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MEASUREMENT OF pH CHANGES IN AN INACCESSIBLE AQUEOUS PHASE DURING BIOCATALYSIS IN ORGANIC MEDIA

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SUMMARY

The pH of the aqueous phase trapped within biocatalyst particles in organic media may be measured using very hydrophobic esters of fluorescein. These remain completely in a pentan-3-one phase, but they ionise there in response to the pH of an equilibrated aqueous phase. They show that the catalyst pH may be significantly shifted from the pre-adjusted value by partitioning of an acidic reactant (N-formyl-tyrosine during chymotrypsin-catalysed esterification) or product (acetic acid during lipase-catalysed ester hydrolysis).

Mainly organic media offer considerable advantages for many bioconversions, as recently reviewed by Dordick (1989), Klibanov (1989) and Khmelnitsky *et al* (1988). The activity and stability of biocatalysts in these systems are undoubtedly affected by pH, as in conventional aqueous media. The pH of the catalyst is usually adjusted to an appropriate value during its preparation, while it is still under high water conditions: see the extended discussion by Zaks & Klibanov (1988). However, it is possible that the effective catalyst pH may change during subsequent use in a mainly organic medium, particularly as a result of acidic or basic reactants. Such changes have been invoked to account for poor rates of lipase-catalysed esterification of the most polar acids, that might be expected to partition most strongly into the aqueous phase and alter its pH (e.g. Cambou & Klibanov, 1984; Abraham *et al*, 1988). The same sort of observations have however also been explained in terms of biocatalyst specificity. The primary problem with the analysis of pH effects on biocatalysts in these systems is the lack of an unambiguous method to determine the pH of the aqueous or polar phase around the catalyst, once the water content has been sufficiently reduced to make this inaccessible to direct measurement (for example when it is purely restricted to the pores of a catalyst particle). Since it has not been possible to determine whether or not pH changes are responsible for the observed slow rates, rational design of methods to avoid these problems is difficult.

Attempts have been reported to make pH measurements in such systems. Cambou & Klibanov (1984) reported pH shifts when using acidic reactants, detected by addition of an indicator to the system. However, the interpretation of their observations in terms of a precise pH value must be uncertain because: i) the indicator used will partition between the aqueous and organic phases, with that in the latter probably dominating the overall appearance; ii) there will be different degrees of ionisation in each phase; and iii) the behaviour in the organic phase will not be as simple as in aqueous solution (see below) and the aqueous pK value they use will

not apply in the organic phase. Cassells & Halling (1989) reported measurements by washing the recovered catalyst in excess water, but acknowledged the uncertainty of this method.

We now report the application of a more reliable method to measure pH in these systems. This uses specially synthesised and very hydrophobic indicators that remain entirely in the organic phase, even in the ionised form, but nevertheless respond to the pH of an adjacent aqueous phase. These indicators have been used to follow pH changes in the aqueous phases completely trapped inside catalyst particles suspended in an organic liquid: i) agarose–chymotrypsin, when the relatively polar N–formyl–tyrosine is used as a reactant in esterification; and ii) resin–lipase (Lipozyme), when acetic acid is released during ester hydrolysis.

EXPERIMENTAL

Indicators were synthesised in our laboratories: farnesol was completely hydrogenated (with hydroxyl protection), the saturated alcohol produced was esterified with fluorescein, and the ester brominated as required. Details are given in a patent (Suckling *et al*, 1990) and a manuscript in preparation.

Dodecyl decanoate was synthesised by stirring 0.25 mole of the alcohol and acid in 0.25 l hexane with porcine pancreatic lipase (600 mg) supported on celite (5 g) as catalyst, at about 20 ^{o}C. After 48 h, analysis showed more than 95% esterification, and the organic phase was passed through 200 g of basic alumina (Brockmann activity 2), eluting with 0.2 l of diethyl ether. The eluate was evaporated under vacuum, and the product analysed by gas liquid chromatography, showing greater than 98% purity with less than 0.01% of residual acid and alcohol.

Reactions with agarose–chymotrypsin catalysts and product analyses were carried out essentially as described previously (Blanco *et al*, 1989). The humidity sensor used to monitor water activity was calibrated by insertion in the vapour space above pure water maintained at different temperatures, which was found to be more reproducible than the previous method using saturated salt solutions. The same reaction vessel and humidity sensor were used for the reactions with lipase.

"Lipozyme" was kindly given to us by Novo Enzyme Products Ltd, Farnham, Surrey, U.K. To estimate the Na^+ concentration in the aqueous phase, 2.6 g of the Lipozyme catalyst was washed with four 20 ml portions of water (by stirring for 5 min). The Na^+ concentration in the combined extracts was determined using an atomic absorption spectrometer; that in the distilled water control was negligible. The $[Na^+]$ in the original catalyst aqueous phase was calculated using its measured water content of 11.1% (by drying at 105 ^{o}C).

