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# Biomimetic recognition and peptidase activities of transition state analogue imprinted chymotrypsin mimics

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ABSTRACT

The first peptidolysis reaction utilizing transition state analogue imprinted polymer was demonstrated utterly in the viewpoint of size and shape-selective substrate recognition. The enzyme mimic polymer was synthesized from the amino acid triad histidine, aspartic acid and serine in presence of phenyl-1-(*N*-benzyloxycarbonylamino)-2-(phenyl)ethyl phosphonate transition state analogue (TSA) by molecular imprinting technique. The polymer catalyst synthesized exhibited high selectivity and performed as a reliable tool for peptidolytic reactions. The peptidase activity of the enzyme mimic polymer catalyst was investigated by following the hydrolysis of dipeptides spectrophotometrically at 207 nm and the kinetic parameters, rate acceleration $k_{acc}$and imprinting efficiency$k_{im}$, were evaluated. The imprinted peptidase displayed a rate acceleration of $1.67 \times 10^3$ contrasted with the uncatalyzed peptidolysis and an imprinting efficiency of 45 over the non-imprinted control polymer. The artificial peptidase amazingly promoted the hydrolysis of dipeptides having Phe/Tyr amino acid as the C-terminal residues discriminating chymotrypsin specific and non-specific substrates. The mimic exhibited higher rate acceleration and substrate specificity towards peptides compared to amino acid *p*-nitroanilides. Despite the fact that natural enzyme is much superior to the MIP catalyst in hydrolase activities, the mimic portrays high thermal stability, prolonged life span and unrivaled recyclability and reusability.

## 1. Introduction

Design of molecularly imprinted polymers (MIPs) has been investigated as an extensively pertinent and expedient method, which fabricates three-dimensional networks with a “memorized cavity” of the shape and functional group positions of the template molecule [1–4]. The substrate binding site in the macromolecular matrix is designed to stabilize the reaction transition state, thereby lowering the activation energy requirements resulting in higher reaction rate acceleration [5,6]. It was rational to combine the two essential features of enzyme action - the binding site and the catalytic functional groups of the enzyme, to produce a miniature organic enzyme model [7–9]. Transition state intermediate of the reaction is known to be recognized specifically by the transition state analogue imprinted cavity and the reaction is accelerated by catalytic moieties within the cavity. Such model enables us to modify the binding specificity as well as the catalytic properties of the polymer catalysts [10–14].

The enzyme catalyzed hydrolysis of polypeptides is of great biological importance. The peptide bond is comparatively inert towards hydrolytic cleavage. The proteolytic enzyme chymotrypsin exhibits specificity towards C-terminal of phenylalanine, tyrosine and tryptophan residues in a polypeptide chain. They accelerate the rate of peptide bond hydrolysis by $\sim 10^{10}$ compared to the uncatalyzed reaction. The practical applications of enzymes are so limited due to their instability to organic solvents, pH and temperature. As a substitute to natural enzymes, TSA imprinted polymer catalysts were reported in esterolytic reactions; however the hydrolysis of peptides utilizing TSA engraved peptidase MIPs is not reported yet. As a preliminary investigation we have carried out the hydrolysis of N-protected phenylalanine *p*-nitroanilides utilizing trifunctional amidase MIPs containing the catalytic triad of ‘imidazole, carboxylic and hydroxyl’ residues at the dynamic destinations engraved with phenyl-1-(*N*-benzyloxycarbonylamino)-2-(phenyl)ethyl phosphonate, a steady transition state analogue of hydrolytic reactions [15–18]. The cooperative effect exhibited by the trifunctional artificial amidase MIP in molecular recognition and shape-selective binding was clearly evidenced in the amidolysis of chymotrypsin specific amino acid *p*-nitroanilides with a rate acceleration of 75 over the uncatalyzed reaction and showed an imprinting efficiency of 4.25 over the non-imprinted control polymer.

