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Effect of Porogen on the Catalytic Activity of Molecular Imprinted Polymers

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A synthetic polymer catalyst was designed and synthesized for enhancing the rate of the hydrolysis reaction of *Z*-L-phenylalanine-PNP ester. Transition state analogues for the ester hydrolysis reaction pathways were used as templates for the synthesis of molecular imprinted *N*-methacryloyl L-histidine(MAH)-ethylene glycol dimethacrylate copolymers. Catalytic studies revealed that the imprinted polymers were selective for the transition state analogue corresponding to the template used in the polymer synthesis. Substrate hydrolysis was measured under pseudo first order reaction conditions (rate constant k). The rates of transition state analogue imprinted polymer catalyzed reactions were compared with those carried out in acetonitrile-*Tris* HCl buffer solution of pH 7.25 at room temperature as well as to the rate of reaction in the presence of non-imprinted polymer. In order to provide comparison between effect of a thermodynamically stable and an unstable solvent on the morphology of the polymer matrix and its catalytic activity the polymerization was carried out using DMSO and chloroform as the porogen. The catalytic activity of the transition state analogue imprinted polymer is found to be substrate specific, solvent and pH dependent. The molecular imprinted polymer synthesized in DMSO showed high selectivity supporting that the nature of the porogen has an effect on the hydrolysis by transition state analogue imprinted catalysts.

Key Words: Molecular imprinting, Transition state analogue, Amino acid ester, Hydrolysis, Porogen, Enantioselectivity.

INTRODUCTION

Molecular imprinting is an emerging technology leading to highly stable synthetic polymers with predetermined selectivity and applications in the field of protein recognition[1,2], biosensor development[3-5], enantiomer separation[6,7] and molecular catalysis[8,9]. The principle of molecular imprinting consists of polymerizing and crosslinking functional monomers, previously positioned by low energy or covalent interactions around a guest or template molecule so as to freeze the imprint. Following extraction of the template molecule, the remaining three dimensional networks presents pores with geometry and positioning of the functional groups complementary to those of the template. This enables it to specifically recognize the template molecule[10,11]. In the area of molecular catalysis, imprinted polymers seem to be particularly attractive with cavities designed to bind to the transition state of a given reaction should function as a selective catalyst for that reaction. In order to prepare molecular imprinted catalyst, molecules that mimic the transition state of a given reaction are used as templates. Furthermore, the system must be designed in such a way that a reactive moiety or a convenient precursor thereof is placed in the cavity in the course of the imprinting procedure. The resulting functionalized cavities are then potentially able to accelerate the rate of a reaction taking place inside them by stabilizing the transition state and/or to impart higher selectivity by favouring the formation of one of the possible transition states[12-14]. The developing understanding of the mechanisms by which enzymic catalysts function has been a major source of inspiration for researchers in molecular imprinted polymer (MIP) catalysis and a number of active imprinted polymer catalysts have now been prepared[15-18].

Even though there are a lot of work has been reported on the transition state analogue imprinted molecular imprinted polymer catalyzed ester hydrolysis, the dependence of the nature of porogen used during polymerization have been less studied[19-22]. The selectivity and efficiency of molecular imprinted polymers depend on the nature of the solvent used for the synthesis of the molecular imprinted polymers[23]. The properties of molecular imprinted polymers like swellability and porosity are often correlated with the solvent conditions employed during polymerization and there are two effects of the polymerization solvent, referred to as the porogen on the formation of molecular imprinted polymers[24-26]. The first is the formation of prepolymerization complex. For weaker non-covalent interactions such as ionic or hydrogen bonds non-polar solvents are preferred in order to drive the template and functional monomer towards complex formation. The second effect of the polymerization solvent is on the formation of the

porous structure of the polymer, which is the reason that it is referred to as porogen. The type of porogen used during the polymerization has a strong influence on the morphology of the imprinted polymer and its catalytic activity. In the present study in order to provide comparison between effect of a thermodynamically stable and an unstable solvent on the morphology of the polymer matrix and its catalytic activity the polymerization was carried out using chloroform and DMSO as the porogen.

