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Monoliths for the purification of whey protein–dextran conjugates

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ABSTRACT

Proteins conjugated to neutral biopolymers are of keen interest to the food and pharmaceutical industries. Conjugated proteins are larger and more charge shielded than un-reacted proteins, making purification difficult using conventional beaded chromatographic supports because of slow mass transfer rates, weak binding, and viscous solutions. Past methods developed for pharmaceuticals are unsuitable for foods. In this work, a food-grade whey protein–dextran conjugate was purified from a feed solution also containing un-reacted protein and dextran using either a column packed with 800 mL of a beaded support that was specifically designed for purification of conjugated proteins or an 8 mL tube monolith. The monolith gave a similar dynamic binding capacity as the beaded support (4–6 g/L), at a 42-fold greater mass productivity, and 48-fold higher flow rate, albeit at somewhat lower conjugate purity. Performance of the monolith did not depend on flow rate. In conclusion, monoliths were found to be well suited for the purification of whey protein–dextran conjugates.

1. Introduction

Conjugated proteins have revolutionized the biopharmaceutical industry. The development of protein–polysaccharide conjugate vaccines (*Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitides* glycoconjugates) created a new era in vaccine design, saving lives in infants and children worldwide [1–3]. Conjugation of therapeutic proteins to polyethylene glycol (PEGylation) reduces immunogenicity and increases plasma half-life [4]. Success of PEGylated proteins in clinical trials, has led to substantial improvements in healthcare for patients suffering from diseases such as hepatitis, rheumatoid arthritis, neutropenia, severe combined immunodeficiency, acromegaly, and acute childhood lymphoblastic leukemia, among others [4,5].

In the food industry, protein–polysaccharide conjugates are useful because of improved functional properties over proteins [6–11]. Conjugates have improved heat and pH stability, solubility, emulsification, and gelation properties and may offer lower astringency and allergenicity compared to unmodified proteins [12–14]. Food grade methods must be used to manufacture conjugated proteins destined for human consumption. Conjugation using traditional protein chemistry reactions such as carbodiimide is not allowed. Food grade conjugates can be formed using the Maillard reaction producing non-enzymatic glycosylation between amino acids or proteins and reducing sugars.

The Maillard reaction has usually been conducted using a dry heating step employing temperatures up to 80 °C and taking up to 3 weeks for significant conjugate formation. Recently, an aqueous heating method using mild heating conditions (60 °C) was developed to limit the Maillard reaction to the very initial stage of Schiff base formation prior to color formation [12–14]. The aqueous method was adapted in the present work to covalently attach whey protein to dextran without the use of toxic chemicals and materials.

Purification of conjugate from the mixture of un-reacted whey proteins and dextran was a major challenge. No food-grade purification methods for conjugates were published. Previous work used two conventional packed-bed chromatography columns in series: a beaded cellulose weak cation exchange column to separate conjugate from un-reacted protein and un-reacted dextran followed by dialysis to remove salt, and then a concanavalin A column to bind and elute the conjugate only. Sodium azide, an extremely toxic preservative, was used because solutions were adjusted to pH 6.8 where microbial growth was rapid. Furthermore, the conjugate was eluted from the concanavalin A column using D-mannopyranoside, which is not food grade and is too expensive for food use. Finally, the procedure took many days to produce mg quantities of conjugate [12–14], and the beaded supports used have severe limitations when used for large-scale production and at higher flow rates [15].

A new method had to be developed to manufacture food-grade protein–polysaccharide conjugates. Food production and pharmaceutical production have very different constraints: food manufacture must be inexpensive, use only certain approved buffers and materials, and purity can be lower than for pharmaceuticals. Increasing the linear velocity and conjugate binding capacity,

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and reducing buffer washing volumes, albeit at the expensive of purity, is an acceptable tradeoff for foods.

Most chromatographic separations use columns packed with adsorptive beads mainly composed of functionalized soft matrices such as dextrans, agarose [15], silica or organic polymer beads [16]. These supports have low capacity for large conjugated proteins and cannot be operated at high linear velocities due to compression and compaction [5,17,18].

A new family of supports, porous polymer monoliths, was introduced in the late 1980s and the early 1990s. These early monolithic columns were designed for the ion exchange separation mode [17,19–21]. Monoliths consist of a unique polymeric support matrix that enables extremely fast, highly efficient separations of proteins. Monoliths have faster mass transfer rates because the solute transfer takes place by convection rather than diffusion as found in conventional bead based-columns [22–24]. Chromatographic monoliths exist as three types of compositions – inorganic (silica-based), synthetic (polymethacrylate, polyacrylamide and polystyrene-divinylbenzene) and natural (agarose and cellulose) [25].

The purpose of the present study was to examine the use of traditional chromatography beads and polymethacrylate monoliths for the purification of protein–polysaccharide conjugates for food use. The use of acidic pH to inhibit microbial growth and avoid the use of toxic preservatives, the use of food grade buffers, and low cost were goals in developing a process. To reduce the cost of manufacture of conjugates for food use, productivity must be increased and buffer and capital costs must be decreased compared to analytical separations or separations for the production of pharmaceuticals. Conversely, purity requirements for food use are less stringent than for pharmaceutical applications. Monoliths were compared to traditional beaded supports in a packed bed chromatography column because monoliths offered increased throughput and have been found to be well suited for the purification of large biomolecules such as protein–polysaccharide conjugates.

