Enhancement of tyrosinase activity by macrocycles in the oxidation of *p*-cresol in organic solvents

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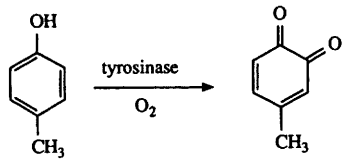
The activity of tyrosinase, suspended in dry halogenated solvents, can be considerably increased upon the addition of macrocycles to the reaction medium.

Non-aqueous enzymology is an important new area in biotechnology.[1,2] One of the advantages of using dry organic solvents instead of water as the reaction medium is that water sensitive reactions can be catalysed enzymatically. An example of such a reaction is the enzymatic oxidation of *p*-cresol to the corresponding *o*-quinone (Scheme 1). This reaction cannot be



Scheme 1

performed in an aqueous medium since *o*-quinones polymerize spontaneously under these conditions. Moreover, the polymerization reaction inactivates the tyrosinase used as the enzyme for the oxidation. Quantitative yields were reported by Kazandjin and Klibanov[3] when the reaction was performed in dry chloroform although the substrate conversion rate was slow. In later reports it was shown that the tyrosinase activity can be increased when the organic solvent is saturated with water.[4–6] However, under these wet conditions the stability of *o*-quinones is considerably decreased, and therefore it is desirable to enhance the enzyme activity under reaction conditions as dry as possible. Previously we have demonstrated that in the presence of crown ethers the enzymes α-chymotrypsin, subtilisin Carlsberg and trypsin, suspended in dry organic solvents, show a much higher transesterification activity.[7–11] This enhancement of enzyme activity by macrocycles is not restricted to protease enzymes only. In this paper we report that the activity of tyrosinase, an oxidase enzyme that operates by a very different mechanistic pathway, can be considerably increased by addition of macrocycles. The effect of several crown ethers, a cryptand (kryptofix 2.2.2) and the podand pentaglyme on the tyrosinase activity in organic solvents under dry conditions has been investigated.

Results and discussion

Klibanov and coworkers[1,3,4] have found that in organic solvents enzyme suspensions show the largest activity when the enzyme is previously lyophilized from an aqueous buffer adjusted to the pH of its optimal activity in water. Since tyrosinase in water shows its optimal activity at pH values around 6.5, the enzyme preparation was pretreated by dissolving tyrosinase in 0.1 M KH_2PO_4 buffer at pH 6.5 and lyophilization.[4] The resulting enzyme powder was used in the studies of the oxidation of *p*-cresol. The reactions were performed in flasks shaken in a thermostatted waterbath. Since part of the enzyme powder tends to adhere to the glass wall, the tyrosinase was immobilized on glass beads[3] to overcome this problem. Furthermore, since the activity of tyrosinase is very sensitive to the water content of the reaction medium,[4] both the solvent containing the substrate and the macrocycle, and the immobilized enzyme were equilibrated prior to use[5,6] above a saturated NaBr solution (a_w = 0.33).‡[,12] In this way, the activity of water (a_w) under the various reaction conditions is always the same.[12] The effect of addition of different types of macrocycles on the initial rate of the tyrosinase-catalysed 4-methyl-1,2-benzoquinone production in chloroform is given in Table 1. Initial rates (to 5% conversion) were measured by following the change in absorbance spectra of samples taken at periodic time intervals from the reaction mixture. The spectra showed that clean product formation occurred during the measurements. In all cases the *p*-cresol concentration in the reaction mixture was 20 mM which is the substrate concentration where the enzyme shows its highest activity in the presence of macrocycles. Of the various compounds tested, kryptofix 2.2.2 has the largest enhancement of the tyrosinase activity. Furthermore, 18-crown-6 increases the initial rate 21 times, and the effect is absent with its open chain analogue pentaglyme. This indicates that similar to the effect of

Table 1 Influence of various macrocycles on the initial rate (V_o) of the oxidation reaction of *p*-cresol catalysed by tyrosinase in chloroform at a_w = 0.33. *Conditions:* 20 mM *p*-cresol, 50 mg cm^{-3} immobilized enzyme, 3 mM macrocycle, 25 °C. Reagents previously equilibrated with a saturated NaBr solution

Macrocycle	V_o(macrocycle) / V_o[a]
Kryptofix 2.2.2	59.4 ± 0.9
Diaza-18-crown-6	1.1 ± 0.3
Monoaza-18-crown-6	18.9 ± 0.1
18-Crown-6	21.0 ± 2.0
Dicyclohexyl-18-crown-6	17.0 ± 2.0
Pentaglyme	0.49 ± 0.05

[a] V_o = 1.45 × 10^{-7} M min^{-1}.

