Protein Recovery from Enzyme-Assisted Aqueous Extraction of Soybean Oil

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Introduction

Because of the high transportation cost of bio-based feedstocks, the transition from a petroleum-based to a bio-based economy will require the development of small-scale distributed production centers rather than large centralized facilities seen in industry today. In vegetable oil production, the use of hazardous petroleum solvents drives up capital costs, resulting in larger processing plants and, therefore, increased transportation costs. In contrast, enzyme-assisted aqueous extraction processing (EAEP) uses only water as a solvent and is potentially less capital intensive.

One EAEP process developed at the Center for Crops Utilization Research (CCUR) at Iowa State University uses extrusion to disrupt cells of soy flakes, from which oil and water soluble components are extracted with the assistance of proteases, resulting in three fractions: residual solids, skim (an aqueous fraction with emulsified oil stable toward creaming), and cream (emulsified oil) [1].Protein values, which potentially account for 65% of total EAEP revenues, are in the solid and skim fractions. The skim fraction differs from the traditional protein-containing fractions and characterization and recovery of this protein is critical to implementation of this alternative process.

We have investigated methods to recover the hydrolyzed proteins of the skim fraction of the CCUR process with an additional benefit of reduction of antinutritional factors, such as oligosaccharides and trypsin inhibitors [1]. Traditional protein concentration methods such as pH precipitation, ultrafiltration, and ion exchange chromatography are complicated not only by the increased solubility and small molecular weight of hydrolyzed polypeptides, but also by the presence of emulsified oil in the skim fraction. Results from various recovery methods will be presented.

Materials and Methods

Skim was produced following a process developed by [1]. Extruded pellets were provided by the Center for Crops Utilization and Research. Two hundred grams of extruded flake pellets were added to 2L of deionized water with 0.5% (w/w flour) endoprotease Protex 6L, (Genencor Int., Rochester, NY) in a 2L jacketed reactor (Model 4742, Chemglass Inc., Vineland, NJ) held at 50 °C by a circulating water bath and agitated at 500 rpm by a stirrer (Model BDC 3030, Caframo, Ltd., Wiarton, Ontario) with a 1-inch, 3-bladed screw impeller. Constant pH 9 was maintained by an autotitrator (Model 718 Stat Titrino, Metrohm, Ltd., Herisau, Switzerland) by adding 2M sodium hydroxide. Following extraction, the slurry was centrifuged at 3000 g, 15 min, 20 °C to remove the solids. Skim and cream fractions were separated overnight in a separatory funnel at 4 °C.

Ultrafiltration of skim was carried out with a YM3 3 kDa nominal molecular weight cut off (NMWCO) regenerated cellulose membrane, (Millipore, Inc. Billerica, MA) in an Amicon 50 ml, 43mm diameter stirred cell (also by Millipore) at 40 psi. For this experiment, skim

fractions were produced under various hydrolysis conditions: 0.5% (w/w solid) Protex 7L (P7L 0.5) for lower hydrolysis, 0.5% Protex 6L (P6L 0.5) for medium hydrolysis, and 1.0% Protex 6L (P6L 1.0) for greater hydrolysis. The three treatments resulted in molecular weight profiles having 28%, 14%, and 10% of the peptides of a molecular weight greater than 10 kDa, respectively, as determined by size exclusion chromatography [1]. Fifty grams of skim were weighed into the membrane cell. Permeate was collected, weighed, and aliquots were withdrawn for saccharide analysis. Retentate composition was calculated as the difference between material content in the permeate and the content of the starting material. Moisture content was determined by weight loss after freeze drying. Protein content of the resulting solids was based on total nitrogen analyzed according to AOAC method 993.13 [2] using a RapidN III combustion analyzer (Elementar Americas, Inc. Mt Laurel, NJ) and a nitrogen to protein conversion factor of 6.25.

Protein recovery by pH precipitation was carried out by placing 50 ml skim in a beaker, adjusting to desired pH by adding 2M hydrochloric acid and agitating for one hour with a magnetic stirrer (pH varied by less than 0.1 pH unit over the course of the experiments). Skim was then transferred to pre-weighed 50 ml centrifuge tubes and centrifuged at 4500 g, 30 min, 20 °C. Supernatant was collected and dried at 130 °C in pre-weighed containers to determine solids content. Protein content was based on total nitrogen content, determined as above. Supernatant protein content was based on the difference between protein present in precipitate and protein present in the untreated skim.

