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Effect of temperature upon the chromatography of proteins on porous glass, chemically coated with N-isopropylacrylamide copolymer

A.E. Ivanov*, L.S. Zhigis, E.V. Kurganova, V.P. Zubov

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Miklukho-Maklaya 16/10, 117871, V-437 Moscow, Russian Federation

Abstract

Wide-pore glass chemically coated with a copolymer of N-isopropylacrylamide (NIPAA) and N-hydroxyethylacrylamide (70:30) was studied as a weak-hydrophobic sorbent for chromatography of proteins. The temperature dependence of the lysozyme chromatographic retention points to the maximum near a lower critical solution temperature of the copolymer (LCST, 41°C). Nevertheless, log k' vs. $[(NH_4)_2SO_4]$ plots found for lysozyme at 25°C and 45°C are only slightly different and indicate almost zero free energies of interaction between the protein and the copolymer in 0.01 *M* potassium phosphate solution, pH 7.5. No temperature-modulated desorption of immunoglobulin G adsorbed to the copolymer-coated glass at 45°C was observed when cooling the column to 30°C. Changes in protein interactions with the polymer grafts are apparently too weak to ensure an effective control of protein adsorption with temperature shift near LCST. © 1997 Elsevier Science B.V.

Keywords: Polyisopropylacrylamide sorbents; Stationary phases; Phase transitions; Temperature effects; Proteins

1. Introduction

Poly(N-isopropylacrylamide) displays a lower critical solution temperature (LCST) within the range 32 to 34°C in water [1,2]. Cross-linked gels of copolymerized N-isopropylacrylamide (NIPAA) undergo a volume phase transition under the same conditions [3,4]. Thermoresponsive properties make these materials promising for preparation of temperature-modulated bioconjugates [5], biosorbents [6,7] and supports for cell cultivation [8,9]. Although many applications of NIPAA for the above purposes are known and many grafting techniques proposed, only a few studies of poly(NIPAA)-coated inorganic sorbents and their interaction with proteins have been carried out [7,10].

Porous glass chemically modified with poly(NIPAA) was studied as a support for gel permeation chromatography of dextrans. Their elution volumes strongly depended on temperature near 30°C, perhaps due to a change of effective porosity of the bonded phases [10]. Narrow-pore glass (pore diameter of 156 Å) grafted with longer poly(NIPAA) molecules (M_r $3.4 \cdot 10^3$) displayed a stronger effect on the elution behaviour of dextrans than wide-pore glass (408 Å). Pressumably, transition of the end-grafted poly(NIPAA) chains from coils to globules might enhance the internal pore volume of the particles accessible to dextran penetration.

Poly(NIPAA)-grafted silica gel was prepared by coupling the NIPAA oligomer ($n<14$), bearing a primary amino group at the end of its chain, to the glutaraldehyde-activated carrier [7]. After reduction of the residual aldehydes to alcohols by sodium

*Corresponding author.

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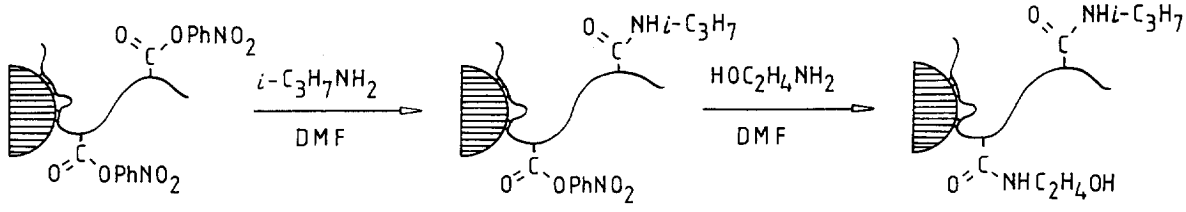

Fig. 1. Synthesis of isopropyl-PA-glass from the carrier chemically coated with poly(*p*-nitrophenyl acrylate).

borohydride, the prepared sorbent bound bovine γ-globulin in 0.067 *M* phosphate solution (pH 7) at 37°C and 62% of the adsorbed protein was released into the same solution at 24°C, presumably due to the diminished hydrophobic interactions. Incomplete protein desorption was ascribed by the authors to its denaturation proceeding in contact with poly(NIPAA) at 37°C.

