Creating a Macromolecular Receptor by Affinity Imprinting

A. A. VAIDYA, B. S. LELE, M. G. KULKARNI, R. A. MASHELKAR

Chemical Engineering Division, National Chemical Laboratory, Pune, India 411 008

Received 4 July 2000; revised 25 September 2000; accepted 7 October 2000

ABSTRACT: A molecularly imprinted polymeric receptor for trypsin was synthesized by employing a novel technique that is a combination of affinity separation and molecular imprinting. An enzyme–inhibitor complex of trypsin and *N*-acryloyl *para*-aminobenzamidine was polymerized with acrylamide and *N*,*N*'-methylene bis-acrylamide. Template trypsin was extracted out to obtain an affinity-imprinted polymer. Control experiments were performed to demonstrate the synergistic affinity-imprinting effect. The percentage of crosslinker used was the crucial factor in determining the imprinting efficacy of the polymers. Imprinted polymer containing 50% crosslinker exhibited a linear Scatchard plot. Unlike non-imprinted gel, the receptor exhibited almost exclusive recognition of trypsin in an individual batch experiment as well as from a mixture of trypsin and chymotrypsin. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 1075–1083, 2001

Key words: affinity; imprinting; trypsin receptor

INTRODUCTION

Over the past two decades, molecular imprinting has emerged as an attractive technique to separate variety of bioactive molecules.[1–4] In this technique, functional monomers are pre-organized around template molecules using covalent or noncovalent interactions between the two. The monomer–template assembly is polymerized with crosslinker, and the template is extracted to obtain molecularly imprinted polymer (MIP). MIPs preferentially adsorb substrate molecules that are structurally similar to the template. Among small bioactive molecules, MIP-based receptors for β-adrenergic antagonists (e.g., atenolol, acebutol,[5] amino acids and peptides,[6] phenyl phosphoric acid,[7] DNA and RNA bases,[8] theophylline,[9] nicotine,[10] herbicides like prometryn,[11] pesticides like atrazine,[12] sterols like androst-5-ene-3β,17β-diol, testosterone,[13,14] and cholesterol[15]) have been reported. In most of these examples, acrylic/methacrylic acid was used as the functional monomer in MIPs that exhibited weak hydrogen bonding interactions with substrates during the binding.

Hydrogen bonding has also been exploited in synthesizing MIPs for bioactive macromolecules (i.e., proteins and enzymes). Shi et al.[16] synthesized thin polymeric films comprised of disaccharide molecules imprinted with various proteins. The polymers exhibited selective recognition of imprinted proteins (albumin, Immunoglobulin G, lysozyme, RNAase, and streptavidine) through H-bonding with the disaccharides. Umeno et al.[17] reported poly(*N*-isopropylacrylamide) grafted with DNA. The DNA on the polymer was imprinted with restriction endonuclease EcoRI for its re-recognition through H-bonding. A different strategy (i.e., metal chelation by proteins) was used in MIPs by Arnold et al.[18] In this case, silica beads comprising Cu(II) iminodiacetate groups were imprinted with proteins having exposed surface histidine groups for their selective rebinding on the beads.[18] Bayerl et al.[19] imprinted bovine

Correspondence to: M.G. Kulkarni (mgk@che.ncl.res.in).

Journal of Applied Polymer Science, Vol. 81, 1075–1083 (2001)

trypsin inhibitor on silica beads coated with vesicles of dimyrystoyl phosphatidylcholine. Hjerten et al.[20] synthesized polyacrylamide gels in the presence of human growth hormone, RNAase, and myoglobin. The imprinted gels exhibited selective recognition for the respective proteins through H-bonding. Mosbach et al.[21] reported MIPs for RNAase and soybean trypsin inhibitor by mixing them with methacrylate silica, vinylimidazole, and acrylamide, and crosslinking the assembly. Functionalized silica gels exhibiting hydrogen bonding have also been imprinted with urease, RNAase, and transferrin to create macromolecular receptors.[22–25] As mentioned earlier, hydrogen bonding is a weak nonspecific secondary valence interaction. This interaction often results in very low uptake (micrograms per gram of the polymer) of proteins or enzymes by MIPs. Selectivity is also limited by the fact that non-imprinted proteins can also exhibit H-bonding with the polymers.

In contrast to H-bonding interactions, affinity interactions between active site of enzymes and inhibitors (affinity ligands) are very strong and selective for a given pair of enzyme and the ligand.[26] Thus, affinity chromatography, affinity precipitation, and affinity ultrafiltration have been extensively used for separation of enzymes. However, affinity polymers also exhibit some nonspecific interactions with proteins other than the desired ones[27–30] The capacity and selectivity of polymers bearing affinity ligands could be enhanced by imprinting the same by the desired enzyme/protein. To our knowledge, there is no report on use of enzyme–inhibitor interactions for the synthesis of MIPs. In this communication, we report a trypsin-imprinted receptor comprising the trypsin inhibitor *p*-aminobenzamidine (PABA). This receptor exhibited almost exclusive uptake of trypsin over chymotrypsin, a closely related enzyme. On the other hand, non-imprinted polymer containing PABA exhibited nonspecific uptake of chymotrypsin.

