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# Analysis of peptides and proteins by temperature-responsive chromatographic system using *N*-isopropylacrylamide polymer-modified columns[1]

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**Abstract**

A new method of HPLC using packing materials modified with a temperature responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), was developed. Homogeneous PIPAAm polymer and its copolymer with butyl methacrylate (BMA) were synthesized and grafted to aminopropyl silica by activated ester-amine coupling and they were used as packing materials. The surface properties and functions of the stationary phases are controlled by external temperature. Isocratic elution by aqueous mobile phase alone is the basis for separation of peptides and protein. The separation of the mixture of three peptides, insulin chain A and B and $\beta$-endorphin fragment 1–27 was achieved by changing the column temperature with 0.9% NaCl aqueous solution as the sole eluent. Retention of peptides and proteins was controlled both by column temperature and by NaCl concentration in the aqueous mobile phases in this chromatographic system. © 1997 Elsevier Science B.V.

*Keywords:* Peptide; Poly(*N*-isopropylacrylamide); Protein; Temperature-responsive chromatography

## 1. Introduction

Poly-*N*-isopropylacrylamide shows (PIPAAm) unique characteristics in aqueous media. It is soluble in water at temperatures lower than 32°C whereas it becomes insoluble above this temperature owing to dehydration of the isopropyl side group. This soluble–insoluble transition is quite sensitive, reversible and reproducible.

The properties of the solid surface modified by PIPAAm have been investigated [1–5]. The surface shows hydrophilic to hydrophobic property alteration with temperature.

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A new chromatography system was developed utilizing the surface property alteration [6]. PIPAAm molecules with a carboxyl terminal were introduced to the surface of aminopropyl silica support by activated ester-amine coupling. In this system, the surface property and function of the stationary phase are controlled by external temperature. The separation of steroids with different hydrophobicity was achieved by changing temperature using the PIPAAm-modified silica column with water as the sole mobile phase. HPLC with this new packing material was expected to be applicable for the separation of peptides and drugs with aqueous solutions as mobile phases.

In the reversed-phase chromatography of proteins and peptides, it is sometimes necessary to avoid the use of organic solvents in the mobile phase as these cause denaturation. With conventional reversed-phase columns, the use of organic solvents is necessary to prevent excessively long retention times. In this chromatographic system using temperature-responsive packing material, total avoidance of organic solvent is possible; this would be advantageous for environmental reasons.

This paper describes new hydrophobic interaction chromatography of peptides and protein in which the properties of the stationary phase were changed by external temperature under isocratic conditions.

## 2. Experimental

### 2.1. Materials

*N*-Isopropylacrylamide (IPAAm) supplied by Kojin (Kumamoto, Japan) was purified by recrystallization from a toluene–hexane mixture and dried at room temperature *in vacuo*. Butyl methacrylate (BMA) was obtained from Wako Pure Chemicals (Tokyo, Japan). BMA was distilled under reduced pressure and the fraction of bp. 61°C (5 mmHg) was used. 3-Mercaptopropionic acid (MPA; Wako Pure Chemicals, Tokyo, Japan) was distilled under reduced pressure and fraction boiling at 95°C (5 mmHg) was used. 2,2′-Azobisisobutyronitrile (AIBN), *N*,*N*-dimethylformamide (DMF), ethyl acetate (EtOAc), and dioxane were obtained from Wako Pure Chemicals (Tokyo, Japan) and purified by conventional methods. Aminopropyl silica (average diameter 5 μm, pore size 120 Å) was purchased from Nishio Kogyo, (Tokyo, Japan). Sulfo-succinimidyl-4-O-(4,4′-dimethoxytrityl)-butyrate) (s-SDTB) was obtained from Pierce (Rockford, IL, USA). Cortisone acetate, hydrocortisone, hydrocortisone acetate were from Wako Pure Chemicals (Tokyo, Japan). Insulin chain A, β-endorphin fragment 1–27 and insulin chain B were from Sigma (St. Louis, MO., USA). Ribonuclease A (M.W. 13 700; bovine pancreas) and chymotrypsinogen A (M.W. 25 000; bovine pancreas) were from Pharmacia (Uppsala, Sweden).

Milli-Q (Millipore, MA, USA) grade water was used for preparation of sample solutions. Other reagents and solvents were commerically obtained and used without further purification.