This paper describes preparation of porous glass chemically coated with NIPAA copolymer. The synthetic route includes chemisorption of poly(*p*-nitrophenyl acrylate), PNPA, (a reaction studied by us previously [11]), followed by amidation of the reactive esters with isopropylamine (see Fig. 1). The *n*-butyl group containing analogous sorbent, was prepared for comparison. Both the sorbents have a weak-hydrophobic character, partially investigated by us elsewhere [12]. The present study is aimed at registering changes in elution behaviour of standard proteins induced by thermal phase transition in the polymer coating of the stationary phase. It seems interesting to outline distinctions between the protein adsorption below and above LCST and thereby to better understand the mechanism of poly(NIPAA) interaction with proteins.

2. Experimental

Wide-pore glass MPS-2000 VGKh (mean pore diameter 2000 Å, particle size 0.16–0.31 mm) was purchased from GOZ VNII NP (N. Novgorod, Russia). Isopropylamine was from Riedel-de Haen, Seelze, Germany. Ammonium sulfate, potassium dihydrogen phosphate, potassium hydroxide and dimethylformamide (DMF), all analytical grade, were provided by Reakhim, Moscow, Russia; lysozyme and immunoglobulin G were from Serva, Heidelberg, Germany.

Poly(*p*-nitrophenyl acrylate), PNPA, $\bar{M}_w = 46\,200$, $\bar{M}_w/\bar{M}_n = 3.4$ was synthesized by radical polymerization of the monomer as described in Ref. [11].

Weak-hydrophobic sorbent coated with copolymer of NIPAA, isopropyl-PA-glass was prepared from the wide-pore glass chemically coated with PNPA (PNPA-glass) [11] as follows. 5 g of PNPA-glass (the ester group content of 184 μmol/g or ca. 6 μmol/m^2) was added to the solution of isopropylamine (260 μl, 3-molar excess of amine) in 25 ml DMF. The reaction was allowed to proceed for 1 week at room temperature, then the second portion of isopropylamine was added and the reaction mixture was incubated in a water bath at 80°C for 2 h. 70% of *p*-nitrophenyl esters were substituted by isopropylamine as estimated by photometric assay of *p*-nitrophenol ($\lambda_{max} = 400$ nm, $\epsilon = 15\,300\ M^{-1}\ cm^{-1}$), the product of the reaction. The residual esters were amidated by adding ethanolamine (1 ml) during overnight incubation at room temperature. The prepared sorbent was washed by distilled water and stored as a wet cake at 8°C. The glass sorbent with N-butylpolyacrylamide coating was prepared as described in Ref. [12].

To prepare a water-soluble copolymer of N-isopropylacrylamide, co(NIPAA), 30 mg of PNPA was dissolved in dimethylformamide (1 ml), then a 3-molar excess of isopropylamine (50 μl) was added to the solution and left for overnight at room temperature. The second portion of isopropylamine (50 μl) was then added and the reaction mixture incubated in water bath at 70°C for 2 h. 70% of *p*-nitrophenyl groups were substituted by isopropylamine, as estimated by photometric assay of *p*-nitrophenol ($\lambda_{max} = 400$ nm, $\epsilon = 15\,300\ M^{-1}\ cm^{-1}$), the product of the reaction. The residual

esters were amidated by adding ethanolamine (250 μl) during overnight incubation at room temperature. The prepared copolymer was separated from low-molecular-mass compounds by gel-permeation chromatography on a 44×1.5 cm column packed with Bio-Gel P6 (Bio-Rad Labs., USA), equilibrated with 10 m*M* NH_4HCO_3, and then lyophilized. IR spectrum of the copolymer was registered by a Shimadzu IR-435 apparatus (Japan).