EXPERIMENTAL

Solid state ^{13}C NMR spectra were recorded on AMX-400 NMR spectrometer. ^{1}H NMR spectra were taken using Bruker Avance DPX-300MHz FT-NMR spectrometer in $CDCl_3$. ^{31}P-NMR spectra was taken on AC200 NMR spectrometer. Infrared spectra were recorded on a Shimadzu FTIR-8400S spectrophotometer. Kinetic determinations were performed using a Shimadzu UV-visible 2450 spectrophotometer equipped with a thermostated cell. SEM analyses were performed on JEOL JSM 6390 SEM analyzer.

Synthesis of monomer: The monomer *N*-methacryloyl L-histidine was prepared from methacryloyl chloride, L-histidine monohydrochloride and NaOH following the reported procedure[20]. FTIR (cm^{-1}): 1612 (HC=CH), 1651 (amide carbonyl), 1705 (acid carbonyl). ^{1}H NMR ($CDCl_3$) δ ppm: 7.6 (s, ^{1}H, imidazole NH), 7.37 (s, 1H, imidazole CH), 6.76 (s, 1H, imidazole CH), 3.03, 2.56 (s, CH_2), 4.29 (d, 1H, C=CH), 1.83 (s, 3H, CH_3).

Synthesis of transition state analogue (phenyl 1-benzyloxycarbonyl amino-4-methoxybenzyl phosphonate): Triphenyl phosphite (13.2 mmol), *p*-methoxy benzaldehyde (19.8 mmol), benzyl carbamate (13.2 mmol) and glacial acetic acid (2 mL) were stirred for 4 h at 100 ºC in an oil bath. The diphenyl phosphonate formed was hydrolyzed with NaOH (0.4 N) at room temperature for 2 days (Fig. 1). It was acidified with conc. HCl and the product formed was purified by column chromatography using 9:1 chloroform-methanol mixture. FTIR (KBr, ν_{max}, cm^{-1}): 1300 (P=O stretching), 945 (P-OH stretching), 1250 (P-O-benzyl stretching). ^{1}H NMR ($CDCl_3$) δ ppm: 1.73 (s, H, OH), 3.77 (s, 3H, OCH_3), 5.49 (d, 2H, CH_2), 6.73-7.42 (m, 13H, aromatic CH). ^{31}P NMR δ ppm: 24.99 P(O)(OPhe)(OH),16.50 (P-O-P angle).

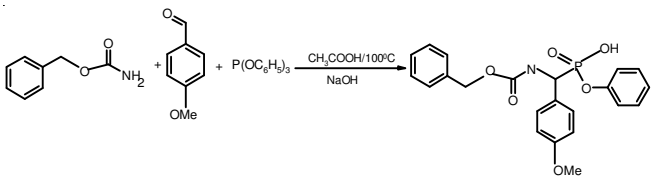

Fig. 1. Synthesis of transition state analogue

Preparation of *N*-protected phenylalanine esters: *N*-protected phenylalanine esters were prepared with coupling between the corresponding protected amino acids and *p*-nitrophenol in presence of DCC[27].

Synthesis of transition state analogue imprinted and non-imprinted polymers: The transition state analogue (0.319 g, 0.748 mmol) and monomer *N*-methacryloyl L-histidine (0.329 g, 1.495 mmol) were dissolved in chloroform (30 mL) and strirred under nitrogen atmosphere for 1 h at room temperature. Ethylene glycol dimethacrylate (2.75 cm^3, 14.95 mmol) and 50 mg initiator AIBN were added and polymerized at 60 ºC for 24 h. The process for the preparation of non-imprinted polymers was the same as that of the molecular imprinted polymers except that the imprinted transition state analogue molecules were not added during polymerization. The polymer (P-1) formed was collected by filtration, washed with acetone, Soxhlet extracted with chloroform for complete removal of transition state analogue and dried in vaccum. The same procedure was followed by using DMSO as porogen instead of chloroform resulted in polymer (P-2).

