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Atrazine transformation using synthetic enzymes prepared by molecular imprinting†

Toshifumi Takeuchi,*[a,b] Satoshi Ugata,[c] Shuichi Masuda,[c] Jun Matsui[c] and Masayoshi Takase[b]

[a] *Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, 657-8501, Japan. E-mail: takeuchi@scitec.kobe-u.ac.jp; Fax: +81-78-803-6158; Tel: +81-78-803-6158*

[b] *PRESTO, Japan Science and Technology Agency (JST), Kawaguchi, 332-0012, Japan*

[c] *Faculty of Information Sciences, Hiroshima City University, Hiroshima, 731-3194, Japan*

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Atrazine imprinted synthetic polymers were prepared using a combination of methacrylic acid and 2-sulfoethyl methacrylate that bound and converted atrazine and other 6-chlorotriazine herbicides to less toxic compounds. A chloride at the 6-position of the triazines was converted to a methoxy group by the polymer in a methanol-containing solvent, where the imprinting effects enhanced the catalytic activity. Competitive inhibition of the atrazine methanolysis was observed in the presence of a structurally related binder, ametryn, suggesting that the catalytic reaction proceeded in the binding sites generated by the molecular imprinting process.

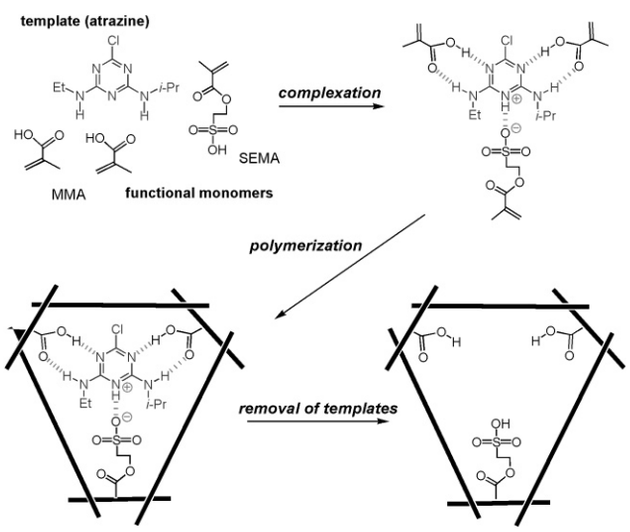
Introduction

The design and synthesis of synthetic materials with specific binding sites and catalytic functions are an area of increasing current interest.[1] Selective recognition and subsequent chemical reactions within binding pockets are instructive as well as of interest not only to supramolecular chemists but also to synthetic chemists. In particular, synthetic enzymes that selectively convert triazine herbicides into to less or non-toxic compounds would be useful catalytic systems as triazine herbicides are acknowledged widespread environmental pollutants.[2]

To date we[3] and others[4] have reported atrazine binding polymers prepared by molecular imprinting for the purpose of detection, separation, and purification.[5] The technique is now known as a means to prepare tailor-made synthetic polymers that are highly selective for target substances. In our recent study on employing automated polymer preparation/evaluation systems for molecularly imprinted polymers,[3a] we found that the use of 2-sulfoethyl methacrylate (SEMA) provides atrazine transformation polymers. Herein we show the selective binding and subsequent transformation of 6-chlorotriazine herbicides, using imprinted polymers that were prepared with methacrylic acid (MAA) as the binding site-constructing monomer and SEMA as the catalytic monomer. The resulting imprinted polymer was demonstrated to act as a novel synthetic enzyme with catalytic ability.

Results and discussion

To prepare polymers with catalytic activity for triazine herbicides, atrazine was used as a template molecule with two functional monomers: MAA and SEMA. The choice of MAA and SEMA as functional monomers was based on their abilities to form complementary hydrogen bonds with atrazine and to act as an acid catalyst for the nucleophilic aromatic substitution of atrazine with MeOH.[3a,6] The former constructs binding sites and the latter provides catalytic sites (Scheme 1). For the characterization of binding sites induced by the imprinting process, binding behavior of the imprinted polymers was examined by injecting triazine herbicides and other pesticides onto a column packed with the polymer prepared using 2 equiv. of SEMA and 6 equiv. of MAA and imprinted against atrazine (IP(S2M6)).[7] Chemical structures of the triazine herbicides and other pesticides used in this study are shown in Fig. 1. Similar to previously reported atrazine-imprinted polymers,[3] only triazine herbicides were strongly adsorbed and other pesticides were hardly retained (Fig. 2). These results clearly showed that the polymers prepared in this study have the selectivity for triazine herbicides.



Scheme 1 Schematic illustration of the imprinted polymer preparation.

