Abstract Focusing on bio-recognition, highly crosslinked TSA–built-polymer catalysts were synthesized as chymotrypsin mimics by molecular imprinting technique for substrate specific amidolysis of p-nitroanilide of amino acids based on the principle of cooperative catalysis. The polymers having enzyme-like behaviour were prepared using methacryloyl-L-histidine, methacryloyl-L-aspartic acid and 4-vinylpyridine as the functional monomers, phenyl-1- (N-benzyloxy carbonylamino) -2-(phenyl) ethyl phosphonate, the analogue of transition state of amide hydrolysis as the template and methacrylic crosslinker EGDMA as the crosslinking agent. The easy insertion of the substrate followed by selective stabilization of the T.S of amidolysis in the “3-D memorized cavity” in the polymer matrix is responsible for the enhanced substrate specificity of the imprinted chymotrypsin mimics. Cost effective synthesis, ease of preparation, high thermal stability, reusability and higher shelf life make the enzyme mimics highly efficient.

Keywords: Chymotrypsin mimics, bio-recognition, transition state analogue, 3-D memorized cavity and amidase activity.

Introduction

Design of molecularly imprinted polymers (MIPs) have been investigated as an extensively pertinent and convenient method, which fabricates three-dimensional networks with a “memorized cavity” of the shape and functional group positions of the template molecule (Mosbach 1994, Haupt 2003, Toorisaka et al. 2003, Sagawa et al. 2004). The immense catalytic activity and specificity of the natural enzymes are the consequences of a complex three-dimensional array of functional groups responsible for catalysis (Sellergren et al. 2000). Nevertheless, the practical relevance of these biocatalysts faces many difficulties due to their instability against high temperature, organic solvents and drastic pH conditions. As an alternative, the principles of enzyme catalysis have been used to design artificial enzyme analogues. The technique of molecular imprinting can be applied for much more stable polymeric mimic of
biological enzymes (Leonhardt and Mosbach 1987, Robinson and Mosbach 1989, Wulff 2002). To synthesize enzyme like polymers by molecular imprinting a stable transition state analogue (TSA) of a reaction must be selected as the template (Sellergren and Shea 1994). The imprint of TSA acts as an active center and exhibits catalyzing effect by reducing the activation energy of the specific reaction (Ohkubo et al. 1996, Ohkubo et al. 2001, Mathew et al. 2016). Natural chymotrypsin is specific to amides or peptides of phenylalanine, tyrosine or tryptophan – having aromatic hydrophobic side chains which could be accommodated in its hydrophobic pocket. Enzymes selectively recognize proper substrates over other molecules. Specificity is controlled by structure - the unique fit of substrate with enzyme controls the selectivity for substrate and the product yield. Since the active site of chymotrypsin protease comprises of hydroxyl group from serine, carboxyl group from aspartic acid and imidazole group from histidine, imidazole-containing monomers were usually used as a key catalytic functionality for preparing MIPs to investigate hydrolytic activity (Ohkubo et al. 1995, Sellergren et al. 2000). There is lot of reports on metal ion (Ohkubo et al. 2001) mediated molecularly imprinted polymer (MIP) catalysts for esterolysis, while MIP catalyzed amidolysis are less reported. We have reported the amidolysis of Z-L-Phe-PNA using chymotrypsin mimics of amino acid monomers methacryloyl-L-histidine/aspartic acid/serine. In the present work, in the framework of Michaelis-Menten kinetics, the specificity of chymotrypsin mimics on Z-L-Phe/Tyr/Trp/Ala-PNA was investigated.

**Methods**

Dicyclohexylcarbodiimide (DCC) and ethylene glycol dimethacrylate (EGDMA) were purchased from Sigma Aldrich, USA. Z-L-phenylalanine, Z-L-tyrosine, Z-L-tryptophan, Z-L-alanine, α-chymotrypsin, L-histidine, L-serine, L-aspartic acid, benzyl carbamate, triphenyl phosphate and phenylacetaldehyde were purchased from SRL, Mumbai. Other chemicals available from local suppliers were purified prior to use by following the standard procedures.