Catalytic activity measurement of imprinted and non-imprinted polymers: *Z*-L-phenylalanine *p*-nitrophenyl ester was chosen as the substrate to evaluate the hydrolytic activity of the imprinted and non-imprinted polymers P-1 and P-2 in a 9:1 (v/v) solution of *tris*-HCl buffer and acetonitrile at 25 ºC. A 10 mg each molecular imprinted polymer and non-imprinted polymers of polymers P-1 and P-2 was placed in a sealed test tube, to which a 2 mL acetonitrile-*tris* HCl buffer (pH = 7.25) added and allowed to equilibrate for 30 min. Acetonitrile solution (0.2 mL) containing 3.42×10^{-3} mmol *Z*-L-phenylalanine *p*-nitrophenyl ester as a substrate [functional host molecule/substrate = 1:0.36 (molar ratio)]. The hydrolysis activity of the polymers was determined by measuring the hydrolytic product (*p*-nitrophenol) produced in the reaction mixture. The produced *p*-nitrophenol was detected at 400 nm with UV-VIS spectrophotometer. The degree of self-hydrolysis of the substrate was also measured without the functional host molecule under the same conditions. The rate of hydrolysis was determined by measuring the concentration of *p*-nitrophenol released at definite time intervals. Rates were determined by calculating the slope of $\ln(A_\alpha - A_t)$ (where A_α is the measured absorbance at infinity and A_t is the absorbance at time t) *vs*. time, after correcting for background hydrolysis without polymer and dividing by the molar concentration of imidazole present.

RESULTS AND DISCUSSION

Spectral analysis of transition state analogue imprinted and non-imprinted polymers: FTIR spectroscopy and solid state ^{13}C NMR spectroscopy were used to characterize the polymer structure. From the FTIR spectrum of molecular imprinted polymer and non-imprinted polymers, the peak at 1712 cm^{-1} represents the C=O double-bond stretching vibration in the carboxylic acid groups of poly *N*-methacryloyl L-histidine. The peak at 1549 cm^{-1} indicates the N-H deformation vibration in the amide groups and a peak at 3417 cm^{-1} corresponds to NH streching frequency of amide. The small peaks at 1387 and 1367 cm^{-1} indicated the stretching vibration of methyl group protons.

The ^{13}C NMR spectrum (Fig. 2) give the information about the chemical structure of polymer backbone. The peak at 178.7 ppm is due to the ester carbonyl carbon of EGDMA. The small peak at 113.1 ppm corresponds to the -CH carbon of imidazole ring. The peaks at 63.9 ppm and 26.9 ppm are due to the -CH_2 carbon atom of ethylene-glycol dimethacrylate copolymer and peak at 20.3 ppm was appeared by the -CH_3 group.

Morphological studies: The morphology of the transition state analogue imprinted and non-imprinted polymers prepared in two solvents was assessed by SEM analysis (Fig. 3). The images of SEM analysis reveals a smooth surface for molecular imprinted polymer and non-imprinted polymers of polymer prepared in chloroform (P-2) comparable to that prepared in DMSO (P-1). This nonporous morphology is very different from the porous morphologies that have been typically observed in molecular imprinted polymers that were prepared in the solvent DMSO. The roughness of the surface should be considered as a factor providing an increase in the surface area. It is a strong evidence for the importance of the use of a thermodynamically stable solvent during the preparation of an efficient imprinted polymer catalyst.

