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Research Article

Selective Separation of Kunitz Trypsin Inhibitors Using Molecularly Imprinted Polymers-Based Approach

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ABSTRACT

The technique of molecular imprinting is used to create molecularly imprinted polymers (MIPs). This process involves the co-polymerization of monomers and crosslinkers along with a specific targeted molecular template. MIP represents a promising method for the selective extraction of different micro and macromolecules from a mixture. In the present work, MIP was synthesized using 2-hydroxyethyl methacrylate (HEMA) functional monomer, p-aminobenzamidine (p-ABA) anchoring monomer, and N,N'-ethylenebis acrylamide (EbAM) as crosslinker, for the selective separation of Kunitz trypsin inhibitors (KTI). The scanning electron microscope (SEM) reveals the rough and cavities-based morphology. These cavities are responsible for the selective removal of KTI from the solution. Further, MIPs were examined by performing a selective binding assay to determine the binding capacity and selectivity. The high selectivity, stability, recovery, repeatability and reusability of MIPs have made them effective for real-time analysis and separation. By using MIPs to selectively remove KTI from different soy products, nutrient absorption can be improved, enhancing enzyme activity by lowering inhibitor concentrations.

Keywords: Molecularly imprinted polymers, Kunitz trypsin inhibitors, Functional monomer, Selective separation, Binding assay.

INTRODUCTION

Molecularly imprinted polymers (MIPs) have fascinated world researcher for the last few decades because of their potential application in various fields. MIP shows an analogy to the phrase 'lock and key', which depicts the preferential interaction between the enzyme receptor and ligand. Just like the receptor and ligand interaction mechanism, MIP selectively binds with a template of interest.[1,2] The construction of MIPs involves a wide range of templates, including small organic molecules, nucleic acids, peptides, proteins, enzymes, drugs, and other micro and macromolecules. MIPs play a very crucial role in micro-extraction, purification, analytical separation, drug delivery, artificial antibodies, catalysis, chemical sensing, enantiomeric recognition and degradation. It has numerous advantageous properties, including selectivity and specificity, high stability, aqueous solubility, automation compatibility, reusability, quick manufacture, low cost and capacity to reuse immobilized templates.[1-4]

MIPs are formed by co-polymerizing functional molecules, referred to as monomers and crosslinkers, in the presence of a specific template. The functional groups present in these molecules, including -OH, -NH_2, -COOH, -CHO, and -CO, etc., enable them to interact and bind effectively with the chosen template within the polymeric structure. The widely used functional monomers for MIP synthesis are methacrylic acid (MAA), acrylic acid (AA), 2-hydroxyethyl acrylate, 2- hydroxyethyl methacrylate (HEMA), glycidyl acrylate, poly (-ethylene glycol) methacrylate, styrenic (styrene), p-aminobenzamidine (p-ABA), N,N'-ethylenebis (acrylamide) (EbAM) and were reported earlier. In addition, many initiators such as tetramethylethylenediamine (TEMED), 2,2-dimethoxy-2- phenylacetophenone (DMPA), benzoyl peroxide (BPO), and 2,2' -azobis (2-methylpropionitrile) (AIBN), etc., were used in the presence of UV radiation for rapid precipitation of the polymer.[1-7] Besides functional molecules and initiators, template plays a crucial role in MIP syntheses. The desired template allows its imprinting in the form of cavities within polymeric structures. Such synthesized MIPs show a higher affinity of binding to selected templates. The potential of selectivity and specificity for binding unique targeted molecules makes this technique popular among researchers and similar product developers. Several studies have been demonstrated in which MIPs are used for selective peptide or protein recognition. Researchers have developed proteins-based MIPs liketrypsin[6], hemoglobin[7], bovine serum albumin (BSA)[8,9], bovine hemoglobin (BHb) [10], immunoglobulin G (IgG)[11] for their potential application in *in-vivo* and *in-vitro* atmosphere.

