water that is necessary for success.

In a biocatalytic esterification water will be formed during the reaction. This means that the amount of water will change during the process. This very often leads to a lag phase where the amount of water is too low for optimal conditions.[3] After a period with optimal conditions the rate will decrease again, due to the water formed in the reaction, favouring the reverse reaction

i.e. hydrolysis. During a transesterification this is not a problem since water is not formed during acyl transfer. However, also in this case the equilibrium will be influenced due to the reverse reaction, although not a hydrolysis. If the water level is too high rates can also be reduced because the suspended catalyst particles aggregate together, leading to diffusional limitation of reaction. Furthermore, the level of water may often tend to change unless the vessel is very well sealed, due to exchange with the environment. The range of water contents giving near optimal behaviour is often quite narrow. Previous investigations conclude that less than a monolayer of water is necessary to retain the activity of chymotrypsin and subtilisin,[4] however optimal reaction conditions were not evaluated.

The optimum level of water may be determined empirically for each reaction. It would clearly be desirable, however, to make some predictions. Unfortunately this is hard to do in terms of the water content by weight or volume. Problems arise because these reaction mixtures contain at least two distinct phases, and the water will be distributed between them. Some will be dissolved in the bulk organic liquid phase, some will be in the more polar phase, sometimes as liquid water, sometimes just bound to the solid-state enzyme. Changing the volume ratio of these phases for example, will change the total water content without affecting the immediate environment of the enzyme, and hence its behaviour.

We now report a convenient and simple method for setting water levels in these reaction mixtures, by just including a suitable pair of solid salt hydrates. This approach maintains near-optimal conditions when changing solvent, reactants and catalyst concentration. The hydrate pair can also take up or release water as required to keep constant conditions during the reaction. A chymotrypsin-catalysed reaction in various organic solvents in the presence of sodium carbonate decahydrate showed that the salt hydrate acted to control water level in the reaction mixture.[5,6]

THEORY

Thermodynamic activities are in theory preferable to concentrations for the characterisation of all equilibria, but their practical use is often limited by the relative difficulty to measure them. However, in systems with more than one phase, like most organic reaction mixtures for enzymatic synthesis, treatements in terms of concentrations require special care. At least for water, it often becomes easier to measure the activity than the relevant concentrations.

The thermodynamic activity af water (a_w) is conventionally given relative to pure water at the same temperature and pressure, as standard state.[7] In a gas phase it is essentially equal to the ratio of the partial pressure of water vapour to that above pure water at the same temperature. Like other activities, it is by definition equal in all phases at mutual equilibrium. Hence, an organic reaction mixture may be characterised by a single a_w value, instead of separate water

concentrations or contents in each phase. In contrast, the "total concentration" has little useful meaning. Several advantages follow: The a_w may be measured or controlled by means of the water vapour pressure in an equilibrated gas phase. The a_w value is a good predictor of the water bound to the enzyme, which in turn seems to be a good predictor of its catalytic activity if for instance solvent is changed. If water is a reactant in the desired conversion, or a side reaction, then a_w determines the water mass action effect on the position of this equilibrium, again by definition. The use of a_w in analysing these systems has been described previously.[8,9]

When a hydrated salt and its corresponding lower hydrate or anhydrous form are present together, ideal behaviour implies a fixed equilibrium water pressure and hence a_w, whatever the relative quantities of the two forms. Many real salt pairs seem to approximate this behaviour, giving a step-shaped graph of water content against a_w. Hence the pair of forms act to buffer a_w at this characteristic value for a given temperature, releasing or taking up water as required, as long as some of each form remains present. Some salts do show non-ideal behaviour, for example because of non-stoichiometric hydrates or very slow equilibration. Data on a number of salt hydrates of possible application for a_w control in organic media have been compiled.[10]