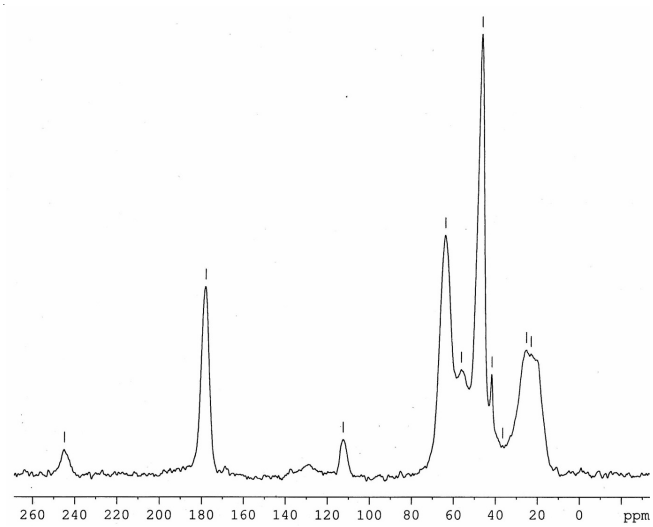

Fig. 2. ^{13}C NMR spectrum of transition state analogue imprinted polymer

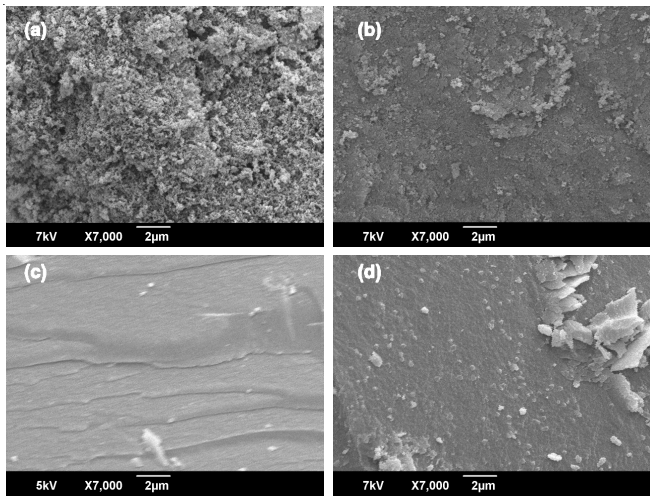

Fig. 3. SEM analysis of (a) transition state analogue imprinted and (b) non-imprinted polymers in DMSO (P1) and (c) transition state analogue imprinted and (d) non-imprinted polymers prepared in chloroform (P2)

Swelling studies: Swelling studies of the imprinted polymer and non-imprinted polymer is an important tool to differentiate the morphological difference between the polymers. Through the molecular imprinting procedure macroporous polymers obtained are permanently porous even in the dry state and solvent can be used to access the pore network. By measuring the swelling ratio of the polymers in different solvents an estimate can be made of the porous structure. The swelling ratios of imprinted polymer and non-imprinted polymer of P-1 and P-2 are tabulated in Table-1. Porogenic solvents play an important role in the formation of the porous structure of molecular imprinted polymer, which known as macroporous polymers. Swelling ratio of the imprinted polymer was found to be higher than that of the non-imprinted polymer because of the porous structure of the molecular imprinted polymer. The studies showed that as the polarity of solvent increases the swelling of both imprinted polymer and non-imprinted polymer increased. Different swelling properties of different solvents may play a role in determining shape and distance parameters that are locked into the imprinted polymer. The swelling ratio of polymer P-1 prepared in DMSO and P-2 prepared in chloroform show considerable difference. The highest swelling ratio exhibited by polymer P-1 is indicative of highly porous structure of polymer formed using thermodynamically more stable solvent DMSO than chloroform.

TABLE-1
SWELLING RATIO OF POLYMERS P-1AND P-2

		Swelling ratio				
Polymer		Chloroform	Acetonitrile	Methanol	DMSO	Water
P-1	MIP	8.29	3.84	5.99	16.30	14.59
	NIP	2.20	1.91	5.90	15.59	13.99
P-2	MIP	0.96	1.87	2.56	9.40	9.76
	NIP	0.52	0.55	1.43	8.34	7.38

Catalytic activities of imprinted polymer and non-imprinted polymer: The catalytic hydrolysis of molecular imprinted polymer is higher than non-imprinted polymer and there is a striking difference in the hydrolysis of esters by imprinted polymer depending upon the porogen used for the polymerization (Fig. 4). The data indicate that the catalytic rate for hydrolysis of *Z*-L-Phe-PNP by transition state analogue imprinted polymer prepared in DMSO (P-1) is very high as compared to the corresponding polymer prepared in chloroform (P-2). The transition state analogue imprinted polymer P-1 exhibited catalytic activity -55 % of the hydrolysis of *Z*-L-phenylalanine *p*-nitrophenyl ester while transition state analogue imprinted polymer P-2 showed less than 10 % hydrolysis of the ester. It is known that the nature and level of porogenic solvents determines the strength of non-covalent interactions and influences polymer morphology which, obviously, directly affects the performance of molecular imprinted polymer. The rate of hydrolysis catalyzed by transition state analogue imprinted polymer is higher than that of the non-imprinted polymer and it represented the imprinting effect.