In order to estimate the affinity of the binding sites for triazine herbicides, a binding isotherm was drawn using ametryn which is structurally similar to atrazine and is more resistant to acid-catalyzed methanolysis. A Scatchard plot from the binding data (ESI†) showed a non-linear profile, suggesting that the binding sites are heterogeneous with respect to the affinity for ametryn. A dissociation constant could be estimated to be 6.5×10^{-6} M from the low concentration range (ESI†). Although this figure is not precisely calculated because of the non-linear profile, it gives an idea for the possessing affinity.

Transformation of atrazine to the less toxic derivative atraton (2-ethylamino-4-isopropylamino-6-methoxy-1,3,5-triazine)[2] was demonstrated by incubation of the polymers with atrazine in the mixed solvent $CHCl_3$–MeOH, 9 : 1, v/v (Fig. 3). A proposed mechanism is the protonation of the atrazine by a sulfonyl group of SEMA

† Electronic supplementary information (ESI) available: (1) Binding isotherm and Scatchard plot for ametryn in IP(S2M6). (2) Transformation of simazine and propazine by IP(S2M6). (3) GC–MS of a transformation reaction mixture with IP(S2M6). See http://www.rsc.org/suppdata/ob/b4/b407002j/

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within the binding pockets, followed by the nucleophilic substitution at the 6-position by methanol yielding atraton (Scheme 2).[2] The obtained atraton was identified by comparing with the authentic sample on GC-MS (ESI†).

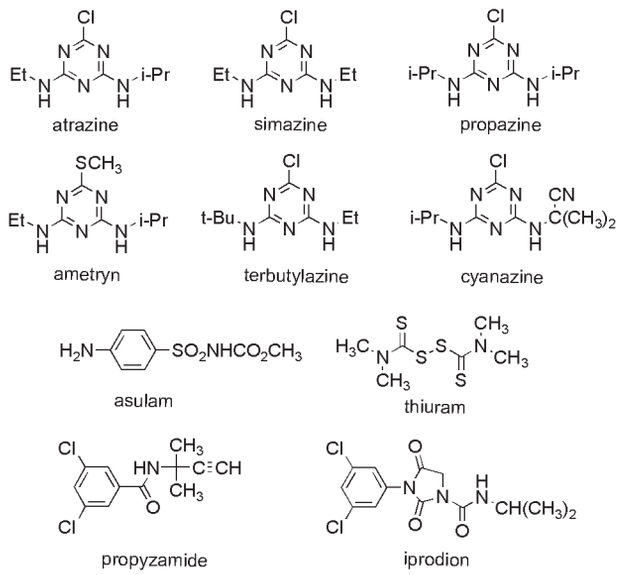


Fig. 1 Chemical structures of the triazine herbicides and pesticides used in this study.

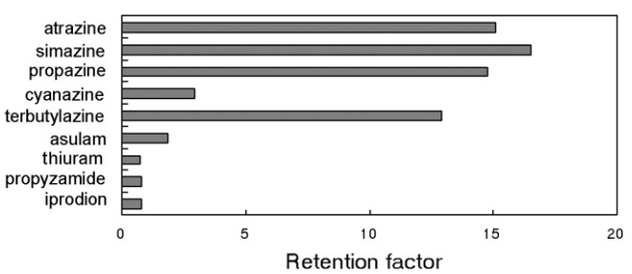


Fig. 2 Retention factors in IP(S2M6); IP(S2M6) was ground and wet-sieved (methanol) to obtain polymer particles with a size of 32–63 μm, then packed into a stainless column (4.6 × 100 mm, i.d). The eluent used was CH_2Cl_2–CH_3CN, 3 : 1, v/v (1.0 mL min^{-1}) and the detection was carried out at 254 nm.

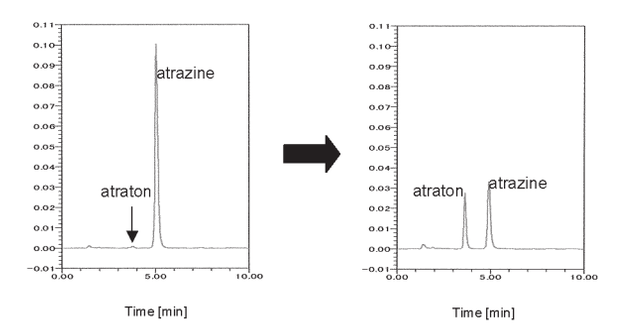
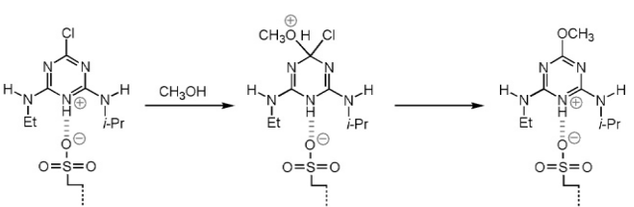


