Reactive and Functional Polymers 121 (2017) 45–50

Contents lists available at ScienceDirect

Reactive and Functional Polymers

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

Recovery of functionally active trypsin inhibitor (SBTI) and lipoxygenase (LOX) from soymilk via multimodal technique

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ARTICLE INFO

Keywords:
Soybean trypsin inhibitor
Lipoxygenase
Multimodal chromatography
Bovine serum albumin
Immobead-350

ABSTRACT

Commercially available epoxy activated polymers Immobeads and Dilbeads were functionalized to 1,2-amino alcohol derivatives and screened for binding and release of soybean trypsin inhibitor (SBTI) and lipoxygenase (LOX) in soymilk. Moderately hydrophobic polymer DILBEAD-VWR functionalized with diethylamine (DIL-DEA) was found to bind SBTI and LOX ($3.5 \pm 0.3\ mg{\cdot}g^{-1}$) without causing protein denaturation. Bound proteins were selectively released by changing the pH of eluting buffer. LOX was obtained in 70 ± 5% overall yield with a purification factor of 5 ± 0.5. The eluent containing SBTI was treated with bovine serum albumin coated polymer (Immobead 350) to obtain SBTI in 61% overall yield and a purification factor of 13.5. The recovered DIL-DEA polymer as well as BSA coated polymer were recycled at least 10 times without any appreciable change in performance.

1. Introduction

Soymilk, prepared from soybean seeds (*Glycine max*) is an excellent health supplement. Although the antinutritional factors such as the soybean trypsin inhibitors (SBTI) [1] and lipoxygenases (LOX) [2] are destroyed during soymilk processing [2-4], these are in fact, high value products. For example, SBTI is a known chemopreventive agent [5-7]. It is also well demonstrated that soybean extracts containing high concentration of SBTI are useful in skin care products. These extracts reduce pigment production and deposition in photo-damaged skin, commonly referred to as “age spots”, [8,9] and also delay hair growth [10,11]. Soybean LOX [12] is employed in production of hydroperoxide derivatives useful in manufacture of soaps, dyes, resins, varnishes and plastic products. Some other applications of LOX are: whitening of bread by oxidizing the carotenoids, increasing the volume of loaf and formation of volatile flavor compounds [13,14]. LOX can also be used as a biosensor for fatty acid determination [15,16]. Thus recovery of active SBTI and LOX from soybeans is an economically attractive proposition.

Several methods describe isolation of SBTI and LOX from soymilk. Standard laboratory procedures involve extraction in buffer, precipitation with ammonium sulfate and ion-exchange chromatography of the crude protein mixture. The protein is finally purified by affinity chromatography [17-19]. Other techniques such as isoelectric focusing [20] and use of peptide probes coupled with high gradient magnetic separation [21] have been described for isolation of SBTI from soybeans. LOX has been extracted from soymilk by employing aqueous two-phase extraction and polyethylene glycol precipitation systems [22-24]. On large scale preparations, the required proteins are usually separated by ultrafiltration using membranes of different molecular weight cut-off [25]. The protein concentrate is then purified by chromatography techniques. However, in case of soymilk, the problem of membrane fouling is often encountered due to presence of fats and fatty acid derivatives [26-28]. A multi-modal chromatography, also called as mixed-mode chromatography technique, can be a useful alternative in such a case. Here, the support comprises of both hydrophobic domains and ionic functional groups. The protein is bound to the support via ion-exchange as well as hydrophobic interactions. The adsorption is independent of salt concentrations and adsorbed protein is easily eluted by changing pH [29,30]. Herein, we describe an application of this technique for processing of soymilk. By reacting an epoxy-activated copolymer with different amines, a series of copolymers possessing a net positive charge at operating pH 7–7.5 and varying hydrophobicity is obtained (Scheme 1). The proteins are bound to the polymer through a combination of ionic and hydrophobic interactions and released by changing pH.