EXPERIMENTAL

Materials

Acrylic acid and *p*-aminobenzamidine dihydrochloride (PABA · 2HCl) were obtained from Aldrich Chemical Company Inc., Milwaukee, WI. Trypsin (type II S) and α-chymotrypsin (type II), both from bovine pancreas, *N*-α-benzoyl-DL-arginyl-*para*-nitroanilide (DL-BAPNA), and *N*-benzoyl-L-tyrosyl-*para*-nitroanilide (L-BTPNA) were obtained from Sigma Chemical Company, St. Louis, MO. Thionyl chloride, acrylic acid, acrylamide, *N*,*N*'-methylene bis-acrylamide, ammonium persulfate (APS), and N,N′,N″,N‴ tetramethylene ethylene diamine (TEMED) were obtained from local suppliers. All the chemicals were used as received. Acryloyl chloride was synthesized from the reaction between acrylic acid and thionyl chloride.

Electronic absorption measurements were made on UV-1601PC Shimadzu spectrophotometer. Mean pore radii of polymers were determined on Quanta chrome mercury porosimeter SP-33B.

Methods

Synthesis of N-Acryloyl *para*-aminobenzamidine · Hydrochloride (Ac.PABA · HCl)

N-**A**cryloyl *para*-aminobenzamidine · hydrochloride (Ac.PABA · HCl) was synthesized according to our earlier reported procedure:[31] yield, 50%; mp, 237–239 °C.

Synthesis of Trypsin-Imprinted Polymers

A typical procedure for the synthesis of imprinted polymers was as follows: 50 mg of Ac.PABA · HCl was dissolved in 3 mL of dilute NaOH to free the guanidine group from its hydrochloride form. To this solution, 50 mg of trypsin was added, and the resulting solution was shaken gently on a shaker bath for 15 min to form a trypsin–Ac.PABA complex. The formation of trypsin–Ac.PABA complex was monitored by estimating the activity of the inhibited trypsin against standard substrate DL-BAPNA. It was observed that 94% trypsin activity was inhibited due to the formation of trypsin–Ac.PABA complex. The rationale for the selection of 1:1 w/w ratio of Ac.PABA to trypsin is discussed later under Results and Discussion. Co-monomer acrylamide (800 mg), crosslinker *N*,*N*'-methylene bis-acrylamide (100 mg), and initiator ammonium persulfate (10 mg) were dissolved in 1 mL of dimethylformamide (DMF) and added to an aqueous solution containing the trypsin–Ac.PABA complex. Nitrogen gas was passed through this solution for 15 min, and 40 μL of TEMED was added. Polymerization was conducted by keeping the tube containing this solution in a water bath at 37 °C for 18 h. The gel that was obtained was treated with acetone to remove water. It was then crushed to fine particles.

Table I Synthesis and Characterization of Imprinted and Nonimprinted Gels

No.	Type of Polymer[a]	Acrylamide (mg)	Methylene bis-Acrylamide (mg)	Swelling Ratio[b]	Mean Pore Radius (Å)[c]	Trypsin Uptake[d]	Uptake Ratio[e]
1	I(10)	800	100	3.5	1.012	0.984 ± 0.010	
2	NI(10)	850	100	3.3	1.026	1.272 ± 0.013	0.77
3	I(30)	600	300	1.9	0.938	1.044 ± 0.008	
4	NI(30)	650	300	1.9	0.911	0.680 ± 0.011	1.53
5	I(50)	400	500	1.8	0.816	0.620 ± 0.004	
6	NI(50)	450	500	1.8	0.835	0.212 ± 0.006	2.92

[a] I = imprinted polymer; NI = nonimprinted polymer; figures in parenthesis denote the percentage ratio of crosslinker; acryloyl PABA was 50 mg in all experiments; imprinted polymers contain trypsin (50 mg each) as the template.
[b] Swelling ratio = (weight of swollen polymer − weight of dry polymer)/(weight of dry polymer).
[c] Mean pore radius = Å × 10^4.
[d] Trypsin uptake expressed in mg of trypsin/g of polymer; the values expressed are the average of three experiments.
[e] Uptake ratio = trypsin uptake [imprinted]/trypsin uptake [nonimprinted].

The template trypsin was extracted from the particles by degrading it with alternative treatments of acetone and chloroform 3–4 times.[32] Imprinted polymers that were synthesized as just described were dried in a vacuum oven at 50 °C for 12 h. Dried polymer particles were then sieved through standard test sieves, and particles in the size range 250–500 μm were used for all further studies. Details of the polymer synthesis are summarized in Table I.

Synthesis of Non-imprinted Polymers

Non-imprinted gels containing Ac.PABA were synthesized as described in the previous section except that the template trypsin was not used during synthesis. Details are listed in Table I.

