Journal of Chromatography A, 1324 (2014) 181–189

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Adsorption behavior of proteins on temperature-responsive resins

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ARTICLE INFO

Article history:
Received 11 July 2013
Received in revised form
29 September 2013
Accepted 20 November 2013
Available online 25 November 2013

Keywords:
Bioseparations
N-Isopropylacrylamide
Thermo-responsible resins

ABSTRACT

The adsorption behavior of proteins on thermo-responsible resins based on poly(*N*-isopropylacrylamide) and its copolymer containing an anionic co-monomer has been investigated. The influence of the polymer composition, i.e., the content of the co-monomer and crosslinker on the thermo-sensitivity of the protein adsorption has been quantified. The properties of ungrafted polymer as well grafted onto the agarose matrix have been analyzed and compared. Batch and dynamic (column) experiments have been performed to measure the adsorption equilibrium of proteins and to quantify the phase transition process. As model proteins lysozyme, lactoferrin, α-chymotrypsinogen A and ovalbumin have been used. The adsorption process was found to be governed by ionic interactions between the negatively charged surface of resin and the protein, which enabled separation of proteins differing in electrostatic charge. The interactions enhanced with increase of temperature. Decrease of temperature facilitated desorption of proteins and reduced the salt usage in the desorption buffer. Grafted polymers exhibited markedly higher mechanical stability and, however, weaker temperature response compared to the ungrafted ones.

1. Introduction

Various chromatographic techniques can be used for separation and purification of proteins, which are based on different types of interactions between the functional groups of the macromolecule and the adsorbent surface. Often, a combination of few chromatographic stages is integrated; such as ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) or mixed mode chromatography exploiting both types of interactions [1–6].

In both processes the adsorption properties of proteins are altered by the composition of the mobile phase. In IEC the sample with the protein mixture is loaded at low concentration of salt containing counter-ions with respect to the column matrix at pH differing from the isoelectric point, while elution is imposed by increase of the salt concentration or proper pH change in the direction of pI. In HIC the sample loading is realized at high concentration of a cosmotropic salt (e.g., ammonium sulfate), which enhances hydrophobic interactions between the adsorbent surface and proteins and, thus, the adsorption strength. The elution is executed by lowering the salt concentration.

A common drawback of both IEC and HIC processes stems from the necessity of using highly concentrated salt solutions to promote adsorption or elution of proteins. High viscosity of the mobile phases containing salts results in increase in the pressure drop on chromatographic columns. This reduces flowrates and, in consequence, productivity of the process. Moreover, in the final stage of the purification process the target protein has to be isolated out of the salt solution, e.g., by use of a membrane process. Because the membrane permeability reduces significantly in the presence of highly concentrated salt solutions the performance of the whole separation process is strongly affected by the salt concentration [7].

In case of HIC processes, to overcome this problem the manipulation of the salt concentration can be combined with temperature to alter the adsorption properties of proteins. Typically, increase of temperature enhances adsorption of proteins, whereas its reduction promotes their elution. Therefore, the effect of temperature can partly offset that of the salt concentration [8–10]. However, stability of typical HIC media drops rapidly with increase of temperature already above 30 °C, at which most of proteins still preserve their native properties. Moreover, a number mesophilic and thermophilic proteins exhibit high thermostability and can be separated at elevated temperatures without denaturation.

Therefore, as an alternative to traditional resins stimuli-sensitive hydrogels, termed also as smart polymers, have been developed. These polymers exhibit changes in the physicochemical properties according to external stimuli such as pH, ionic strength, interaction with the molecules of chemical compounds as

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http://dx.doi.org/10.1016/j.chroma.2013.11.040

well as temperature [8,11,12]. Among stimuli-sensitive hydrogels the thermo-responsive polymers gained interest as chromatographic media for purification of proteins under mild and environmental friendly conditions. The mechanism of separations is based on temperature-mediated cycles of adsorption–desorption, where a change in temperature is used to manipulate the adsorption behavior of proteins indirectly, i.e., by altering properties of the resin [13,14].

The most studied temperature-responsive polymer is poly(*N*-isopropylacrylamide) (PNIPA) which has a lower critical solution temperature (LCST) of approximately 32 °C [15].

At temperatures below the LCST the PNIPA is hydrophilic due to hydrogen bonding with water. At a temperature above (LCST) water molecules dissociate from polymer leading to aggregation of the PNIPA chains, which form a hydrophobic surface.

The LCST of temperature-responsive polymers can be manipulated by copolymerization of *N*-isopropylacrylamide monomer (NIPAm) with hydrophilic or hydrophobic monomers. Integration of hydrophilic monomers such as acrylamide into the molecular structure of polymer results in an increase in LCST and the polymer hydrophilicity, whereas copolymerization of NIPAm with hydrophobic monomers, such as butyl acrylamide, imparts hydrophobicity and decreases the LCST of the copolymer [12]. Copolymerization of NIPAm with anionic acrylic acid [16] or cationic *N*,*N*′-dimethylaminopropylacrylamide [17] produces co-polymers capable of hydrophobic and ionic interactions, the same which are active in HIC, IEC or mixed mode chromatography.