### 2.2. Transmittance measurements

The lower critical solution temperatures (LCST) of IPAAm polymers were determined by change of optical transmittance. The transmittances at 500 nm of IPAAm polymer solutions (5 mg/ml) were measured at various temperatures using a spectrophotometer (Shimadzu, UV-240). The temperature of observation cell was controlled with a LAUDA RC20 waterbath with a deviation of $\pm 0.02$°C. Cloud points of polymers were measured by a CPM-100 cloud-point monitor (Asahi Techneion, Fukuoka, Japan).

### 2.3. Preparation of PIPAAm modified column

Synthesis of PIPAAm and modification of aminopropyl silica with IPAAm polymer by activated ester–amine coupling were described in a previous report [6]. Synthesis of poly(IPAAm-co-BMA) was as follows: IPAAm (0.44 mol) and BMA (2.2 mmol) were dissolved in DMF. AIBN (3.9 mmol), and MPA (12 mmol) were used as an initiator and a chain transfer agent, respectively. The reaction mixture was degassed by subjecting to freeze–thaw cycles and an ampoule containing the monomer solution was sealed under reduced

pressure. The reaction was then performed at 70°C for 5 h. After evaporation of the solvent, the reactant was poured into diethyl ether to precipitate the polymers. The polymers was further purified by repeated precipitation from THF into diethyl ether. The molecular weight of the polymers was determined by end group titration with 0.01 M NaOH using phenolphthalein as an indicator. An average of molecular weights of the polymers used was 5000.

### 2.4. Glycidol end-capping reactions

Glycidol (15 ml, 226 mmol) was allowed to react with PIPAAm-modified silica (3 g) in 1,4-dioxane at room temperature for 24 h. After the end capping, PIPAAm modified packing material was washed with $H_2O$ and methanol. The protocol used in the glycidol end-capping reaction was based on the method of previous reports [7,8].

### 2.5. Quantification of amino groups on supports

The amount of amino groups on silica supports was determined by a spectrophotometric method using s-SDTB [9] as described in a previous report [6]. The amount of residual amino groups on the PIPAAm-modified silica support was 64.2 μmol/g solid support. After the end capping, the amount decreased to 34.3 μmol/g solid support.

### 2.6. Chromatographic apparatus

The polymer-grafted silica support was packed into a stainless-steel column (150 × 4.6 mm i.d.). The column was connected to an HPLC system (Hitachi Model L-6200 intelligent pump; L-4000 UV-monitor; and D-2500 data processor). Milli-Q grade water and aqueous NaCl solutions were used as the mobile phases. The elution behaviour of samples were recorded at a flow-rate of 1 ml $min^{-1}$ at various temperatures. The column temperatures was controlled within ± 0.02°C by a Lauda RC20 water-bath.

## 3. Results and discussion

### 3.1. Effect of temperature on peptide separation

On the IPAAm homogeneous polymer-modified column, the retention times of insulin chain A, β-endorphin fragment 1–27 and insulin chain B were 4.82, 7.13 and 13.44 min, respectively with 0.9% NaCl (0.17 M) as a mobile phase with a flow-rate of 0.5 ml $min^{-1}$ at 50°C. To increase retention of the peptides at lower temperature, copolymer of IPAAm was used for modification of support. Fig. 1 shows chromatograms of the mixture of insulin chain A, β-endorphin fragment 1–27 and insulin chain B on the poly(IPAAm-co-BMA) modified column. Temperature-dependent peptides separation was achieved with 0.9% NaCl (0.17 M) as a mobile phase with a flow-rate of 0.5 ml $min^{-1}$. The three peptides were not separated at 5°C, which was lower than the LCST (Fig. 1(a)). As the column temperature was raised to 40°C, they were well resolved (Fig. 1(b)). These peptides consist of 21–30 amino acid residues. The elution order of the three peptides should reflect their hydrophobic properties. The effect of temperatures on capacity factors of peptides and proteins were measured on the poly(IPAAm-co-BMA) modified silica column (Tables 1 and 2).

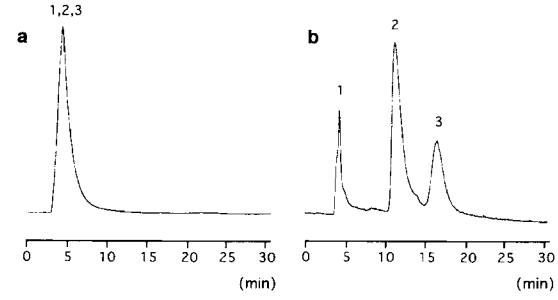


Fig. 1. Chromatograms of a mixture of insulin chain A, β-endorphin fragment 1–27 and insulin chain B on the poly(IPAAm-co-BMA) modified column. Column temperature, (a) 0°C, (b) 40°C. Eluent, aqueous 0.9% NaCl (0.17 M) solution. Flow-rate, 0.5 ml $min^{-1}$. Detection, 215 nm.

