УДК 577.1+577.151.7+577.152.1+543.068

MOLECULARLY IMPRINTED POLYMERS – TYROSINASE MIMICS

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За допомогою методу молекулярного імпринтингу синтезовано полімери, що імітують активний центр ферменту тирозинази. Молекулярно-імпринтовані полімери було одержано шляхом співполімеризації комплексу між Cu (II), катехолом, етиловим ефіром уроканової кислоти та (етиленгліколь)диметакрилатом. Полімери з оптимальною композицією демонстрували подібні до тирозинази грибів (КФ 1.14.18.1) каталіз, кінетику Міхаеліса–Ментен та конкурентне інгібування. На відміну від природного ферменту синтезовані молекулярно-імпринтовані полімери характеризувалися високою хімічною та механічною стабільністю.

Ключові слова: молекулярний імпринтинг, тирозиназа, полімерний каталізатор, полімери-біоміміки.

The concept of using organic chemical templates for directing organic synthesis [1], together with the impact of supramolecular chemistry [2,3], stimulated development of systems capable of mimicking the unique characteristics of biological enzymes. In this context the development of molecularly imprinted polymer (MIP) synthetic receptors and polymer catalysts has proven an area of significant interest [4–6]. The molecular imprinting strategy entails using of functionalized monomers which can bind reversibly to a template molecule; the resultant complex is subsequently incorporated into a network polymer by copolymerized with an excess of cross-linking monomer in the presence of an inert solvent (porogen) and a free radical initiator. Removal of the template leaves sites of complementary shape and functionality capable of selective rebinding of the template. By analogy to the preparation of catalytic antibodies [7], catalytic MIPs can be prepared using template substances with structures mimicking the transition states of reactions [8–11]. The superior chemical and physical stability of polymeric analogues of natural enzymes and receptors [12], relative in structure and functions to their biological counterparts, makes them of particular interest for use in chemical manufacturing, *in-vivo* applications and in environmental clean-up requiring robust materials.

Materials and Methods

Materials. Catechol, sodium benzoate, ethylene glycol dimethacrylate (EGDMA), 2,2′-azobisobutyronitrile (AIBN), resorcinol, dimethylformamide (DMF) were purchased from Acros. All the other chemicals were of analytical grade and were used as received without purification. Urocanic acid ethyl ester was kindly provided by Dr. A. Terentyev, IMBG, Ukraine. Mushroom tyrosinase (EC 1.14.18.1), activity 2,000 U/mg was received from «Sigma».

Polymer preparation. A series of EGDMA cross-linked polymers was prepared using a range of catechol-functional monomer-Cu (II) relative concentrations (Table). The thermal polymerisation was initiated by addition of 2,2′-azobisisobutyronitrile (AIBN) and performed at 80 °C for 12 h. The resultant polymer was crushed, ground, sieved (63 μm) and sedimented in water. In order to remove the template the polymer powder was washed successively with aqueous disodium EDTA solution, DMF and water, then dried prior to evaluation of catalytic properties.

Measurements. In evaluations of polymer catalytic activity 50 mg of EDTA-washed polymer samples in 20 ml of 100 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CuCl_2$, were incubated at 25 °C with intensive stirring in a reaction flask containing no head-space (atmosphere). Dissolved oxygen (255 mM) was the sole source of oxidant for the reaction. Reactions were initiated by the injection of catechol solution (100 μl, 1 M), in the same buffer, into the reaction vessel. The change in the oxygen concentration in solution due to catechol oxidation was monitored using an oxygen selective electrode. The catechol concentrations measured using HPLC (15 cm C_{18} column, gradient of acetonitrile in water) showed

a concomitant decrease in direct proportion to oxygen consumption. Due to the fact that the polymers were prepared with various quantities of Cu (II), which is able to catalyse catechol oxidation itself, polymer-catalysed catechol oxidations were performed in solutions with the same concentration of copper ions. Under such conditions differences in the activity can be related to differences in the polymer structure *per se*.

Results and Discussion

The active site of the enzyme tyrosinase possesses a binuclear copper centre capable of oxidizing two molecules of *o*-diphenol to *o*-quinone with concomitant 4 e^--reduction of molecular oxygen to two molecules of water (Fig.1) [13]. This reaction requires the simultaneous coordination of the two Cu (II) ions by the *o*-phenolic oxygens, with a copper-copper distance of 0.36 nm (Fig. 2). Several attempts have been made to mimic the active site of enzymes for the conversion of *o*-diphenols (catechols) to *o*-quinones [14–16], though most have involved multi-step organo-metallic syntheses and results thus far have been modest.

