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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

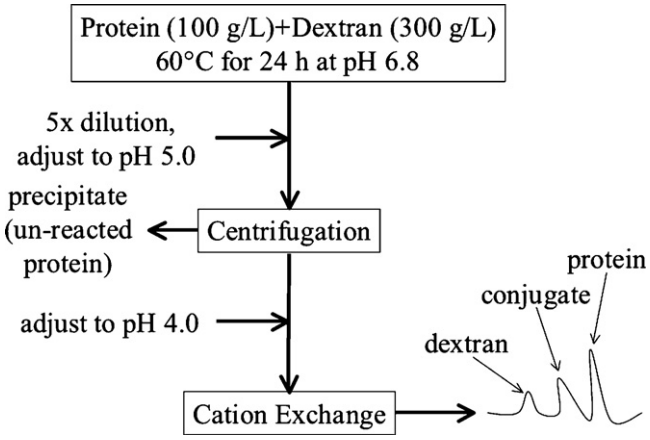

Fig. 1. Schematic diagram of conjugate reaction and purification procedure.

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-

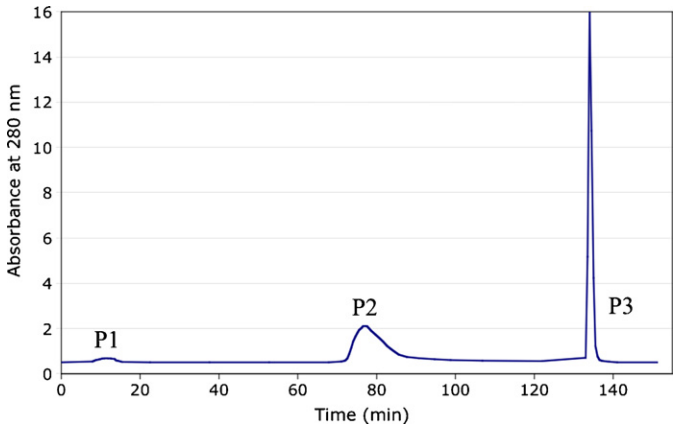

Fig. 2. Chromatogram for 800 mL of beaded support in a 10-cm diameter chromatography column. Unbound material in flow through (peak P1). Conjugate eluted at low salt (peak P2). Un-reacted protein eluted at high salt (peak P3).

PAGE analysis was carried out in parallel on a two identical Ready Gels (Tris–HCl Gel, 4-20% linear gradient, 15 wells). Samples were concentrated before application to the wells to normalize the protein concentration (Amicon Ultra-4 Centrifugal Filter Units, 3 kDa, Millipore, Bedford, MA). Electrophoresis was for 35 min at 200 V in 25 mM Tris–HCl buffer (pH 8.3, including 0.192 M glycine and 0.1% w/w SDS) at room temperature. After electrophoresis, one gel was stained for protein using Coomassie Blue G-250 stain, and the other gel for glycoprotein using the GelCode glycoprotein staining kit.

3. Results

First, the new process for conjugate formation, iso-electric precipitation, and purification using a single ion exchange chromatography step was evaluated using the beaded support. The chromatogram contained three peaks (Fig. 2): unbound material in the flow through solution (P1), the low salt elution peak (P2), and the high salt elution peak (P3). The composition of each peak was characterized by SDS-PAGE (Fig. 3). Protein staining is shown in panel (a) and glycoprotein staining in panel (b).

Before reaction (lane 0 h, panels (a) and (b), Fig. 3), the protein and dextran mixture contained no conjugate and consisted of the primary whey proteins beta-lactoglobulin (BLG, 18.6 kDa) and alpha-lactalbumin (ALA, 14.4 kDa). After reaction (lane 24 h), conjugate was produced as indicated by the wide distribution of protein sizes (smeared band of apparent size 20–100 kDa), all larger than the unmodified whey proteins, which are also present. After the iso-electric precipitation step, the feed solution (lane FS) contained little to no ALA, and a greater ratio of conjugate to un-reacted BLG.

After chromatography, the flow through solution (lane P1, panels (a) and (b), Fig. 3) was devoid of un-reacted protein and conjugate. Dextran alone does not stain using protein or glycoprotein stain, and although likely present in the flow through peak P1, did not show in lane P1. The low salt elution peak (lane P2) consisted of conjugate primarily and some BLG. The high salt elution peak (lane P3) contained primarily BLG and traces of conjugate. Glycoprotein staining in panel (b) revealed that the conjugate was present in the feed solution and peak P2 only. The conjugate had a broad size range of about 20–100 kDa, with the majority of the conjugate falling within the range 25–60 kDa.