RESULTS AND DISCUSSION

The indicators and their behaviour

The indicators used are esters of fluorescein with a long chain, very non–polar alcohol, 3,7,11–trimethyldodecanol. Their acid strength, and hence the pH range over which they respond, may be altered by bromination, giving either the 4,5–dibromo or 2,4,5,7–tetrabromo derivatives.

Both the free acid indicators and their sodium salts are soluble in organic solvents. When solutions in relatively polar but water–immiscible solvents like pentan–3–one are equilibrated with aqueous buffers, the indicators do not partition measureably into the aqueous phase. In these systems, the ionisation state of the indicator is affected by the pH of the aqueous phase, and may be determined by absorbance measurements. Figure 1 shows the response of the dibromo and tetrabromo indicators to the pH of an aqueous phase large enough to allow direct measurement.

Though these curves bear some resemblance to an aqueous titration curve, there are some important differences. The slope is less steep than for the aqueous system, and the position of the curve is shifted by changes in aqueous phase $[Na^+]$. This behaviour may be explained in terms of a theoretical model allowing for the various processes that can occur in these two–phase systems. Even though the indicator is present only at low concentrations, the relatively poor solvation of ions in the organic phase makes it necessary to consider counter–ion movements to maintain electroneutrality, and ion pair formation (manuscript in preparation).

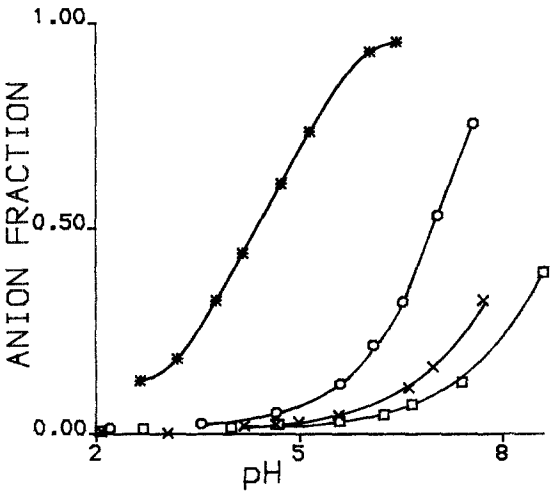

Figure 1. Response of indicators in pentan-3-one to pH of an equilibrated aqueous phase.

The aqueous phase was a solution of $Na_4P_2O_7$, adjusted to the pH shown with H_3PO_4. It was emulsified to equilibration with an equal volume of pentan-3-one based organic phase, and the aqueous pH confirmed by direct measurement with a glass electrode. The absorbance of organic phase samples was measured at 3 wavelengths. A_{650} was used to apply a small correction for turbidity, then the indicator acid and anion concentrations calculated using absorbances at 465 and 530 nm (dibromo) or 475 and 540 nm (tetrabromo), and separately measured extinction coefficients.

The anion form has a much higher extinction coefficients than the acid at 530 nm, so that small anion fractions may be measured fairly accurately. (Hence the measurement of pH with these indicators is more sensitive on the acid rather than the alkaline tail of the titration curve.) Curves were constructed with between 3.2 and 3.7 X 10^{-5} M of the dibromo indicator in the organic phase, and 0.4 M Na^+ in the aqueous phase (X); with 1 M phenylethanol included in the organic phase (O); with the aqueous [Na^+] reduced to 0.025 M (□); and substituting 1.18 X 10^{-5} M of the tetrabromo indicator (✳).

Importantly, though, the ionisation state of the indicator is not affected by reduction in the volume fraction of the aqueous phase, provided its pH is unchanged. We have shown this experimentally for organic:aqueous ratios up to 1000. This is again in accord with the theoretical analysis. The indicator also responded correctly to the pH of the aqueous phase trapped inside in celite particles suspended in pentanone (pH varied by pre-washing with appropriate buffers). With agarose beads the indicator showed the expected trend of response, though the errors were somewhat increased; we are not sure whether this reflects a change in response or real changes in pH. The response was not affected by 0.25 M dodecanol in the organic phase, very slightly by 0.25 M decanoic acid, but as can be seen significantly by 1 M phenylethanol (Fig. 1). This probably reflects a contribution to solvation of the indicator anion in the organic phase by such a high concentration of alcohol. Overall, these observations make us confident that the indicators will respond correctly to aqueous pH during the reaction experiments.

pH measurement during esterification of N-formyl tyrosine

Agarose-chymotrypsin has been shown to be a useful catalyst for organic media (Blanco *et al*, 1989). The catalyst beads contain a substantial amount of trapped water, but there is no free water in the reaction system: the moist beads are suspended directly in the solvent. In the present experiments the amount of water was sufficient to maintain the thermodynamic water activity (a_w) between 0.98 and 1.00 throughout, as the behaviour of the indicators has yet to be investigated at lower a_w. The beads were also pre-equilibrated with a buffer containing 0.4M NaCl, to give a high and known [Na^+] in the aqueous phase.