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‡ No tyrosinase activity was observed after equilibration above a saturated LiCl solution (a_w = 0.012), probably because the reaction conditions were too dry. However, addition of minor amounts of water immediately resulted in the formation of 4-methyl-1,2-benzoquinone in the reaction mixture containing kryptofix 2.2.2, whereas product formation was much slower in the absence of macrocycle. This experiment also indicates that previous incubation of the enzyme with kryptofix 2.2.2 has not resulted in enzyme inactivation.

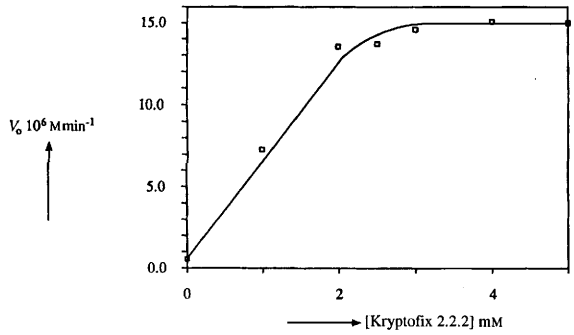

Fig. 1 Influence of the kryptofix 2.2.2 concentration on the initial rates (V_o) of the oxidation reaction of *p*-cresol catalysed by tyrosinase. *Conditions:* 20 mM *p*-cresol, 1 mg cm^{-3} tyrosinase, 25 °C.

Table 2 Influence of various macrocycles on the initial rate (V_o) of the oxidation reaction of *p*-cresol catalysed by tyrosinase in chloroform at $a_w = 0.72$. *Conditions:* 20 mM *p*-cresol, 50 mg cm^{-3} immobilized enzyme, 3 mM macrocycle, 25 °C. Reagents previously equilibrated with a saturated Na_2CO_3 solution

Macrocycle	V_o(macrocycle)/V_o[a]
Kryptofix 2.2.2	1.02 ± 0.09
Monoaza-18-crown-6	1.51 ± 0.09
18-Crown-6	1.20 ± 0.04

[a] $V_o = 6.14 \times 10^{-5}$ M min^{-1}.

macrocycles on proteases this reaction also shows an increase in enzyme activity due to a macrocyclic effect.

The dependency of the activity effect of the kryptofix 2.2.2 concentration is shown in Fig. 1. Addition of the first 2 mM kryptofix 2.2.2 significantly increases the enzyme activity, but further addition of kryptofix 2.2.2 has almost no effect. Such saturation behaviour was also observed for the activating effect of 18-crown-6 on α-chymotrypsin in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester in organic solvents.[8]

Enhancement of the enzyme activity from 1.45×10^{-7} M min^{-1} (Table 1) to 6.14×10^{-5} M min^{-1} (Table 2) can also be achieved by increasing the water activity in chloroform from 0.33 to 0.72.§ At this water activity the addition of macrocycles appeared to have hardly any effect on the enzyme activity (Table 2). However, enhancement of the water activity is not an effective method to increase the conversion rate since the changes in the UV absorbance spectra show that under these relatively wet conditions products other than 4-methyl-1,2-benzoquinone are also formed at higher conversions, most probably due to polymerization of the initially formed quinone product.

Table 3 shows that in tetrachloromethane ($a_w = 0.33$) also, large effects on the tyrosinase activity can be observed upon addition of kryptofix 2.2.2, dicyclohexyl-18-crown-6 and 18-crown-6. However, with hexyl acetate as the solvent ($a_w = 0.33$) no significant effect of macrocycle addition could be detected.

The above results indicate that, similar to the effects found for a number of serine proteases, the activity of tyrosinase in organic solvents can also be strongly accelerated upon addition of macrocyclic compounds. Some similarities in the results found for both type of enzymes are evident. Both in the case of tyrosinase and serine proteases like α-chymotrypsin the effect is dependent on the macrocycle structure, the concentration of the macrocycle and the solvent used.[8] Furthermore, a high water activity in the organic solvent reduces, or even annihilates, the

§ A water activity of 0.72 is obtained by previous equilibration of solvent and solutes above a saturated Na_2CO_3 solution.[12]

Table 3 Influence of various macrocycles on the initial rate (V_o) of the oxidation reaction of *p*-cresol catalysed by tyrosinase in tetrachloromethane at $a_w = 0.33$. *Conditions:* 20 mM *p*-cresol, 50 mg cm^{-3} immobilized enzyme, 3 mM macrocycle, 25 °C. Reagents previously equilibrated with a saturated NaBr solution