In the present work, we proposed a MIP synthesis using 2-hydroxyethyl methacrylate (HEMA), N, N'-ethylenebis (acrylamide) (EbAM) and p-aminobenzamidine (p-ABA) in the presence of tetramethylethylenediamine (TEMED) as initiator.[6-9,11-13]

This synthesized MIP is employed for the selective separation of Kunitz trypsin inhibitors (KTI) from the aqueous solution. KTI is one of the antinutritional factors present in nutritionally rich crop plants.[12,13] KTI has gained significant importance for its potential inhibiting activity against trypsin-like serine proteases. A selective binding assay was performed for MIPs to determine the binding efficacy and selectivity. This method is found simple and effective for the removal and real-time analysis of antinutritional factor KTI.[1-3,7-9,11-14]

MATERIAL AND METHODS

Materials

KTI from soybean, trypsin from bovine pancreas, ammonium persulfate (APS), N,N,N′,N′ -tetramethylethylenediamine (TEMED) and phosphate buffer saline (PBS) were purchased from Himedia, Thane. 2- hydroxyethyl methacrylate (HEMA), N,N'-ethylenebis(acrylamide) (EbAM) and p-aminobenzamidine (p-ABA) were procured from Tokyo Chemical Industry (TCI), India. Dimethyl sulfoxide (DMSO), purchased from Merck, Mumbai. Bradford reagent from SRL, India, tris-HCl, calcium chloride dihydrate ($CaCl_2.2H2O$), acetic acid, n-hexane, Milli-Q water.

Synthesis of Trypsin Inhibitor MIPs

The outline represented forthe synthesis of MIP is shown in Fig. 1. Typically, 12 mg KTI and a stoichiometric amount of p-ABA (1.5 mg) in 1-mL PBS pH 7.3 were incubated at 6°C for 45 minutes. Then, 57 µL (0.47 mmol) of HEMA, 120 mg (0.71 mmol) EbAM, 8.7 µL PBS, 5 µL of APS (5%) and TEMED were added.[6,13-15] The mixture was maintained on ice and purged with nitrogen for 5 minutes before being allowed to polymerize under UV irradiation at 6°C for 12 hours. Once polymerization was complete, water was introduced, and the mixture was subjected to ultrasonication to achieve a uniform suspension. The polymers were then centrifuged at 12,000 rpm for 15 minutes and subsequently washed multiple times with a 5% sodium dodecyl sulfate solution in a 9:1 water/acetic acid mixture, followed by ethanol and water. A non-imprinted polymer (NIP) was synthesized using the same procedure, except without the presence of KTI.[6,14-16]

Characterization Techniques of MIP

Characterisation of MIP was carried out by sophisticated techniques involving spectroscopy and microscopy. Fourier transform infrared spectroscopy (FTIR) was performed on Shimadzu spectrometers (IR Affinity-1/MIRacle 10) with a range of wavenumber between 400 to 4000 cm^{-1}. Surface morphological investigation was carried out with a JSM-76108 field emission scanning electron microscope (FESEM). In addition, thermogravimetric and differential thermal analysis (TG-DTA) were analysed on TG-DTA 7200 (Hitachi, Japan).

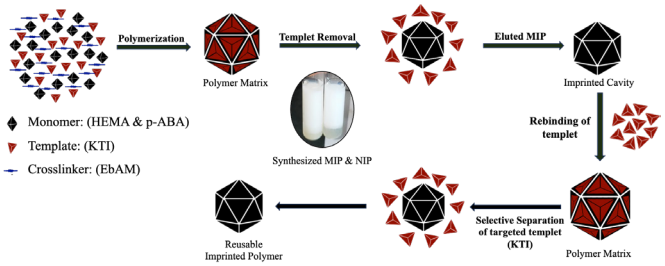

Fig. 1: Schematic representation of KTI-based MIP

Rebinding Assay

The rebinding experiment for synthesized MIP was carried out in triplicate to study the selective binding to a protein molecule KTI. Prepared concentrations of MIP and NIP (1, 3 and 5 mg/mL) were allowed to bind by incubating at 6°C and also at room temperature separately with 15 mL of standard KTI (30 µg/mL) and kept for 12 hours on a rotational shaker along with control KTI concentration without polymer was performed in parallel. The samples were centrifuged at 15000 rpm for 20 minutes. After centrifugation, the unbound KTI from the supernatant was quantified by the Bradford method. The activity of KTI was assessed by observing how KTI proteins bind to Coomassie Brilliant Blue G250, forming a complex with a significantly higher extinction coefficient than the unbound dye. The absorbance monitor at 595 nm, and binding efficacy was quantified in the equation