Substrate selectivity of transition state analogue imprinted polymers: In order to study the substrate selectivity of transition state analogue imprinted polymers catalytic hydrolysis of structurally related esters *t*-Boc-L-Phe-PNP and *N*-Phth-L-Phe-PNP were also carried out. The polymer imprinted with transition state analogue exhibited the highest activity with the rate constant ratio of $k_{MIP}/k_{uncat} = 2.33$ for the

TABLE-2
KINETIC PARAMETERS FOR THE HYDROLYSIS OF VARIOUS *N*-PROTECTED L-Phe-PNP ESTER BY MIP AND NIP OF POLYMERS P1 AND P2

Polymer	Substrate	10^3k_{uncat}	10^3k_{NIP}	10^3k_{MIP}	k_{MIP}/k_{uncat}	10^2k_{cat}
P1	*Z*-L-Phe-PNP	0.56	4.97	7.76	13.86	81.77
	t-Boc-L-Phe-PNP	0.59	2.24	3.47	5.88	36.56
	N-Phth-L-Phe-PNP	0.41	0.99	1.23	3.15	12.96
P2	*Z*-L-Phe-PNP	0.45	0.62	1.05	2.33	11.04
	t-Boc-L-Phe-PNP	0.41	0.515	0.664	1.62	6.99
	N-Phth-L-Phe-PNP	0.34	0.456	0.476	1.4	5.02

TABLE-3
KINETIC PARAMETERS FOR THE HYDROLYSIS OF Z-(L)/(D)-Phe-PNP BY EGDMA CROSSLINKED MIP AND NIP OF POLYMERS P-1 AND P-2

Polymer	Substrate	10^3k_{uncat}	10^3k_{NIP}	10^3k_{MIP}	k_{MIP}/k_{uncat}	$10^2k_{MIP}/[Im]$	k_L/k_D
P-1	*Z*-L-Phe-PNP	0.56	4.97	7.76	13.86	81.77	4.51
	Z-D-Phe-PNP	0.34	1.15	1.72	5.06	18.12	
P-2	*Z*-L-Phe-PNP	0.45	0.62	1.05	2.33	11.04	1.81
	Z-D-Phe-PNP	0.44	0.46	0.58	1.32	6.11	

Z-L-Phe-PNP hydrolysis, but it indicated the lower activity for the hydrolysis of other amino acid *p*-nitrophenyl esters possessing small sized t-Boc-L-Phe-PNP and more sterically hindered N-Phth-L-Phe-PNP (Table-2). Hence, the imprinted polymer catalyst was capable of exhibiting efficient selectivity in its esterolytic catalysis. The polymer catalyst recognized the structure of imprinted molecule and selectively catalyzed the substrate that has a similar structure of the imprinted transition state analogue molecule than the non-imprinted molecule. The transition state analogue imprinted polymer prepared in DMSO (P-1) retains higher substrate selectivity than that prepared in chloroform (P-2).

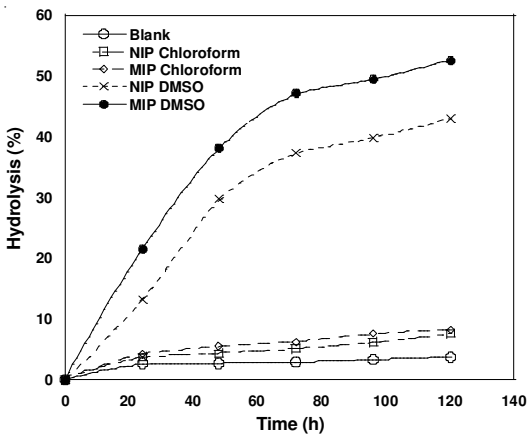

Fig. 4. Comparison of catalytic activity with time by TSA imprinted and non-imprinted polymers prepared in chloroform (P2), DMSO (P1) and uncatalyzed reaction