It was perceived that molecular imprinting may offer an alternative strategy for generation of a synthetic tyrosinase mimic, as the imprinting of metal ions *per se* has been achieved in a number of studies [17–20]. On the basis of molecular model studies, it was considered that a catechol molecule, coordinated to two Cu (II) ions separated by a distance of 3.6–0.4 nm as in the natural enzyme [13], could be imprinted using the imidazole moiety of the functional monomer urocanic acid ethyl ester (Scheme). It was envisaged that the functional monomer would be capable of fulfilling the remaining coordination sites of the binuclear complex. The removal of catechol and Cu (II) from the imprinted polymer should leave a three-dimensional network polymer with cavities containing imidazole groups oriented for complexation with Cu (II) ions and catechol.

Elemental analysis of polymer P9, which demonstrated the highest rate acceleration in subsequent studies, showed that the polymer contained the same proportion of nitrogen and carbon as in the monomeric mixture, indicating the quantitative inclusion of monomers into this polymer.

The polymers producing the highest rate enhancements, P9 and P12, which were prepared after optimization of polymerization mixture compositions, show activities similar to that of the enzyme mushroom tyrosinase (Table), though with higher Michaelis constants (K_m). Addition of the competitive tyrosinase inhibitor sodium benzoate led to a proportional decrease in catalytic activity (Fig. 3). By analogy to the natural enzyme, the decrease in activity was interpreted in terms of direct coordination of benzoate by copper. The observed rate constants were proportional to polymer concentration (data not shown).

Figure 4 shows the influence of imidazole concentration on the catalytic oxidation of catechol

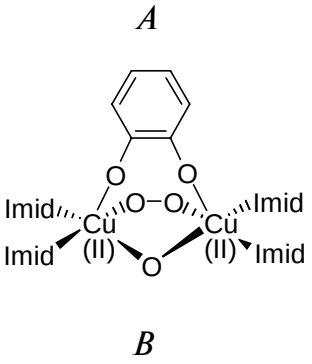


Fig. 2. A: Proposed transition state for the oxidation of catechol. B: Schematic representation of the tyrosinase-mimicking active site of molecularly imprinted polymer for oxidation of o-diphenol to o-quinone.

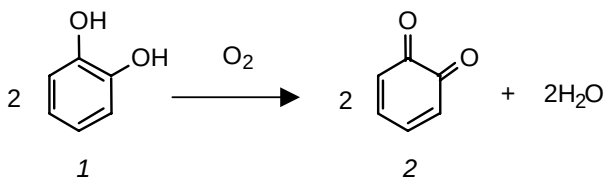
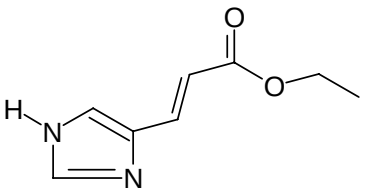


Fig. 1. Oxidation of catechol (1) to o-quinone (2).



Scheme. Structure of urocanic acid ethyl ester.

*Composition and catalytic properties of the polymers mimicking the tyrosinase active site**

Polymer	Im[a] (μmol)	Cu[b] (μmol)	Catechol[c] (μmol)	K_m[d] (mM)	k_{cat}[e] (x 10^{-2} s^{-1} g^{-1})
P9	250	1000	62.5	4.3 ± 1.1	4.2 ± 0.5
P12	375	1000	62.5	7.6 ± 2.5	4.2 ± 0.9
P7	375	125	62.5	6.4 ± 2.1	3.6 ± 0.9
P1	250	125	62.5	6.9 ± 3.5	2.8 ± 0.9
P10	500	500	62.5	3.1 ± 1.1	2.2 ± 0.1
P5	250	750	62.5	1.8 ± 0.5	2.1 ± 1.2
P8	250	2000	62.5	2.3 ± 0.3	1.9 ± 0.1
P6	250	250	62.5	2.6 ± 0.7	1.7 ± 0.2
P2	250	125	0	3.5 ± 1.0	1.5 ± 0.2
P4	250	500	0	1.7 ± 0.1	1.5 ± 0.1
P11	250	1000	62.5	3.9 ± 1.4	0.8 ± 0.2
P3	250	250	0	1.3 ± 0.1	0.4 ± 0.1
P0	250	0	62.5	0.9 ± 0.3	0.6 ± 0.6
Tyrosinase				0.9 ± 0.4	8.5 ± 2.1