Hydrophobic-interaction chromatography was performed on 9×1 cm glass columns, equipped by thermostatted water jackets at a flow-rate of 0.5–0.7 ml/min controlled by peristaltic pumping. A 1 mg sample of lysozyme in 0.5 ml of 0.01 *M* phosphate buffer (pH 7.5), containing varied concentrations of ammonium sulfate, was applied to the column and eluted with the same solution. LKB Uvicord II was used for detection of absorbing fractions (λ=280 nm) during chromatography.

Effect of temperature on chromatographic retention of lysozyme on isopropyl-PA-glass and butyl-PA-glass was studied in 0.01 *M* phosphate buffer (pH 7.5), containing 1.5 *M* ammonium sulfate at temperatures from 7°C to 55°C, controlled by a U1 thermostat (VEB MLV, Germany).

Dynamic light scattering by co(NIPAA) aqueous solutions (15 mg/ml) at various temperatures was registered by a laser light scattering analyser, registration angle 90°, model N4SD, Coulter Electronics, USA.

3. Results and discussion

3.1. Characterization of the NIPAA copolymer

Fig. 2 shows IR spectra of poly(*p*-nitrophenyl acrylate), PNPA and copolymer of N-isopropylacrylamide, co(NIPAA), prepared by amidation of PNPA by N-isopropylamine. Strong absorbance of *p*-nitrophenyl ester group carbonyl (1760 cm^{-1}) presented in PNPA spectrum can not be found in the spectrum of co(NIPAA). Characteristic absorbances of the nitrogroup (1340 and 1520 cm^{-1}) and aromatic ring (1480 and 1590 cm^{-1}) are also absent, the facts testify to complete substitution of the reactive PNPA esters for N-substituted amides. The absorbances of the latter (1650 and 1550 cm^{-1},

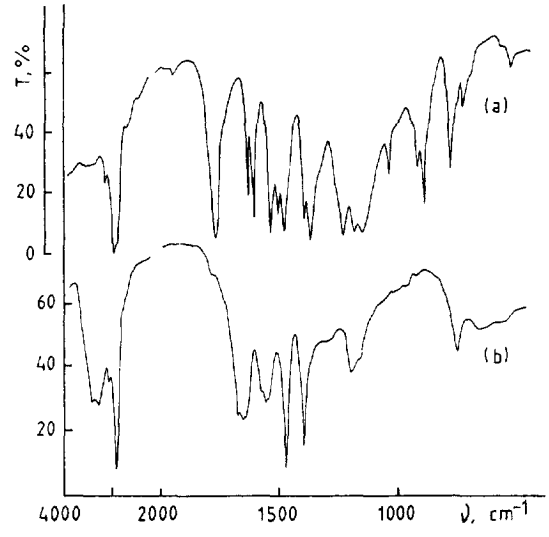

Fig. 2. IR-spectra of polymers: (a) poly(*p*-nitrophenyl acrylate), PNPA; (b) copolymer of NIPAA prepared by amidation of PNPA with isopropylamine and ethanolamine.

i.e., amide I and amide II, respectively) are both present in the co(NIPAA) spectrum. No absorbance is observed within the range 1700 to 1750 cm^{-1}, indicating no free carboxyl group in the copolymer. Therefore, co(NIPAA) contains only N-alkylamide functions, concretely, 70% of NIPAA units and 30% of N-hydroxyethylacrylamide units as estimated by the amount of liberated *p*-nitrophenol (see Section 2).