IR spectra were recorded on a Shimadzu FT-IR-8400S spectrophotometer. Kinetic studies were performed using Shimadzu UV 2450 spectrophotometer. JEOL JSM6390 SEM analyzer was used for SEM analysis. $^1$H NMR spectra were taken using Bruker Advance DPX-300 MHz FT-NMR spectrometer in CDCl$_3$.

**Synthesis of the substrate Z-L-phenylalanine $p$-nitroanilide (Z-L-Phe-PNA)**

The substrate Z-L-Phe-PNA was synthesized by dissolving 2.99 g (0.01 mol) Z-L-phenylalanine and 1.38 g (0.01 mol) $p$-nitroaniline in 30 mL ethyl acetate and the solution was stirred on a magnetic stirrer in an ice-water bath for half an hour. A solution of 2.06 g (0.01 mol) DCC in 30 mL ethyl acetate was added drop wise and the reaction mixture was stirred for 30 min in the ice water
bath and the stirring was continued for further 3 h at room temperature. The DCU formed was filtered off and the filtrate was evaporated in vacuum. The residue obtained was recrystallized from hot ethanol containing 1% acetic acid. The substrates, Z-L-tyrosine/tryptophan/alanine p-nitroanilide were also prepared following the same procedure.

**Catalytic amidolysis- General procedure**

A suspension of 10 mg chymotrypsin mimic CM (0.00542 mmol) was suspended in 5 mL acetonitrile-Tris HCl buffer (1:9 by volume, pH 7.75) in a reagent bottle and 227 mg, 0.542 mmol of the substrate Z-Phe-PNA in 50 mL acetonitrile was added. The reaction mixture was placed in a water bath shaker at 45 °C and shaken gently. Amidolysis of Z-Phe-PNA was followed by monitoring the absorbance of released p-nitroaniline spectrophotometrically at 374 nm in the framework of Michaelis–Menten kinetics and the reaction was monitored for two days. A blank reaction was also carried out in the absence of the enzyme mimic. From the absorbance data, the rate constants and percentage amidolysis were evaluated. Amidase activity of the mimic towards Tyr/Trp/Ala-PNAs was also evaluated in a similar manner.

**Results and Discussion**

![Scheme 1: Synthesis of TSA.](image)

**Synthesis of transition state analogue (TSA)**

The TSA, which has more structural resemblance with the substrate, was synthesized using triphenyl phosphite, benzyl carbamate and phenylacetaldehyde. The TSA synthesized possesses Z group of the substrate and the “specificity determinant”- C₆H₅CH₂ group - of chymotrypsin (scheme 1).

The TSA synthesized was characterized by FTIR and NMR spectroscopic techniques. FTIR of the TSA exhibited bands at 1301, 946 and 1252 cm⁻¹ corresponding to P=O stretching, P-OH stretching and P-O-benzyl stretching respectively. The ¹H NMR spectra showed singlets at δ 1.73 and 5.21 corresponding to OH group and CH₂ of Z group respectively. The methylene protons (CH-CH₂) appeared at δ 2.81 as doublet and the alkyl CH resonated at δ 4.09 as multiplet. The NH proton appeared as doublet at 6.01 ppm. The 15 aromatic protons showed a multiplet at 6.5-7.5 ppm.

**Synthesis of TSA imprinted and non-imprinted polymers**

The molecularly imprinted polymers were prepared by radical initiated bulk polymerization method using the functional monomers, template TSA and the cross linker EGDMA. Trifunctional
chymotrypsin mimic CM was obtained from the three monomers, the crosslinker, CoCl$_2$·6H$_2$O and TSA in the molar ratio 0.33:0.33:0.33:0.33:9:0.5 (scheme 2). The non-imprinted control polymer (CP) was also synthesised in the same molar ratios of monomers and the cross linker without the TSA molecule. The total functional groups in the mimics-imidazole, carboxyl and pyridine-were estimated by cleaving the amino acids from the methacrylic backbone by refluxing with HCl followed by treating with ninhydrin reagent (Mathew et al. 2016).