Mass balances on the peaks (Table 1) confirmed SDS-PAGE analysis in that the flow through solution contained almost no protein; less than 3% of the protein in the feed solution. The bound protein was split equally between peaks P2 and P3. This result corresponded to about half the protein in the feed solution was conjugate and the other half was un-reacted protein. The mass balance closed; there was no statistical difference between the protein loaded in the feed solution and the sum of the protein that emerged in peaks P1, P2, and P3 ($p > 0.05$). The dynamic binding capacity of the beaded support was about 6 g/L, similar to the value reported by the manufacturer of 4–6 g/L.

The chromatogram for the monolith was measured at three different flow rates (Fig. 4). Flow rate had no impact on the shape of the chromatograms; only time was shortened as flow rate increased. Flow rate had no impact on SDS-PAGE analysis (data not shown). Only the result for the highest flow rate (480 CV/h) is shown (Fig. 5). The loading volume and salt elution steps used for the monolith were the same as those used for the beaded support. The low salt elution peak (lane P2) contained the majority of the conjugate, and some BLG. The high salt elution peak (lane P3) contained primarily BLG and a small amount of conjugate. Although peak P1 appeared to contain a substantial amount of conjugate in panel (b) of Fig. 5, this was not true as shown by mass balance below. Each sample was concentrated to different extents by ultrafiltration before SDS-PAGE analysis to equalize the protein concentration applied to each lane to 2 g/L. Although all lanes appeared equally dark in panel (a), the protein concentration in each sample before concentration was different by more than 10-fold.

Mass balances (Table 1) revealed no significant difference between the protein mass in peaks P1, P2 or P3, or the total amount eluted versus flow rate ($p > 0.05$). Nearly all the protein in the feed solution emerged in peaks P2 and P3, and total bound protein (conjugated plus un-conjugated) was split 70:30 between peaks P3 and P2 for the monolith. Less than 8% of the protein loaded in the feed solution did not bind and emerged in the flow through in peak P1. The dynamic binding capacity of the monolith was about 5–6 g/L based on absorbance at 280 nm and BCA.

Protein concentration was measured by A_{280} and BCA to provide two independent measures of protein for the mass balance calculations (Table 1). There was no significant difference ($p > 0.05$) between A_{280} and BCA for the protein concentration in the feed solution; peaks P2 and P3; or the total amount of protein emerging from the monolith. There was a statistically significant difference ($p < 0.05$) between A_{280} and BCA for flow through peak P1 that was attributed to the high dextran content of peak P1. Dextran was found to be a weakly interfering substance for BCA when present at high concentrations and low protein concentrations. This was only an issue for peak P1 and of little consequence because peak P1 contained a negligible amount of protein. Examination of the BCA data from all peaks revealed that 1 absorbance unit at 280 nm corresponded to about 1 g/L protein.

In summary, a sulfonic acid functionalized monolith and a beaded support were used successfully to purify protein–polysaccharide conjugates from a mixture of un-reacted protein and dextran. The conjugate eluted at low salt and the un-reacted protein at high salt. Both chromatographic media had a dynamic binding capacity of about 4–6 g protein per Liter of media.

The main difference between the two chromatographic media was speed; it took about 140 min per chromatogram for the beaded support compared to about 2 min for the monolith. Flow rate for the beads was limited by pressure drop to about 10 CV/h, but not for the monolith, where flow rates of 480 CV/h were possible. Flow rates greater than 480 CV/h were not examined solely because the experimental system would not allow it. The performance of the monolith did not depend on the flow rate.

4. Discussion

For food versus pharmaceutical applications, throughput and buffer consumption are relatively more important than purity. The mass productivity of the monolith and beads can be cal-