Trypsin Uptake Studies

A typical procedure for trypsin uptake studies was as follows: In a 50-mL capacity conical flask, 250 mg of polymer (particles of 250–500 μm size) was suspended in 25 mL of 10 mM Ca^{+2} aqueous solution containing trypsin (2 mg/mL). The flask was allowed to shake at a speed of 50 rpm at 37 °C on a rotary shaker for 1 h. An independent experiment was conducted that showed that at this Ca^{+2} concentration, there was no loss of trypsin activity due to self proteolysis. The swollen gel particles were filtered, and the trypsin content in the filtrate was estimated according to the Lowry procedure.[33] Uptake of trypsin by the gel was determined from the difference between the initial amount and that present in the filtrate. Results of trypsin uptake by imprinted and nonimprinted polymers are summarized in Table I.

Estimation of Swelling Ratios

Polymer particles (200 mg) were placed in a stoppered conical flask. To this, 25 mL of distilled water was added and the flask was kept at 37 °C for 72 h. The swollen gel was then filtered, wiped with tissue paper, and weighed. This procedure was repeated until constant weight was obtained. The swelling ratio was determined by dividing the difference between the weights of the swollen gel and the dry gel by the weight of the dry gel. The results are summarized in Table I.

Estimation of Dissociation Constant (K_d) and Number of Binding Sites

The trypsin uptake by imprinted polymer–trypsin uptake by nonimprinted polymer ratio was maximum when the polymers were synthesized with 50% crosslinker. Hence, trypsin imprinted polymer I (50) and nonimprinted polymer NI (50) were evaluated for the determination of K_d (dissociation constant) and total number of binding sites as follows: 200 mg of I (50) or NI (50) was allowed to equilibrate with 5 mL of trypsin solution of varying concentrations, ranging from 100 to 700 μg/mL, and containing 10 mM Ca^{+2} at 37 °C for 18 hr. Trypsin-bound particles were filtered. Trypsin present in the filtrate (S_f) was estimated and bound trypsin (S_b) was calculated from the difference between initially added trypsin and S_f. The Scatchard plot (S_b/S_f) versus (S_b) was plotted (Figure 2). From the slope of the graph, the dissociation constant K_d (−1/slope) was obtained, and from the intercept on the x axis, the total number of binding sites was calculated. Similarly, Scatchard plots for all other polymers were plotted.

Table II Synthesis and Uptake of Trypsin and Chymotrypsin Imprinted Gels

No.	Type of Polymer[a]	Acrylamide (mg)	Methylene bis-Acrylamide (mg)	Uptake of Trypsin[b]	Uptake of Chymotrypsin[c]	Selective Uptake Ratio[d]
1	T(50)	400	500	0.600 ± 0.010	No uptake	Exclusive trypsin uptake
2	C(50)	400	500	0.186 ± 0.008	0.096 ± 0.007	1.94
3	NI(50)	450	500	0.212 ± 0.006	0.090 ± 0.005	—

[a] Acryloyl-PAB was 50 mg in all experiments; the percent crosslinker was 50% for all the polymers; 50 mg of trypsin and chymotrypsin were incorporated as template for T(50) and C(50), respectively.
[b] mg of trypsin/g of polymer; these are average of three readings.
[c] mg of chymotrypsin/g of polymer; these are average of three readings.
[d] Selective uptake ratio = uptake of trypsin by imprinted polymer/uptake of chymotrypsin by imprinted polymer.

Synthesis of Trypsin and Chymotrypsin Receptors

Trypsin receptor T(50) and chymotrypsin receptor gel C(50) were synthesized using 50% crosslinker following the procedure already described in the section about synthesis of trypsin-imprinted polymers (vide supra). The details of synthesis are listed in Table II.

Trypsin and Chymotrypsin Uptake from Individual Batch Experiments

First, 250 mg of T(50) or C(50) was equilibrated with 25 mL of 10 mM Ca^{+2} solution containing trypsin or chymotrypsin (2 mg/mL) on a shaker bath for 1 h at 37 °C. The gel particles were filtered off, and the amount of trypsin or chymotrypsin present in the filtrate was determined by the Lowry method.[33] The uptake of respective enzymes by receptors was estimated from the difference between initially added amounts and those present in the filtrates. Relevant data are listed in Table II.

Trypsin versus Chymotrypsin Uptake from their Mixture of Identical Initial Activities

To demonstrate selective uptake of trypsin in the presence of chymotrypsin, a mixture of identical initial activities of trypsin and chymotrypsin was allowed to shake with trypsin imprinted polymer T(50) as described in the previous section. The filtrate was assayed for individual enzymes using standard substrates.[34] DL-BAPNA and L-BTPNA were used for trypsin and chymotrypsin, respectively. From the percentage of substrate hydrolyzed, the residual enzyme activity was estimated. The data are shown in Table II.