Batch adsorption experiments were carried out by adding 2 to 13.7 g of Streamline Q XL (GE Healthcare, Chalfont St. Giles, United Kingdom), a strong anion-exchange EBA resin, to 50 ml skim in a beaker and agitating 40 min with a magnetic stirrer (such agitation was only used for initial screening as this resin is prone to shear damage from the stir bar). To avoid size segregation, resin aliquots had been obtained by pouring resin slurry into a sintered glass vacuum funnel, rinsing several times with deionized water, draining liquid, and transferring full-depth portions of the cake into a beaker with a spatula. After adsorption, resin was allowed to settle, and the supernatant was collected, dried, and analyzed as above for solids and protein content. Protein concentration was adjusted by diluting skim with a phosphate buffer of pH and conductivity matching that of the skim. Adsorbed protein was calculated by difference from the initial and final liquid phase protein concentrations. Resin was regenerated by adding 1M sodium chloride solution, agitating several minutes, decanting liquid, and repeating until the supernatant was clear.

Breakthrough experiments were conducted with a Streamline 25 expanded bed adsorption column from GE Healthcare using between 20 and 30 g Streamline Q XL resin. The column was equilibrated by pumping a phosphate buffer at a pH and conductivity equal to that of the skim through the column at 15 ml/min (3 cm/min), resulting in an expanded bed height of 12-16 cm. After feed loading, the column was rinsed with equilibration buffer, and then proteins were eluted with 1M sodium chloride solution. Resin was cleaned and regenerated by recycling a 1M sodium chloride, 1M

sodium hydroxide solution (after discarding the first 3 column volumes of effluent) for several hours, and then rinsing for three column volumes with a 20% ethanol solution. Effluent fractions were collected into 15 ml centrifuge tubes. Because of the size of the samples collected, total nitrogen determination was not practical. Protein content was therefore determined by measuring the absorbance of samples at 215 nm with an Ultrospec 4000 UV/visible spectrophotometer (Pharmacia Biotech, now GE Healthcare). Samples were diluted with phosphate buffer to have an absorbance of less than 1 AU/cm, which also reduced sample opacity to negligible levels.

Protein fractions were characterized by size exclusion chromatography (SEC) using a 300 mm x 7.8 mm Biobasic SEC 120 column (BioRad Laboratories, Ltd.) following methods described previously [1] Raffinose and stachyose concentrations were determined by cation-exchange chromatography, also described previously [1].

Results and Discussion

Optimum selectivity between protein and non-protein components, in particular the stachyose/protein ratio, is desired. Of the hydrolysis treatments, the P6L 0.5 treatment gave the best protein-stachyose separation (Figure 1). This treatment is milder hydrolysis than the P6L 1.0 skim [1] providing a larger size difference of selective filtration. The P7L skim has still lower hydrolysis but also lower initial protein concentration that was not offset by any selectivity advantage.