Enantioselectivity of transition state analogue imprinted polymer: The enantioselectivity studies of the transition state analogue imprinted polymers prepared in two solvent systems were carried out using *p*-nitrophenyl *Z*-L-phenylalanine and *p*-nitrophenyl *Z*-D-phenylalanine. The results of the enantioselectivity studies of the transition state analogue imprinted polymer catalysts for the hydrolysis of *p*-nitrophenyl *Z*-L-phenylalanine and *p*-nitrophenyl *Z*-D-phenylalanine are given in Table-3. The imprinted polymer catalyzed the hydrolysis of *p*-nitrophenyl *Z*-L-phenylalanine more than that of *p*-nitrophenyl *Z*-D-phenylalanine. This result indicates that the transition state analogue imprinted polymer exhibits efficient enantioselectivity. The ratio k_L/k_D for imprinted polymer made with DMSO as porogen (P-1) is 4.51 and for the polymer synthesized in chloroform as porogen (P-2) is only 1.81. This explains the role of porogen in creating the macroporous structure in the polymer matrix and thus enhancing catalytic activity and enantioselectivity.

Optimization of conditions of catalytic hydrolysis: For optimizing the conditions of catalytic hydrolysis the effect of parameters like substrate concentration, solvent and pH on catalytic hydrolysis were carried out.

a) Effect of substrate concentration: The catalytic hydrolysis of the transition state analogue imprinted and non-imprinted polymers were carried out using different molar concentrations of *Z*-L-phenylalanine *p*-nitrophenyl ester. The studies with different concentrations by polymers P-1 and P-2 showed highest rate of hydrolysis for 1.14 mM concentration of ester (Fig. 5). The rate of hydrolysis was found to be increased with increase in concentration upto 1.14 mM and beyond this concentration catalytic activity decreased due to substrate inhibition.

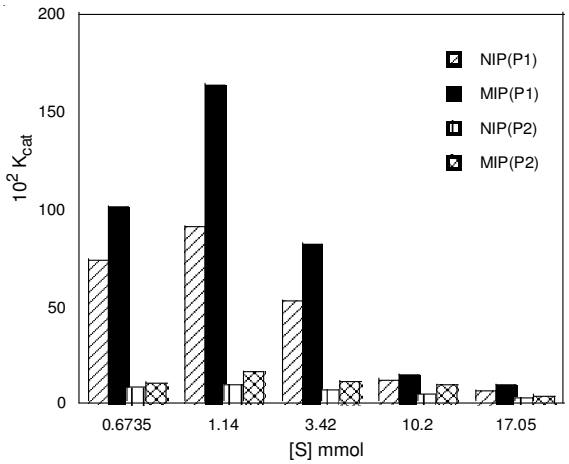

Fig. 5. Effect of substrate concentration on the hydrolysis of *Z*-(L)/(D)-Phe-PNP by EGDMA-crosslinked molecular imprinted polymer and non-imprinted polymer of polymers P-1 and P-2

TABLE-4
CATALYTIC ACTIVITY OF MIP AND NIP PREPARED OF POLYMERS P-1 AND P-2
WITH DIFFERENT RATIOS OF SOLVENT-BUFFER SYSTEM

