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Influence of cross-linking monomer and hydrophobic styrene comonomer on stereoselective esterase activities of polymer catalyst imprinted with a transition-state analogue for hydrolysis of amino acid esters

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Abstract

Polymer catalysts, cross-linked with *N*,*N*′-ethylene (C_2) {or butylene (C_4)}-bisacrylamide containing L-histidine and quaternary trimethylammonium groups were imprinted with a racemic transition-state analogue of phenyl 1-benzyloxycarbonyl-3-methylpentylphosphonate for the hydrolysis of *p*-nitrophenyl *N*-(benzyloxycarbonyl)-L (or D)-leucinate {*Z*-L (or D)-Leu-PNP}. Maximal stereoselectivity (L/D = 8.4) was obtained by using *N*,*N*′-C_4-bisacrylamide cross-linked polymer catalyst, which was copolymerized with hydrophobic styrene monomer.

Keywords: Molecularly imprinted polymer; Transition-state analogue; Esterase

Preparations of molecularly imprinted polymers (MIPs) have been investigated as a widely applicable and convenient method, which creates three-dimensional networks with a "memorized cavity" of the shape and functional group positions of the template molecule [1–8]. In this respect, MIPs have been applied for creating transition-state imprinted enzymes as artificial "catalytic antibodies" in recent years [9–13]. We previously reported polymer catalysts [12,13] which were imprinted with a racemic transition-state analogue (*rac*-TSA) of phenyl 1-benzyloxycarbonylamino-3-methylpentylphosphonate for the stereoselective hydrolysis of amino acid *p*-nitrophenyl ester of *p*-nitrophenyl *N*-(benzyloxycarbonyl)-L (or D)-leucinate {*Z*-L (or D)-Leu-PNP}. In our previous work, acceleration factors were evaluated by the ratios of the pseudo-first-order reaction rate constants obtained with and without the catalyst (k_{cat} and k_{uncat}, respectively); k_{cat}/k_{uncat} = 2–12 [12,13]. Although the attainable acceleration factors (k_{cat}/k_{uncat}) were improved by Wulff et al. k_{cat}/k_{uncat} = 18–102 [11] compared to ours, the factors of the substrate-stereoselectivity is not systematically investigated in detail. Therefore, further investigations of novel TSA-imprinted polymer catalysts seem to be of interest and significant to design efficient "plastic enzymes" as a simplified model reaction of esterase. Here we wish to describe our improvement on the substrate-stereospecific properties of a cross-linked L-histidyl group (L-His)-introduced polymer catalyst, which were imprinted with *rac*-TSA for the substrate-stereoselective hydrolysis of *Z*-L (or D)-Leu-PNP, by following two methods: (1) examination of different lengths of two cross-linkers at the copolymerization (TP-1 and TP-2 in Fig. 1) in order to alter the polymer swelling; and (2) copolymerizations with hydrophobic styrene comonomer (TP-3 in Fig. 1) so as to introduce the additional cooperative effects of catalytically active groups in memorized cavity against the hydrophobic substrate.

Rac-TSA was obtained as described previously [12]. Cross-linked polymers were prepared by radical polymerization. Equivalent amounts (0.195 mmol) of methyl *N*-acryloyl-L-histidinate (L-His monomer) and *rac*-TSA were mixed in DMSO (5.0 cm^3) for 1 h at room temperature in N_2 for making some interaction between the L-His monomer and TSA before the polymerization,[1] followed by the addition of acrylamide (1.95 mmol), *N*-(3-trimethylaminopropyl)acrylamide chloride (0.875 mmol), a cross-linker of *N*,*N*′-C_n-bisacrylamide (0.389 mmol, $n = 2$ or 4) and AIBN (0.130 mmol) into the DMSO solution, and then

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[1] In the 400 MHz ^{1}H NMR spectra of the DMSO-d_6 solution including L-His monomer and *rac*-TSA, the chemical shift of the imidazolyl NH proton (in L-His monomer) from 6.60 to 7.20 ppm and that of the amide C(=O)NH proton (in TSA) from 7.53 to 6.90 ppm suggested hydrogen bond formation or electrostatic interaction between them.