RESULTS AND DISCUSSION

The objective of the present work was to synthesize a polymer comprising an affinity monomer that is molecularly imprinted for its corresponding enzyme so that the resulting polymer will exhibit high selectivity and capacity for the imprinted enzyme. We selected trypsin as a model enzyme for synthesizing such an affinity-imprinted polymer. The guanidine group present in PABA exhibits strong affinity for the aspartate group in the active site of trypsin. We therefore selected *N*-acryloyl PABA (Ac.PABA; K_i = 67 $\times 10^{-6}$ M) as the trypsin-specific affinity monomer for the use in the synthesis of imprinted polymers.[27] In the following sections, we describe the synthesis of trypsin-imprinted polymers containing PABA and evaluation of affinity imprinting.

Choice of Acrylamide-Based Polymers

Trypsin–Ac.PABA complex formation takes place in aqueous medium. Therefore, water-soluble acrylamide and *N*,*N*'-methylene bis-acrylamide were selected as co-monomer and crosslinker, respectively, for synthesizing the polymers. Here it may also be noted that poly(acrylamide) gels are routinely used in gel electrophoresis technique for the separation of enzymes and various other proteins in their active form.

Affinity-Imprinting Methodology

In brief, our methodology is a combination of affinity-based separation and molecular imprinting, which is shown schematically in Figure 1. The steps involved include the formation of affinity complex between Ac.PABA and trypsin, polymerization of the complex with acrylamide and *N*,*N*'-methylene bis-acrylamide, and the removal of the template–trypsin from the gel.

Trypsin–Ac.PABA Complex

Conventionally, stoichiometric amounts of functional monomers and small template molecules

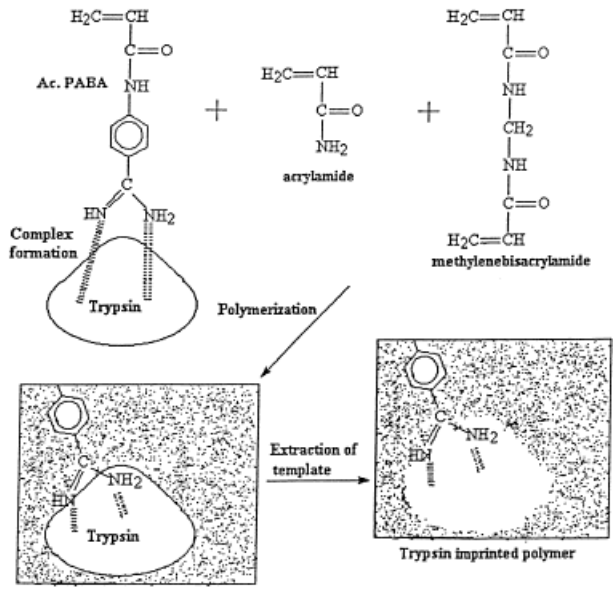

Figure 1 Affinity-imprinting methodology.

have been used to form the monomer–template assembly. But in the case of macromolecular templates like enzymes, the molar ratio required for the formation of such a monomer–template assembly is best determined by the active-site titration of the enzyme with its inhibitor. We performed the active-site titration of trypsin with Ac.PABA by monitoring the inhibition of trypsin activity against the standard substrate DL-BAPNA. Data listed in the Table III shows trypsin is completely inhibited at a trypsin:Ac.PABA molar ratio of 1:200. White et al.[37] reported 1:236 molar trypsin:benzamidine ratio for the complete inhibition of trypsin. Thus, it can be concluded that in the present case the ideal molar ratio for trypsin–Ac.PABA complex formation would be 1:200. Yet, any inadvertent excess of Ac.PABA in the complex would result in this fraction of Ac.PABA acting as merely an affinity chromatography ligand. To eliminate any such possibility, we selected a slightly lower trypsin:Ac.PABA ratio that consumes all the affinity monomer for complex formation and only 6% residual activity of trypsin remains. For this result, the trypsin: Ac.PABA molar ratio is 1: 127. On a weight basis, this ratio is 1:1. Thus, 1:1 w/w trypsin and Ac.PABA were used to form the complex between the two.

Synthesis of Imprinted Polymers and Removal of Template

The trypsin:Ac.PABA complex (1:1 w/w) was polymerized with acrylamide and *N*,*N*'-methylene bis-acrylamide as already described (vide supra). First, we attempted to extract the template trypsin out of the gels by the conventional treatment of polymer particles with dilute HCl, which dissociates the complex into free trypsin and inhibitor.[36] But we observed that dilute HCl treatment could not extract out trypsin completely. Therefore, we opted for organic solvent treatment of the polymer particles. Chloroform/acetone treatment degrades trypsin into smaller fragments and elutes them efficiently.[32] This method of template removal resulted in 94% removal of trypsin from the imprinted polymers. Because trypsin so extracted is in degraded form, it does not show any activity. Here it may be noted that once the cavity specific for trypsin is formed and frozen by crosslinking, subsequent removal of trypsin by its degradation and drying the gel particles is not expected to affect the cavity. Further, when trypsin rebinds the polymer, it is not expected to show any activity because it is inhibited by Ac. PABA. This work aims at demonstrating the receptor concept; that is, selective uptake (recognition) of imprinted enzyme and not the recovery. However, as already mentioned, active trypsin may be recovered from the polymers by repeated treatments with dilute HCl.