RESULTS AND DISCUSSION

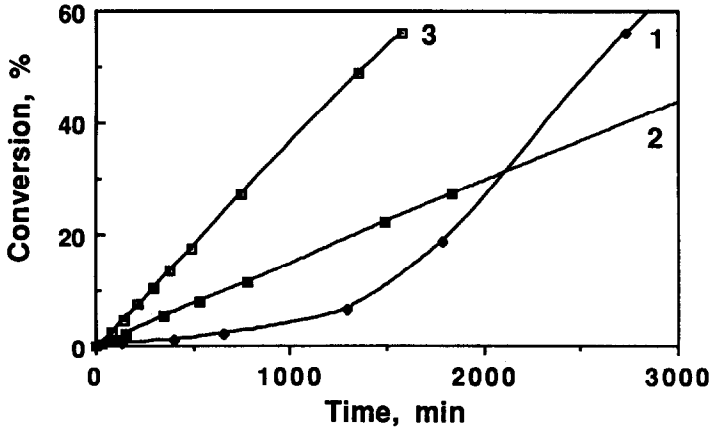

Figure 1. Esterification of butanoic acid (0.50 mmol) with butanol (1.0 mmol) catalysed by lipase (5.0 mg) from *Candida rugosa* in hexane (8 mL) (Model reaction). Nothing added (1), 6 μL water added (2) and in the presence of 1 g of $Na_2SO_4 \cdot 10 H_2O/Na_2SO_4$ (3).

The formation of butyl butanoate from butanoic acid and butanol, catalysed by lipase from *Candida rugosa* (formerly *C. cylindracea*) in hexane was chosen as a model reaction. The time course of the reaction showed, as expected, a sigmoid shape when water was not added (1, figure 1). The initial acceleration is due to the water produced in the reaction which causes the enzyme activity to increase, while the later decline is partly due to the water level now becoming excessive. During this period the enzyme was observed to be progressively smeared over the surface of the reaction tube. By adjusting the initial water level it would be possible to shorten the initial acceleration phase, but only at the expense of slower progress later in the reaction. As the reaction is very sensitive to the amount of water present, it will easily become too much, here represented by the addition of 6 μL (2, figure 1). The optimal water level for the initial stage of this reaction is approximately 3 μL (not shown). As also shown, if water is supplied by adding a salt hydrate pair (50% sodium sulphate decahydrate and 50% anhydrous sodium sulphate) directly to the reaction mixture, the progress curve becomes smooth, with an initial linear section (3, figure 1). In this case the decahydrate can initially give up water, converting to non-hydrated sodium sulphate, at constant water activity, but later take up water which is formed as a reaction product. Hence the hydrate pair acts to "buffer" the water activity at a value near-optimal for the reaction.

In the presence of a salt hydrate pair, a near-optimal progress curve is maintained when the composition of the reaction mixture is changed. The change can be in the quantity of enzyme, concentrations of reactants or in the solvent.

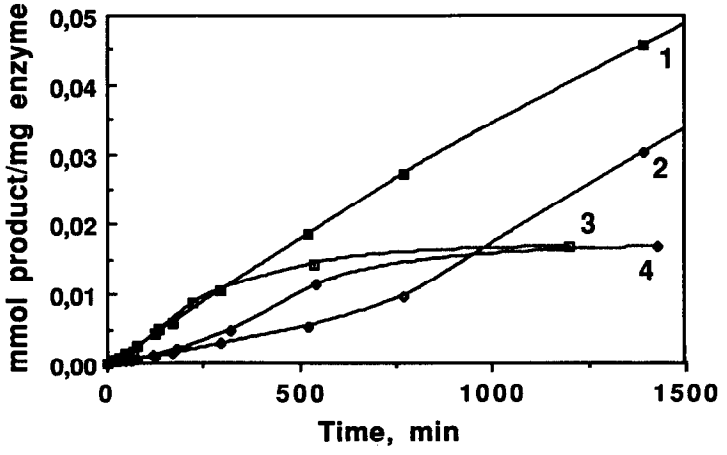

Figure 2. The effect of presence of salt hydrates ($Na_2SO_4 \cdot 10\ H_2O/Na_2SO_4$) when increasing the quantity of enzyme for the esterification reaction of fig. 1. 5 mg enzyme with salt hydrate (1) and without (2), 30 mg enzyme with salt hydrate (3) and without (4).