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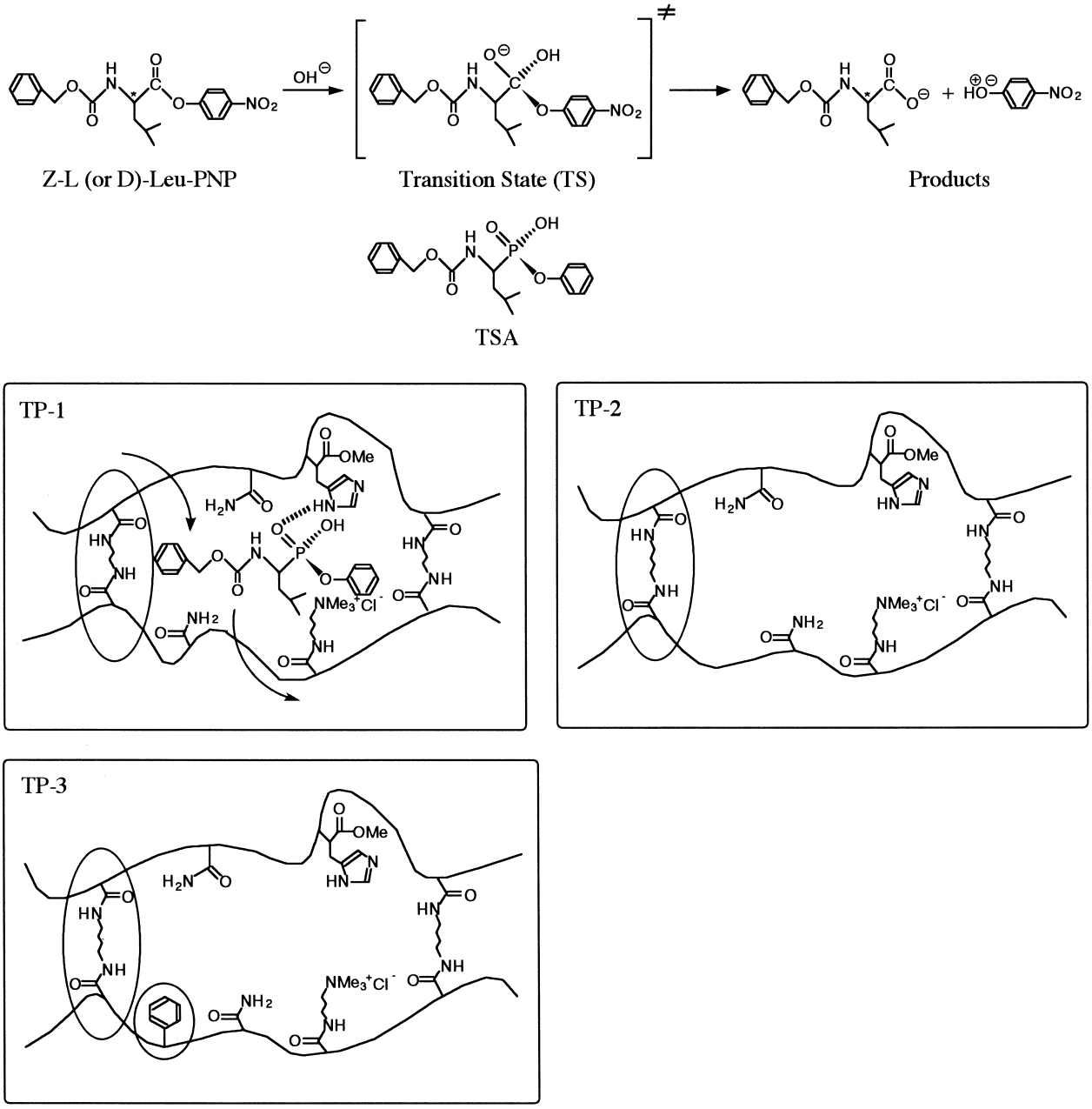

Fig. 1. Schematic illustration of the *rac*-TSA-imprinted polymers for stereoselective hydrolysis of *Z*-L (or D)-Leu-PNP.

