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Tridodecylamine and its hydrochloride salt: an organo-soluble buffer for subtilisin Carlsberg in non-polar organic solvents

Neil Harper,† Barry D. Moore and Peter J. Halling *

Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, Scotland, UK

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Abstract

Mixtures of commercially available tridodecylamine and its hydrochloride dissolved in hexane or toluene have been used as acid-base buffers for a suspended immobilised enzyme. The activity of subtilisin Carlsberg increases with the proportion of free amine, to a similar extent in both solvents. This indicates that the buffer fixes the ionisation state of the enzyme in the range appropriate for catalytic activity. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

There is growing knowledge of the factors which affect the activity of enzymes in low-water media (for recent reviews, see Refs. 1–5). As in aqueous systems the enzyme activity is dependent on the enzyme's ionisation state. It has been shown that the enzyme activity is dependent on the last aqueous pH to which the enzyme was exposed prior to drying and suspension in the organic solvent. The dependence on the aqueous pH is very similar to that observed in an aqueous medium.[6] However, acid-base conditions may subsequently change due to, for example, acidic or basic reactants (e.g. Refs. 7 and 8). In order to control the ionisation state after preparation, organo-soluble buffers have been added to the reaction mixture. These buffers are composed of either an acid and its sodium salt or an amine and its hydrochloride.[9,10]

In such low dielectric media where opposite charges interact more strongly, the presence of counter-ions needs to be taken into account in addition to H^+. Therefore, whereas pH alone is sufficient to fix the ionisation state in aqueous systems, in low dielectric media new parameters which take account of the

* Corresponding author. Tel: +44 141 548 2683; fax: +44 141 548 4822; e-mail: p.j.halling@strath.ac.uk (P. J. Halling)
† Present address: ITQB, Universidade Nova de Lisboa, 2780 Oeiras, Portugal.

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counter-ion must be introduced. Eqs. (1) and (2) show the equilibria for the control of the ionisation state of the enzyme by the two different classes of buffer.[11]

$$\text{Enz-COOH} + \text{A}^-\text{Na}^+ \rightleftharpoons \text{Enz-COO}^-\text{Na}^+ + \text{AH} \quad (1)$$

$$\text{Enz-NH}_2 + \text{BH}^+\text{Cl}^- \rightleftharpoons \text{Enz-NH}_3^+\text{Cl}^- + \text{B} \quad (2)$$

In Eq. (1) the acid and sodium salt together control the ionisation state of the enzyme's carboxyl groups. The parameter controlled is the ratio of the thermodynamic activities of the H^+ and Na^+ ions (a_{H^+}/a_{Na^+}). In Eq. (2) the amine and hydrochloride control the ionisation state of basic (e.g. amino) groups on the enzyme. The relevant parameter here is the product of the thermodynamic activities of H^+ and Cl^- ions ($a_{H^+} \cdot a_{Cl^-}$).

Thus far, two organo-soluble buffers of $a_{H^+} \cdot a_{Cl^-}$ have been tested in enzyme reactions in organic solvents. Tri-isooctylamine and its hydrochloride were dissolved in pentan-3-one and shown to buffer in the range suitable for activity of *Rhizomucor miehei* lipase.[9] 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and its hydrochloride were also dissolved in pentan-3-one to control the activity of subtilisin Carlsberg.[11] As yet, such buffers have not been used in less polar solvents such as toluene and hexane.

Here we demonstrate the application of a very hydrophobic tertiary amine, tridodecylamine (TDA) and its hydrochloride (TDA·HCl) as a buffer of $a_{H^+} \cdot a_{Cl^-}$, dissolved in toluene and hexane. Tridodecylamine has been shown to be useful as a basic extractant in several solvents, e.g. extraction of HCl from the aqueous phase into *n*-decanol.[12] It was important to use a tertiary amine as primary and secondary amines could act as nucleophiles (in competition with the alcohol in a transesterification), yielding an amide rather than an ester.

2. Results and discussion

There was concern that the solubility of the hydrochloride salt of tridodecylamine might not be sufficient to allow its use in non-polar solvents. However, the measured solubilities of TDA·HCl were 0.475 M in hexane/1 M propan-1-ol and 0.378 M in toluene/1 M propan-1-ol (both solvent systems at a_w 0.70). These concentrations are well in excess of that required for effective buffering. In dry hexane the solubility of TDA·HCl was approximately 3 mM; therefore, the addition of polar compounds such as propanol is necessary to give adequate solubility. Conveniently propanol is present at 1 M as a substrate in our model reaction.