N-formyl-tyrosine was used as one reactant in these experiments. The formyl group is of considerable interest as an economical protecting group in peptide synthesis. This compound is soluble in solvents like pentanone, but is rather polar and partitions extensively into an equilibrated aqueous phase. In this it will dissociate, so naturally there is a risk that the pH will be shifted to a lower value. In our experiments analysis of organic phase samples showed that most of the added

N-formyl-tyrosine disappeared quickly, presumably by partition into the aqueous phase; giving an estimated concentration of about 380 mM, much greater than the buffer concentrations that would normally be used. We have studied an esterification rather than a peptide synthesis reaction, as a significant limitation of our current indicators is that they are expected to fail to respond correctly in the presence of basic reactants (like an amino acid derivative with a free amino group).

The progress of esterification of N-formyl-tyrosine with phenylethanol catalysed by agarose-chymotrypsin is shown in Fig. 2. With a catalyst that had been prepared with 10 mM phosphate buffer pH 7.0 in the trapped aqueous phase inside the beads, dibromo indicator in the organic phase gave an anion fraction rising slightly from 0.088 to 0.113 during the course of the reaction. Using the calibration curve for 1 M phenylethanol in the organic phase, and 0.4 M Na^+ in the aqueous phase (Fig 1), we estimate pH values of 5.2 and 5.5 for the aqueous phase of the catalyst in use. Clearly, partitioning of N-formyl-tyrosine has caused a substantial fall in aqueous phase pH. Even if the buffer concentration was raised to 100 mM, the indicator anion fraction was 0.130 after initial equilibration, implying a pH of 5.6 (from a calibration curve for the appropriate [Na^+], not shown); again, the anion fraction rose later to 0.152 (pH 5.7). The change from the originally adjusted catalyst pH is still large, though slightly reduced. The slight rises in pH during the course of each reaction, which were reproducible, may reflect N-formyl-tyrosine consumption.

As may be seen from Fig. 2, the somewhat lower pH in the less buffered catalyst is accompanied by a substantial fall in observed reaction rate. The activity of chymotrypsin in simple hydrolysis reactions is known to be often very dependent on pH below 7, sometimes following a more or less theoretical titration curve (e.g. Hess, 1972). The relative rates in our experiments are consistent with the measured pH difference of about 0.4 units; and our absolute pH values suggest that in both cases the enzyme may be well below its optimal activity.

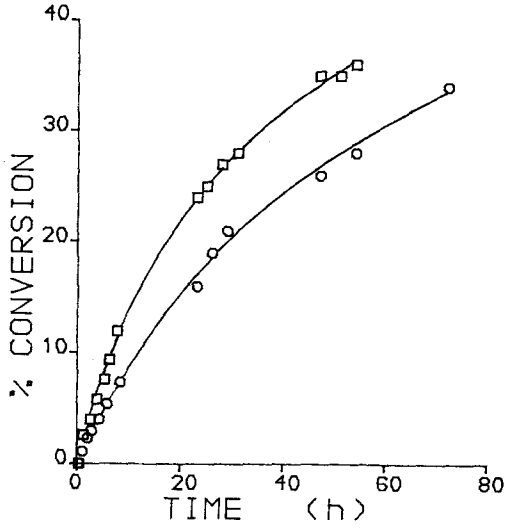

Figure 2. Esterification of N-formyl-tyrosine with catalysts of different buffer capacity.

25 mM N-formyl-tyrosine, 1 M phenylethanol, about 1.3 X 10^{-5} M dibromo indicator dissolved in 15 ml pentan-3-one at 25 °C. Added 1 g wet weight agarose-chymotrypsin catalyst, pre-equilibrated with 0.4 M NaCl containing 0.1 M (□) or 0.01 M (○) Na-phosphate buffer pH 7.0. Indicator anion fraction was determined as for Fig. 1. The progress of the reaction was not affected by the presence of the indicator.

pH measurement during hydrolysis of dodecyl acetate

Another possible cause of pH changes is the release of a polar acid as a reaction product. Lipase-catalysed hydrolysis of esters could have this effect, and is of interest particularly where specificity is required; organic media are often desirable because the reactants are poorly water-soluble. For experimental convenience we have studied a model reaction, the hydrolysis of dodecyl acetate, as a representative of many others in which an acetate ester is hydrolysed.

