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Amidase activity of phosphonate analogue imprinted chymotrypsin mimics in shape-selective, substrate-specific and enantioselective amidolysis of L-phenylalanine-*p*-nitroanilides

Divya Mathew [a], Benny Thomas [a,b], K.S. Devaky [a,*]

[a] School of Chemical Sciences, Mahatma Gandhi University, Kottayam 686 560, India
[b] Department of Chemistry, St. Berchmans College, Changanacherry, 686 101, India

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ABSTRACT

Focusing on chymotrypsin mimics, highly crosslinked enzyme mimics are synthesized by molecular imprinting technique for the amidolysis of *p*-nitroanilide of phenylalanine, using phenyl-1-(*N*-benzyloxycarbonylamino)-2-(phenyl)ethyl phosphonate – the transition state analog of amidolysis – as the template, *N*-methacryloyl-L-histidine, *N*-methacryloyl-L-aspartic acid, and *N*-methacryloyl-L-serine as the functional monomers and EGDMA as the crosslinking agent. The amidase activity of the enzyme mimics follows pseudo first order kinetics. The transition state analog provides a tetrahedral geometry complementary to the transition state intermediate, which is responsible for the catalytic activity of the imprinted enzyme mimics. The enzyme mimics show stereospecificity and substrate selectivity in the amidolysis of phenylalanine *p*-nitroanilide. The proper orientation of the reactive functionalities in the super crosslinked macroporous polymer matrix for selective binding of the substrate through H-bonding is responsible for the high imprinting efficiency and substrate specificity of the imprinted polymer catalysts. Low cost, ease of preparation, high thermal stability, reusability and higher shelf life make the polymer catalysts better chymotrypsin mimics.

1. Introduction

The fabrication of synthetic enzyme mimics by molecular imprinting technique – the creation of tailor made binding sites with memory of the shape, size and functional groups of the template molecule – has spanned the area of research to conquer the practical borders of biological enzymes like denaturation in organic solvents, instability against high temperatures, severe pH conditions, difficulties in isolation, loss of activity on recycling etc [1–3]. In the molecular imprinting process, the template is of vital significance, which directs the organization of the functional groups pendent to the functional monomers, the complete removal of the template molecule creates specific recognition sites complementary to the size, shape, and chemical functionalities of the template molecule [4–7]. Super-crosslinked nature of the three-dimensional recognition site is responsible for the binding specificity and lot of applications like chromatographic separation, sensors, drug delivery, recognition of peptides etc [8–10].

Most of the studies have focused on the enzyme chymotrypsin, which specifically hydrolyses esters or amides comprising of phenylalanyl or tyrosyl residues as a part of the carbonyl group [11,12]. The environment of the active site and mechanism of its hydrolytic action are well understood, that the nucleophilicity of serine hydroxyl group is enhanced by the co-operative action of the imidazole moiety of histidine and carboxylic group of aspartic acid, which are buried in a hydrophobic pocket [13,14]. Even though there are many reports on imprinted and non-imprinted polymer catalyzed esterolytic reactions, amide or peptide hydrolysis reactions using artificial enzyme mimics are less reported. In the present paper, we report the synthesis of a series of chymotrypsin mimics and studies on their catalytic activity in the amidolysis of *p*-nitroanilides of phenylalanine highlighting the co-operative effect of functional monomers in the imprinted polymer. The substrate specificity and stereo selectivity of the enzyme mimics, effect of the structure of the imprinted TSA on the catalytic activity etc. are illustrated.

* Corresponding author at: School of Chemical Sciences, Mahatma Gandhi University, Priyadarshini Hills P.O., Kottayam 686 560, Kerala, India. Fax: +91 481 2731002.
E-mail address: ksdevakyscs@yahoo.com (K.S. Devaky).

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2. Experimental

2.1. Materials and methods

Dicyclohexylcarbodiimide (DCC), ethylene glycol dimethacrylate (EGDMA) and phenethyl boronic acid were purchased from Sigma–Aldrich, USA. α-Chymotrypsin, Z/Boc/Nphth/Fmoc-L-phenylalanine, *Z*-L-alanine, L-histidine, L-serine, L-aspartic acid, benzyl carbamate, triphenyl phosphite 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 $CDCl_3$.