However, despite promising adsorption properties of this polymer poor mechanical stability often restricts its use in column chromatography. To tackle this problem different methods of the polymer grafting to a rigid matrix have been proposed, e.g., in several works [18–27] PNIPA and related temperature-responsive polymers were grafted onto a silica matrix and used as a stationary phase for HPLC analysis of micro as well as macro-molecules. For the conduction of this analytical technique salt free mobile phases were used, while the separation selectivity was altered by temperature changes. However, the applicability of those resins was mostly limited to analytical procedures.

Maharjan et al. [28] developed temperature-responsive ion-exchange resins (for IEC) by grafting a PNIPA-based polymer onto cross-linked agarose. The agarose matrix is characterized by large specific surface area and pore size and it is very often used in manufacturing chromatographic media for protein separations in a preparative and industrial scale. The authors suggested a cyclic adsorption–desorption process for the fractionation of whey proteins.

Data presented in the literature listed above have mainly been based on qualitative description of adsorption properties of different compounds on the thermo-responsive resins.

The goal of this study was detailed analysis of the pattern of adsorption and desorption of proteins on PNIPA-based resins versus temperature and the polymer composition. Few model proteins differing in hydrophobicity and the ionic charge have been studied such as lysozyme, lactoferrin, α-chymotrypsinogen A and ovalbumin. Static and dynamic experiments have been performed to evaluate the adsorption behavior. Moreover, the swelling properties and rate of the phase transition have been determined, analyzed and compared.

PNIPA-based beads were prepared by polymerization with a cross-linking agent. Copolymers were obtained by copolymerization of NIPAm, acrylic acid or its sodium salt and, optionally, with *tert*-butylacrylamide.

The properties of both ungrafted polymer and grafted onto the agarose matrix have been analyzed and compared.

2. Materials, instrumentation and methods

2.1. Materials

2.1.1. Proteins

Lysozyme from chicken egg white (LYZ) (pI = 11.4, molecular mass 14.4 kDa), lactoferrin from bovine milk (LTF) (pI = ca. 8.7, molecular mass 87 kDa), α-chymotrypsinogen A from bovine pancreas (CHTG A) (pI = 9.1, molecular mass 25.7 kDa) and ovalbumin (OVA) (albumin from chicken egg white) (pI = 4.6, molecular mass 44.3 kDa) were obtained from Sigma–Aldrich (Poland).

2.1.2. Chemicals

N-Isopropylacrylamide (NIPAm), acrylic acid (AAc), sodium acrylate (SA), chlorotrimethylsilane (TMS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), sodium stearate (SS), 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydro-quinoline (EEDQ), *tert*-butyl-acrylamide (tBAm), *N*,*N*-dimethylformamide (DMF), 4,4′-azobis(4-cyanovaleric acid) (ACV) were obtained from Sigma–Aldrich (Poland). Sodium borohydride, *N*,*N*′-methylenebisacrylamide (BIS), toluene, sodium hydroxide, pyridine were obtained from Fluka (Poland). Sodium chloride, chloroform, sodium phosphate dibasic, methanol, ammonia solution, epichlorohydrin were obtained from POCH (Poland). Ethanol, phosphoric acid, ammonium persulfate (APS) were obtained from Chempur (Poland). Sepharose 6Fast Flow (highly cross-linked agarose 6%), mean particle size 90 μm, was obtained from GE Healthcare (Sweden). Silica gel (Si), particle size 40–63 μm, pore size 60 Å, surface area 530 m^2/g, was obtained from Merck (Germany).

2.2. Instrumentation

The HPLC instrument Äkta purifier with UV, and conductometric detectors and a data station (GE Healthcare Life Sciences, Uppsala, Sweden) were used. The injector was a Rheodyne sampling valve with 100 μL sample loop. The system was equipped with glass columns (40 mm × 16 mm I.D.). Low temperature thermostat Lauda Re110 (Lauda, Lauda-Königshofen, Germany) was used for the column thermostatting.

2.3. Methods

2.3.1. Preparation of ungrafted polymer PNIPA

The synthesis was performed according to the procedure described in [29].

The following reagents have been used: NIPAm, BIS – cross-linking agent, SA – ionizing agent, APS – initiator, TEMED – catalyst.

Polymers with different chemical composition were synthesized. The quantity of SA was changed within the range 2–10% [g/g] (gram SA per gram of NIPAm) and BIS 2–30% [g/g].

1 g of NIPAm, BIS, SA with adequate proportions and water (8 mL) were mixed at ambient temperature under a nitrogen atmosphere to dissolve. In the next step, temperature was lowered to 5 °C. TEMED (0.8 mL) and APS solutions (1.26 g APS in 1.6 mL water) were added to the gel slurry. The gelation process lasted about 2 min. The gel was then stored in water at ambient temperature for 24 h. PNIPA was chopped into small pieces and rinsed with water over the next 48 h four times.

The same procedure as described above was performed in a suspension of silica gel to produce the resins meant to be used in dynamic experiments, i.e., to be packed into the column. Surfactant, sodium stearate (SS), was added to the suspension to reduce the surface tension between phases. The hydrophobic phase of silica gel was mixed with a hydrophilic environment of the reaction. NIPAm,