Proficient pre-organization is essential for successful imprinting; from the rac-TSA synthesized, the methacryloyl-L-amino acid monomers selectively forms TSA-Monomer pre-polymerization complex with L-TSA over D-TSA (Ohkubo et al. 2001, Ohkubo et al. 1996). After copolymerization in the presence of large excess of DVB crosslinker, the crosslinked polymer underwent thorough extraction to leach out the TSA molecules leaving behind the “3D memory pocket” of L-configuration lined with catalytic entities as depicted in Scheme 2. The specificity determinant group of TSA, C$_6$H$_5$CH$_2$ group designs the hydrophobic pocket in the polymer matrix (Ohkubo et al. 2001, Ohkubo et al. 1995).

Amidolysis is believed to proceed through selective binding of the substrate and a higher energy tetrahedral oxyanion intermediate and the “shape-selective-stabilization” of the unstable intermediate in the memorized cavity complementary to the L-TSA leads to the rate enhancement of the proteolytic enzyme mimics.

Evaluation of catalytic amidolysis

Amidolysis of Z-L-Phe-PNA using the trifunctional chymotrypsin mimic was found to follow pseudo first order kinetics and observed to be stereoselective, substrate specific and selective to the structure of the substrate. The amidolysis of Z-L-Phe/Tyr/Trp/Ala-PNA (Fig. a) was carried out in the framework of Michaelis-Menten kinetics and the substrate specificity of the mimics was compared. The amidolysis is found to obey pseudo first order kinetics as in the case of MIP catalyzed esterolysis and the rate constant for the amidolysis was evaluated as per the equation:

$$kt = \ln\left(\frac{A_\alpha}{A_\alpha - A_t}\right)$$

where, $k$ is the pseudo first order rate constant, $A_\alpha$ is the absorbance corresponding to the released $p$-nitroaniline at infinite time, $A_t$ is the absorbance at time $t$.

The observed value of rate constant for the uncatalyzed amidolysis of Phe-PNA is found to be 0.52 x $10^{-4}$ min$^{-1}$ in ACN-Tris HCl buffer of pH 7.75 at 45°C. The value of $k_{acc}$ observed for CM and CP are 75.38 and 16.15 respectively. It is mainly due to the co-operative action of imidazole, carboxylic and pyridine moieties in the polymer matrix. The imprinting efficiency $k_{im}$ is 4.22. In the imprinted polymer, the imprint of the “phosphonate TSA” fabricates a site complementary to the tetrahedral transition state
of amidolysis. The effective hydrogen bonding between the transition state, imidazole units, carboxylic functions and pyridine moieties makes the imprinted polymer catalytically more efficient than the non-imprinted polymer in which the functional monomers are randomly distributed due to the lack of the TSA.

The rate acceleration, imprinting efficiency and catalytic efficiency were evaluated in the catalytic amidolysis of Tyr-PNA, Trp-PNA and Ala-PNA also and the results are listed in table 1.

Table 1: Kinetic parameters from catalytic amidolysis.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$k_{\text{acc}}$</th>
<th>$k_{\text{im}}$</th>
<th>$k_{\text{app cat}}$</th>
<th>$S_i$</th>
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</thead>
<tbody>
<tr>
<td>Z-L-Phe-PNA</td>
<td>75.38</td>
<td>4.22</td>
<td>62.70</td>
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<tr>
<td>Z-L-Tyr-PNA</td>
<td>69.43</td>
<td>3.98</td>
<td>57.52</td>
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<tr>
<td>Z-L-Trp-PNA</td>
<td>34.76</td>
<td>1.34</td>
<td>28.89</td>
<td>48</td>
</tr>
<tr>
<td>Z-L-Ala-PNA</td>
<td>24.52</td>
<td>1.21</td>
<td>20.42</td>
<td>-</td>
</tr>
</tbody>
</table>