Fig. 3 Atrazine transformation by IP(S2M6). The polymer particles were incubated with atrazine in $CHCl_3$–MeOH (1.5 mL, 9 : 1, v/v) at 25 °C. After 24 h, the suspension was filtered (0.2 μL) and the filtrate was dried *in vacuo*. The residues were dissolved in CH_3CN (1.0 mL) and analyzed (Waters HPLC system) with a reversed phase column (Supelco LC-8-DB) and an eluent of CH_3CN–0.1 M ammonium acetate buffer (47 : 53, v/v, pH 6.0, 1.0 mL min^{-1}).



Scheme 2 A possible mechanism of atrazine transformation with MeOH activated by SEMA residues.

Since atrazine is essentially transformed under acidic or basic conditions,[2] reaction kinetics were examined to evaluate the effects of changing imprinting conditions on the catalytic activity. IP(S2M6) showed higher activity than the polymer prepared with 1 equiv. of SEMA and 7 equiv. of MAA against the amount of initial atrazine (IP(S1M7)). The differences between the imprinted and reference polymers was larger in IP(S2M6) than IP(S1M7) (Fig. 4). The activity of the corresponding reference polymers (RP(S2M6) and RP(S1M7)) was lower than that of the imprinted polymers, revealing that the binding sites formed during the imprinting process appear to enhance the catalytic activity. It is obvious that more SEMA residues provide higher activity but too many SEMA residues may lead to non-specific binding and to less selective catalytic reactions, therefore IP(S2M6) was used for the following experiments.

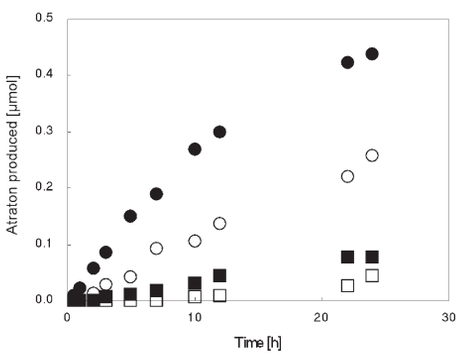


Fig. 4 Transformation velocity in the imprinted and reference polymers: IP(S2M6) (●), RP(S2M6) (○), IP(S1M7) (■) and RP(S1M7) (□).

Attempts were made to evaluate the rate enhancements in the polymer by comparing transformation velocities of atrazine, *i.e.* the velocity of atraton production in imprinted polymers against that in free SEMA solutions. Firstly, effects of substrate concentrations on the reaction velocity were investigated in the imprinted polymer. As seen in Fig. 5, saturation kinetics was obtained, suggesting that the atrazine transformation reaction proceeds within discrete binding sites within the polymer. From a Lineweaver–Burk plot using the kinetic data, we estimated a velocity constant of this reaction; a maximal velocity V_{max} of 1.0×10^{-7} M min^{-1} and a Michaelis constant K_m of 5.2×10^{-4} M. A k_{cat} value was also estimated to be 1.0×10^{-6} M min^{-1} (Fig. 5). According to the elemental analysis,[8] the polymer contained 0.87% of S, meaning that there was 0.815 μmol of SEMA residues in 3 mg of the polymer and the concentration of total catalytic sites can be estimated as 0.54 mM in the 1.5 mL incubation solutions. It should be noted that this figure is probably an overestimation because not all SEMA residues will be incorporated into a catalytic site, and there is no way to distinguish SEMA in the catalytic sites from randomly located SEMA in the polymer. However, the maximum number of binding/catalytic sites should not exceed the amount of atrazine added in the polymerization process, which was half the SEMA concentration.

Secondly, the transformation was carried out in homogeneous solutions as a reference, using free SEMA solutions and V'_{max}, K'_m, and k'_{cat} were also estimated to be 2.4×10^{-7} M min^{-1}, 3.1×10^{-4} M,