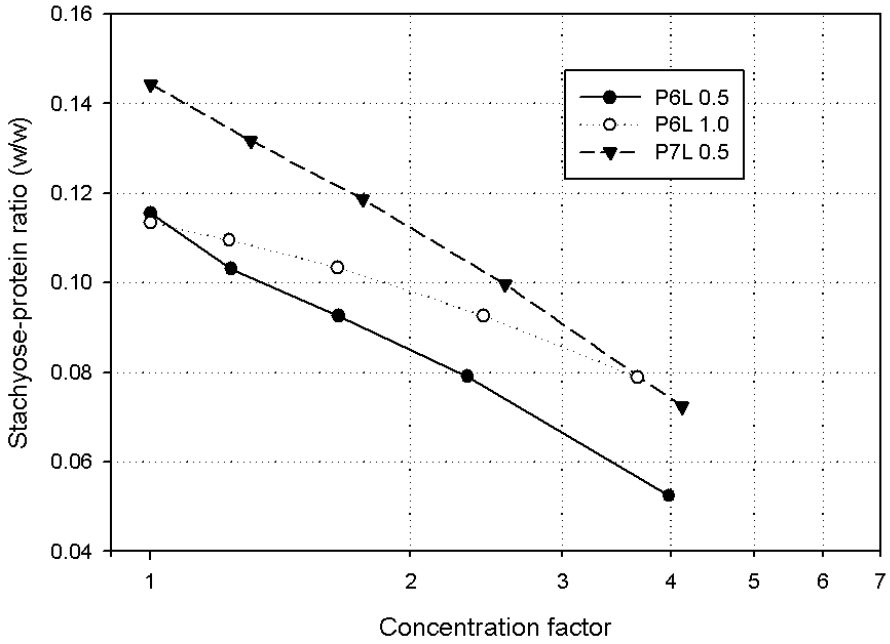

Figure 1- Stachyose-protein ratio of the retentate from skim ultrafiltration with a 3,000 Da NMWCO membrane up to a concentration factor of 4 for skims obtained with three different hydrolysis conditions.

For skim from P6L 0.5, at a concentration factor of four, the dry-basis protein concentration of the retentate had increased from 55% to 70%, while stachyose

decreased from 6% to about 2%, shown in Figure 2. Along those lines, ultrafiltration also achieved significant dewatering with total solids of the retentate increasing from 5% to 14%. The major drawback of this treatment is the loss of valuable protein, which for P6L 0.5 skim was 26%. Experiments with a 1,000 Da NMWCO resulted in decreased protein losses, but the stachyose retention increased as well, resulting in no substantial protein-stachyose separation (data not shown).

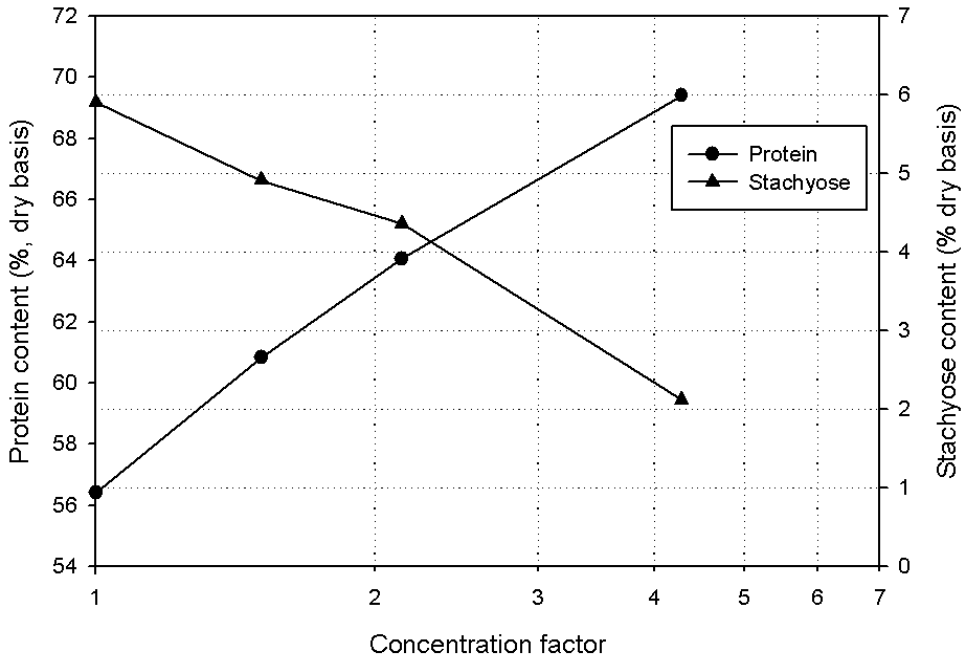

Figure 2- Dry-basis protein and stachyose composition of skim retentate from P6L 0.5 extraction with a 3,000 Da NMWCO membrane.