In this paper, we have extended our investigations on the hydrolase

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activity of TSA imprinted chymotrypsin mimic targeting dipeptides, containing chymotrypsin specific amino acids, as the substrates. All the catalytic parameters were evaluated in the framework of Michaelis-Menten kinetics. Substrate selectivity, enantioselectivity and substrate specificity of the TSA imprinted polymer catalyst and non-imprinted control polymer in terms of rate acceleration, imprinting efficiency, saturation time and Michaelis-Menten constant are demonstrated in detail.

## 2. Experimental

### 2.1. Materials and methods

Ethyleneglycol dimethacrylate (EGDMA) and dicyclohexylcarbodiimide (DCC) were purchased from Sigma Aldrich, USA. α-Chymotrypsin, benzyl carbamate, triphenyl phosphite, phenylacetaldehyde, Z/Boc/Nphth/Fmoc-L-phenylalanine, Z-L-alanine, Z-L-leucine, Z-L-tyrosine, Z-L-tryptophan, glycine, L-histidine, L-serine and L-aspartic acid were purchased from SRL, Mumbai. Other chemicals available from local suppliers were purified prior to use by following the standard procedures.

FTIR 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 $CDCl_3$. BET analyzer used was Thermo Fisher Scientific Surface Analyzer V-230 50/60 Hz.

### 2.2. Synthesis of phenyl-1-(N-benzyloxycarbonylamino)-2-(phenyl)ethyl phosphonate TSA

The TSA was synthesized by refluxing triphenyl phosphite (4.10 mL, 13.20 mmol), benzyl carbamate (2 g, 13.2 mmol), phenylacetaldehyde (2.38 mL, 19.80 mmol) and glacial acetic acid (2 mL) for 4 h at 100 °C in an oil bath. The diphenyl phosphonate formed was hydrolyzed with NaOH (0.4 N), acidified with conc. HCl, filtered, and purified by column chromatography using 9:1 chloroform-methanol mixture [15–18]. FT-IR: 1244 $cm^{-1}$ (P=O stretching), 1029 $cm^{-1}$ (P-OH stretching) and 932 $cm^{-1}$(P—*O*—phenyl stretching).

### 2.3. Synthesis of artificial peptidase as chymotrypsin mimic

A mixture of methacryloyl L-histidine (MALH, 74 mg, 0.33 mmol), methacryloyl L-aspartic acid (MALA, 67 mg, 0.33 mmol) and methacryloyl L-serine (MALS, 58 mg, 0.33 mmol) and the phosphonate TSA (0.5 mmol, 205.5 mg) in DMSO (40 mL) was stirred under nitrogen atmosphere to form a pre-polymerization complex (PPC). The PPC was then subjected to radical polymerization using AIBN (50 mg) as the radical initiator and EGDMA (1.8 g, 9 mmol) as the crosslinker by stirring at 80 °C for 6 h. The solid polymer formed was washed with DMSO and acetone, and subjected to Soxhlet extraction with chloroform and finally dried in vacuum. The corresponding non-imprinted control polymer CP was also synthesized keeping the molar ratio of the functional monomers and crosslinker, but without the TSA molecule [18].

### 2.4. Solution phase synthesis of dipeptides: Synthesis of Z-L-Phe-Gly-OH (P1)

To a suspension of Z-L-phenylalanine (299 mg, 1 mmol) in $CHCl_3$ (10 mL) glycine (75 mg, 1 mmol) in $CHCl_3$ (10 mL) and DCC (206 mg, 1 mmol) were added. A saturated solution of $CsCO_3$ was added to this mixture till the pH was 7.75. The reaction mixture was stirred for 3 h, filtered off the precipitated DCU, washed with $CHCl_3$ (3 × 5 mL) and added the washings to the filtrate. The combined filtrate and washings were washed with 5% $NaHCO_3$ solution and then with a saturated solution of NaCl (4 × 5 mL). The organic layer was dried over anhydrous $Na_2SO_4$, filtered and evaporated under vacuum. The crude product was recrystallized from a mixture of $CHCl_3$ and ice cold petroleum ether [19].