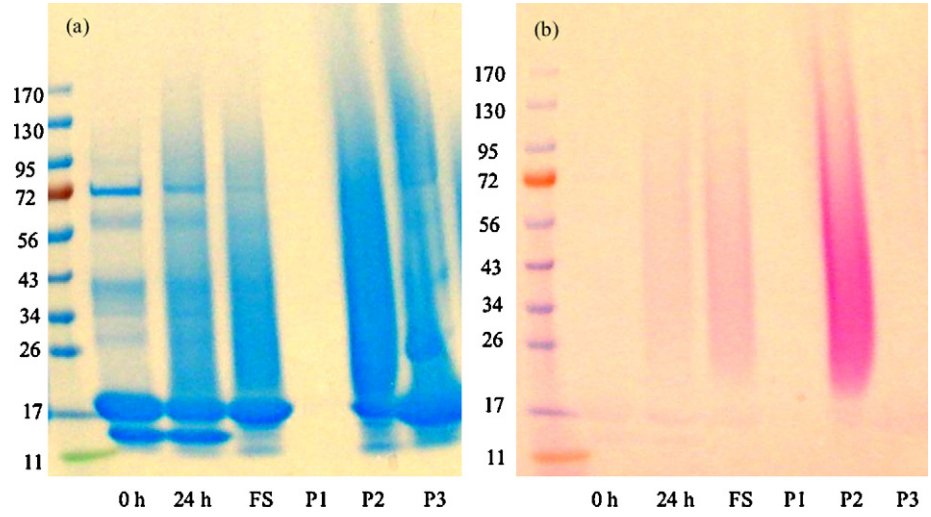

Fig. 3. SDS-PAGE for fractions from the beaded support. Coomassie Blue protein stain in panel (a), and glycoprotein stain in panel (b). Lane 0 h = reaction mixture before heating. Lane 24 h = reaction mixture after heating. Lane FS = feed solution. Lane P1 = unbound material in flow through of peak P1. Lane P2 = conjugate eluted at low salt in peak P2. Lane P3 = un-reacted protein eluted at high salt in peak P3. Molecular mass markers in kDa.

Table 1
Mass balance calculations for beaded support and monolith.

	Beads		Monolith				
	Amount collected (CV[a])	A_{280} (AU × CV)	Amount collected (CV)	A_{280} (AU × CV)			BCA (g/L × CV)
Flow rate		10 CV/h		120 CV/h	240 CV/h	480 CV/h	120 CV/h
Feed solution	0.8	5.66 ± 0.06[b]	0.8	5.66 ± 0.06	5.66 ± 0.06	5.66 ± 0.06	5.99 ± 0.04
P_1 peak	10	0.18 ± 0.01	4	0.49 ± 0.03	0.39 ± 0.02	0.44 ± 0.05	0.97 ± 0.03
P_2 peak	10	2.94 ± 0.02	6	1.67 ± 0.09	1.63 ± 0.05	1.76 ± 0.06	1.7 ± 0.1
P_3 peak	2	2.9 ± 0.2	6	3.9 ± 0.6	4.00 ± 0.03	4.15 ± 0.08	3.2 ± 0.4
Equilibration buffer			2	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.00	0.01 ± 0.00
0.1 M NaOH			2.5	0.05 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.06 ± 0.03
Total		6.0 ± 0.3		6.2 ± 0.5	6.14 ± 0.07	6.5 ± 0.2	5.9 ± 0.3

[a] Column volumes.
[b] Mean ± SD, $n = 2$.

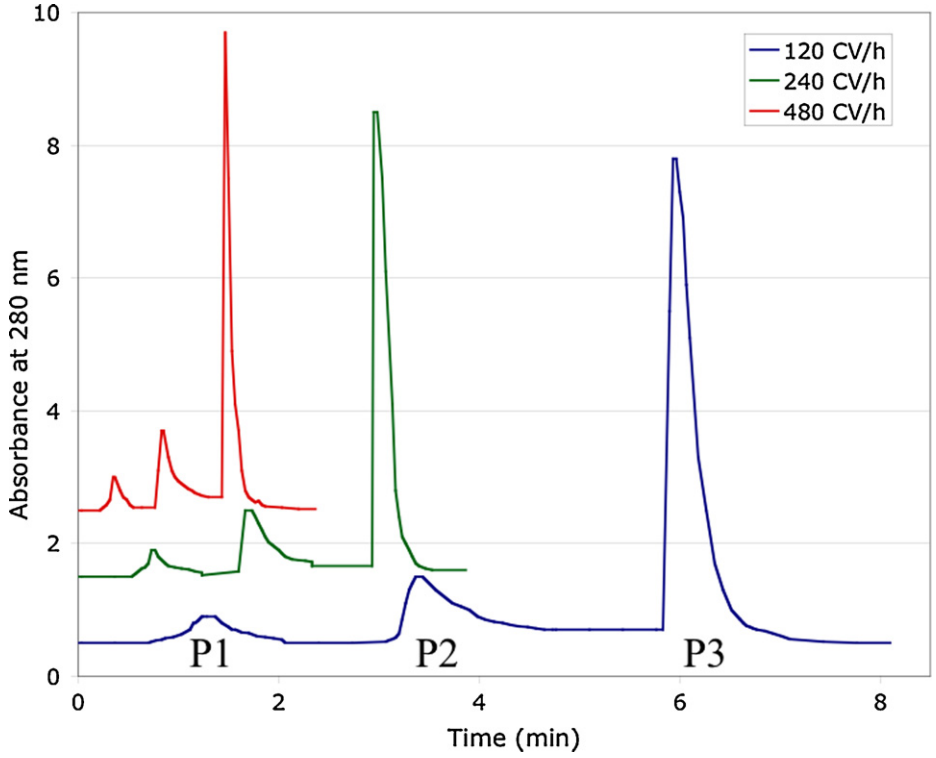

Fig. 4. Chromatograms for the 8 mL monolith at three different flow rates. Unbound material in flow through (peak P1). Conjugate eluted at low salt (peak P2). Un-reacted protein eluted at high salt (peak P3).