The solubility profile of skim over a range of pH for skim containing 26 mg/ml and 50 mg/ml protein is shown in Figure 3. Unhydrolyzed soy protein aqueous extracts from soy flours and extrudates have very low solubility (less than 10%) at or near the pI of soy proteins, between pH 4 and 5 [3-5]. The hydrolyzed skim proteins in this case appear to have a minimum solubility at the same pH; however, the percentage of the protein that remains soluble has been dramatically increased, with 70% of the skim proteins remaining in solution at pH 4. This is high compared to other studies of hydrolyzed soy protein. The minimum solubility of a 10 mg/ml extruded soy protein aqueous extract with a degree of hydrolysis of 4% was reported to be about 30% [5]. The reason for the increased solubility appears to be entirely attributable to the hydrolysis. Molecular weight profiles, as determined by SEC (Figure 4), show that no polypeptide with a molecular weight greater than 30 kDa remains in solution at a pH of 4. The large peak to the left on Figures 4 A and B represents large, presumably unhydrolyzed, proteins of molecular weights greater than 30 kDA [1]. While this demonstrates that pH precipitation would be a beneficial first step for a protein recovery process mirroring traditional SPI or SPC production, recovery of the remaining 70% is still necessary for economic success. Furthermore, the high solubility at low pH of the

hydrolyzed fraction would be a potentially valuable functional property setting this apart from traditional SPI.

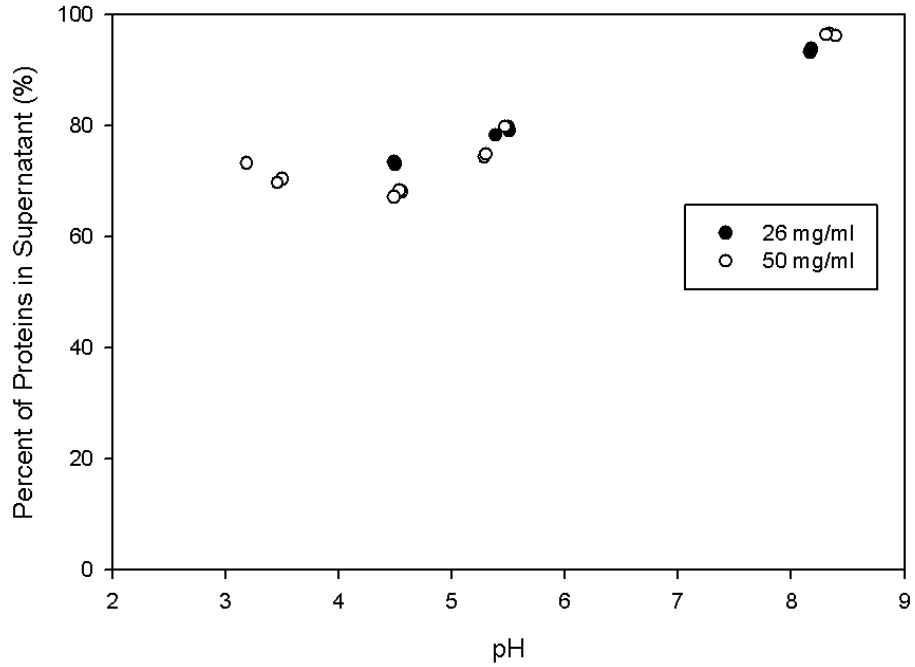

Figure 3- pH solubility profile of skim proteins for trials using 26 mg/ml and 50 mg/ml protein in skim.

It would be logical to suspect that many polypeptides resulting from hydrolysis would have surface charge properties similar to the intact proteins. Since soy proteins have an acidic isoelectric point, and therefore have a net negative charge at high pH, anion exchange chromatography was investigated as a means of recovering a large portion of the hydrolyzed proteins. For batch adsorption experiments, very good repeatability was achieved where the same mass concentration of resin was used, while the specific protein adsorption increased as the resin concentration decreased, as shown in Figure 5. This can be attributed to different binding constants of different protein fractions in the skim. A linear adsorption isotherm model with two different adsorption coefficients fits the data quite well in the linear region of the adsorption isotherms. With low resin concentrations, specific protein adsorption as high as 100 mg/ml was achieved.