• *Increase in the quantity of enzyme.* The effect of variation in the amount of enzyme is shown in figure 2. Curves 1 (5 mg) and 3 (30 mg) show that when varying the amount of enzyme optimal initial reaction rate is obtained when hydrate salt is present. Similarly curves 2 (5 mg) and 4 (30 mg) show a much lower reaction rate at the initial stage as no hydrate salts are present. The decline in curves 3 and 4 simply reflects that the conversion is complete. To maintain optimal conditions when increasing the amount of enzyme would require an increase in the water content in weight terms because of the water adsorbed by the enzyme particles. However, the increase is not simply proportional because there will be no change in the required weight of water dissolved in the organic phase.

• *Increase in the concentration of reactants.* This will increase the solubility of water in the organic phase, but there will be no change in the water bound to the enzyme. Figure 3 shows the effect of a threefold increase in the substrate concentration with (1) and without (2) addition of salt hydrate. The advantage of control of the water level is evident.

• *Change of organic solvent.* This will alter the solubility of water in the organic phase, and hence again the optimal weight content of water. Comparison between hexane, toluene and diisopropyl ether as solvent is shown in figure 4.

In each of these cases, it seems that the salt hydrate can maintain near-optimal hydration conditions, by keeping water activity constant. A more theoretical consideration of the system would lead to the same conclusion. In the first of the three cases it is unlikely that the individual enzyme particles interact, so constant water activity would clearly keep each one optimally

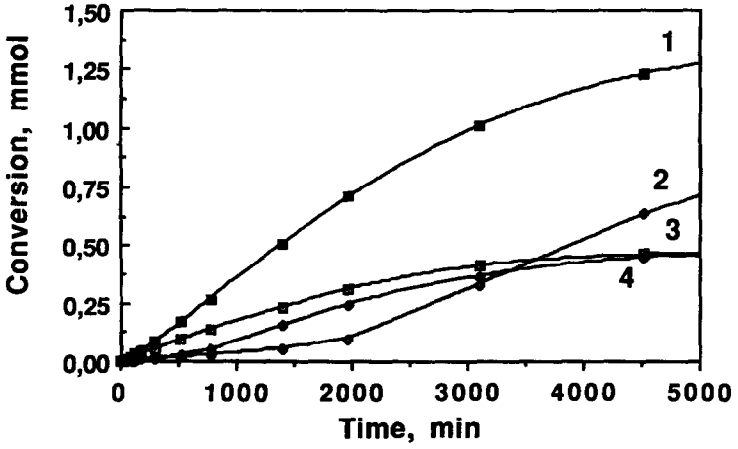

Figure 3. The effect of presence of salt hydrates ($Na_2SO_4 \cdot 10 H_2O/Na_2SO_4$) when increasing the concentration of reactants in the esterification reaction of fig. 1. Three times concentration of fig. 1 with salt hydrate (1) and without (2). Same concentration as in fig. 1 with salt hydrate (3) and without (4).