By similar procedure dipeptides P2-P10 were synthesized.

### 2.5. Kinetic evaluation of peptidase activity of the polymer catalysts

To a suspension of the polymer catalyst (CM,10 mg, 0.00542 mmol) in acetonitrile-Tris HCl buffer (1:9 by volume, 5 mL, pH 7.50) Z-L-Phe-Gly-OH, (**P1**, 201 mg, 0.542 mmol) in acetonitrile (50 mL) was added. The reaction mixture was placed in a water bath shaker at 45 °C and shaken gently for a period of 120 h [18]. Aliqotes of the reaction mixture was withdrawn at definite intervals of time, filtered to remove the spent polymer catalyst and the clear solution was collected. To 5 mL of the filtrate ethanolic solution of ninhydrin reagent (5 mL) was added and heated solution to 90 °C in a water bath for 30 min and then cooled to room temperature. A purple color was developed in the solution and the absorbance was recorded on a UV–Vis spectrophotometer at 550 nm and the total glycine content was calculated using a relevant standard calibration plot as reference. The reaction was monitored for two days. The experiment was repeated with the control polymer under the same conditions. A blank hydrolysis was also carried out in the absence of the enzyme mimic.

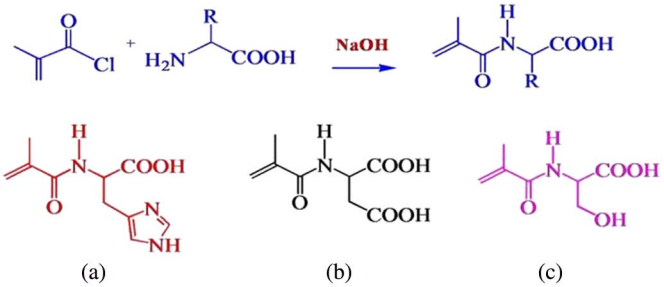
Hydrolytic activity of both the polymers towards the dipeptides Boc/Fmoc/Nphth-L-Phe-Gly-OH, Z-L-Tyr/Trp-Gly-OH and the free peptide Phe-Gly was also investigated in a similar manner.

## 3. Results and discussion

### 3.1. Choice of the substrates, templates and monomers

We have previously reported the preparation of phosphonate TSA imprinted artificial amidases and the dependence of amidase activity of the mimic on various physico-chemical factors in the hydrolysis of amino acid *p*-nitroanilide [15–17]. The investigations were extended to the catalytic application of the polymer catalysts in chymotrypsin specific dipeptides. The dipeptides **P1-P10** were synthesized by solution phase strategy by DCC coupling of $N^{\alpha}$ -protected amino acids (Table 3). The $N^{\alpha}$-protecting groups were removed by suitable methods to afford free dipeptides. The amino acids Phe, Tyr, Trp, Ala, Gly and Leu were used for the peptide synthesis. The methacryloyl-L-amino acids synthesized by Schotten-Baumann reaction were used as the functional monomers to mimic the catalytic triad of native chymotrypsin (Scheme 1).

The phosphonate TSA, phenyl-1-(*N*-benzyloxycarbonylamino)-2-(phenyl)ethyl phosphonate equipped for designing *N*-benzyloxycarbonyl (Z) protecting group of the substrate and the "specificity determinant" - $C_6H_5CH_2$ assemble- complementary to the hydrophobic pocket of chymotrypsin CT [19,20] in the polymer matrix was successfully synthesized, characterized and utilized as the print molecule in molecular imprinting (Scheme 2).




**Scheme 1.** Synthesis of amino acid monomers: (a) $N^{\alpha}$-methacryloyl-L-histidine, (b) $N^{\alpha}$-methacryloyl-L-aspartic acid & (c) $N^{\alpha}$-methacryloyl-L-serine.