Optimization of Crosslinker

Poly(acrylamide) gels swell substantially in water. This swelling disturbs the imprinted cavity

Table III Active-Site Titration of Trypsin in the Presence of Ac.PABA

No.	Mole Ratio of Trypsin:Ac.PABA	% Residual Activity of Trypsin[a]
1	1 : 0	100
2	1 : 1	98
3	1 : 25	32
4	1 : 60	17
5	1 : 127	6
6	1 : 200	0

[a] 50 mg of trypsin was dissolved in 2 ml of aqueous solution containing 10 mM Ca^{+2}. To it different concentrations of Ac.PABA were added and this mixture was incubated at 37°C for 10 min. Then, 1 mL of BAPNA (6 mM) was added to it, and the activity of trypsin was monitored by the release of paranitroaniline at 410 nm. The change in absorbance (ΔA_{410}) at 410 nm was followed for 1 min. The same experiment was performed in the absence of Ac.PABA (control). The percent residual activity of trypsin in the presence of Ac.PABA was calculated by assuming the activity of trypsin in the control as 100%.

and results in poor imprinting effect. This effect has been overcome by using a high percentage (>90%) of crosslinker, which reduces the swelling and thus improves the specificity of the imprinted cavity. However, this procedure is effective only for small substrate molecules (e.g., rhodaniline blue, safranine, etc.[35]). For macromolecular substrates, like trypsin in the present case, the degree of crosslinking has to be optimized because small pore sizes of highly crosslinked polymers limit the access of high molecular weight proteins towards the imprinted sites.[38] We therefore performed a detailed study of the effect of percent crosslinker on the imprinting efficacy. Here it may be noted that that mean pore radii of various imprinted and/or nonimprinted polymers are in the range 8000–10,000 Å (Table I). Shape dimensions in angstroms for trypsin and chymotrypsin molecule in aqueous solution are 50 × 40 × 40 and 49.3 × 67.3 × 65.9, respectively.[39,40] Thus, the pore radii of polymers are large enough to allow the access of both the proteins inside the polymer matrix.

The imprinting efficacy was quantified in terms of the uptake ratio of trypsin by imprinted and nonimprinted gels prepared with same percentage of crosslinker ($\text{uptake}_{\text{imprinted}}/\text{uptake}_{\text{nonimprinted}}$).

Trypsin imprinted and nonimprinted polymers, both containing affinity monomer Ac.PABA, were synthesized with 10–90% crosslinker of the weight of the monomers. The feed compositions and other relevant data are summarized in Table I. The polymers were evaluated for the uptake of trypsin. It was observed that at lowest percentage of crosslinker (10%), imprinted polymer I(10) exhibited lower uptake of trypsin (0.984 mg/g) than did the nonimprinted polymer NI(10) (1.272 mg/g). Swelling ratios of I(10) and NI(10) were very high (3.3–3.5). Lower trypsin uptake by I(10) suggest that the high swelling caused the loss of the imprinted cavity. Also, Scatchard plots for I(10) and NI(10) were curvilinear, indicating that both the polymers behaved as simple affinity chromatography gels (data not shown). In summary, I(10) did not exhibit the imprinting effect ($\text{uptake}_{\text{imprinted}}/\text{uptake}_{\text{nonimprinted)}} = 0.77$).

With further increase in the percentage of crosslinker, swelling ratios of both the polymers decreased. This decrease in swelling helped maintaining the shape of the imprinted cavity, and the imprinting effect was then evident. Uptake ratios ($\text{uptake}_{\text{imprinted}}/\text{uptake}_{\text{nonimprinted}}$) increased from 0.77 for I(10) to 2.92 for I(50). But, higher amounts of crosslinker in the polymers also decreased their pore radii and the net uptake of trypsin by either imprinted or nonimprinted polymers, as seen from the data listed in Table I. This result is consistent with the trend reported by Venton and Gudipati[38] for urease-imprinted silica gels of decreasing pore radii that restricted the free access of urease to the imprinted cavities. When crosslinker was increased above 50%, uptakes of trypsin and chymotrypsin by both imprinted and nonimprinted polymers were very close. This similarity could be attributed perhaps to the small pore radii of both the polymers that restrict the protein uptake to the outer surface of polymer particles. In summary, at 50% crosslinker, maximum selectivity was exhibited by imprinted polymers ($\text{uptake}_{\text{imprinted}}/\text{uptake}_{\text{nonimprinted}} = 2.92$).