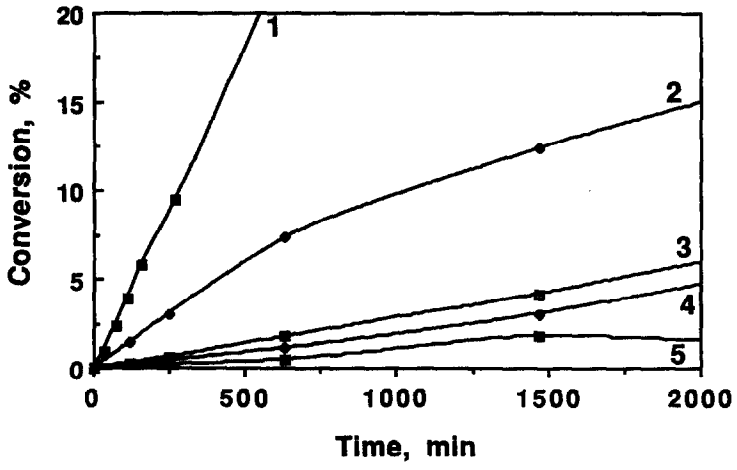

Figure 4. The effect of presence of salt hydrates ($Na_2SO_4 \cdot 10\ H_2O/Na_2SO_4$) when changing the organic solvent in the esterification reaction of fig. 1. Hexane with salt hydrate (1), toluene with salt hydrate (2) and without (4), diisopropyl ether with salt hydrate (3) and without (5).

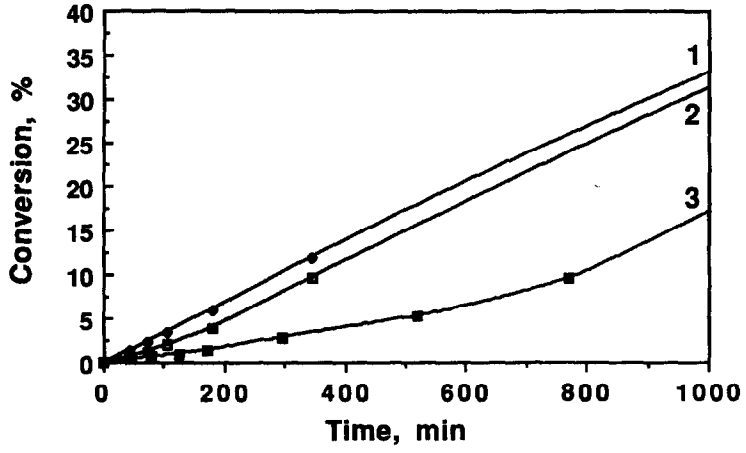

Figure 5. The effect of the amount of the salt hydrate present ($Na_2SO_4 \cdot 10\ H_2O/Na_2SO_4$) for the esterification reaction of fig. 1, 1g (1), 10 mg (2) and 0 mg (3).

hydrated. In the two latter cases there is some possibility that changes in the reactants or solvent could affect the activity of the enzyme by influencing its hydration or in other ways. However, it has been demonstrated experimentally that at least one lipase shows very similar optimal water activity in a wide variety of different solvents.[8]

For the salt hydrate pair to successfully maintain optimal water conditions (*i.e.* water activity), enough salt must be added so that some of each form remains present throughout the reaction. It may often be sufficient to add excess of the components, where this does not interfere mechanically with mixing or eventual recovery of the organic phase. Figure 5 shows that the progress is essentially unchanged as the quantity of salt added is varied over a considerable range. However, there will be a return to sigmoid behaviour at very low levels.

The minimum quantity of salt(s) required may be estimated from a "water budget" for the system. Apart from the water associated with the salt hydrates other sources or sinks of water are:

- *Water dissolved in the organic phase.* In our experiments the initially dry organic phase will take up water from the higher hydrate. For example with sodium sulphate decahydrate at 20 ^{o}C (a_w 0.76) the organic phase contains 100 mg dissolved water pr. L. (measured by Karl Fischer titration), *i.e.* 46 µmol in 8.1 ml total.
- *Water associated with the catalyst (enzyme and support, if any).* In our experiments the initially dry lipase will take up water. At a_w 0.76 this will be about 0.2 g/g[11] *i.e.* 56 µmol in total.
- *Water vapour in the head space.* The direction of equilibration will here depend on the ambient humidity in the laboratory. The water content of a saturated gas phase (a_w=1) is 0.96, 1.68, 2.84 and 4.59 mmol L^{-1} at temperature 20, 30, 40 and 50 ^{o}C respectively. In our experiments at a_w 0.76 and 20 ^{o}C the 2 mL headspace will contain 1.46 µmol water.
- *Water formed or consumed in the reaction or in a side reaction.* In our system complete reaction will produce 500 µmol water.
- *Continuing exchange between headspace and the room air (e.g. by incomplete seals, opening of vessel to sample).* As the figures above show, this is not important in this reaction, but will become pronounced for small reaction volumes if the headspace is comparatively larger.