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

The transition state analog was synthesized by refluxing 4.10 mL, 13.20 mmol triphenyl phosphite, 2.0 g, 13.20 mmol benzyl carbamate, 2.38 mL, 19.80 mmol phenylacetaldehyde and 2.0 mL glacial acetic acid for 4 h at 100 °C in an oil bath. The diphenyl phosphonate formed was hydrolyzed with 0.40 N NaOH, acidified with conc. HCl, filtered, and purified by column chromatography using 9:1 chloroform–methanol mixture. FTIR:- 1301 cm^{-1} (P═O stretching), 946 cm^{-1} (P—OH stretching) and 1252 cm^{-1} (P—O—benzyl stretching).

The transition state analog phenyl-1-benzyloxycarbonylamino-4-methoxybenzyl phospohonate was also prepared by the same method using 4-methoxybenzaldehyde instead of phenylacetaldehyde for a comparison [15].

2.3. Synthesis of TSA imprinted enzyme mimics and non-imprinted control polymers

The enzyme mimic polymer **C1** was prepared by radical initiated bulk polymerization of 223 mg, 10 mmol, of monomer *N*-methacryloyl-L-histidine (MALH) and 3.4 mL, 90 mmol, of the crosslinking agent EGDMA in presence of 205.5 mg, 0.50 mmol TSA-A in 40 mL DMSO for 6 h at 80 °C. The template was completely leached out by washing with methanol and then subjected to Soxhlet extraction with chloroform. The polymer obtained was collected and dried over vacuum.

Enzyme mimics **C2–C7** were also synthesized as per the same procedure. The corresponding non-imprinted control polymers **CPs** were also prepared by the same procedure in the absence of TSA. The total amino content in the polymer mimics were estimated by ninhydrin reagent. The morphology of the polymers was characterized SEM analysis.

2.4. 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 mg (0.01 mol) *Z*-L-phenylalanine and 1.38 mg (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 mg (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, t-Boc/Nphth/Fmoc-L-phenylalanyl-*p*-nitroanilide and *Z*-L-alanyl-*p*-nitroanilide were also prepared following the same procedure.

2.5. Amidolysis of Z-L-*phenylalanine*-p-*nitroanilide* (Z-L-*Phe-PNA) using TSA imprinted and non-imprinted enzyme mimics: general procedure*

A suspension of 10 mg chymotrypsin mimic **C1** (0.00647 mmol) was suspended in 5 mL acetonitrile–Tris HCl buffer (1:9 by volume, pH 7.75) in a reagent bottle and 271 mg, 0.647 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 mimics **C2–C7** was evaluated in a similar manner.

2.6. Regeneration and reusability of the spent polymer

The spent enzyme mimics were collected by filtration, washed simultaneously with distilled water and Tris–HCl buffer (pH 7.75) and dried under vacuum. In the second cycle the amidolytic reaction was carried out in fresh Tris–HCl buffer solution. Catalytic amidolysis was repeated for 6 cycles.

3. Results and discussion

3.1. Synthesis of TSA- A: *phenyl-1-(*N-*benzyloxycarbonylamino)-2-(phenyl)ethyl phosphonate*

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" – $C_6H_5CH_2$ group – of chymotrypsin.

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 ^{1}H NMR spectra showed singlets at δ 1.73 and 5.21 corresponding to OH group and CH_2 of Z group respectively. The methylene protons (CH—CH_2) 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.

3.2. 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. The monofunctional mimics **(C1–C3)** were synthesized using *N*-methacryloyl-L-histidine (MALH)/*N*-methacryloyl-L-aspartic acid (MALA)/*N*-methacryloyl-L-serine (MALS) respectively with EGDMA and TSA-A in the molar ratio 1:9:0.5.

Similarly the bifunctional enzyme mimics **(C4–C6)** were prepared using functional monomers *N*-methacryloyl-L-histidine, *N*-methacryloyl-L-aspartic acid and *N*-methacryloyl-L-serine**—C4** with MALH and MALA, **C5** with MALH and MALS and **C6** with MALA and MALS. The monomers, EGDMA and TSA-A were in the molar ratio 0.5:0.5:90:0.5 (Table 1). Trifunctional mimic **C7** was obtained