

# Peptide Separation using Molecularly Imprinted Polymer Prepared by Epitope Approach

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Peptide separation in the aqueous-rich mobile phase using the molecularly imprinted polymer prepared by the epitope approach as a stationary phase for HPLC was investigated. Usually, for the preparation of stable molecularly imprinted polymers of predetermined ligand selectivity, only relatively low molecular weight compounds are used as templates. According to the early proposed epitope approach, we have synthesized polymers, which are able to recognize the template as well as some larger peptides possessing the same structural fragment as that of the template. After optimization of the composition of chromatographic mobile phase, a clear base-line separation of the equimolar mixtures of template YPLG or oxytocin possessing the same C-terminus sequence as the template, with other peptides was shown. The MIP demonstrated reliable resolution of mixtures containing YPLG up to 75  $\mu\text{M}$  ( $R_s > 1.3$ ), and mixtures containing oxytocin up to 50  $\mu\text{M}$  peptide load ( $R_s > 1.5$ ).

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Chemically and mechanically stable materials, which are able to mimic recognition properties of biological structures such as antibodies can find numerous applications for the separation and analysis of a vast variety of biologically active or harmful substances in industry, health services and environmental monitoring. Such materials with predetermined ligand selectivity can be produced using molecular imprinting technology. Preparation of molecularly imprinted polymers (MIPs) relies on the presence of a template (imprint molecule) during the formation of a new material. At first, in solution, so-called functional monomers arrange around the template via either non-covalent or covalent interactions and then these complexes are fixed by polymerization with a high degree of cross-linking. Subsequent removal of the template leaves cavities with a complementary size, shape and arrangement of functional groups. Therefore, resulting polymers can specifically interact with template during rebinding procedure and can single it out from a mixture with other compounds.<sup>1-5</sup>

Usually, for the preparation of MIPs, only relatively low molecular weight compounds are used as templates and organic solvents are primarily used as a reaction medium. The conformation of protein molecules is very changeable and sensitive to solvent, temperature, pH and ionic strength. Therefore, attempts to involve the protein itself in the polymerization process<sup>6-9</sup> have been characterized by low specificity and unsatisfactory reproducibility. Recently, plasma deposition was applied to form polymeric films around proteins coated with disaccharide molecules.<sup>10</sup> However, in this case, no significant difference was detected between the amount of protein adsorbed on different protein imprints, which is consistent with non-specific protein adsorption. On the other hand, Mosbach and coauthors<sup>11-13</sup> have demonstrated the efficient recognition of short oligopeptides by MIPs imprinted with these oligopeptides.

This leads us to the following idea: If a short peptide representing only the small exposed fragment of a protein structure is used as a template, then the resulting molecularly imprinted polymer should also be able to retain the whole protein molecule. We use the term "epitope approach"<sup>14,15</sup> from the fact that small fragments of antigens responsible for the specificity of antibody-antigen interaction are called epitopes. It was proved that the MIPs prepared by the epitope approach recognize not only the template, but also some larger peptides possessing the same structural fragment as that of the template (e.g., oxytocin). These polymers can be used not only in organic solvents, but in the aqueous-rich media as well. The materials with such properties could be used for detection and separation of practically important peptides and proteins. Moreover, some epitopes can correspond to specific features or even functions of proteins. Therefore, all proteins can be grouped into certain categories based on this criterion. Consequently, the analytical systems developed on the basis of MIP synthesized by the epitope approach can be used for proteomic investigations.

The aim of the present work is the investigation of peptide separation ability of the MIP prepared by the epitope approach in an aqueous-rich mobile phase.

## Experimental

### Materials

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), 2,2'-azobis (2,4-dimethylvaleronitrile) (ABDV) and acetic acid were obtained from Wako (Osaka, Japan). Tyr-Pro-Leu-Gly amide (YPLG), oxytocin, tocinoic acid, and Gly-Leu-Tyr (GLY) were obtained from Sigma (St. Louis, U.S.A.). Acetonitrile used was of HPLC grade. Water was of Milli Q grade.

### Polymerization

A radical polymerization was carried out at 40 °C for 16 h using ABDV as the initiator according to the procedure described earlier.<sup>15</sup> The polymers obtained were ground and sieved to collect the 20- to 53- $\mu\text{m}$  fraction. The polymers were washed several times with acetonitrile and water containing acetic acid until the template could no longer be detected in the supernatant. Control (nonimprinted) polymers were synthesized under the same conditions, though in the absence of template.