For comparison with the above, 10 mg sodium sulphate decahydrate will release 310 µmol water on complete dissociation. Thus the minimum quantity of salt found experimentally is in reasonable agreement with that expected from the water budget. *Note that it is not necessary to add both hydrate forms of a salt. The lower hydrate may be produced in sufficient amounts by initial water migration to other (dry) components of the reaction mixture, while a higher hydrate can be formed if other components are initially relatively wet.*

The water activity in the reaction mixture is in theory affected only by the choice of the salt hydrate pair. Figure 6 shows the progress curves obtained with four different hydrate pairs

expected to give different water activity values. As can be seen the reaction rate declines when the water activity decreases.

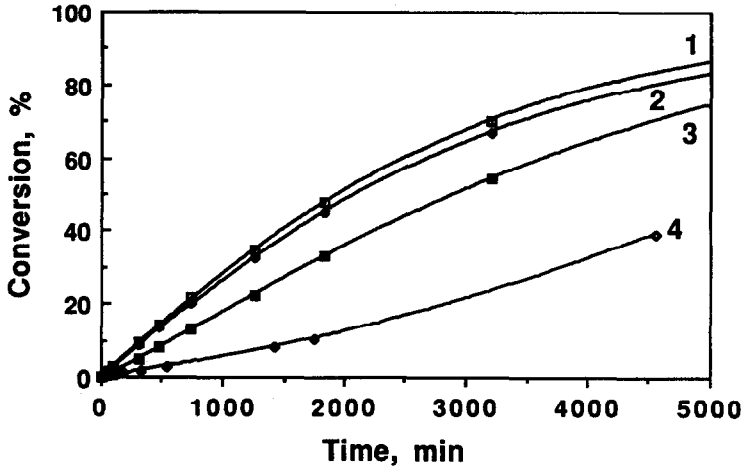

Figure 6. The effect of presence of different salt hydrates in the esterification reaction of fig. 1, $Na_2SO_4 \cdot 10\ H_2O/Na_2SO_4$, a_w 0.76 (1), $Na_2HPO_4 \cdot 12\ H_2O/Na_2HPO_4 \cdot 7\ H_2O$, a_w 0.74 (2), $Na_2HPO_4 \cdot 7\ H_2O/Na_2HPO_4 \cdot 2\ H_2O$, a_w 0.57 (3), $Na_2HPO_4 \cdot 2\ H_2O/Na_2HPO_4$, a_w 0.15 (4).

The rate at which water can re-distribute in the reaction mixture may affect the behaviour of these systems. To investigate this, we have tried pre-equilibrating the organic phase and the enzyme separately. Both were equilibrated overnight with the same salt hydrate with direct contact and through the vapour phase respectively, and then mixed to start the reaction. When the phases were pre-equilibrated, but salt hydrates were not included in the reaction mixture, the initial rate was not significantly altered. This shows that the presence of the hydrate pair does not significantly interfere with the action of the catalyst.