2. Experimental

2.1. Materials

Trypsin (cat. no. C7762), N_{α}-benzoyl-L-arginine-*p*-nitroanilide

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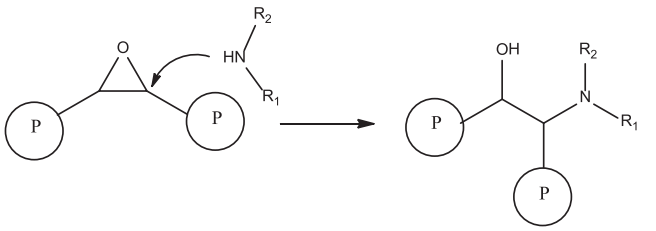
http://dx.doi.org/10.1016/j.reactfunctpolym.2017.10.009
Received 18 April 2017; Received in revised form 10 October 2017; Accepted 17 October 2017
Available online 18 October 2017
1381-5148/



Scheme 1. Preparation of amine substituted polymers. R_1 = H; R_2 = Me, Et, *n*-Pr, *n*-Bu, cyclohexyl, benzyl, 2-aminobutanol, 3-aminobutanol; R_1 = R_2 = *n*-Bu, isopropyl, benzyl. Hydrophobicity of polymer increases with hydrophobicity of amine.

(BAPNA), soybean trypsin inhibitor (cat. no. T9128) and lipoxygenase (cat. no. L7395) were purchased from Sigma-Aldrich, Bangalore, India. Soy beans, variety MACS124, were procured from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad. Immobead IB-350, obtained from Chiral Vision, Leiden, The Netherlands, http://chiralvision.com, are macroporous methacrylate copolymers with average particle size of 150–300 μm. DILBEADS were obtained from Fermenta Biotech, Mumbai, India, http://www.fermentabiotech.com. Dilbead VWR have the following properties: average particle size 100–500 μM, pore size 0.06 μm, epoxide content 6 mmol·g^{-1}, swelling capacity in water 2.5 times the dry volume, pore volume 1.05 $cm^3 \cdot g^{-1}$ and surface area of 140 $m^2 \cdot g^{-1}$. Ammonium iron (II) sulfate hexahydrate and Xylenol orange were purchased from Sigma Aldrich. All other reagents were of analytical grade obtained from HiMedia, India and used as received. Curve fittings were performed with Origin version 8.0, Microcal Software Inc. USA and GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All experiments were performed thrice and were reproducible within ± 5%. Enzyme activity was measured on Cary-100 (Agilent Technologies) UV–visible spectrophotometer equipped with temperature control and magnetic stirring. Atomic force microscopy (AFM) was employed to characterize the morphology of the polymer particles using Digital Nanoscope IV (Veeco Instruments, Santa Barbara, CA, USA). The microscope was vibration-damped. Commercial phosphorous (n) doped silica tips on an I-tape cantilever with a length of 115–135 μm and resonance frequency of 260 kHz were used. SDS PAGE was performed using Bio-Rad unit Mini-PROTEAN Tetra Cell Module.

2.2. Methods

2.2.1. Preparation of soymilk

Soy beans (10 g) were soaked in distilled water (100 mL) overnight in cold at 10 °C, husked and ground for 10 min in a blender. The mixture was then filtered through double layered cheesecloth and centrifuged at 5000 rpm for 10 min. The supernatant soymilk had total protein content of 16.5 mg·mL^{-1}. This was diluted to required protein content using distilled water for studies with protein binding to the polymer.

2.2.2. Polymer functionalization

The epoxy polymer (2 g) was stirred with a 25% solution of appropriate amine in methanol-water (20 mL, 50% v/v) overnight. The supernatant was decanted, the polymer was washed with methanol (5 × 10 mL) till the supernatant did not show any absorbance at 220 nm (complete removal of unreacted aromatic amine) and then with distilled water till pH was neutral. The beads were dried under vacuum at 80 °C for 12 h. BSA coated IB-350 polymer beads were prepared as described earlier [31].

2.2.3. Naphthalene binding

The polymer (25 mg) was added to naphthalene solution in distilled water (0.2 mM, 10 mL) in falcon tubes and the contents were shaken in a cold chamber at 10 °C for 1 h. Absorbance of the supernatant was measured at 274 nm ($\Delta\varepsilon$ = 4545 M^{-1} cm^{-1}).