The idea of protein fractions of varying adsorption coefficients is supported by chromatography breakthrough curves, shown in Figure 6. Initial experiments used the column in reverse flow (packed bed) mode, and the breakthrough appears very similar to expanded bed mode. During the protein elution, however, severe channeling was observed, apparently caused by resins forming a compact plug during application. Operating the column in expanded bed mode during application and elution seemed to eliminate this problem. Although some early breakthrough is evident, the tracer profile shows that column flow approaches plug flow. Hence, deviation from this behavior for protein breakthrough indicates either selective binding of only portions of the peptides or slow-binding kinetics. The latter should not be the case given the size of the proteins, the resin, and the flow rate. Overall protein balances have closures of around 95%, with

19% and 17% of total applied protein remaining bound after rinsing for 2 and 4.5 bed volumes applied, respectively. Based on this, the specific protein adsorption after rinsing was 24 mg/g and 46 mg/g for the two respective trials, much lower than would be expected based on batch adsorption data. Protein and resin conditions used in these two EBA trials correspond with those used in two of the batch trials, which resulted in 44 mg/g and 74 mg/g adsorbed protein, respectively. In other words, had these EBA experiments been conducted as batch adsorption experiments with the same masses of skim and resin, the specific protein adsorption would have been almost double the mass adsorbed under plug flow operation.