Fig. 3 shows the intensity of light scattering by aqueous solutions of co(NIPAA) as a function of temperature. The light scattering is rather slight at temperatures lower than 40°C and displays a steep increase at 41°C. The registered LCST is, therefore, higher than that of poly(NIPAA), known to fit a narrow range between 32 and 34°C [2] depending on a polymer chain length. The LCST increase may be ascribed to the presence of hydrophilic N-hydroxyethylacrylamide units in the co(NIPAA) chains. The similar effect was recently found for NIPAA and N,N-dimethylacrylamide copolymer [13], the latter monomer being more hydrophilic than NIPAA.

Solubility of co(NIPAA) in water becomes poorer in the presence of ammonium sulfate. The copolymer cannot be dissolved in 1 *M* ammonium sulfate, whereas its solutions in 0.3 *M* and 0.8 *M* ammonium sulfate are somewhat cloudy. They scatter light more

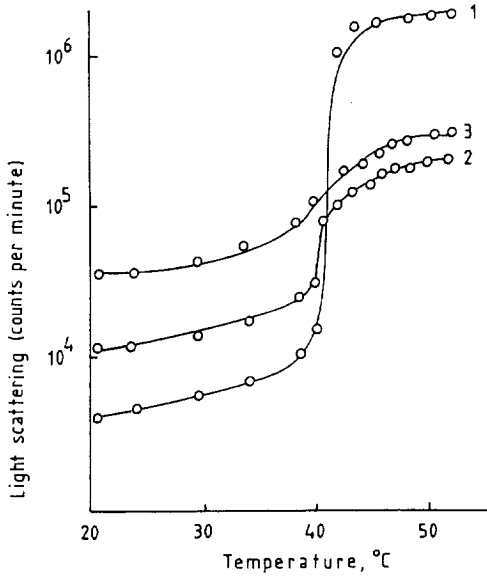

Fig. 3. Intensities of dynamic light scattering by 15 mg/ml solution of co(NIPAA) in water (1), 0.3 *M* (2) and 0.8 *M* (3) ammonium sulfate as a function of temperature.

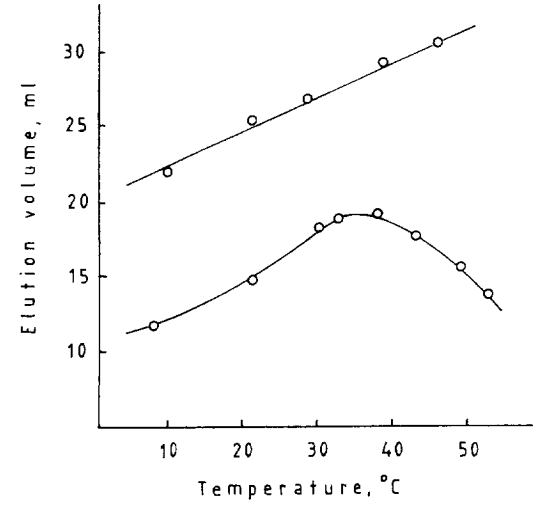

Fig. 4. Effect of temperature on chromatographic retention of lysozyme on isopropyl-PA-glass and butyl-PA-glass. For conditions see Section 2.

intensively even below 41°C (see curves 2 and 3 in Fig. 2), probably due to a partial association of co(NIPAA) caused by a salting-out effect. This may underlie the reason why the increase of light scattering at 41°C is not so steep in the solutions of co(NIPAA), containing ammonium sulfate: the copolymer is already involved in the other type of association. We did not notice, however, any shift of LCST to lower temperatures promoted by the high salt concentration, unlike reported for poly(NIPAA) by other researchers [2].

3.2. Chromatography of proteins

Retention of proteins in hydrophobic-interaction chromatography usually increases with increasing temperature [14]. We have also found a linear dependence of elution volume of lysozyme on the temperature on butyl-PA-glass column (Fig. 4, line 1). The dependence obtained for the same protein on isopropyl-PA-glass column is quite different, with the broad maximum nearby LCST (Fig. 4, line 2). As lysozyme is known to be stable in aqueous solution up to 70°C [14], one may ascribe the change in retention mechanism to a thermal transition of the coating copolymer within the bonded phase. The abnormal temperature dependence registered for lysozyme on isopropyl-PA-glass column prompted us to study the influence of salt concentration on retention of the protein below and above its LCST.