The natural chymotrypsin exhibits specificity towards anilides of phenylalanine, tyrosine or tryptophan – having aromatic hydrophobic side chains which could be accommodated in its hydrophobic pocket. The enzyme mimic CM exhibits greater specificity towards Phe-PNA, which is attributed to the more structural similarity between the print molecule and the substrate. The ratios $k_{\text{Phe}}/k_{\text{Tyr}}$ (1.09), $k_{\text{Phe}}/k_{\text{Trp}}$ (2.17) and $k_{\text{Phe}}/k_{\text{Ala}}$ (3.07) observed for CM clearly indicates the specificity of CM as a consequence of TSA imprinting. Moreover, the corresponding values noticed for CP are 1.03, 1.02 and 1.04 respectively.

**Michaelis-Menten kinetics**

The Michaelis-Menten kinetics of CM was investigated by following the kinetics of the amidolysis of Z-L-Phe/Tyr/Trp/Ala-PNA. The plot of percentage amidolysis vs. time for chymotrypsin mimic CM supports its substrate
specificity towards Phe-PNA and Tyr-PNA (fig. b). The reaction obeying Michaelis–Menten kinetics exhibits initial burst kinetics and reaches saturation (Ohkubo et al. 1995). The mimic CM follows Michaelis–Menten kinetics under excess substrate condition (1:100) in the amidolysis of Phe-PNA and Tyr-PNA, which is accountable for the structural complementarity of the substrates to the reaction cavity and depicts typical plot of catalytic turnover vs. time for an enzyme-
catalyzed reaction under the excess substrate condition. The cooperative action amongst pyridine, carboxyl and imidazole groups is responsible for the enzyme catalysis shown by CM similar to chymotrypsin in which the catalytic triad comprises of imidazole, carboxyl and hydroxyl moieties. Unlike natural chymotrypsin that shows specificity towards Trp, the mimic CM exhibits lower affinity towards Trp-PNA due to incompatibility of indole ring in Trp with the hydrophobic pocket furnished by the catalytic centers. Even though there is no initial burst in the case of Trp-PNA, saturation in catalytic activity is obtained at 24 h. The enzyme mimic CM behaves like a nucleophilic catalyst towards Ala-PNA.

The reaction was carried out with different substrate concentrations. Fig. c. shows Michaelis–Menten plots of initial velocity ($V_0$) vs. substrate concentration (S) for the enzyme mimic CM with different catalyst to substrate ratios in the range 1:25–1:200.

The hyperbolic curves obtained for the initial velocity ($V_0$) vs. substrate concentration (S) data shows good adherence to the Michaelis-Menten saturation model for the mimic CM. Such hyperbolic curves are obtained for Phe/Tyr-PNA only. The mimic CM behaves like a nucleophilic catalyst towards Ala-PNA. The polymer catalysts obeying Michaelis-Menten kinetics are described as chymotrypsin mimics and the kinetic parameters $1/K_m$ and $V_{max}/K_m$ are cited to characterize their amidase activity. The double reciprocal (Lineweaver-Burk) plots of $1/V_0$ vs. $1/S$ for Phe/Tyr/Trp/Ala-PNA are shown in fig. d. The imprinted mimic CM showed remarkable difference in substrate affinity ($1/K_m$) towards the substrates which is mainly due to the well-defined 3-D memorized catalytic cavity in the polymer matrix.

The $K_m$ values noticed for CM are 0.273 mM (Phe-PNA), 0.346 mM (Tyr-PNA), 2.692 mM (Trp-PNA) and 4.416 mM (Ala-PNA). The mimic
CM exhibited higher affinity towards Phe-PNA and Tyr-PNA because of the structural resemblance to the benzyloxy carbonyl protecting group and the aromatic hydrophobic side chain. Even though natural chymotrypsin is specific to Trp, the mimic CM exhibited comparatively lower affinity towards Trp-PNA because the indole side chain may not fit properly in the hydrophobic pocket fabricated by the “TSA and catalytic functionalities”.