*Polymers were prepared using the monomer and template compositions shown above with EGDMA (6.06 mmol), DMF (1.25 g) and AIBN (50 mg). [a]Im – urocanic acid ethyl ester (3). [b]Copper (II) chloride. [c]All imprinted polymers were prepared using catechol as template, except for P11 for which resorcinol was used. [d]K_m – Michaelis constant. [e]Reaction rates (per gram dry weight polymer) of 50 mg polymer samples in 20 ml of 100 mM Tris-HCl buffer pH 8.0 containing 5 mM $CuCl_2$, or a solution of 10 mg/ml of mushroom tyrosinase (2000 U/mg). Data were derived from a minimum of three experiments. Errors reflect linear regression deviations from the calculation of initial reaction rates. The apparent k_{cat} was calculated using the relationship $k_{cat} = V_{max}$/number of active sites. The number of active sites was based upon the copper concentration present in each assay (0.1 mmol).

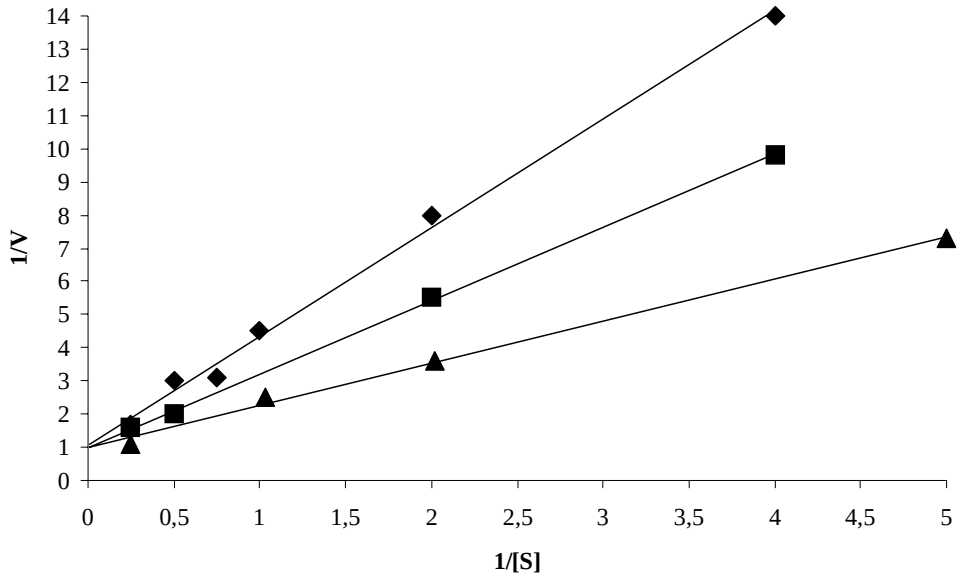


Fig. 3. Double reciprocal Lineweaver-Burk plot for initial reaction rate, v_0^{-1} (s μmol^{-1}) versus substrate concentration s^{-1} (L $mmol^{-1}$). Inhibitor concentrations (sodium benzoate): ◆ 7.5 mM, ■ 2.5 mM, ▲ no inhibitor.

by Cu (II) in solution. This clearly shows that higher imidazole concentrations lead to a higher rate of reaction in solution. When examining the influence of various polymers on catechol oxidation, however, the observed rate enhancements cannot be attributed to mere non-specific substrate adsorption effects, as polymers P2, P4, P5 and P8 have lower K_m values than the more active polymers P7 and P12.

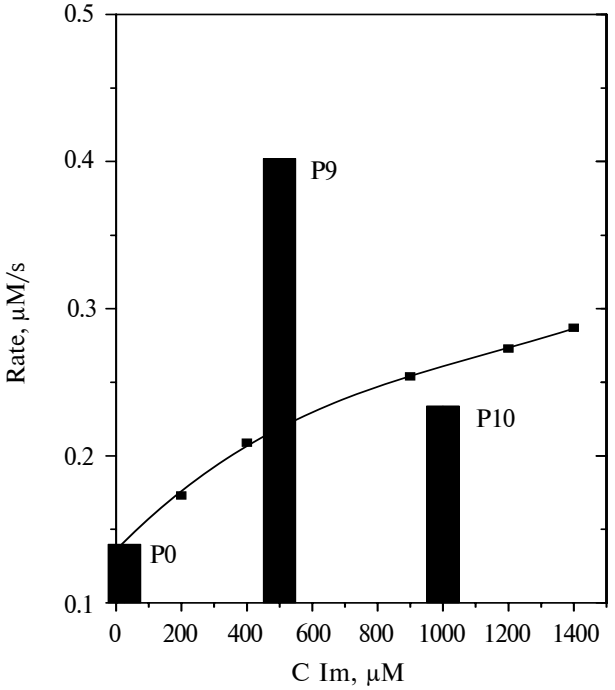


Fig. 4. Influence of imidazole concentration on concentration of o-quinone as a product of copper (II)-catalysed oxidation of catechol. The increase in the product concentration in solution reaction is shown by the curve. Measurements were carried out in the 50 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CuCl_2$. Black bars reflect the reaction rates of catechol oxidation in the presence of three polymers as a function of their corresponding imidazole content.