### Chromatographic Evaluation

Chromatographic analyses were performed using a Tosoh 8010 HPLC system (Tosoh, Tokyo, Japan), that included a system controller and UV detector. Polymers were slurry-packed into 100  $\times$  4.6-mm stainless steel columns. Samples (20  $\mu\text{L}$ ) were analyzed at a flow rate of 1.5 mL $\cdot\text{min}^{-1}$  and monitored with the UV detector set at 225 nm (using  $\text{NaNO}_3$  as a void marker). The capacity factor ( $k'$ ) was calculated as  $(t - t_0) / t_0$ , where  $t$  is the retention time of the solute and  $t_0$  is the retention time of the void marker; the resolution  $R_s$  of two adjacent bands was defined equal to the distance between the two band centers, divided by average band width.<sup>16</sup> The imprinting factor was defined as  $I = k'_{\text{imp}} / k'_{\text{non}}$ , where  $k'_{\text{imp}}$  and  $k'_{\text{non}}$  are the capacity factors of the same compound on the imprinted and nonimprinted polymer, respectively.<sup>17</sup> The aqueous-rich mobile phases were prepared by addition of different quantity of acetic acid to a mixture of water and acetonitrile (4:1), then pH was adjusted by NaOH to 6.5.

## Results and Discussion

Separation schemes for peptides are often complex and include different steps and chromatographic modes: size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), and reversed-phase high-performance liquid chromatography (RPHPLC). The initial steps usually aim at isolating the molecular weight fraction of interest from proteins and low molecular weight components. The following steps capitalize on differences in ionic charge or hydrophobicity.<sup>18</sup> It is possible to say that chromatography based on MIPs can to some extent combine the above-mentioned features, because the size of recognition sites within MIPs corresponds to dimensions of template and in an aqueous-rich mobile phase molecular recognition is primarily determined by ionic and hydrophobic interactions.<sup>14,19,20</sup> Therefore, molecular imprinting technology can provide the possibility to develop one-step purification procedure for different compounds including amino acid derivatives and polypeptides.

Our previous investigation demonstrated the successful application of the proposed method. Using a small tetrapeptide YPLG as a template, we synthesized MIPs that were able to recognize both the template and some other peptides possessing the same structural fragment as that of the template, 3-amino acid C-terminus PLG sequence.<sup>14,15</sup> However, as it was observed in the course of some other investigations,<sup>5</sup> the amount of peptides adsorbed to the control polymer – although much lower than the amount adsorbed to the peptide-imprinted polymer – correlated to some extent with the amount adsorbed to the MIP. In order

to reduce the level of non-specific binding we added the increasing amount of acetic acid to the aqueous-rich mobile phase. One can see that along with increased concentration

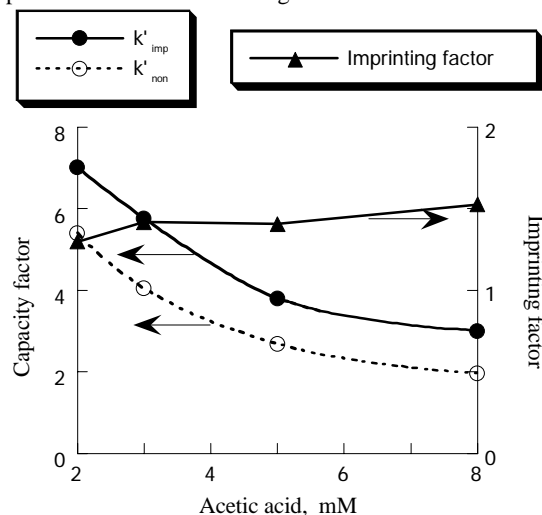


Fig. 1 Influence of concentration of acetic acid in mobile phase on HPLC parameters of YPLG.

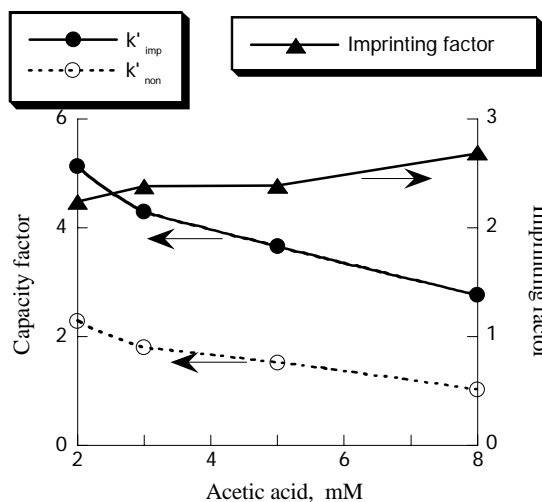


Fig. 2 Influence of concentration of acetic acid in mobile phase on HPLC parameters of oxytocin.

of acetic acid until 8 mM, the retention of YPLG and oxytocin (and the corresponding values of capacity factor) were brought down by both polymers, while the values of imprinting factor somewhat raised (Fig. 1, 2). A further increasing of concentration of acetic acid appears to be undesirable, because it will result in a too weak retention even by the MIP.