Table 1
Amino acid sequence of peptides

| Peptide | Amino acid sequence |
|---|---|
| Insulin Chain A | Gly-Ile-Val-Glu-Gln-$CysSO_3$-$CysSO_3$-Ala-Ser-Val-$CysSO_3$-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-$CysSO_3$-Asn |
| Insulin Chain B | Phe-Val-Asn-Gln-His-Leu-$CysSO_3$-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-$CysSO_3$-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala |
| β-endorphin fragment 1–27 | Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr |
| α-endorphin | Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr |
| β-endorphin | Thr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu |
| α-neoendorphin | Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys |
| Leucine enkephalin-Lys | Tyr-Gly-Gly-Phe-Leu-Lys |

### 3.2. Effect of salt concentration in mobile phase on peptide and protein separation

It was reported previously that the LCST decreased with increasing concentration of NaCl in water. In 1 M NaCl, the transition temperature shifted remarkably to 20°C from 32°C in water. For the separation of steroids, the retention times were greater with 1 M NaCl mobile phases at 25°C than with water. The results are attributed to lowering of the LCST by the concentration of the salt in the mobile phases. Fig. 2, shows the effect of salt concentration on the LCST of PIPAAm and poly(IPAAm-BMA).

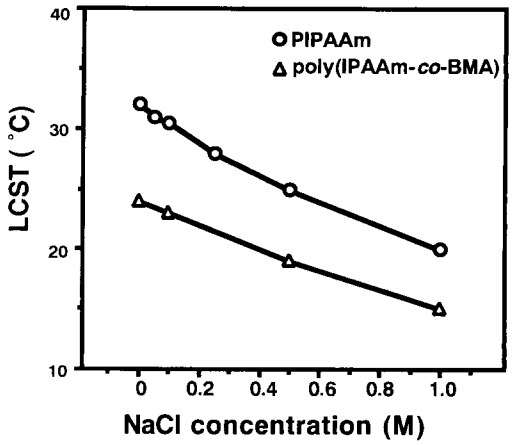


Fig. 2. Effect of salt concentration on LCST of PIPAAm (O) and poly(IPAAm-co-BMA) (△).

Separation of proteins on PIPAAm-silica column was examined. The effects of salt concentration on the chromatographic behaviour of ribonuclease A and chymotripsinogen A are shown in Fig. 3. The increase of resolution with an increase of the salt concentration may be explained in terms of the effect of salting-out.

The effects of salt concentration in mobile phase on the retention times of the two proteins are listed in Table 3. The retention times of the proteins were increased with increasing salt concentration. Ammonium sulphate showed larger effects of salting-out and prolonged retention times more than the same concentration of NaCl.

The effects of salt concentration were also observed on the separation of peptides. Fig. 4 shows the effects of salt concentration and temperature on the retention times of insulin chain A, insulin chain B and β-endorphin fragment 1–27. Retention times of insulin chain B and β-endorphin fragment 1–27 were remarkably increased as the column temperature was raised. Salt concentration also affected peptide retention time; longer peptide retention times was observed with a higher NaCl concentration at any given temperature.

Table 2
Effect of column temperature on capacity factor[a]

| | 5°C | 30°C | 50°C |
|---|---|---|---|
| Insulin Chain A | −0.46 | 0.01 | 0.02 |
| Insulin Chain B | −0.25 | 1.68 | 4.46 |
| β-endorphin fragment 1–27 | −0.33 | 0.92 | 2.46 |
| α-endorphin | −0.22 | — | 0.19 |
| β-endorphin | −0.25 | — | 0.69 |
| α-neoendorphin | 0.00 | — | 0.60 |
| Leucine enkephalin-Lys | −0.02 | — | 0.27 |
| Ribonuclease A | −0.26 | — | 0.15 |

[a]Mobile phase, 0.9% NaCl aqueous solution; detection (215 nm or 280 nm depending on sample); other conditions were the same as for Fig. 1.