TDA and TDA·HCl were therefore studied as a buffer during the transesterification of *N*-Ac-L-Tyr-OEt with PrOH in hexane and toluene, catalysed by subtilisin Carlsberg. Reactions at a_w 0.85 were carried out over a range of buffer compositions. *N*-Ac-L-Tyr-OH was formed as a by-product due to the water present. Figs. 1 and 2 show the relationship between the initial rate of transesterification and the logarithm of the ratio of buffer amine to hydrochloride concentration, in hexane and toluene, respectively. Both profiles show a strong trend of increasing enzyme activity with increasing basicity, as expected since the catalytic triad of subtilisin has to be deprotonated in the active enzyme. The same trend was observed with DBU and its hydrochloride as a buffer for subtilisin Carlsberg in pentan-3-one.[11] Clearly TDA and its hydrochloride can control the catalytic activity of subtilisin in non-polar solvents, presumably by altering the ionisation state of basic groups on the enzyme (Eq. (2)). H^+ and Cl^- are exchanged together between the enzyme and the buffer, with the ratio of hydrochloride to free base determining $a_{H^+} \cdot a_{Cl^-}$.

Comparison of Figs. 1 and 2 shows that the factor by which the rate increases over the buffer range is similar in the two solvents. With a buffer dissolved in the organic phase, the values of the $a_{H^+} \cdot a_{Cl^-}$ set will be solvent-dependent, affected by the relative solvation of the free base and hydrochloride ion

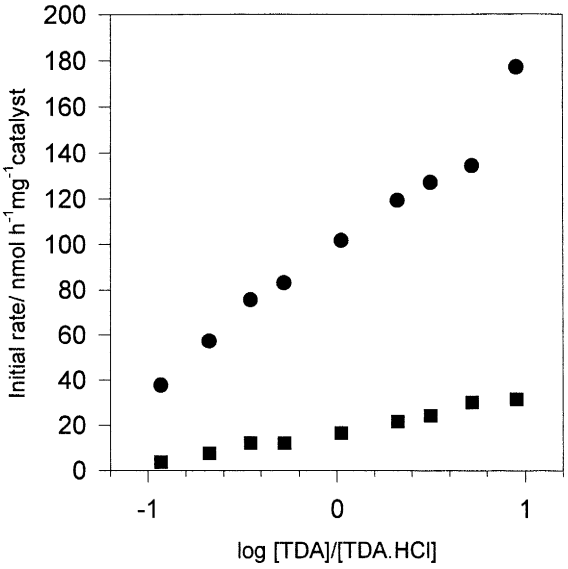

Fig. 1. Initial rate in hexane versus logarithm of ratio of concentration of TDA to TDA·HCl, for transesterification by subtilisin Carlsberg at a_w 0.85. The initial rate is expressed per mg of dry immobilised enzyme preparation. Rates of transesterification (●) and hydrolysis (■).

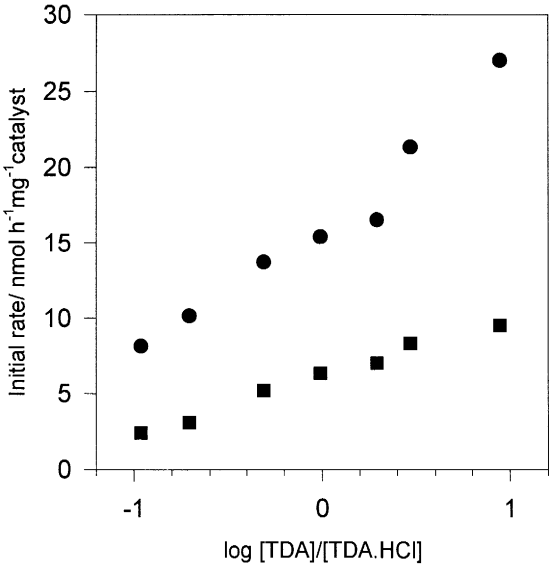

Fig. 2. Initial rate in toluene versus logarithm of ratio of concentration of TDA to TDA·HCl, for transesterification by subtilisin Carlsberg at a_w 0.85. The initial rate is expressed per mg of dry immobilised enzyme preparation. Rates of transesterification (●) and hydrolysis (■). In the absence of any buffer, the reaction rate in toluene was about 10 nmol h^{-1} mg^{-1} catalyst, which corresponded to that obtained at a TDA·HCl to TDA ratio of 5:1, so for most buffer ratios there is a rate enhancement

pair. However, the difference between hexane and toluene containing 1 M propanol may be less than might be expected, as indicated by the similar measured solubilities of TDA·HCl reported above. In both cases the rate did not reach a maximum, unlike the observation for subtilisin Carlsberg with some other organo-soluble buffers.[9,11] It may therefore be possible to obtain even higher activity by using a more

'basic' buffer. To find the true optimum, it would also be necessary to fix or buffer the complementary acid-base parameter a_{H^+}/a_{Na^+}.