A few salt hydrates tested gave anomalous behaviour (not shown). Sodium carbonate decahydrate, which was successfully used in peptide synthesis,[6] reacted in this case with butanoic acid to give its sodium salt, removing it from solution, so no esterification occurred. With $Na_4P_2O_7 \cdot 10\ H_2O$, $CaSO_4 \cdot 2\ H_2O$ and $K_4Fe(CN)_6 \cdot 3\ H_2O$ the progress curves showed a lag of about 1000 min, and then accelerated to a rate similar to that at higher water activity. This sigmoid behaviour is not necessarily related to the rates of water equilibration, as for $CaSO_4 \cdot 2\ H_2O$ it persisted to a certain extent even with pre-equilibration. Water dissociation from this hydrate is

known to be particularly slow.[12] Hydrate forms of $Zn(NO_3)_2$ also showed anomalous behaviour and hence we consider these salt hydrates unsuitable for this application.

Different salt hydrates, giving different buffered water activity, would be expected to give different final equilibrium positions for the esterification. This follows directely from the law of mass action. In the reaction we have tested, with excess butanol, the residual butanoic acid concentration at equilibrium is too small to measure since the conversion was more than 99.9%. Hence the equilibrium position is not of practical significance here, and changes in it cannot be detected. However, in some other cases, it may be significant.

Salt hydrates can equally be used for water activity buffering with other lipases, though the optimal value may change significantly. We have obtained good progress curves with salt hydrates while the catalyst is either free porcine pancreatic lipase or lipase from *Candida rugosa* adsorbed to macroporous polypropylene. In each case a sigmoid progress curve was obtained in the absence of salt hydrates, for the reasons discussed above. It should be noticed that some immobilised lipases do not show such problems, even in the absence of water buffering. With catalysts such as Lipozyme Novo (lipase from *Rhizomucor miehei* adsorbed on anion exchange resin) the water binding capacity of the support acts as a partial water buffer and the enzyme activity is not so sensitive to water activity.[8] Even here, however, water activity effects on the equilibrium position can be seen, and salt hydrates could be useful to ensure optimal conditions for this purpose.

CONCLUSION

Addition of appropriate solid salt hydrates is a simple and convenient way of "buffering" optimal water conditions during enzymatic synthesis in mainly organic media. In the presence of a given pair of salt hydrates the fixed water activity value maintains near-optimal conditions even when changes are made in enzyme concentration, solvent used or reactant concentrations. The hydrate pair is also able to take up or release water (at constant water activity) to maintain optimal conditions as the reaction proceeds.

ACKNOWLEDGEMENT

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EXPERIMENTAL

Materials. Lipase from *Candida rugosa* (formerly *C. cylindracea*, EC 3.1.1.3), 900 units pr. mg was purchased from Sigma Chemical Co. All chemicals including organic solvents were of analytical grade.

Analytical Methods. GLC was performed using a Varian 3400 instrument equipped with autosampler 8100 and a Vista 402 data system for integrations. A capillary column, J&W Scientific, DB-1701, 30 m 0.255 mm, film thickness 0.25 μm, was used with a temperature program, 60 - 120 °C, 10 °C pr. min. The increase of products was calculated from the internal standard. Calibration curves were established from 7 independent concentrations.

Determination of water in the organic phase. The concentration of water in the standard organic phase of the reaction mixture (1.0 mmol BuOH, 0.50 mmol butanoic acid, 8 mL hexane) after equilibration at 20 °C with $Na_2SO_4 \cdot 10\ H_2O$ (a_w 0.76) was determined by coulometric Karl Fischer titration using a Metrohm Coulometer 684 KF as 100 mg L^{-1} (5.7 mmol L^{-1}).

General experimental procedure for enzymatic esterification. A typical reaction mixture consisted of lipase (5.0 mg, when not otherwise stated), n-hexane (8 mL), n-butanol (1.0 mmol), butanoic acid (0.50 mmol) and, as internal standard, n-decane (0.31 mmol). Salt hydrates in pairs, (0.5 g - 1.5 g) were added to the reaction mixtures, when not otherwise stated. The reactions were carried out at room temp. in closed vials (10 mL, i.d. 20 mm) with shaking 130 strokes/min. All experiments were conducted at least twice and the maximum deviations were less than 10%.

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