I(50) exhibited trypsin uptake of 0.62 mg/g. This uptake capacity of the gel is indeed low compared with the large molar excess of Ac.PABA over that of trypsin used in the gels (1:127, trypsin:Ac.PABA). This result could be attributed to the heterogeneous binding mode in which a large fraction of affinity ligand in crosslinked polymer matrix is unavailable to bind with the enzyme. However, this uptake (mg/g) is hundreds-fold higher than the results reported for glucose oxidase-imprinted silica gels.[25] Here we would like to emphasize that I(50) exhibited three times higher trypsin uptake (0.62 mg/g) than did NI(50) (0.212 mg/g) because of the imprinting effect. Furthermore, to discern between the affinity and imprinting and validate our methodology, we performed additional control experiments using the optimized 50% crosslinker and 1:1 w/w trypsin: Ac.PABA complex. These experiments are detailed next.

Affinity Imprinting: Concept Validation

Trypsin-imprinted polymer was synthesized using 450 mg of acrylamide as the functional monomer, 500 mg of methylene bis-acrylamide (50% of the total feed) as the crosslinker, and 50 mg of trypsin as the template molecule. This gel did not exhibit any uptake of trypsin because there are no sites (affinity groups) within the gel to which the enzyme could bind. From this result it is clear that voidage created by the mere presence of trypsin during polymerization is not adequate for the uptake of trypsin. This result also points towards the limitations of hydrogen bonding as a rebinding mechanism in synthesizing MIPs for enzymes.

We also synthesized a polymer containing a blocked guanidine group using 400 mg of acrylamide, 500 mg of *N*,*N*′-methylene bis-acrylamide, 50 mg of trypsin, and 50 mg of Ac.PABA · HCl. This gel also did not exhibit any trypsin uptake because the guanidine group that binds to the active site of trypsin has been blocked by hydrochloride.

Nonimprinted polymer NI(50) containing Ac.PABA was synthesized according to the composition shown in Table I. The data show that trypsin uptake by this gel was 0.212 mg/g, despite the fact that it was not imprinted for trypsin. This result is because of the inherent ability of Ac.PABA to bind to trypsin.

Trypsin-imprinted polymer I(50) containing Ac.PABA was synthesized according to the composition shown in Table I. Trypsin uptake by this gel was 0.620 mg/g, which is almost three times higher than that of NI(50). This difference is because this gel contains affinity monomer Ac.PABA, which has its inherent trypsin-binding capacity as well as an imprinted cavity specific for trypsin that results in a synergistic "affinity-imprinting" effect.

Finally, I(50) was treated with 1 N HCl to block the active guanidine groups. The gel did not exhibit any uptake of trypsin, lending further support to the hypothesis that the uptake of trypsin by affinity-imprinted gel is indeed due to the synergistic effect.

Affinity-Imprinted Receptors for Trypsin

To highlight the difference between affinity chromatography gels (nonimprinted polymers) and receptors (trypsin-imprinted polymers), Scatchard plots were constructed for I (50) and NI (50). The Scatchard plot for I (50) was linear, as shown in Figure 2. The total number of binding sites calculated was 0.165 μM/g and K_d = 0.0375 μM. Scatchard plot for NI (50) was curvilinear, indicating thereby that this polymer did not exhibit the characteristics of a selective receptor. In fact, the curvilinear nature of the plot shows that NI (50) indeed functions as an affinity chromatography gel. Thus, although both the gels were synthesized using same amounts of Ac.PABA, only I(50) exhibited trypsin binding like a true receptor.[41]

Exclusive Recognition of Trypsin by Receptor

Trypsin-imprinted polymer T(50) and chymotrypsin-imprinted polymer C(50) comprising Ac.PABA