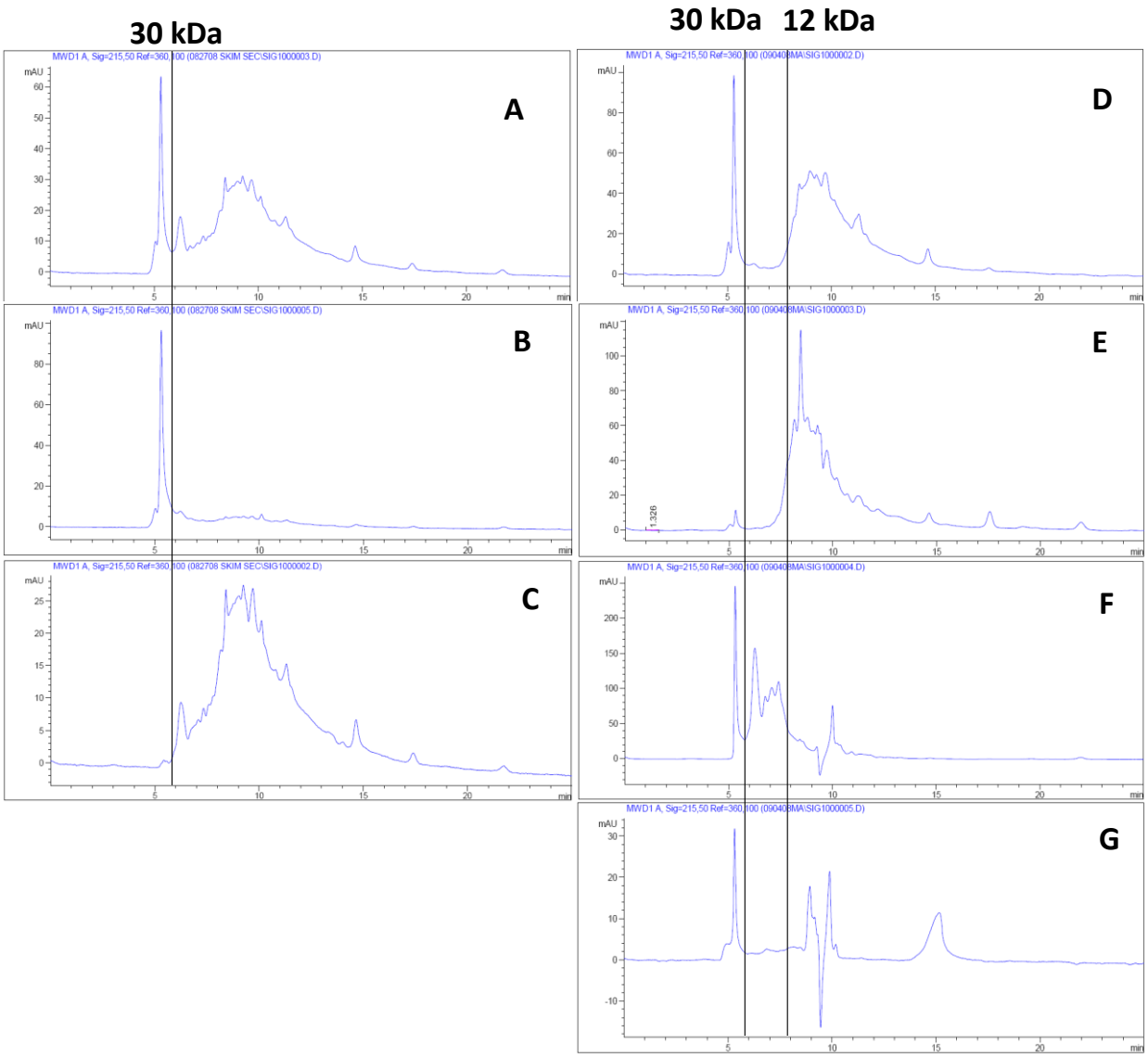

Figure 4-Size exclusion chromatography profiles for (A) untreated skim at pH 9, (B) proteins precipitated at pH 4.0, (C) supernatant proteins at pH 4, (D) EBA column breakthrough at applied volume of 4 column volumes, (E) proteins rinsed from column using equilibration buffer (40mM phosphate buffer, pH9), (F) proteins eluted from column using 1M NaCl, and (G) proteins eluted from column during cleaning cycle using 1M NaOH with 1M NaCl. Elution times corresponding to molecules of 30 kDa and 12 kDa molecules are indicated. Sample dilution and injection volumes were all such that approximately 10 μg of protein were injected for each chromatogram.

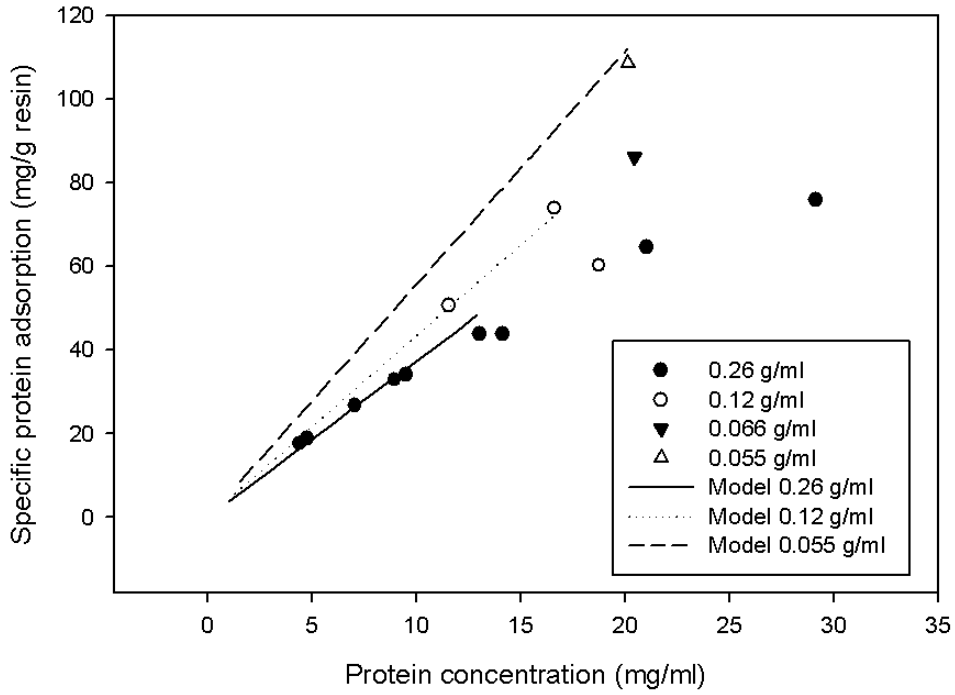

Figure 5- Adsorption isotherm for Streamline Q XL resin with skim using different resin concentrations for batch adsorption experiments with preliminary model fit.

SEC profiles of selected EBA eluent fractions are shown in Figure 4 D through G. The profile for column effluent collected after 4 column volumes had been applied appears to be practically identical to the profile of the initial skim (Figure 4 A), except for polypeptides between 12 and 30 kDa, which bind strongly to the resin with no breakthrough. Even though the >30 kDa proteins appear in the breakthrough chromatograph, they do not appear in the rinse fraction (Figure 4 E), indicating strong adsorption. The presence of these proteins in the breakthrough indicates that either adsorption sites specific to these have become saturated or their binding equilibrium coefficients are less than the 12-30 kDa fraction, or both. Another fraction of strongly binding proteins is seen between 3.9 kDa and 7.4 kDa, in Figures 4 F and G.