2.2.4. SBTI assay via trypsin inhibition

Trypsin activity was assayed using N_α-benzoyl-L-arginine *p*-nitroanilide (BAPNA) as its specific substrate. BAPNA stock solution (2.5 mM) was prepared in Tris-HCl buffer (0.05 M, pH 8.2) containing $CaCl_2$ (1 mM). Trypsin stock solution (1 mg·mL^{-1}) was prepared in 1 mM HCl and stored at 5 °C. For measurement of trypsin activity, the substrate solution (2 mL) was placed in quartz cuvette at 25 °C, trypsin solution (10 μL) was injected and activity was measured through change in absorbance at 410 nm ($\Delta\varepsilon$ 8800 M^{-1} cm^{-1}).

For assay of SBTI, soymilk containing SBTI (10–50 μL) was first mixed with the substrate solution (2 mL) in cuvette. Trypsin solution (10 μL) was then injected and its activity was measured. The difference in activity of native trypsin (A_0) and that of trypsin in presence of SBTI (A_s) gave the value for trypsin inhibitor activity. Control experiments were performed with commercially available soymilk trypsin inhibitor. Based on trypsin inhibition assay, the soymilk sample with protein content of 16.5 mg·mL^{-1} contained 1 mg·mL^{-1} SBTI.

2.2.5. Lipoxygenase assay

Lipoxygenase was assayed by using linoleic acid as substrate. The amount of linoleic acid hydroperoxide formed due to enzymatic oxygenation was measured with Ferrous Oxidation-Xylenol orange (FOX) reagent [32]. Stock substrate solution (14.3 mM) was prepared in 0.05 M borate buffer containing 20% ethanol, pH 9.0. Typically, soymilk containing LOX (500 μL) was stirred with linoleic acid solution (500 μL) for 1 h. Aliquots of reaction mixture (10 μL) were removed periodically (every 10 min) and added to FOX reagent (2 mL). After incubation for 15 min, absorbance was measured at 560 nm ($\Delta\varepsilon$ = 4.25 × 10^4 M^{-1} cm^{-1}). Control experiments were performed with commercially available soymilk lipoxygenase as standard. Lipoxygenase (LOX) activity for commercially available LOX was found to be 2980 kunits·mg^{-1}. Based on this assay, the soymilk sample prepared by us contained 2380 kunits·mL^{-1} (0.76 mg·mL^{-1}) LOX.

2.2.6. Protein adsorption experiments

The functionalized polymer (0.1–1 g) was shaken with: a) solution of soymilk (10 mL); b) commercial SBTI (20 mg in 10 mL water); and c) standard lipoxygenase (20 mg in 10 mL water) on an orbital shaker at 100 rpm for 1 h at room temperature. The polymer was separated from the supernatant by centrifugation at 5000 rpm for 10 min. The supernatant was filtered through 0.2 μ filter and used for assay of LOX and SBTI. The polymer was washed with distilled water (2 × 10 mL) and bound proteins were recovered by desorption.

2.2.7. Protein desorption

LOX desorption from the polymer was performed by shaking the recovered polymer with 0.01 M citrate-phosphate buffer, pH 8.0 (10 mL) while SBTI was recovered by shaking the polymer with 1% acetic acid solution (pH 3.0) on an orbital shaker at 150 rpm for 1 h. The supernatant was collected and analyzed for protein content using Bradford assay.

2.2.8. Polymer recycle

After elution of SBTI, the DIL-DEA polymer (1 g) was washed with 0.1 M sodium phosphate buffer (2 × 5 mL, pH 7.0) followed by distilled water (2 × 5 mL) and reused for protein binding. The BSA coated polymer after SBTI elution (1 g) was washed with ice cold 0.1 N NaOH (2 × 5 mL) to remove adsorbed proteins. The beads were washed once with distilled water (5 mL) then suspended in distilled water (10 mL) and pH of supernatant was adjusted to 7.0 by addition of 1 N acetic acid. The supernatant was discarded and the beads were washed with distilled water (2 × 5 mL) till the pH of supernatant was neutral.