$$(A_b - A_s)/ A_b \times 100$$

Where A_b: KTI activity in the control (incubated without polymer); A_S: KTI activity in the sample(incubated with polymer).[6,14-16]

RESULTS AND DISCUSSION

Synthesis and Characterization of MIPs

MIP was successfully synthesized using KTI as a targeted template molecule with 2- hydroxyethyl methacrylate (HEMA), N, N'-ethylene bis (acrylamide) (EbAM) and p-aminobenzamidine (p-ABA), as functional monomers, crosslinker and anchoring monomer respectively. TEMED initiated the under-UV polymerization with few optimizations.

Synthesis and Characterization of MIPs

FTIR analysis

FTIR spectra of unleached-MIP, MIP and NIP are shown in Figs 2 (a, b and c) respectively. FTIR study was conducted within a range of 4000 to 400 cm^{-1} and describes the surface groups of polymers. The starting materials of the NIP and MIP, such as the monomer, crosslinker, initiator, etc., were the same. Therefore, the overall data of both NIP and MIP graphs has an approximate similarity. The weak transmittance intensity peaks at 3649 and 3290 cm^{-1} were caused by the presence of –NH and –OH groups, respectively. The presence of two significant peaks at 1629 and 1546 cm^{-1} corresponds to the C=O and C=C bond, which confirms the existence of the EbAM crosslinker. The weak, intense peaks that occurred in the range of 1400 to 1000 cm^{-1} correspond to C-C, C-O and C-N bonds which are derived from the functional monomer and crosslinker chain. The peak appeared at 2922 cm^{-1}, which corresponded to the vibration of C-H stretching due to the presence of an aromatic ring in the polymer structure. The stretching frequency of unleached-MIP shifts to lower intensities, which may relate to the van der Waals interactions between monomer and template.[6,13,16-19]

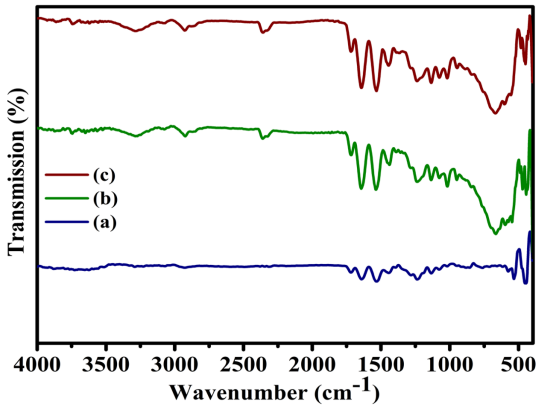

Fig. 2: FTIR spectra of (a) Unleached-MIP (b) MIP (c) NIP

SEM investigation

SEM may be used to figure out the size and shape of both the MIP and NIP. Several studies have highlighted the effectiveness of SEM in analyzing the MIP and NIP.[6,13,16-19] Examining the structural morphology and smoothness of the sample, SEM stands out as a crucial and valued analytical method. Fig. 3 (a) and (b) depict the different morphologies of the NIP and MIP samples. The NIP polymer appears to have a smoother surface (Fig. 3 (a)) compared to the MIP, which, after the template was removed, showed rough surfaces (Fig. 3(b)). This roughness can be attributed to the formation of cavities during the synthesis process. It has been reported that the roughness of MIP particles can result in a larger surface area compared to NIP, allowing MIP to better adsorb the template of interest than NIP.[6,13,16-19]