Polymer	Acetonitrile-*Tris* HCl ratio	10^3k_{uncat}	10^3k_{NIP}	10^3k_{MIP}	k_{MIP}/k_{uncat}	10^2k_{MIP}/[Im]
P-1	1:9	0.56	4.97	7.76	13.86	81.77
	3:7	0.84	1.54	1.67	1.99	17.56
	5:5	0.96	1.15	1.19	1.24	12.54
P-2	1:9	0.45	0.62	1.05	2.33	11.04
	3:7	0.48	0.72	1.37	2.85	14.44
	5:5	0.47	1.26	1.52	3.23	16.02

b) Effect of pH on the catalytic hydrolysis: The pH effect on the activities of imprinted and non-imprinted polymers P-1 and P-2 for ester hydrolysis was studied in the pH range 6.0 and 7.25 in acetonitrile and *tris* HCl buffer and the results are presented in Fig. 6. The catalytic activity of imprinted polymer prepared in DMSO (P-1) is higher than those prepared in chloroform (P-2). The pH profile follows the same manner in the polymers P-1 and P-2 that it exhibits an optimum at pH 7.25. The shrinkage or expansion of the polymer matrix took place on varying the pH of the reaction medium due to changes in the number of protonated imidazole residues and it will also resulted in decreased catalytic activity.

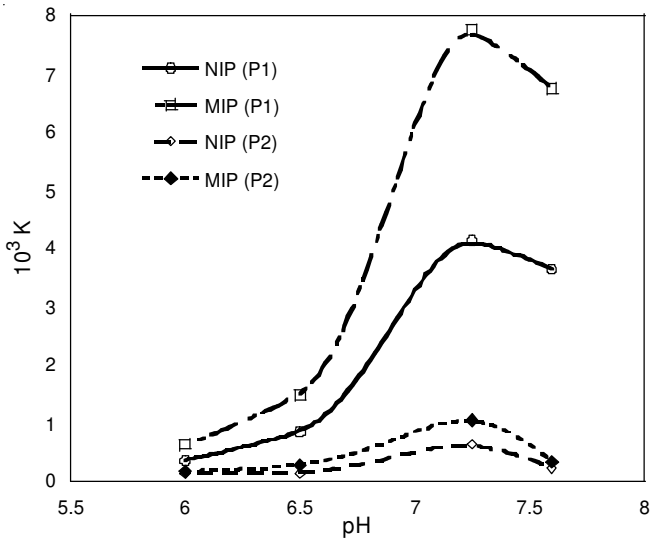

Fig. 6. Effect of pH on catalytic hydrolysis by molecular imprinted polymer and non-imprinted polymer of polymers P-1 and P-2

c) Rate of hydrolysis and solvent effect: The nature of the solvent used during the reaction has a great effect on the catalytic activity of the imprinted polymer. For studying the effect of solvent on catalytic hydrolysis the reaction was carried out with different ratios of acetonitrile-*tris* HCl buffer at pH 7.25 at room temperature. In contrast to the polymer prepared in DMSO (P-1), the polymer prepared in chloroform (P-2) showed higher catalytic activity on increasing the ratio of acetonitrile in the acetonitrile-*tris* HCl buffer (Table-4). The origin of specificity in the imprinted polymer is postulated to arise from the positioning of the complimentary functional groups which are covalently locked into place during polymerization. The polymer prepared in chloroform as porogen resulted in less porous structure. When the ratio of acetonitrile increased swelling of the imprinted polymer increased and then only the polymer can retain their cavity and increased catalytic activity. The polymer prepared in chloroform resulted in more rigid polymer matrix with low swelling for rebinding the template.

Conclusion

The molecular-imprinting technique provide new catalytic systems with tremendous performances for reactions for which enzymes cannot be efficient at all and under the reaction conditions where enzyme systems cannot be applied. We have successfully demonstrated that a transition state analogue imprinted polymeric catalysts can be prepared in a bulk polymerization protocol utilizing *N*-methacryloyl L-histidine as functional monomer, DMSO and chloroform as porogen. The morphological studies of the imprinted polymer and non-imprinted polymer synthesized in DMSO exhibited highly porous morphology while those prepared in chloroform exhibited nonporous structure. Results of catalytic studies of polymer prepared with DMSO displayed the highest catalytic activity, substrate selectivity and enantioselectivity. The drastic behaviour of the imprinted polymers could be attributed to the different kinetics of polymerization in the two systems. The high catalytic activity, efficiency and selectivity together with the strong chemical, mechanical and thermal stability give the catalysts a real advantage compared to catalytic antibodies and also provide an alternative compared to natural enzymes.

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