polymerized at 60°C to produce a polymer possessing a cross-linker content of 11 mol%. Copolymerization with styrene monomer was performed in the TP-2 type synthesis (TP-3 type). Three kinds of the TP-3 type-polymer catalysts were prepared by adjusting the initial molar ratio; TSA:L-His monomer:styrene monomer:acrylamide:*N*-(3-trimetylamino-propyl)acrylamide chloride:*N*,*N*′-C_4-bisacrylamide = 1:1:2:9:3.9:3.9 (TP-3A), 1:1:2:9:3.9:5.7 (TP-3B), and 1:1:2:9:3.9:7.3 (TP-3C). Removal of TSA from the polymer was performed with 5 vol% Et_3N-AcOEt. Recovers of the template from the cross-linked polymers were 70.5 (TP-1), 65.6 (TP-2), 72.7 (TP-3A), 69.3 (TP-3B), and 58.9 mol% (TP-3C). These polymer catalysts possess randomly distributed quaternary trimethylammonium groups through their framework and were found to be very soluble in water. These positive trimethylaminopropyl groups probably attract negative phosphonate groups and/or hydroxide ions.

The hydrolyses of *Z*-L (or D)-Leu-PNP (20.0 μmol dm^{-3}) by the soluble polymer catalyst {methyl L-histidinate (His) unit concentration = 0.10 mmol dm^{-3}} were carried out in 10 vol% MeCN–Tris buffer (pH 7.15) at 30°C. The pseudo-first-order reaction rate constants obtained with and without the catalyst (k_{cat} and k_{uncat}, respectively) were determined by monitoring the produced amount of *p*-nitrophenolate (PNP) anion spectrophotometrically at 400 nm. The second-order catalytic rate constant k_{cat}^{app} was evaluated by the equation

Table 1
Catalytic activities ($k_{cat}^{app\ L}$) and stereoselectivities ($k_{cat}^{app\ L}/k_{cat}^{app\ D}$) of *rac*-TSA-imprinted polymer catalyst (TP-1, TP-2, TP-3A, 3B, or 3C) for the hydrolysis of *Z*-L (or D)-Leu-PNP ([Substrate] = 20.0 mmol dm^{-3} in 10 vol% MeCN–Tris buffer (pH 7.15) at 30°C. The histidyl group contained in the catalyst was 0.100 mmol dm^{-3} in the reaction)

Catalyst	Cross-linker content (mol%)	k_{cat}^{app} (mol^{-1} dm^3 min^{-1})		
		L	D	L/D
TP-1	11	33.6	10.0	3.36
	20	9.60	3.60	2.67
TP-2	11	23.0	10.1	2.28
	20	22.5	2.88	7.81
	35	13.6	2.30	5.91
TP-3A	20	31.0	3.72	8.33
TP-3B	25	26.0	3.09	8.41
TP-3C	30	19.4	2.57	7.54

$k_{cat}^{app} = (k_{cat} - k_{uncat})/[His]$, where [His] denotes the concentration of His unit in the catalyst.

Kinetic parameters for the hydrolyses of *Z*-L (or D)-Leu-PNP by TP-1, TP-2, or TP-3 with the various cross-linker contents are summarized in Table 1.

Predominant reaction for the L-form of the substrate was observed compared with the D-form. We previously reported that the water-soluble polymer catalyst has a reaction cavity which recognizes the skeleton of *rac*-TSA, especially that of L-TSA [13]. This implies that the complex formation of L-His monomer with L-TSA in DMSO through hydrogen bonding or electrostatic interaction was predominant rather than with D-TSA in the preliminary mixed system of L-His monomer and *rac*-TSA for the synthesis of the polymer catalyst. In the case of the cross-linker content of the polymer catalyst was 11 mol%, the catalytic activity for L-form of the substrate became lower with increasing length of alkyl chain of the cross-linker from C_2 to C_4. On the contrary, the catalytic activities for the D-form of the substrate were almost constant with k_{cat}^{app} = 10.0 (C_2) and 10.1 (C_4) mol^{-1} dm^3 min^{-1}. The catalytic activities of TP-1 containing C_2-cross-linker for both L- and D-forms of the substrate decreased remarkably when the cross-linker content increased from 11 to 20 mol%. On the other hand, significant decrease of the catalytic activity of TP-2 containing C_4-cross-linker was observed for only the D-form of the substrate and resulted in an increase of L-form predominant stereoselectivity with L/D value from 2.28 (11 mol%) to 7.81 (20 mol%). Greater stereoselectivity was observed with TP-2 compared to TP-1 when the cross-linker contents were 20 mol%. We deduce that appropriate cavity was formed by using the longer C_4-cross-linker compared to the shorter C_2 one. Further increase of the cross-linker-content from 20 to 35 mol% resulted in a decrease of the catalytic activities of TP-2 against both L- and D-forms of the substrate. This result implies that plenty of cross-linker causes the decrease of the "fluctuations" of the cavity. Therefore, substrate-stereospecific properties of cross-linked polymer catalyst can be improved by changing the length and the content of the cross-linker. Addition of styrene comonomer to the copolymerization system led to an enhancement of the catalytic activities and L-form predominant stereoselectivities of TP-3A, 3B, and/or 3C. The maximal enantiomeric selectivity was 8.41 with TP-3B when the cross-linker content was 25 mol%.