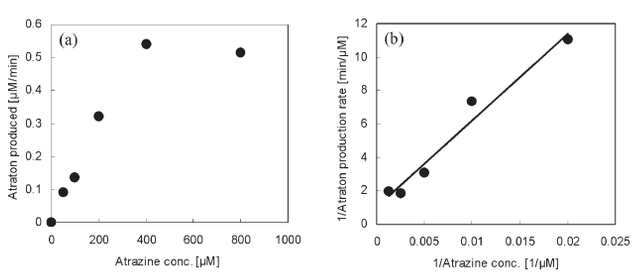


Fig. 5 Effects of substrate concentrations on the atraton production (a) and Lineweaver–Burk plot (b) in IP(S2M6): $V_{max} = 1.0 \times 10^{-7}$ M min^{-1}, $K_m = 5.2 \times 10^{-4}$ M, $[E]_{total} = 5.4 \times 10^{-4}$ M, $k_2 = V_{max}/[E]_{total} = 1.9 \times 10^{-3}$ min^{-1}, $k_{cat} = k_2 K_m = 1.0 \times 10^{-6}$ M min^{-1}. [E]: IP(S2M6) conc.

and 1.3×10^{-7} M min^{-1}, respectively (Fig. 6). Thus, a ratio of the two k_{cat}, *i.e.* imprinting effects for the transformation, was evaluated to be at least 7.6. Unfortunately, the increment by the imprinting effect was not as high as previously reported MIP catalyzed reactions,[5] this may be partly due to the influence of improperly located SEMA residues and the overestimation of SEMA in the polymer involved in the catalytic reaction. Product inhibition may also occur because atraton is structurally similar to atrazine, contributing to the low k_{cat} value.

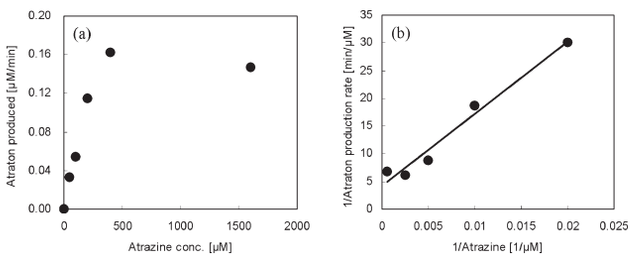


Fig. 6 Effects of substrate concentrations on atraton production (a) and Lineweaver–Burk plot (b) in free SEMA: $V_{max} = 2.4 \times 10^{-7}$ M min^{-1}, $K_m = 3.1 \times 10^{-4}$ M, $[E]_{total} = 5.9 \times 10^{-4}$ M, $k_2 = V_{max}/[E]_{total} = 4.1 \times 10^{-4}$ min^{-1}, $k_{cat} = k_2 K_m = 1.3 \times 10^{-7}$ M min^{-1}.

To confirm that the reaction proceeds in the binding sites of the imprinted polymers, inhibition experiments were conducted using an inactive compound. Ametryn was ideal for this purpose because it has almost the same structure as atrazine except for the methylthio group at the 6-position. Inhibition of the reaction by ametryn would indicate that it was competing for the binding sites in the atrazine-imprinted polymers, and the nucleophilic substitution with MeOH would not occur even if in the presence of the SEMA catalytic side chains. The reaction velocities were measured for three different atrazine concentrations at a variety of inhibitor concentrations. The activity was inhibited with increasing ametryn concentration, and a Dixon plot showed that ametryn was a competitive inhibitor. Also calculated from these plots was the inhibition constant K_i, indicating that the dissociation constant for the enzyme-inhibitor complex was 4.7×10^{-7} M (Fig. 7). Thus, it was confirmed that atrazine methanolysis reactions proceeds within the binding sites that were constructed during the molecular imprinting process.

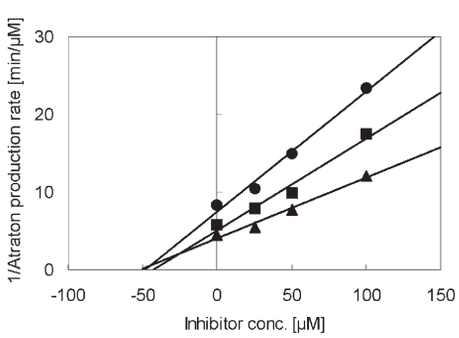


Fig. 7 Inhibition of the transformation activity by ametryn. Atrazine (200 (▲), 300 (■) and 400 (●) μM) and ametryn (0–100 μM) were incubated with the polymers (3 mg in methanol–chloroform, 1 : 9, v/v, 1.5 mL) at 25 °C. The K_i value was estimated to be 4.7×10^{-7} M.

Other triazine herbicides that also contained a Cl leaving group at the 6-position, such as simazine and propazine, showed similar reactivity in the presence of the imprinted polymer (ESI†). This demonstrates that the binding sites produced from the molecular imprinting of atrazine showed selectivity not only in binding but also in catalytic activity.