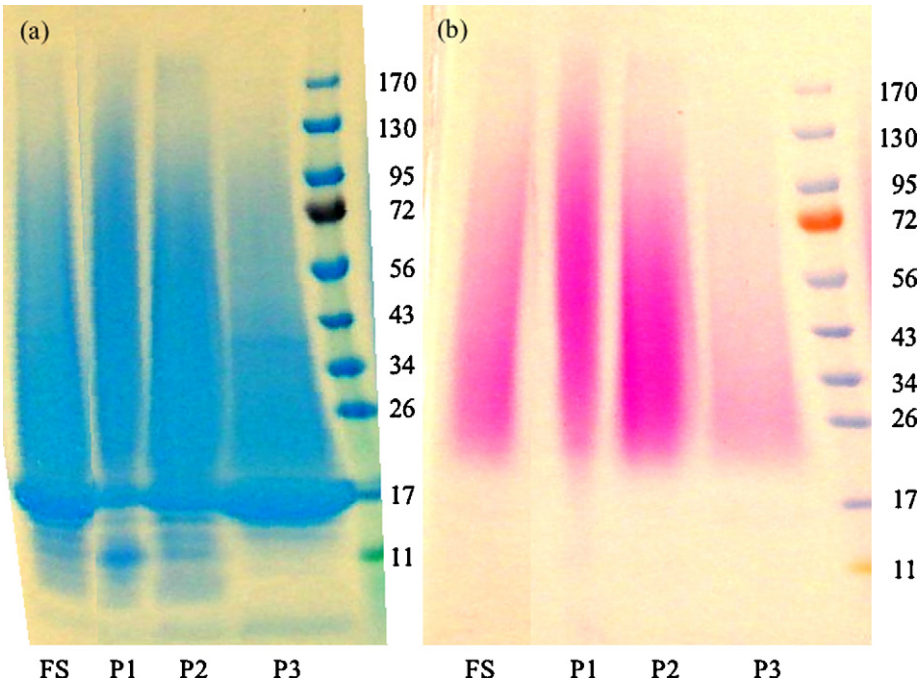

Fig. 5. SDS-PAGE for fractions from the monolith. Coomassie Blue protein stain in panel (a), and glycoprotein stain in panel (b). Lane FS = feed solution. Lane P1 = unbound material in flow through of peak P1. Lane P2 = conjugate eluted at low salt in peak P2. Lane P3 = un-reacted protein eluted at high salt in peak P3. Molecular mass markers in kDa.

culated from the data in Table 1. The 800 mL chromatography column produced 2.94 AU × CV of conjugate protein per 140 min chromatogram. This corresponded to 2.35 g of conjugate protein (2.94 AU − CV × 0.8 L = 2.35 g). The 8 mL monolith produced 1.76 AU × CV of conjugate protein or 14 mg in 2 min. To compare on an equal basis, an 800 mL monolith would produce 1.4 g of conjugate protein in 2 min. This corresponds to a mass productivity of 42 g/h of conjugate protein for the monolith compared to 1 g/h for the chromatography beads. Buffer consumption was 22 CV for the beaded support and 16 CV for the monolith (Table 1). However, less buffer could have been used for the beaded support as shown by the long baseline segments in the chromatogram (Fig. 2). Therefore, buffer consumption was about the same for the two different supports.

Although it appears from the beaded support chromatogram (Fig. 2) that excessive elution volumes were used, dextran was not detected at 280 nm. Reducing the elution volumes caused unreacted dextran to contaminate the conjugate peak (P2), and loss of some conjugate into the P3 peak. Dextran trapped in the beaded support by inadequate washing led to reduced dynamic binding capacity in subsequent chromatograms. Fine adjustments to the salt concentration used for peak P2 shifted the balance between yield and purity, but were not better overall. Reducing the elution volumes would not substantially reduce the 42-fold increase in productivity going from the beaded support to the monolith.