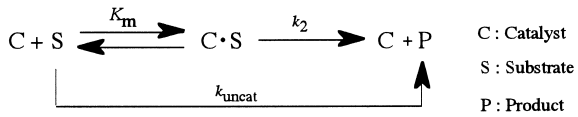

Fig. 2. Simplified reaction process of esterolysis with the TSA-imprinted polymer catalyst.

There are two possible enantiomer differentiating processes in the present substrate-stereoselective reactions; the substrate-binding process (viz. the catalyst–substrate complex formation pathway) and the reaction step of the catalyst–substrate complex to form the product, as shown in Fig. 2.

The substrate dissociation constant (K_m) and the rate constant (k_2) for the stereospecific esterolysis were obtained from the linear relation $1/(k_{cat} - k_{uncat}) = K_m/(k_2 - k_{uncat})[C] + 1/(k_2 - k_{uncat})$. Kinetic parameters for the esterolysis of *Z*-L (or D)-Leu-PNP with TSA-imprinted polymer catalyst (TP-2 or TP-3B) are summarized in Table 2.

It is worth emphasizing that the polymer catalyst actually incorporates the L-form of the substrate predominantly over the D-enantiomer to form the catalyst–substrate complex with $K_m^D/K_m^L = 4.46$ or 9.33 and hydrolyzes the L-substrate efficiently with $k_2^L/k_2^D = 1.10$ or 2.54. Thus, the present TP-2 and TP-3B have reaction cavities which seem to be predominantly recorded by the shape of L-TSA. Especially the TP-3 polymer catalyst, which was copolymerized with hydrophobic styrene monomer, exhibits the efficient

Table 2
Kinetic parameters for the hydrolysis of *Z*-L (or D)-Leu-PNP by *rac*-TSA-imprinted polymer catalyst (TP-2 or TP-3B) ([Substrate] = 20.0 mmol dm^{-3} in 10 vol% MeCN–Tris buffer (pH 7.15) at 30°C. The histidyl groups in the catalyst were 0.025, 0.050, 0.075, or 0.100 mmol dm^{-3} in the reaction. Cross-linker content of TP-2 was 20 mol%)

Catalyst	Substrate	$10^{-3}Km^{-1}$ (mol dm^{-3})	K_m^D/K_m^L	$10^4 k_2$ (s^{-1})	k_2^L/k_2^D	k_2/k_{uncat}
TP-2	*Z*-L-Leu-PNP	2.29	4.46	1.16	1.10	5.51
	Z-D-Leu-PNP	0.513		1.05		4.99
TP-3B	*Z*-L-Leu-PNP	9.52	9.33	0.684	2.54	3.41
	Z-D-Leu-PNP	1.02		0.269		1.41

substrate-stereospecific hydrolysis of *Z*-L-Leu-PNP through substrate incorporation into its reaction cavity owing to the predominant hydrophobic effect for the L-form. The k_2 and k_2/k_{uncat} values of TP-3B were smaller than those of TP-2. This result probably came from the addition of the styrene units into TP-3B, which caused a depression of the diffusion of *p*-nitrophenolate anion (product) from the inner reaction cavity to the outside of the polymer catalyst. Although the magnitude of the hydrolysis in the reaction (k_2) step with TP-3B was not so different from that with TP-2, that in the substrate-binding (K_m^{-1}) process was much greater with TP-3B than with TP-2.

Further experiments to examine the effect of different molar ratios of styrene comonomer and different lengths (from C_2 to C_4) of cross-linkers at the copolymerization are now in progress.

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