Conclusion

We have succeeded in the preparation of synthetic polymers with binding and catalytic sites for triazine herbicides with Cl at the 6-position using molecular imprinting. As compared to homogeneous SEMA, this synthetic enzyme exhibited higher catalytic activities. At the present stage, the activity seems to be low even though the k_{cat} values were underestimated because the number of catalytic sites was overestimated. The low rate accelerations may be caused by randomly positioned SEMA that arises from the use of non-covalent imprinting strategy, in which an excess of functional monomer is used to shift the equilibrium in favor of complex formation with the template molecule. We found that the product inhibition occurred at a high substrate concentration range. This may be due to the structure similarity of the substrates and the products. Therefore the construction of more precise binding/catalytic sites will be necessary to improve this system, and polymers that organize the catalytically active site more precisely using combined use of covalent and non-covalent imprinting systems are currently being studied in our group.

Experimental

Materials

Atrazine was kindly donated by Nissan Chemical Industries, Ltd. (Tokyo, Japan). Propazine, ametryn, prometrine, terbutylazine, cyanazine, simazine, propazine, asulam, thiuram, propyzamide, iprodion, methacrylic acid and 2,2′-azobis(isobutyronitrile) were purchased from Chemical Industry (Tokyo, Japan). 2-Sulfoethyl methacrylate (SEMA) was purchased from Polysciences (Warrington, PA). Ethylene glycol dimethacrylate (EGDMA) and other solvents were obtained from Katayama Chemical (Osaka, Japan).

Preparation of atrazine imprinted polymers using SEMA and MAA as functional monomers

Atrazine (1.67 mmol), functional monomers (SEMA 1.67 mmol/MAA 11.69 mmol or SEMA 3.34 mmol/MAA 10.02 mmol) and EGDMA (47.2 mmol) were mixed in 25 mL of chloroform. After the polymerization was initiated by photo-irradiation using a radical initiator, 2,2′-azobis(isobutyronitrile) (120 mg) for 12 h at 5 °C, the obtained polymer was crushed, washed to remove atrazine first with the mixed solvent (100 mM H_2SO_4 aqueous solution–MeOH, 1/9, v/v) and then with MeOH. The resulting polymers were further ground and wet-sieved in methanol to obtain polymer particles with the size of 32–63 μm. Corresponding reference polymers were prepared with the same recipe without atrazine.

Selectivity and kinetic experiments

For the selectivity experiments, the particles were packed into a stainless steel column (4.6 mm × 100 mm i.d.). A Waters HPLC system consisting of pumps (model 626), an auto-sampler (model 717 plus, sample size: 20 μl), a detector (model 996, 254 nm) and an eluent of dichloromethane–acetonitrile, 3 : 1, v/v (1.0 mL min^{-1}) was used.

In the kinetic experiments, the polymer particles (3 mg) were incubated with atrazine (0, 50, 100, 200, 400 or 500 μM) in methanol–chloroform (1.5 mL, 1 : 9, v/v) at 25 °C. At appropriate intervals, the suspensions were filtered (0.2 μm) and the filtrates were dried *in vacuo*. The residues were dissolved in acetonitrile (1 mL) and analyzed by the Waters system with a reversed phase column (Supelco LC-8-DB) and an eluent of acetonitrile–0.1 M ammonium acetate buffer (47 : 53, v/v, pH 6.0, 1 mL min^{-1}). For the Lineweaver–Burk and Dixon plots, the velocities of atraton production for the first 3 h were measured against each substrate concentration based on the amount of atraton in the supernatants as quantified by HPLC.

As a reference, free SEMA (590 μM) was used instead of the polymer for the kinetics of free SEMA in solution. The amount was adjusted to be the theoretical amount of SEMA in 3 mg of the particles.

Inhibition experiments

The polymer particles (3 mg) were incubated with atrazine (200, 300 and 400 μM) and ametryn (an inhibitor, 0–100 μM) in methanol–chloroform, 1 : 9, v/v (1.5 mL) at 25 °C. At appropriate intervals, the suspensions were filtered (0.2 μm) and the filtrates

were dried *in vacuo*. The residues were dissolved in acetonitrile (1 mL) and analyzed by the Waters HPLC system using a reversed phase column (Supelco LC-8-DB) and an eluent of acetonitrile–0.1 M ammonium acetate buffer (47 : 53, v/v, pH 6.0, 1 mL min^{-1}).

Acknowledgements

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7 The polymer had a surface area and an average pore size of 1.76 m^2 g^{-1} and 15.7 nm as measured by its BET isotherm (Shimadzu MIC-2360).

8 Elemental analysis data of IP(S2M6) (wt%): C, 56.1; H, 7.01; N, <0.1; S, 0.87.