The catalyst was *Mucor meihei* lipase immobilised on anion exchange resin (Novo "Lipozyme"). This contained 11.1% w/w water as supplied, and was found to give a water activity of greater than 0.97 in the reaction mixture. Nevertheless, the catalyst particles are fully dispersed in the organic phase, with no evident free water. The Na^+ concentration in this water was measured as 0.025 M, so a titration curve for the indicator at this [Na^+] was used to estimate pH values (Fig.1).

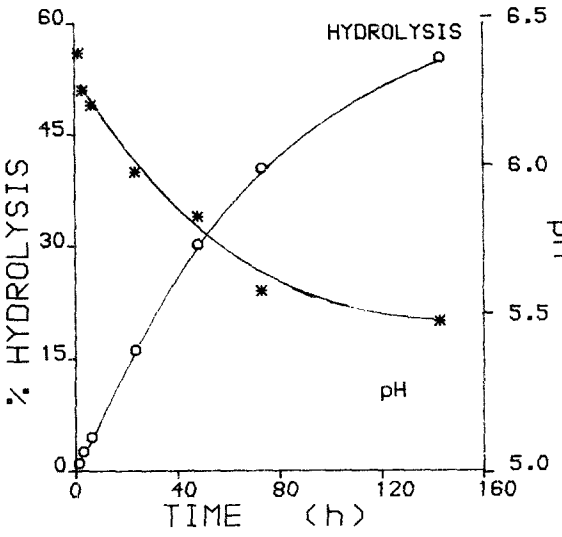

Figure 3. pH change during Lipozyme-catalysed hydrolysis of dodecyl acetate.

Lipozyme (50 g/l) stirred in water-saturated pentan-3-one solution of dodecyl acetate (0.25 M) at 20 °C. Organic phase samples (50 μl) were mixed with internal standard (methyl palmitate) in 100 μl tetrahydrofuran, silylated by addition of 100 μl Bis-trimethylsilyl-trifluoracetamide (BSTFA), then analysed by GLC. When present, the dibromo indicator was 2.86 X 10^{-5} M in the organic phase, the ionization state was determined as for Fig. 1, and the aqueous phase pH estimated from the 0.025 M Na^+ calibration curve. The rate of reaction was the same in the absence of the indicator. The fall in pH shown corresponds to a decline in anion fraction from 0.055 to 0.028.

Fig 3 shows that as the hydrolysis of dodecyl acetate proceeds there is a steady decrease in pH reported by the indicator, from 6.45 to 5.45. In contrast, during the hydrolysis of dodecyl decanoate under otherwise identical conditions (not shown), the anion fraction declines only marginally, from 0.063 to 0.059, corresponding to pH values of 6.55 and 6.45. The hydrolysis of the acetate proceeds at only about 2% of the rate found for the decanoate, but comparison of the progress curves showed no evidence that the pH change affects the enzyme activity in this case.

General application of pH indicators of this type

The theoretical analysis of the behaviour of such indicators shows that their ionisation will almost always be affected by the aqueous concentrations of at least one other ion, either positive or negative, besides H^+. This is apparent from a simple physical picture; another ion movement is needed to maintain electroneutrality when the aqueous H^+ crosses the interface to protonate the indicator in the organic phase. Hence, in order to measure aqueous pH, it is necessary to have some independent means of measuring or predicting the concentration of at least one other ion in the aqueous phase. However, by manipulating indicator properties and other components of the system, there is some freedom to choose which ion this should be.

We have chosen a system in which the counter-ions are Na^+ and/or K^+. This is achieved by use of an indicator itself sufficiently non-polar to extract simple cations from the aqueous phase. (In this respect, our present compounds are limited to use with relatively polar water-immiscible solvents.) The cations Na^+ and/or K^+ will usually be present in the aqueous phase at reasonably high and constant concentrations. At least where the phase remains a dilute solution (even if of very low volume), their activities should be similarly reasonably predictable. In particular, their activities are unlikely to change much during the use of a given catalyst, so that an indicator response should be mainly to pH.

The same indicators may also be used with other means of counter-ion supply, such as addition of organic soluble cations, or of crown ethers to solubilise Na^+ or K^+ (manuscript in preparation).

As mentioned above, there still remain some other limitations to our current method: interference by basic reactants, behaviour at a_w significantly below 1.

Conclusion

These very non-polar indicators offer a means of measuring the pH of an otherwise inaccessible aqueous phase in a mainly organic reaction mixture. Such measurements allow a more rational basis for the selection of optimal conditions for the use of biocatalysts under these conditions.

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