Analytical Biochemistry 525 (2017) 1–7

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Synthesis of hydroxyethyl-methacrylate-(L)-histidine methyl ester cryogels. Application on the separation of bovine immunoglobulin G

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ARTICLE INFO

Article history:
Received 21 November 2016
Received in revised form
26 January 2017
Accepted 7 February 2017
Available online 21 February 2017

Keywords:
Cryogels
Pseudobioaffinity
MAH ligand
Separation
Bovine
IgG

ABSTRACT

In this study cryogels based 2-hydroxyethyl methacrylate (HEMA) functionalized with N-methacryloyl-L-histidine methyl ester (MAH) were synthesized and used for the adsorption and separation of bovine IgG. Two series of cryogels functionalized with 5 and 10 mg of MAH as pseudobioaffinity ligand were prepared and characterized by swelling test, FTIR and SEM analysis. The adsorption efficiency of the bovine immunoglobulin into cryogels is discussed with respect to the following chromatographic parameters: pH, flow rate, initial IgG concentration, adsorption time and ionic strength. Our results show good adsorption of bovine immunoglobulin under mild separation conditions at pH 7.4. The maximum binding capacity was determined (32.4 mg/g of cryogel) and demonstrates the efficiency of the used cryogels. This efficacy is clearly seen upon increasing the maximum binding capacity from 23.2 mg (obtained with cryogels with 5 mg MAH) to 32.4 mg/g (for cryogel with 10 mg MAH ligand concentration). The purity of separated fractions was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Together our observations highlights poly (HEMA-MAH) as an efficient adsorbent for bovine immunoglobulins G separation.

1. Introduction

Separation technology occupies a central position during any biotechnological process and permits the purification of bioactive molecules available for use in medical research as well as numerous fields of science and industry [1,2]. Nowadays, the development of new methods aiming at purifying molecules from biological sources has become a major challenge to scientist [3]. For instance, proteins used for therapeutic and medical purposes must be highly purified and should meet stringent requirements of safety and biological activity [4].

In fact, purification of proteins and peptides is usually performed through a variety of conventional chromatographic adsorbents upon using packing gel beads in a column. These chromatographic procedures share in common several limitations including: high back pressure, low flow rates, risk of ligand leakage, loss of activity and high costs [5,6]. Alternatively monolithic materials such as cryogels have been developed and are currently used as new adsorbents for bioseparation besides their involvement in different biotechnology applications [7].

Cryogels are known for their highly connected porous network structures with enhanced mechanical properties [8,9]. The chemical and physical stability of cryogels, as well as their macroporosity makes them attractive adsorbents in bioseparation technology [10,11]. Interestingly cryogels functionalized with different ligands have proven success for the adsorption and separation of a wide range of proteins and biomolecules [12–14].

Bovine serum contains many bioactive components including growth factors, peptides, oligosaccharides and immunoglobulins which exhibit a variety of biological activities, thus increasing relevance to human health [15]. In bovine serum, bovine IgG occurs predominantly in two subclasses IgG_1 and IgG_2, both of which are present in about equal amounts (IgG_1: 11.2 mg/ml and IgG_2: 9.2 mg/mL) [16]. Several studies [17,18] have provided evidence for the protective and therapeutic effects of bovine immunoglobulin including lowering of cholesterol levels [19], modulating the immune response [20] and oral immunotherapy against helicobacter pylori [21]. Isolation and separation of bovine immunoglobulin represents a key step for their diagnostic and therapeutic uses.

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http://dx.doi.org/10.1016/j.ab.2017.02.003
0003-2697/

Several separation methods have been developed for the separation of bovine immunoglobulins [22–24]. Among these pseudobioaffinity chromatography, during which the adsorption mechanism between ligand and proteins involves electrostatic, hydrogen bonds, and hydrophobic interactions [25]. The versatility of histidine as a pseudobioaffinity ligand in molecular interactions arises from its molecular structure together with some specific properties such as its moderate hydrophobicity, possible weak charge transfer, and wide range of pKa. Such properties allow histidine to interact differently depending on its microenvironment [26]. In previous studies [27–29] histidine was coupled to various media (Epoxy and bisoxirane activated sepharose, hydroxyethyl methacrylate, magnetic polyethylene glycol dimethacrylate beads) and others being applied for the separation of antibodies.

In the present study, we synthesized poly (HEMA) cryogels containing (MAH) as pseudobioaffinity ligand for the adsorption and separation of bovine IgG. Separation of the bovine IgG was achieved in a single step under gentle elution conditions. Our results clearly indicated an increase in the amount of bovine IgG adsorbed upon increasing the MAH concentration from 5 to 10 mg. These observations highlights the feasibility and efficacy of the cryogels used for the separation of bovine IgG from whole bovine serum.