A characteristic feature of hydrophobic-interaction chromatography is the possible isocratic elution of proteins with rather high retention factors k'. By increasing the salt concentration the retention factor increases so that the dependence of log k' on molarity often becomes linear. The limiting slope of this plot is given by the hydrophobic-interaction parameter (HIP) accounting for the interaction area between the protein and the sorbent, the surface tension increment of the salt and the dipole moment of the protein molecule [15]. For a given protein solute eluted at various concentrations of ammonium sulfate HIP is a measure of the contact area between the solute and the sorbent employed. The intercept of the log k' vs. M plots gives the logarithm of the retention factor of the solute in the absence of salt in the eluent, the parameter related to free energy of hydrophobic interaction between the sorbent and the solute.

Chromatographic retention factor k' equals $K(V_s/V_m)$, where K is a distribution coefficient of the solute between the solid and mobile phases, V_m and V_s are volumes of the mobile phase and the solid-

phase available for the solute in the column, respectively [16]. As K is a function of the free energy of interaction: $K=\exp(-\Delta F/RT)$ [17], the logarithmic retention factor may be expressed as follows:

$$\log k' = \log e(-\Delta F/RT) + \log V_s/V_m \quad (1)$$

Therefore, if a limiting value of log k', related to the absence of salt in the eluent, and V_s/V_m are known, one may calculate ΔF and discover if the protein adsorption is thermodynamically favorable due to hydrophobic interactions or not.

Although an accurate calculation of V_s is a somewhat special problem, one may evaluate V_s either from the maximal adsorption capacity of isopropyl-PA-glass (ca. 6 mg lysozyme/ml sorbent) or from the overall volume of the polymeric coating, its thickness is known to be about 5 nm as found by mercury porosimetry [11]. These two methods give V_s values of 21 or 150 μl/g dry sorbent, respectively, the former one seems to be more realistic. V_s is about 3 ml/g dry sorbent, so log $V_s/V_m \sim -2.1$. From Eq. (1) appears that log k' values above -2.1 correspond to adsorption of lysozyme to the sorbent ($\Delta F<0$).

Fig. 5 shows the log k' vs. M plots obtained for lysozyme chromatographed on isopropyl-PA-glass and butyl-PA-glass sorbents at different temperatures. The polymer coatings of both the sorbents are poorly swollen at 45°C and/or self-associated because N-butylpolyacrylamide is insoluble in water, whereas co(NIPAA) is above its LCST. Thus, their contact areas with lysozyme are not much different, that of butyl-PA-glass being slightly larger, perhaps due to a larger volume of butyl radical. Furthermore, at high ammonium sulfate concentrations (above 0.8 M) almost no difference is observed between the isopropyl-PA-glass adsorptivities displayed at 25 and 45°C. This is due to an association of co(NIPAA) at 25°C under these conditions (see Fig. 3, curve 3). The salted-out polymer might not spread its segments far into solution and thereby change the contact area and the energy of interaction with the protein.

The intercepts of log k' vs. M plots found for isopropyl-PA-glass are rather close to -2, that means nearly zero (or only slightly negative) free energy of lysozyme adsorption in the absence of ammonium sulfate. In other words, one may expect insufficient hydrophobic interaction of the protein with co(NIPAA) in 0.01 M potassium phosphate aqueous solution either at 25 or at 45°C.