Purity of the conjugate protein was comparable for the beaded support and the monolith (lane P2, panel (a), Figs. 3 and 5). Both supports contained primarily conjugate protein in peak P2 with traces of un-reacted BLG. Un-reacted protein emerged primarily in peak P3 for both supports, but the salt concentration used for peak P2 was not great enough to elute all the bound conjugate for the monolith. The result was that some of the bound conjugate protein was lost into peak P3 for the monolith, but not for the beaded support (see lane P3, panel (b), Figs. 3 and 5). Slightly increasing the salt concentration for peak P2 for the monolith shifted conjugate protein from peak P3 into peak P2, but also shifted some un-reacted protein into peak P2 (data not shown). There was a tradeoff between purity and yield for the monolith not found for the beaded support.

The conjugate peak P2 was substantially broader than the unreacted protein peak P3 for both supports (Figs. 2 and 4). For example, the peak width at half height was 1.4 CV for peak P2 and 0.2 CV for peak P3 using the beaded support. For the monolith, it was 1.2 CV for peak P2 and 0.6 CV for peak P3. A heterogeneous mixture of conjugate stoichiometries in the feed solution would be expected to broaden peak P2, but not peak P3, which was comprised primarily of the homogeneous un-reacted protein BLG. Furthermore, the lower salt concentration of peak P2 increased selectivity of elution, whereas the high salt of peak P3 disrupted all binding and caused un-selective elution of all remaining bound protein.

The conjugate protein was comprised of more than monodextran conjugates as seen by the wide distribution of sizes in the glycoprotein stained electrophoresis gels (panel (b), Figs. 3 and 5). One BLG (18.6 kDa) conjugated to one dextran (10 kDa) would have given a narrower band on the gel than was observed. The large excess of dextran in the reaction mixture favored formation of a plurality of dextran molecules conjugated to each protein. More dextran molecules per protein molecule would likely cause more charge shielding and require less salt for elution. A similar occurrence was observed with PEGylated conjugates where these species bound weakly to the cation exchanger and this weak binding was attributed to the charge-shielding effect of the neutral polymer, poly(ethylene glycol) (PEG) [26–29]. During the rise in salt concentration from 0.0 to 0.3 M for peak P2, multi-dextran conjugates would elute first, followed by di-dextran conjugates, and lastly mono-dextran conjugates [28]. This selective elution sequence would broaden peak P2. Conversely, the abrupt rise in salt concentration to 1.0 M for peak P3 would unselectively disrupt all binding of the remaining bound protein, which would be predominately the smaller un-conjugated protein. This would sharpen peak P3.

For food applications, the proper balance between purity, throughput, and yield is different than for pharmaceutical products. For example, a mixture of mono-dextran and multi-dextran conjugates containing traces of un-conjugated whey protein may

be acceptable for food use whereas pharmaceutical products may require mono-PEGylated protein pristinely free of different forms of conjugated protein and un-conjugated protein. Food products are generally lower in value than pharmaceutical products. Faster processing can outweigh concerns about lower purity and yield for food products, but a different balance might be struck for pharmaceutical products. The 42-fold increase in throughput of the monolith compared to the beaded support found in this work must balanced against a lower purity and yield. The monolith had a 48-fold higher flow rate than the beaded support. This high flow rate was due to high porosity of the monolith resulting in lower pressure drop than that of conventional packed bed [30,31]. For food applications, monoliths were found to offer a high throughput method to purify whey protein-dextran conjugates from un-conjugated reactants.

5. Conclusions

Conjugation of polysaccharides to proteins increases heat stability, solubility, emulsification properties, and may lessen astringency and allergenicity compared to un-conjugated proteins. This work developed the first food-grade methods to manufacture and purify whey protein–dextran conjugates. Conjugation using the Maillard reaction was followed by iso-electric precipitation to remove un-reacted whey protein prior to chromatographic purification using either a beaded support or a monolith. Both supports successfully fractionated conjugated protein from un-conjugated reactants, had a similar dynamic binding capacity of 4–6 g/L, and a similar buffer consumption of 16–22 CV per chromatogram. The main difference was the monolith gave a 42-fold higher productivity than the beaded support largely because of a 48-fold higher flow rate. This came at the expense of a somewhat lower purity and yield for the monolith.

The whey protein–dextran conjugate appeared as a heterogeneous 20–200 kDa band by gel electrophoresis, much larger than the 14–18 kDa whey proteins or 10 kDa dextran. The broadness of the conjugate elution peak at low salt compared to the un-reacted protein peak at high salt was attributed to this heterogeneity.

Monoliths were found to be well suited for purification of conjugated proteins for food use. The high throughput of monoliths combined with reasonable purity balanced well against the higher purity and yield of the beaded support at a substantially lower throughput.

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