With equimolar amounts of the two forms of the buffer, in hexane the initial rate was about seven times that obtained in toluene. This probably reflects the better solvation of the substrates in toluene, so that formation of the Michaelis complex and/or transition state is less energetically favourable. This type of effect has commonly been observed (e.g. Refs. 13–16). The relative rate of the hydrolysis side-reaction was also affected by solvent, being about twofold greater in toluene (compare square symbols in Figs. 1 and 2). The fraction of hydrolysis is determined by a competition between water and propanol as nucleophiles to attack the acyl-enzyme intermediate. The solvent effect observed probably again reflects substrate solvation. The availability of water remains the same (fixed a_w), but the more polar toluene solvates propanol better (it has a lower thermodynamic activity at the fixed 1 M concentration used in these studies). Hence water is better able to compete as a nucleophile.

It was likely that the TDA ion-paired with the by-product *N*-Ac-L-Tyr-OH formed by reaction with water. This would remove some free base, but the maximum concentration of acid produced in these studies was 1.1 mM, and then only after 100 h with an [amine]/[hydrochloride] ratio of 9. Hence the 10 mM buffer should be adequate, certainly during the initial rate period. The buffer would prevent significant changes in acid-base conditions due to the acidic by-product, which might otherwise affect enzyme activity. It is also possible that buffers such as this could interact directly with the enzyme, although their good solvation in the organic phase should limit this. Thus TDA could form ion pairs with carboxyl groups on the enzyme, which might affect catalytic activity.

3. Conclusion

We have shown the ability of a new organo soluble $a_{H^+} \cdot a_{Cl^-}$ buffer, tridodecylamine and its hydrochloride, to control activity of subtilisin Carlsberg in the non-polar solvents toluene and hexane.

4. Materials and methods

Materials: Tridodecylamine (95%) was obtained from Lancaster Synthesis, UK. Hexane was obtained from Aldrich Chemical Co., toluene from Merck Ltd. and propan-1-ol from Fisons, UK. The substrate, *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-L-Tyr-OEt), was from Sigma Chemical Co.

Tridodecylamine hydrochloride: Concd aqueous HCl (1.8 mol. equiv.) was added to 96 mM tridodecylamine (TDA) in dichloromethane. The mixture was shaken for 25 min and the solvent removed in a rotary film evaporator until brown crystals appeared. The crystals were then dried under vacuum. Microanalysis found: C, 77.37; H, 14.03; N, 2.53; Cl 6.33. $C_{36}H_{76}NCl$ requires: C, 77.41; H, 13.74; N, 2.51; Cl, 6.35.

Solubility of hydrochloride salt: A saturated solution of tridodecylamine hydrochloride was made in toluene and hexane both containing propan-1-ol (1 M) and pre-equilibrated to a_w 0.70 over a saturated solution of potassium iodide. The solutions were filtered and a known volume added to a clean, pre-weighed round-bottom flask. The solution was vacuum-dried overnight and the flask re-weighed. The difference in the weight gave the maximum solubility of the hydrochloride. The solubility of the salt was also determined in dry hexane.

Enzyme reactions: Subtilisin Carlsberg catalysed the transesterification of *N*-Ac-L-Tyr-OEt with propan-1-ol in hexane and toluene. The enzyme was covalently immobilised to PolyHipe SU500, a

porous support with a polydimethylacrylamide surface layer, as described.[9] The immobilised beads were rinsed in deionised water, then stored over molecular sieves. In an aqueous assay the immobilised enzyme showed activity equivalent to 1.73 mg free enzyme per g conjugate.

(1) Solutions of the free amine and hydrochloride (10 mM) in toluene or hexane containing 1 M propanol were mixed to give the required buffer molar ratio. This organic solution and the catalyst were separately pre-equilibrated over a saturated salt solution of KCl to fix the initial a_w at 0.85. The catalyst was pre-equilibrated for a minimum of 2 days, and the organic phase for a minimum of 36 h.

(2) Solid *N*-Ac-L-Tyr-OEt (to give 11.5 mM) was added to the equilibrated organic phase (4 mL) along with the catalyst (2.5 mg mL^{-1}). The reaction vials were shaken (600 min^{-1}) at 20°C, aliquots (50 μL) taken periodically and the solvent removed under nitrogen.

(3) The samples were redissolved in acetonitrile:water (50:50 v/v), filtered and analysed by HPLC with a C_{18} reverse phase column (HiChrom, UK) and UV detection at 280 nm. The mobile phase composition was 55% water (pH 2 by addition of orthophosphoric acid) and 45% acetonitrile. The percent conversion was calculated from the ratio of the area of the propyl ester peak to the sum of peak areas for the ethyl and propyl esters and the acid by-product (*N*-Ac-L-Tyr-OH).

It was possible that the amine or hydrochloride could act as a general acid-base catalyst for the transesterification. However, a control reaction in the absence of enzyme gave no reaction (with a 1:9 molar ratio of TDA·HCl to TDA in hexane, conditions giving the highest enzymic rate).

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