2. Materials and methods

2.1. Chemicals

(HEMA), *N*, *N′* methylenebisacrylamide (MBAAm), ammonium persulfate (APS), *N, N, N′,N′*- tetramethylenediamine (TEMED), whole bovine serum (protein concentration: 45–75 mg ml) and bovine IgG (reagent grade ≥ 95% SDS-PAGE, essentially salt free, lyophilized powder)were supplied by Sigma Chemical Co. (St Louis, Mo., U.S.A.). The following buffers were used as binding buffers: (1) 0.1 M acetate at pH 5, and pH 5.5, (2) 0.1 M phosphate at pH 6.5, pH 7.0 and pH 7.4, (3), 0.1 M Tris-HCl at pH 8.0, (4), 0.1 M carbonate at pH 9.0. Elution buffer: Acetate 0.1 M pH 4.0 + 1 M NaCl.

2.2. Preparation of MAH monomer

The monomer (MAH) was prepared according to the following preparative procedure [30]. 5 g of L-histidine methylester and 0.2 g of hydroquinone were dissolved together in 100 ml of dichloromethane. After dissolution, the mixture were cooled to 0 °C and 12.7 g of triethylamine are added. 5.0 ml of methacryloyl chloride were slowly poured to the mixture under nitrogen atmosphere and magnetic stirring for 2 h at room temperature. The unreacted hydroquinone and methacryloyl were removed by 10% NaOH solution. The aqueous phase was subsequently evaporated in a rotary evaporator at 30 °C. Finally the MAH residues were crystallized in ethanol and ethyl acetate.

2.3. Preparation of poly (HEMA-MAH) cryogels

The monomer MAH was chosen as pseudobioaffinity ligand for the adsorption of bovine immunoglobulins. The cryogel poly (HEMA-MAH) was prepared at two different MAH concentrations as follow: 5 and 10 mg of MAH were separately added to 0.283 g of MBAAm and dissolved in deionized water. 1.3 ml of HEMA were added to each solution followed by degassing under vacuum to remove soluble oxygen. The polymerization of cryogels was initiated by TEMED (30 μl) and APS (24 mg) followed by cooling the reaction mixture in an ice bath for 2–3 min. The mixtures were poured in plastic syringes serve as columns and then frozen at −12 °C for 24 h. Finally the obtained cryogels were abundantly washed by ethanol and water and stored in an adequate buffer containing 0.02% sodium azide at 4 °C.

2.4. Cryogels characterization

The both sets of poly (HEMA-MAH) cryogels were characterized for their morphology, chemical structure and porosity by Scanning Electron Microscopy (SEM), Fourier transform infrared (FTIR) spectrometry and swelling test.

2.4.1. Swelling test

A dry sample of each cryogel was weighed before putting it in a vial containing distilled water. Cryogels samples were subsequently recovered, dried at 60 °C and again weighed. The amount of water retained by the cryogels is calculated by applying the following formula:

$$\text{Water up take ratio } (\%) = [(Wf - Wo) / Wo] \times 100$$

W_o and W_f are the respective weights of cryogel before and after water uptake and are expressed in g.

2.4.2. SEM analysis

A dry cross section of cryogels has initially dried for 7 days at 25 °C before SEM analysis. A fragment of dry cryogel was mounted on a SEM sample holder, coated with a thin gold layer and thereafter subjected to SEM analysis on a (Jeol, JSM 5600 scanning electron microscopy, Tokyo, Japan).

2.4.3. FTIR analysis

The FTIR analysis was performed on FTIR spectrophotometer (FTIR 8000 series, Shimadzu, Japan) equipped with a KBr detector. Samples of cryogels were dispersed in dry KBr pellets and their spectra were recorded between 4000 and 600 cm^{-1}.

2.5. Adsorption study of bovine IgG on poly (HEMA-MAH) cryogels

For the adsorption of bovine IgG, cryogels were early washed with 30 ml of distilled water followed by 10 ml of equilibration buffer. All the adsorption procedure was conducted at room temperature. Purified bovine IgG (Sigma-Aldrich, USA) was applied to the column using a peristaltic pump and the decrease in absorbance at 280 nm was monitored throughout each chromatographic cycle. The influence of the following parameters: pH, flow-rate, ionic strength, initial bovine IgG concentration on adsorption capacity of cryogels was evaluated.

2.6. Adsorption study of bovine IgG from whole bovine serum

A fraction (10 ml) of bovine serum diluted at 1/5, 1/10 and 1/20 was separately injected into the column (plastic syringes contain the cryogel poly (HEMA-MAH), at a flow-rate of 1.0 ml/min, for a 35 min adsorption time. Retained and non-retained fractions obtained after each chromatographic cycle were analyzed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Stacking gel 6%, Resolving gel 10%) under reducing conditions.

2.7. Quantitative measurement of bovine IgG by enzyme linked immunosorbent assay ELISA

The quantitative measurement of bovine IgG was performed by ELISA method as described in the IMMUNO tek® (ZeptoMetrix Corporation, Buffalo, New York, USA) bovine IgG ELISA kit. Microplate strips coated with purified goat anti-bovine IgG form the capture phase of the assay. The preparation of samples was