Thermogravimetric analysis

A thermal analyzer was used to investigate the thermal stability of NIP and MIP. From the graph of TGA shown in Fig. 4, there is a minor drop in mass in relation to temperature, particularly up to 280°C. Further, there is a noticeable mass decrease, indicating stability to 200 to 280°C. These results are further supported by the differential thermogravimetry (TG) graph, which shows that the major weight loss happens after 300°C. These findings illustrate the polymer's behavior and offer important insights about its thermal characteristics.[21,22]

Rebinding assay

The rebinding experiments for synthesized MIP were carried out in triplicate to study the selective binding to a protein molecule KTI, data shown in Fig. 5. Prepared concentrations of MIP and NIP (1, 3 and 5 mg/mL)were allowed to bind by incubating at 6℃ and also at room temperature (30℃) separately with 15 mL of standard KTI (30 µg/mL) and kept for 12 hours on a rotational shaker. Free KTI was quantified by spectrophotometric measurements using the Bradford assay, which reveals that more KTI is bound to MIP than NIP. The average ratio of KTI bound to the NIP compared to MIP is about 1:4.5. In addition, by increasing the concentration of MIP, more KTI would be selectively removed from the solution. The rebinding assay was found to be not significantly influenced by the incubation temperature, but it may be influenced by high temperature.

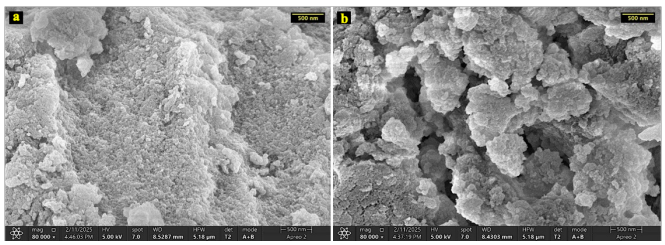

Fig. 3: SEM images of (a) NIP (b) MIP

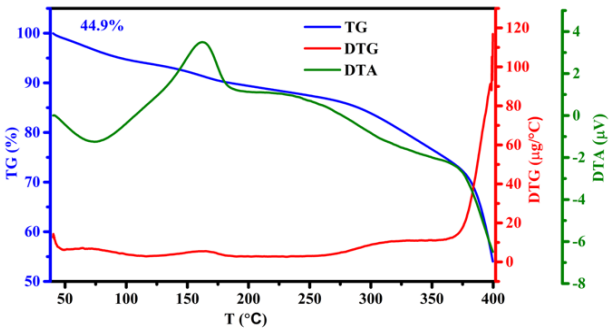

Fig. 4: Thermogravimetric analysis of MIP

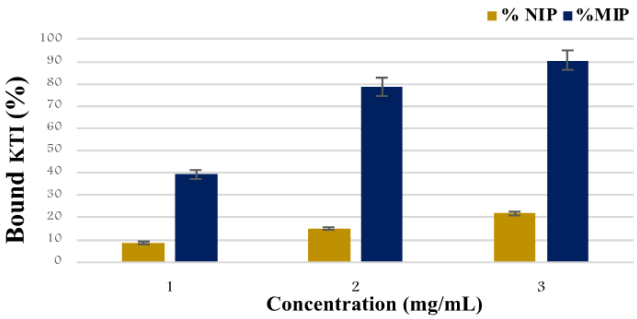

Fig. 5: Rebinding assay of MIP versus NIP

CONCLUSION

In the present work, MIP for selective removal of KTI was synthesized successfully with the help of functional monomer and crosslinker. SEM study reveals that the rough surface of MIP is attributed to the formation of cavities. The thermal stability of MIP was found in the range of 280 to 300°C. The rebinding assay of MIP with KTI was quantified using the spectrophotometric Bradford assay. The average ratio of KTI bound to the NIP compared to MIP is about 1:4.5. Furthermore, by increasing the concentration of MIP, more KTI would be selectively removed from the solution. This method will be promising for the real-time analysis and selective removal of KTI from KTI-based products.

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