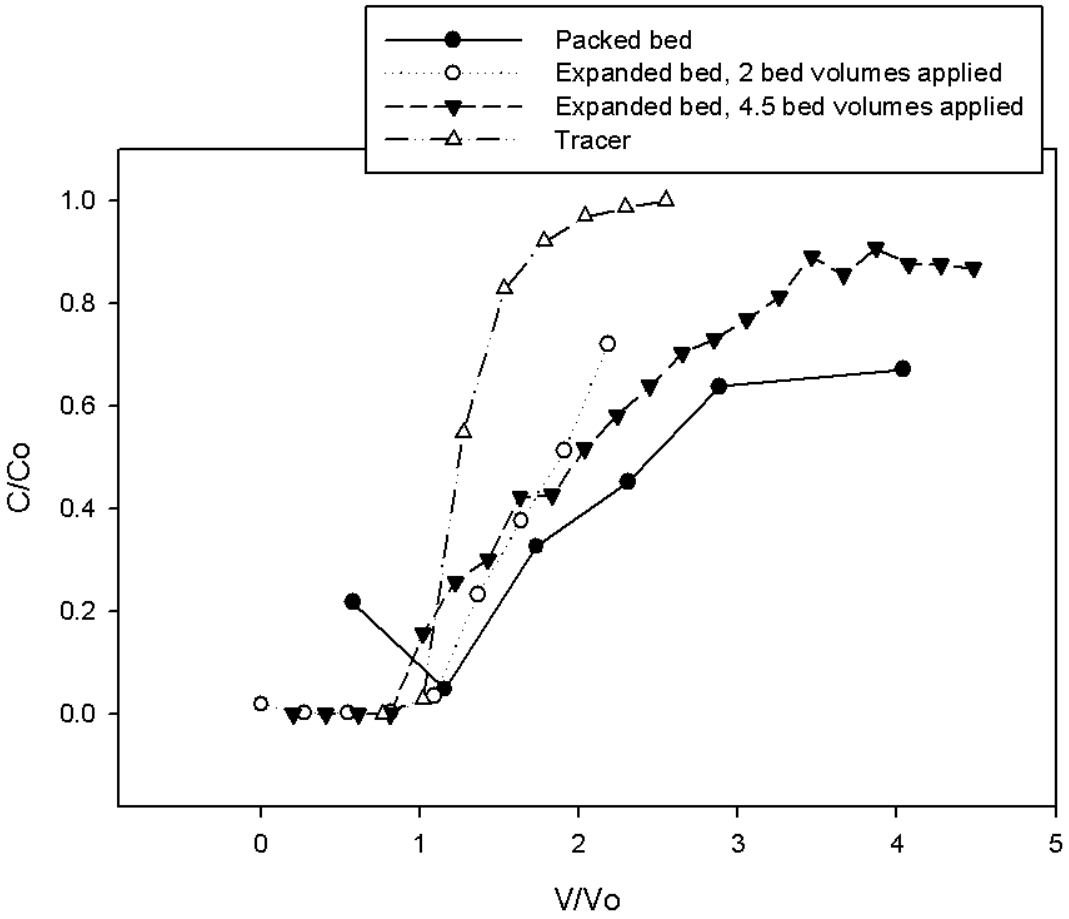

Figure 6- Breakthrough profiles for expanded bed adsorption and packed bed adsorption compared to tracer breakthrough profile. X-axis is volume applied (V) divided by the column volume (Vo).

Conclusions

Hydrolysis complicates the concentration and purification of protein from the soy EAEP skim fraction. Ultrafiltration may work, however it will result in protein losses of at least 25%. Traditional isoelectric precipitation allows the recovery of large, presumably unhydrolyzed proteins. Expanded bed anion exchange chromatography shows promising results from an operational point of view and may provide some interesting polypeptide selectivity; however, high protein recovery yields were not attained. An approach more likely of success will be a multistage process, incorporating isoelectric precipitation, with staged anionic and/or cationic chromatographic separation and this is being investigated.

References

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