Macrocycle	V_o(macrocycle)/V_o[a]
Kryptofix 2.2.2	70.9 ± 3.0
Monoaza-18-crown-6	4.4 ± 0.7
Dicyclohexyl-18-crown-6	19.1 ± 0.0
18-Crown-6	58.6 ± 6.0

[a] $V_o = 1.13 \times 10^{-8}$ M min^{-1}.

effect of macrocycle activation.[8] This indicates that the complexation properties of the macrocycle play an important role in the activation mechanism. A structure–activity study of the effect of different crown ethers on the activity of α-chymotrypsin showed that an increased effect on the enzyme activity was related to an increased affinity of the crown ether for water.¶[8] As we have suggested before this relationship may be rationalized by assuming that the macrocycle acts as a carrier for water molecules which have to be displaced[14] upon complexation of the substrate at the active site. The idea that the macrocycles are involved in the water transport from the active site is further supported by the observation that presence of extra water in the enzyme–organic solvent system (Table 2) results in a decrease in the effect of macrocycles. In this medium a lower amount of macrocycle can enter the active site in an uncomplexed state. Moreover, a higher a_w might give larger hydrated areas on the enzyme surface near the active site to which water molecules can be more easily displaced upon substrate binding, resulting in a diminished role for the macrocycle.

In conclusion, the activity of tyrosinase in halogenated solvents at low water activity can be considerably increased by the addition of macrocycles. In contrast to the introduction of extra water to the system the addition of macrocycles does not decrease the stability of the *o*-quinone products formed. The higher enzyme activity is due to the good complexing capabilities of the macrocycles. Most likely, the water complexing properties of the macrocycles play a key role in the activation mechanism.

Experimental

Tyrosinase was from Sigma with tyrosinase activity of 24 000 units mg^{-1}. Glass beads (150–212 micron) were purchased from Sigma and previously washed with 10% HNO_3, followed with doubly distilled water until neutral pH. Solvents were from Merck (p.a. grade). *p*-Cresol (Merck), 18-crown-6 (Shell), dicyclohexyl-18-crown-6 (Aldrich), diaza-18-crown-6 (Merck), 15-crown-5 (Merck) and kryptofix 2.2.2 (Merck) were used as obtained. Monoaza-18-crown-6 was synthesized according to the literature.[15] Pentaglyme (hexaoxaoctadecane)[16] was synthesized from pentaethylene glycol (Aldrich) by reaction with sodium hydride and methyl iodide.

Unless stated otherwise, solutions with 20 mM *p*-cresol (and if appropriate 3 mM of the macrocycle) were equilibrated above a saturated salt solution in a desiccator at 5 °C for 48 h.

Tyrosinase was previously dissolved (5 mg cm^{-3}) in 0.1 M KH_2PO_4–KOH buffer pH 6.5, frozen and lyophilized.[4] When immobilized on glass beads, tyrosinase (20 mg cm^{-3}) was

¶ The association constants between water and diaza-18-crown-6, dicyclohexyl-18-crown-6, kryptofix 2.2.2, 18-crown-6, and 15-crown-5 in chloroform at 25 °C are 321, 20.3, 14.9, 11.0 and 5.00 dm^3 mol^{-1}, respectively.[13]

dissolved in 0.1 M KH_2PO_4 buffer pH 7.0, to which solution glass beads (2 g cm^{-3}) were added.[3] After drying in air, drying was continued with a lyophilizer, followed by equilibration at 5 °C in a desiccator above a saturated salt solution for 48 h.

Reactions were performed on a 3 cm^3 scale in stoppered 25 cm^3 Erlenmeyer flasks, which were shaken in a thermostatted waterbath at 25 °C. At certain time intervals, samples were taken (if necessary centrifuged and diluted) and the absorbance due to formation of product was measured at 395 nm in a 1 cm quartz cuvette. UV spectra were recorded with a Hewlett-Packerd 8452 A diode array UV-spectrophotometer. The extinction coefficient of 4-methyl-1,2-benzoquinone at 395 nm is 1.4 mM^{-1} cm^{-1}.[3] Reactions were performed at least in duplicate. No reaction occurred in the absence of enzyme or when bare glass beads, previously lyophilized from 0.1 M KH_2PO_4–KOH buffer pH 7.0, were suspended in chloroform with 20 mM *p*-cresol and 3 mM kryptofix 2.2.2.

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