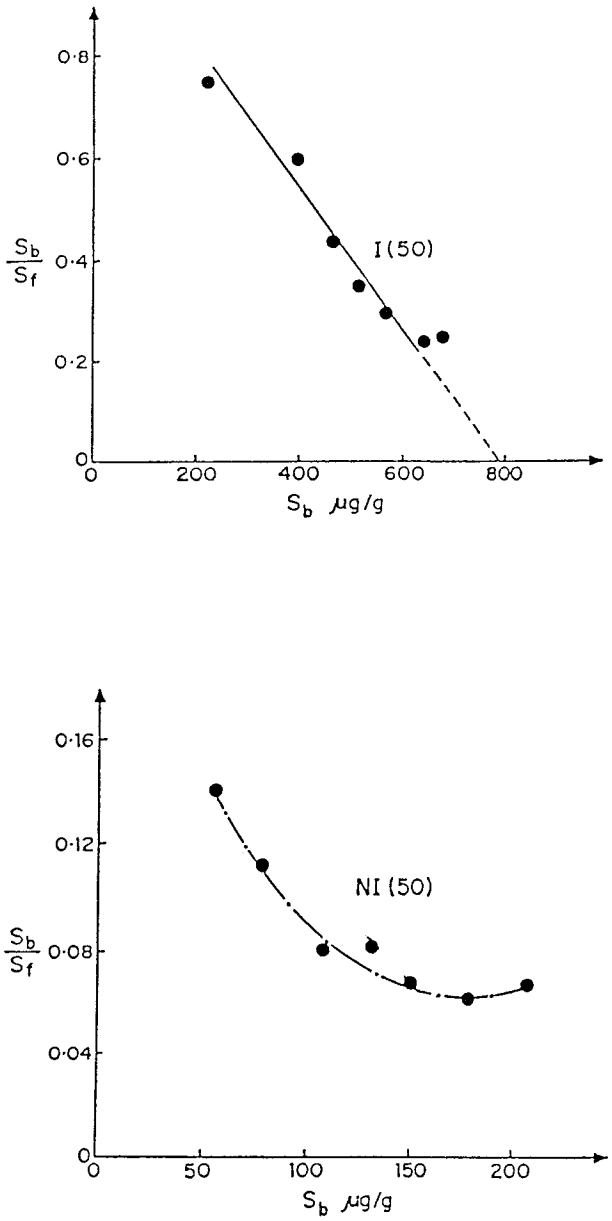

Figure 2 Scatchard plot for I (50) and NI (50).

were synthesized with 50% crosslinker. The composition of these gels is shown in Table II. The ability of these receptors to discriminate between trypsin and chymotrypsin was evaluated in independent batch experiments and also in the mixture of trypsin and chymotrypsin. Both trypsin and chymotrypsin belong to the same class of enzymes (i.e., serine proteases). Both the enzymes contain serine, aspartic acid, and histidine in their respective active sites. Moreover, both the active sites have hydrophobic slits in which their model substrates (arginyl substrate for trypsin and phenyl alanyl substrate for chymotrypsin) can fit.

An independent experiment was conducted to confirm that in the presence of 10 mM Ca^{+2}, the trypsin activity was not lost due to self-proteolysis during the time span of the experiment. Data in Table II show that trypsin uptake for T(50) was 0.68 mg/g gel. However, T(50) exhibited no uptake of chymotrypsin. We confirmed this result by estimating chymotrypsin in the filtrate, which remains after separating the receptor gel particles. The estimation by the Lowry method and activity measurement (double cross checking) confirmed that the amount of protein and the activity of chymotrypsin in the filtrate were the same as in the initial solution. Thus, trypsin-imprinted receptor gel exhibited almost exclusive recognition of trypsin. We believe that this exclusivity stems from imprinted cavity created around inhibitor–enzyme complex, which is very specific for that enzyme.

NI(50) exhibited nonspecific chymotrypsin uptake of 0.090 mg/g. This result is consistent with the nonspecific adsorption of chymotrypsin by Ac.PABA observed in affinity precipitation.[27,28,30] Thus affinity-imprinted polymers reported here have an advantage over simple affinity gels in that they can eliminate the nonspecific adsorption of proteins other than the imprinted one. This property of affinity-imprinted polymers could be exploited in a negative purification technique wherein trace amounts of a particular impurity (in this case trypsin) need to be removed from the protein of interest.

C(50) gel exhibited chymotrypsin uptake of 0.096 mg/g, which is consistent with that for NI(50) just described. C(50) also exhibited trypsin uptake of 0.186 mg/g due to the inherent affinity of Ac.PABA for trypsin. It may be noted here that enzyme uptake studies were carried out using pure aqueous solutions of either trypsin or chymotrypsin.

Encouraged by our success in achieving almost exclusive uptake of trypsin by T(50) in individual batch experiment with trypsin and chymotrypsin solutions, we evaluated the ability of T(50) to discriminate between trypsin and chymotrypsin from a mixture containing identical initial activities of the two. We found that with regard to activity, 40% trypsin and only 2.6% chymotrypsin was taken up by T(50). This negligible uptake of chymotrypsin can be attributed to its nonspecific adsorption on trypsin because of the protein–protein hydrophobic interactions, which is well known in the literature.[42]

CONCLUSIONS

A molecularly imprinted receptor for trypsin was synthesized by employing a novel affinity-imprinting technique. *N*-Acryloyl *para*-aminobenzamidine, which contains the active site inhibitor of trypsin (viz. guanidine group), was used as a functional monomer in the polymers. The affinity-imprinting effect was demonstrated. For an optimum percentage of crosslinker (50%) the imprinted polymer exhibited a linear Scatchard plot, which is a characteristic of a true receptor. But the nonimprinted polymer exhibited a curvilinear plot, indicating that it functions as an affinity chromatography gel. Trypsin receptor T(50) exhibited almost exclusive uptake of trypsin and no uptake of chymotrypsin from individual batch experiment as well as from the mixture of the two. The affinity-imprinting technique could be useful in negative purification of traces of protein impurities from the mixture.

AAV acknowledges the financial support rendered by the Council of Scientific and Industrial Research, New Delhi, India, in the form of a Senior Research Fellowship.