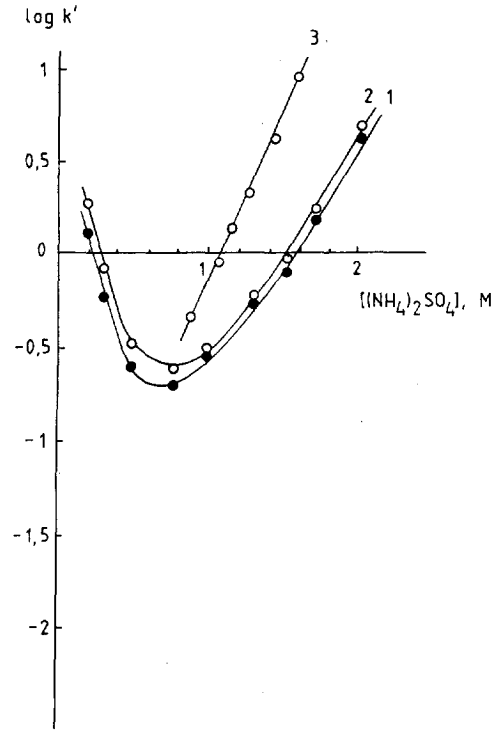

Fig. 5. Logarithm of the retention factor (log k') of lysozyme plotted versus molar concentration of ammonium sulfate. Isocratic hydrophobic-interaction chromatography on isopropyl-PA-glass at 45°C (1) and 25°C (2) and butyl-PA-glass at 45°C (3). For conditions see Section 2.

On the other hand, a small difference in lysozyme retention factors at 25 and 45°C is observed at lower ammonium sulphate concentrations (below 0.8 M). The left upward branches of log k' vs. M dependencies were earlier registered at low salt concentrations for lysozyme chromatographed on polyethylene glycol-coated silicas [15] and ascribed to electrostatic attraction of the positively charged protein to silica matrices. The swollen polymer reduce the interaction of the protein with inorganic support more effectively, thus making the elution volumes registered at 25°C smaller.

Temperature-induced desorption of immunoglobulin G (IgG) adsorbed to a poly(NIPAA)-coated

silica was recently described [7]. We attempted to reproduce this effect with isopropyl-PA-glass. Under the conditions used in the above mentioned study (0.067 *M* phosphate buffer, pH 7.0 [7]), IgG could not be irreversibly adsorbed to isopropyl-PA-glass, but quantitively eluted as an asymmetrical peak nearby the total volume of the column, i.e., a weak reversible adsorption took place. The protein (1 mg) could be partially adsorbed to isopropyl-PA-glass from 0.01 *M* phosphate buffer (pH 7.5), containing 0.15 *M* NaCl and 1 *M* $(NH_4)_2SO_4$ at 45°C. About 50% of the protein was found in a breakthrough fraction. No further desorption was observed when the column was cooled to 30°C and washed with the same solution. The adsorbed IgG fraction could be completely desorbed, however, by 0.01 *M* phosphate buffer (pH 7.5), containing 0.15 *M* NaCl at 30°C, i.e., in the common conditions of hydrophobic-interaction chromatography. The discrepancy with the earlier results of other researchers [7] may arise from different structures of NIPAA polymeric coatings or different experimental conditions, such as use of a batch operation in the cited study.

Thus, the above experiments provide the conclusion that adsorptions of lysozyme and immunoglobulin G to isopropyl-PA-glass are not strongly influenced by thermal phase transition of the grafted polymer. Temperature-modulated adsorption and desorption of proteins might be possible if an additional ligand, capable of a stronger specific binding, is incorporated into the sorbent together with thermoresponsive polymer. Such a temperature-modulated sorbent was recently proposed for enzyme purification, the desorption step was effectively controlled by a slight shift of temperature [18]. The hydrophobic interactions of proteins with co(NIPAA) seem to be not enough strong and/or selective to control protein adsorption by changing a column temperature in the range of LCST. On the other hand, this study suggests a chromatographic method for evaluation the free energy of protein interaction with synthetic polymers, which is partially based on the known relationship between k' and ΔF [17], and may be helpful for characterization of new biomaterials and biosorbents.

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