In order to check the separation ability of the MIP we have chosen the peptide Gly-Leu-Tyr (or using the one-letter code GLY), which possesses amino acid composition close to the template YPLG but with another sequence. For comparison with oxytocin we used tocinoic acid -- hexapeptide containing the same cyclic part as that of oxytocin, but without the C-terminus tail. Our data show that resolution of equimolar mixture of YPLG and GLY by

the MIP is much higher than that by the control polymer under all tested concentrations of acetic acid. Although increasing the concentrations of acetic acid brings down the value of  $R_s$ , the MIP ensures very clear and reliable separation with a high value of  $R_s$  even at 8 mM acetic acid (Fig. 3, 4). At low ionic strength, strong non-specific interactions of YPLG with the control polymer can result in a significant value of  $R_s$ , but reducing of these interactions (at 8 mM acetic acid) does not permit to control polymer to resolve the mixture of YPLG and GLY (Fig. 4).

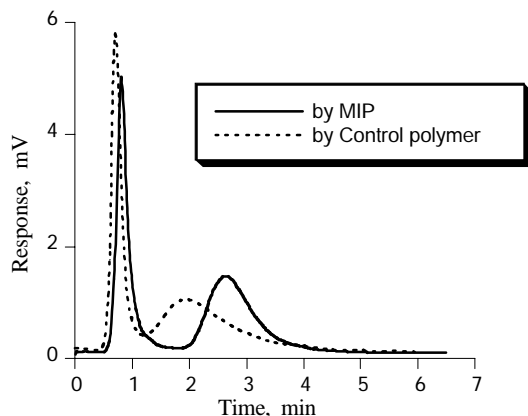


Fig. 3 HPLC traces of 10  $\mu$ M mixtures of YPLG and GLY in mobile phase containing 8 mM acetic acid.

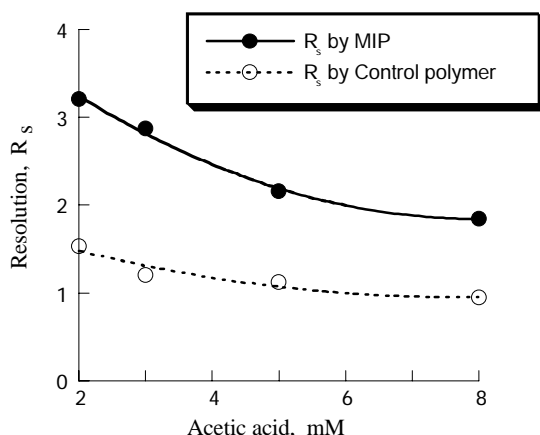


Fig. 4 Dependence of resolution of mixtures of YPLG and GLY (both 10  $\mu$ M) by the MIP and the control polymers on concentration of acetic acid in mobile phase.

The level of non-specific interactions of oxytocin is lower than that of YPLG. Consequently, independently of the ionic strength of the tested range, the non-imprinted polymer could not resolve the mixture of oxytocin and tocinoic acid. Contrary to this, the MIP demonstrates the obvious base-line separation (Fig. 5). Increasing the concentrations of acetic acid from 2 to 8 mM lowered the value of  $R_s$  for the mixture of oxytocin and tocinoic acid, but this change is essentially weaker than that for mixture of YPLG and GLY (Fig. 6).

Taking into account the above-mentioned results, the study of resolution of YPLG and GLY in dependence on peptide load was carried out in the aqueous-rich mobile phase containing 8 mM acetic acid, whereas resolution of

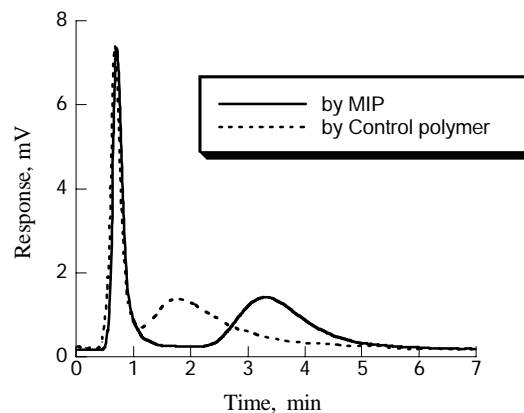


Fig. 5 HPLC traces of 10  $\mu$ M mixtures of oxytocin and tocinoic acid in mobile phase containing 3 mM acetic acid.