2. Materials and methods

Whey protein isolate (WPI) was from Davisco Foods International, Inc. (Le Sueur, MN), Dextran (9000–11,000 Da) and 2-mercaptoethanol were from Sigma–Aldrich (St. Louis, MO). Ready gels (Tris–HCl Gel, 4–20% linear gradient, 10/15 wells), prestained molecular mass standards, Tris/glycine/SDS premixed buffer, Laemmli sample buffer and Coomassie Blue G-250 stain were from Biorad Laboratories (Hercules, CA). Gelcode glycoprotein staining kit and Pierce BCA protein assay kit were from Thermo Fisher Scientific, Inc. (Rockford, IL). Chemicals used in the preparation of the buffers were obtained from Fisher Scientific (Pittsburgh, PA). All buffers were prepared and pH adjusted at room temperature (20–22 °C).

WPI was further purified by ultrafiltration and diafiltration to remove traces of residual lactose. Purified WPI and dextran were dissolved in 10 mM sodium phosphate buffer, pH 6.8 in the mass ratio of 1:3 (Fig. 1). The solution was stirred at room temperature to dissolve large pieces followed by gentle stirring at 4 °C overnight for complete hydration. An aliquot of this mixture was frozen at −20 °C as a control. The remaining mixture was heated in a water bath maintained at 60 °C for 24 h. Next, the mixture was cooled on ice, diluted 5-fold with 10 mM sodium phosphate buffer, pH 6.8 to reduce viscosity, and centrifuged at 10,000 × *g* for 40 min to remove sediment. The supernatant was adjusted to pH 5.0 and centrifuged at 10,000 × *g* for 40 min to remove a portion of the unreacted protein by iso-electric precipitation. Whey proteins have an iso-electric point near pH 5.0. The supernatant thus obtained

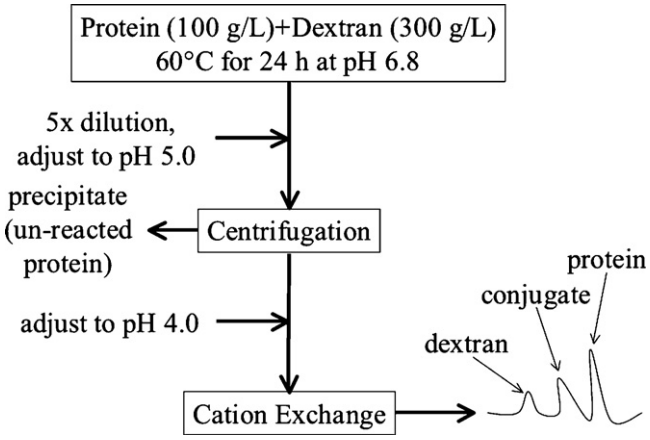

Fig. 1. Schematic diagram of conjugate reaction and purification procedure.

was adjusted to pH 4.0 and used as the feed solution to the chromatography system.

Chromatography experiments were conducted using BPG 100 column containing MacroCap SP cation exchanger from GE Healthcare (Piscataway, NJ) and CIM SO_3-8f tube monolithic column from BIA Separations (Ljubljana, Slovenia).

MacroCap SP is a cation exchanger specifically designed to purify PEGylated proteins and other large biomolecules. It consists of 50 μm diameter beads made of a cross-linked copolymer of allyl dextran and *N*,*N*-methylene bisacrylamide functionalized with a sulfonic acid ($-SO_3$) strong cation exchange moiety. The column had a bed height of 102 mm and a bed volume of 800 mL. The column was connected to a 280 nm detector (model UV-1) and chart recorder (model REC 112) both from GE Healthcare (Piscataway, NJ). A peristaltic pump (Masterflex L/S Easy Load, Cole-Parmer Instrument Company, Vernon Hills, IL) was used to supply the column. The column was equilibrated using 8 L of 50 mM sodium lactate buffer, pH 4 at a flow rate of 136 mL/min. The pH-adjusted feed solution (640 mL) was loaded into the column at a reduced flow rate of 62 mL/min, because it was viscous and caused the pressure to rise. After loading the feed solution, the flow rate was increased back to 136 mL/min. The column was washed with 8 L of equilibration buffer. The WPI–dextran conjugate was eluted using 8 L of 50 mM sodium lactate, 300 mM NaCl, pH 4.0. Un-reacted protein was eluted using 1.6 L of 50 mM sodium lactate, 1 M NaCl, pH 4.0. The column was rinsed using 2 L of equilibration buffer and flushed with 1.6 L of 0.5 M NaOH for storage overnight.

The CIM SO_3-8f monolith consisted of a poly (glycidyl methacrylate-co-ethylene dimethacrylate) support matrix functionalized by a sulfonic acid strong cation exchanger moiety. It had an outer diameter of 15 mm, inner diameter of 6.5 mm, length of 56 mm, and bed volume of 8 mL. It was connected to the flow system mentioned previously except smaller tubing was used that had an inner diameter of 1.6 mm instead of 4.8 mm. The monolith was equilibrated using 32 mL of 50 mM sodium lactate buffer, pH 4. The pH-adjusted feed solution (6.4 mL) was loaded into the monolith at a flow rate of 16, 32, or 64 mL/min. The monolith was washed using 32 mL of equilibration buffer. The WPI–dextran conjugate was eluted using 48 mL of 50 mM sodium lactate, 300 mM NaCl, pH 4.0. Un-reacted protein was eluted using 48 mL of 50 mM sodium lactate, 1 M NaCl, pH 4.0. The column was rinsed using 16 mL of equilibration buffer and flushed with 20 mL of 0.1 M NaOH for storage overnight.

Fractions collected were analyzed by absorbance at 280 nm, bicinchoninic acid assay (BCA) and gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Mini-Protean 3 cell (Bio-Rad). Reducing SDS-