![Double reciprocal plot for CM catalyzed amidolysis.](image)

**Fig. d. Double reciprocal plot for CM catalyzed amidolysis.**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m$</th>
<th>$1/K_m$</th>
<th>$V_{max}/K_m$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe-PNA</td>
<td>0.273</td>
<td>3.663</td>
<td>0.027</td>
<td>4.909</td>
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<tr>
<td>Tyr-PNA</td>
<td>0.346</td>
<td>2.890</td>
<td>0.022</td>
<td>4.038</td>
</tr>
<tr>
<td>Trp-PNA</td>
<td>2.692</td>
<td>0.371</td>
<td>0.002</td>
<td>0.363</td>
</tr>
<tr>
<td>Ala-PNA</td>
<td>4.416</td>
<td>0.226</td>
<td>0.001</td>
<td>0.170</td>
</tr>
</tbody>
</table>

Like natural chymotrypsin, the enzyme mimic CM did not show any specificity towards amino acids with electron releasing side chains. The control polymer CP did not show any substrate specificity and the observed $K_m$ values are 4.79 (Phe-PNA), 4.84 (Tyr-PNA), 4.90 (Trp-PNA) and 4.97 (Ala-PNA), which gives a direct indication of TSA imprinting effect. The lack of the 3D-substrate recognition site designed during TSA imprinting is accountable for the nearly same affinity exhibited by the control polymer CP. Further, most probably the catalytic functionalities are randomly distributed in the polymer matrix making the H-bonding ineffective.

The enzyme mimic CM exhibited 1.27 ($K_{Phe}^{Tyr}$/$K_{Tyr}^{Phe}$), 9.87 ($K_{Tyr}^{Trp}$/$K_{Trp}^{Tyr}$) and 16.21 ($K_{Trp}^{Ala}$/$K_{Ala}^{Trp}$) times substrate affinity ratio towards Phe-PNA than Tyr-PNA, Trp-PNA and Ala-PNA respectively. The best substrates for CM are observed to be Z-L-Phe-PNA and Z-L-Tyr-PNA. The reactivity of enzyme mimic is compared with that of natural chymotrypsin. The kinetics of amidolysis of Z-L-Phe-PNA catalyzed by mimic CM was compared with the amidolysis catalyzed by natural chymotrypsin. The mimic CM shows 62% completion of the reaction with a saturation time of 22 h. With natural chymotrypsin, the reaction reached 80% completion in 45 minutes. The $K_m$ value of chymotrypsin catalyzed amidolysis at room temperature was evaluated from the double reciprocal plot by taking various substrate concentrations in the range of 5-30 μm. The $K_m$ value is found to be 0.1 μm which is 2.7 x 10^3 times the value of the mimic CM at 45 °C. Michaelis-Menten behavior of CM is not significant in the initial period compared to the
natural enzyme due to the heterogenic nature of the reaction and the time required for the solvation of the polymer catalyst.

Conclusion

High thermal stability (up to 140 °C), higher shelf life (2 years), regeneration and reusability (even after seven cycles of experiments) make the enzyme mimic superior over natural chymotrypsin. Moreover, ease and cost effective method of synthesis make the molecular imprinting effectual. The specificity determinant C₆H₅CH₂ group of TSA is responsible for the hydrophobic pocket. The 3D memory cavity with hydrophobic pocket makes CM substrate specific. The cooperative effect through effective H-bonding provides easy insersion of the anilide substrate in the recognition site through shape selective binding.

Acknowledgement

The authors gratefully acknowledge the support from CSIR for JRF and SRF (Divya Mathew). We are also thankful to IIRB-Mahatma Gandhi University for providing facilities for spectral analysis and School of Biosciences for providing facilities for incubation studies.

References