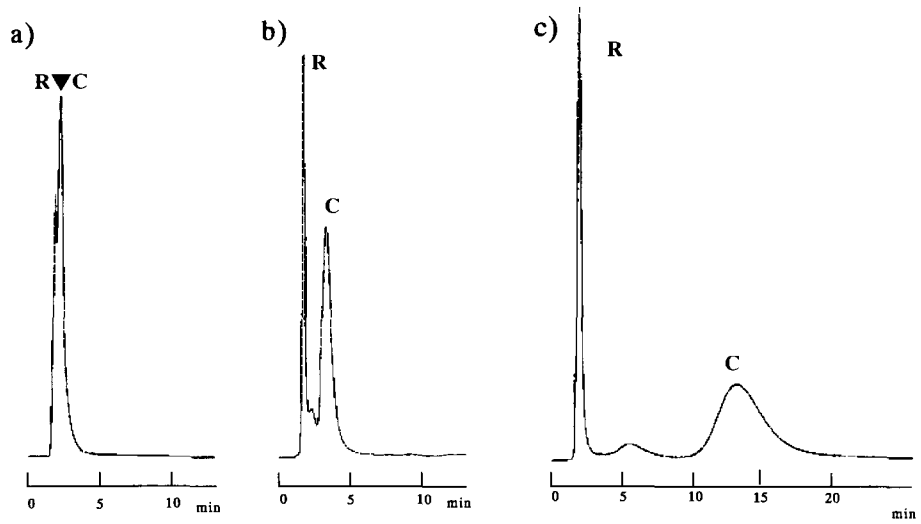


Fig. 3. Effect of salt concentration on chromatographic behaviors of ribonuclease A and chymotripsinogen A. Eluent, (a) 0.1 M, (b) 0.5 M, (c) 1.0 M NaCl aqueous solution. Flow-rate, 1.0 ml $min^{-1}$. Detection, 280 nm. Column temperature; 40°C.

The temperature-responsive interaction between PIPAAm-modified silica and the eluates should be due to changes in the surface properties of the stationary phase by the reversible transition from a hydrophilic to a hydrophobic nature. The results shown above indicate that the present system is a kind of hydrophobic interaction chromatography (HIC).

In conventional HIC systems, proteins are separated by using a descending salt gradient, running from high to low salt concentrations. In contrast, in temperature-resposive chromatography the separation of peptides and protein is achieved by a single mobile phase; a gradient elution-like effect was also achieved by controlling external temperature.

Table 3
Effect of salt concentration in mobile phase on retention times[a]

| | Ribonuclease A | | Chymotrypsinogen A | |
|---|---|---|---|---|
| | 5°C | 40°C | 5°C | 40°C |
| 0.1 M MaCl | 1.46 | 1.80 | 1.46 | 2.10 |
| 0.5 M MaCl | 1.33 | 1.88 | 1.33 | 3.48 |
| 1.0 M MaCl | 1.50 | 2.04 | 1.50 | 13.07 |
| 0.5 M $(NH_4)_2SO_4$ | 1.25 | 1.82 | 1.25 | 8.47 |

[a]Retention times are expressed in min; Flow-rate, 1 ml $min^{-1}$; detection, 280 nm.

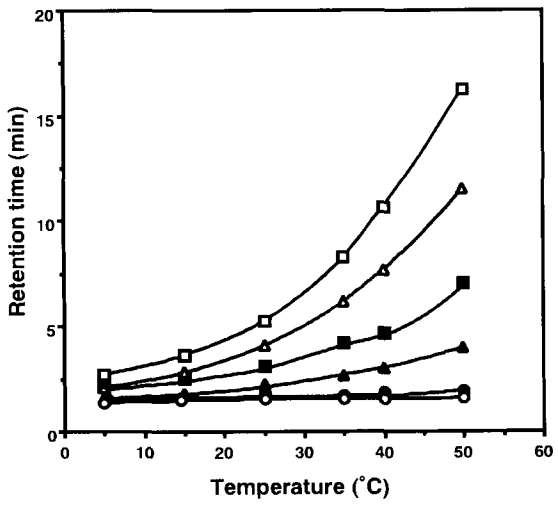


Fig. 4. Effects of salt concentration and temperature on the retention times of insulin chain A, insulin chain B and β-endorphin fragment 1–27. (○) Insulin chain A with 1.0 M NaCl, (●) Insulin chain A with 0.5 M NaCl, (□) Insulin chain B with 1.0 M NaCl, (■) Insulin chain A with 0.5 M NaCl, (△) β-endorphin fragment 1–27 with 1.0 M NaCl, (▲) β-endorphin fragment 1–27 with 0.5 M NaCl.

Consequently, stationary phases based on IPAAm copolymers-modified silica may prove useful in high-performance peptide and protein separations. In such separations, retention on the supports also may involve hydrophobic interaction between protein and polymer chains. The temperature-responsive chromatography of peptides and proteins with larger molecular sizes are currently in progress in this laboratory.

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