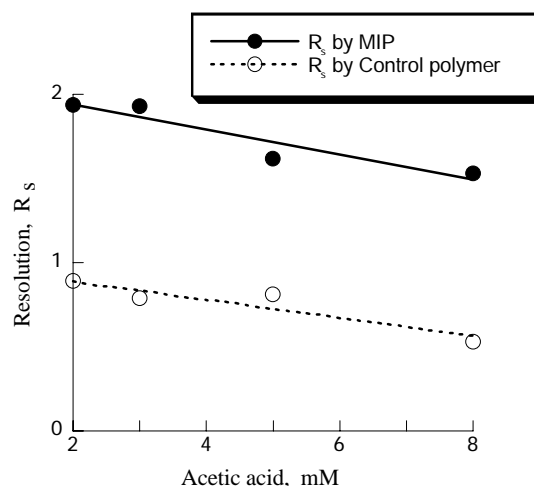


Fig. 6 Dependence of resolution of mixtures of oxytocin and tocinoic acid (both 10  $\mu$ M) by the MIP and control polymers on concentration of acetic acid in mobile phase.

oxytocin and tocinoic acid was performed at 3 mM acetic acid. Fig. 7 shows that until 20  $\mu$ M YPLG and 20  $\mu$ M GLY the value of  $R_s$  is close to constant, further increase of peptide load results in its decreasing, while resolution of YPLG and GLY by the MIP remains satisfactory even when concentration of both peptides are 75  $\mu$ M. Similar data were obtained when studying the dependence of resolution of oxytocin and tocinoic acid on peptide concentration (Fig. 8). Only at 50  $\mu$ M of both peptides, an essential decrease of the  $R_s$  value can be observed, but even this minimum value of 1.53 is high enough and corresponds to clear base-line separation. Our previous investigations of the influence of load of individually injected peptides on the chromatographic performance of the MIP in the aqueous-rich mobile phase containing citrate-phosphate

buffer showed that the peak shape of YPLG changes at about 0.1 mM. An additional shoulder can be observed at about 0.5 mM. Chromatography of oxytocin at the range 0.01 to 0.2 mM showed no essential changes of peak asymmetry.<sup>21</sup> The data of the present study coincide with the previous ones: until 50  $\mu$ M no changes of peak asymmetry of YPLG or oxytocin were observed. However, at 50  $\mu$ M and higher concentration of weakly retained compounds (GLY and tocinoic acid) a significant increase of their bandwidth and peak split were observed.

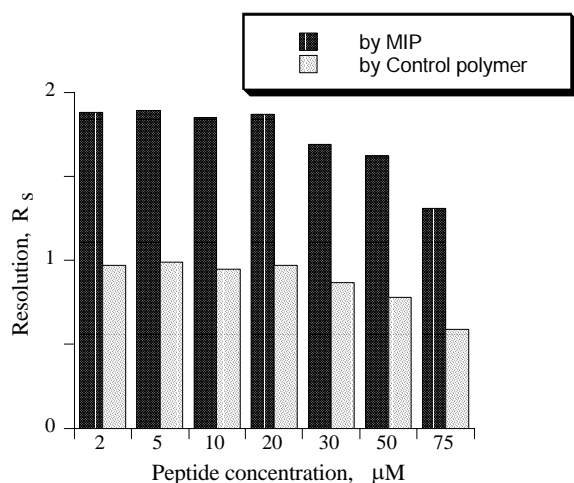


Fig. 7 Dependence of resolution of mixtures of YPLG and GLY by the MIP and control polymers on peptide load.

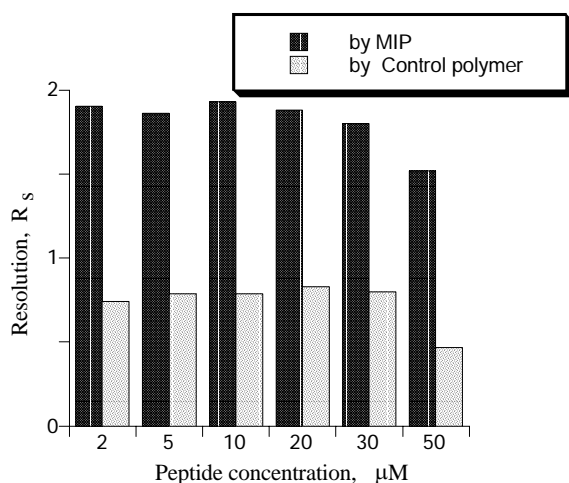


Fig. 8 Dependence of resolution of mixtures of oxytocin and tocinoic acid by the MIP and control polymers on peptide load.

In conclusion, we presented in our paper the results of successful application of the molecularly imprinted polymer prepared by epitope approach for peptide separation in the aqueous-rich mobile phase. The equimolar mixtures of the template YPLG and GLY were efficiently resolved at the range of peptide concentration until 75  $\mu$ M. It is very important that oxytocin (the peptide bearing the same 3-

amino acid C-terminus PLG sequence as that of the template) can be also recognized by the MIP and can be efficiently separated from the mixture with tocinoic acid, which has a structure containing the same cyclic part as that of oxytocin, but without the C-terminus tail. The obvious advantage in the separation of both mixtures by the MIP comparing with the performance of control polymer clearly indicates the main role of the specific recognition sites within imprinted polymer in the process of molecular recognition of the peptides.

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