As illustrated by the lower activities of P4, P5, P8 and P10, the catalytic activity of the polymers is not directly related to the imidazole concentration in the polymer. Practically no direct correlation between the polymer imidazole content and catalytic properties (shown as bars) was evident. Furthermore, the rate of catechol oxidation is significantly higher for P9 than for P0 and P10 (the latter with twice the imidizole content of P9), which showed a level of activity comparable to that of the corresponding solution reaction. It is noteworthy that in order to achieve a reaction rate similar to that demonstrated by P9, in the absence of polymer much higher concentrations of reagents are required: copper ion concentration of 5 mM and at least 5 mM imidazole. The observed activity can therefore be explained as a direct consequence of the molecular imprinting effect, *i.e.* the organization of copper-imidazole catalytic centres with space for substrate binding arising from the presence of catechol during the polymerization process.

Although P7 has copper and imidazole concentrations more representative of the natural enzyme's catalytic centre [13], the catalytic activity of the polymer P12 is significantly higher. This we attribute to the higher copper concentration used in the preparation of polymer P12, which would favour the formation of binuclear copper complex with catechol, and is essential for providing the correct relative geometry of the copper ions. As can be seen from Table, the low activities of polymers prepared in the absence of catechol support the imprinting-based origin of the observed MIP catalytic properties. This implies that catechol is required, in conjunction with imidazole, to coordinate two copper ions in the requisite geometry. The almost negligible activity of polymer P11, prepared with resorcinol instead of catechol, suggests that distorted binuclear copper ion geometry cannot provide the conditions necessary for catalysis. Nonetheless, the possibility that sites selective for the higher order copper ion clusters cannot be excluded, nor that they may contribute to the observed catalytic effects.

Importantly, experiments run in the absence of polymer demonstrated no significant substrate conversion over the time frames studied, though addition of polymer to these solutions immediately initiated the oxidation reaction.

The catalytic turnover observed for best MIPs varied from 3 to 6, calculated from theoretical numbers of catalytic sites.

Tyrosinase has been shown to undergo an irreversible inactivation by reaction with hydroxyl radical during the oxidation of catechol to *o*-quinone [20]. In the case of the polymers reported here no such effect was observed. Indeed, polymer P9 was regenerated after use by washing with aqueous EDTA, water, 1 M NaOH and ethanol, and reloaded with $CuCl_2$. The regenerated polymer samples retained 100% of their original activity. This implies that the mechanism of deactivation of the native enzyme is not relevant to these synthetic enzymes. Furthermore, the stability of the imprinted polymer catalysts was examined by repeated evaluation of P9 activity over a period of two months, with no loss of activity being observed.

The molecular imprinting of complexes mimicking the transition state for catechol oxidation yields the synthetic polymers exhibiting properties comparable to the enzyme tyrosinase. The work on the optimization of polymer composition, especially finding the right quantity and ratio of functional monomers results in defined functional group spatial orientations around template structures. These polymers demonstrate catalytic turnover, Michaelis-Menten kinetics and competitive inhibition properties similar to those of the natural enzyme. The chemical and mechanical stability of molecular imprinted polymers, along with the possibility of introducing catalytic properties not available in nature,

and freedom of choice of templates and monomer functionalities, suggests that molecularly imprinted polymer catalysts provide a powerful complement to their biological counterparts. Furthermore, they should prove useful in biotechnological and chemical sensing applications.

Acknowledgements IN thanks the Swedish Engineering Sciences Research Council (TFR) and the University of Kalmar Research Fund. SP thanks to JSPS for a research support.

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S u m m a r y

Synthetic polymers mimicking the enzyme tyrosinase have been prepared by the molecular imprinting of a complex between Cu (II) and catechol and ethyl ester of urocanic acid in an ethylene glycol dimethacrylate copolymer. Optimised polymer systems demonstrated catalysis, Michaelis-Menten kinetics and competitive inhibition similar to those of mushroom tyrosinase. The polymers benefited from superior chemical and mechanical stability in comparison with natural enzyme.

K e y w o r d s: Molecular imprinting, tyrosinase, polymer catalyst, polymers-biomimics.

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Received 05.07.2005