REFERENCES

1. Wulff, G. Angew Chem Int Ed Engl 1995, 34, 1812.
2. Mosbach, K. Trends Biochem Sci 1995, 19, 9.
3. Steinke, J.; Sherrington, D.C.; Dunkin, I.R. Adv Polym Sci 1995, 123, 81.
4. Shea, K.J. Trends Polym Sci 1994, 2, 166.
5. Nilsson, S.; Schweitz, L.; Peterson, M. Electrophoresis 1997, 18, 884.
6. Kempe, M.; Mosbach, K. J. Chromatography 1995, 691, 317.
7. Sasaki, D.Y.; Alam, T.M.; Assink, R.A.; Hart, B.; Shea, K.J. Book of Abstracts, 217th edition; ACS National Meeting: Anaheim, CA, March 21–25, 1999.
8. Spivak, D.A.; Shea, K.J. Macromolecules 1998, 31, 2160.
9. Baggiani, C.; Trotta, F.; Giraudi, G.; Moraglio, G.; Vanni, A. J. Chromatography 1997, 786, 23.
10. Matsui, J.; Takeuchi, T. Anal Commun 1997, 34, 199.
11. Matsui, J.; Miyoshi, Y.; Takeuchi, T. Chem Lett 1995, 11, 1007.
12. Matsui, J.; Miyoshi, Y.; Doblhoff-Dier, O.; Takeuchi, T. Anal Chem 1995, 67, 4404.
13. Smith, C.R.; Whitcombe, M.J.; Vulfson, E.N. Spec Publ R Soc Chem 1994, 158 (Separations for Biotechnology 3), 482.

14. Cheong, S.H.; McNiven, S.; Rachkov, A.; Levi, R.; Yano, K.; Karube, I, Macromolecules 1997, 30, 1317.
15. Whitcombe, M.J.; Rodriguez, M.E.; Villar, P.; Vulfson, E.N. J Am Chem Soc 1995, 117, 7105.
16. Shi, V; Tsai, W-B ; Garrison, M.D.; Ferrari, S.; Ratner, B.D. Nature 1999, 398, 593.
17. Umeno, V.; Kawasaki, V; Meada, V. ACS Symp. Ser. 703 (Molecular and ionic recognition with imprinted polymers) 1998, 206.
18. Mallik, S.; Plunkett, S.D.; Dhal, P.K.; Robinson, R.D.; Pack, D.; Shenk, D.; Arnold, F.H. New J Chem 1994, 18, 299.
19. Bayerl, T.; Decher, G.; Braunschweig, T. Ger. Offen. DE 19823432 (1999).
20. Hjerten, S.; Liao, J.L.; Nakazato, K.; Wang, Y.; Zamaratskaia, G.; Zhang, H.X. Chromatographia 1997, 44, 227.
21. Glad, M.; Kempe, M.; Mosbach, K. WO 9305068 (1993).
22. Glad, M.; Norrlow, O.; Sellergren, B.; Siegbahn, N.; Mosbach, K. J Chromatogr 1998, 347, 11.
23. Kempe, M.; Glad, M.; Mosbach, K. J Mol Recogn 1995, 8, 35.
24. Venton, D. L.; Guddipati, E. Biochim Biophys Acta 1995, 1250, 126.
25. Burow, M.; Minoura, N. Biochem Biophys Res Commun 1996, 227, 419.
26. Mares-Guia, M.; Shaw, E. J Biol Chem 1965, 240, 1578.
27. Luong, J. H. T.; Male, K. B.; Nguyen, A. L. Biotech-Bioeng 1988, 31, 439.
28. Schneider, M. C.; Guillot, C.; Lamy, B. Ann NY Acad Sci 1981, 369, 257.
29. Sigundsson, K.; Filippusson, H. Polym Int 1996, 41, 355.
30. Luong, J. H. T.; Male, K. B.; Nguyen, A. L. Biotech Bioeng 1988, 31, 516.
31. Vaidya A.A., Lele, B.S., Kulkarni, R.A., Mashelkar, R.A. Biotech Bioeng 1999, 64, 418.
32. Fahien, L. A.; Kmiotek, E. J Biol Chem 1979, 254, 5983.
33. Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. J Biol Chem 1951, 193, 265.
34. Erlanger, B. F.; Kokowsky, N.; Cohen, W. Arch Biochem Biophys 1961, 95, 271.
35. Ekberg, B.; Mosbach, K. Trends Biotechnol 1989, 7, 92.
36. Senstad, C., Mattiasson, B. Biotechnol Bioeng 1989, 33, 216.
37. White, E.; Chen, Y. Biochemistry 1995, 34, 15123.
38. Venton, D.L.; Gudipati, E.. J. Chromatogr 1995, 1250, 126.
39. Squire, P.G.; Himmel, M.E. Arch Biochem Biophys 1979, 196, 165.
40. Sigler, P.B.; Blow, D.M.; Matthews, B.W.; Henderson, R. J Mol Biol 1996, 35, 143.
41. Segel, I. H. In Biochemical Calculations, 2nd edition; John Wiley & Sons: New York, 1973; pp. 241–243.
42. van Oss, C.J. In Protein Interaction Symposium, 201st Annual Meeting of the American Chemical Society; Visser, H., editor; VCH: Weinheim, 1992; pp. 22–55.