The Impact of Dehulling and Germination on the Physiochemical, Protein Solubility and Water and Oil Holding Capacities of Yellow Eye Bean (*Phaseolus vulgaris* L.) Protein Concentrates

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Pulse varieties including Yellow Eye (YE) beans (*Phaseolus vulgaris* L.) are a rich source of protein (~26.5%) that can be utilized to create value-added protein concentrates. Pre-treatments including dehulling and germination have been shown to be effective at improving the nutritional and functional properties of extracted protein concentrates. However, the composition and functionality of these protein concentrates can vary depending on the pre-treatments and the method of extraction used (salt vs. alkaline). Furthermore, little is known about the impact of combining these different processing methods on the properties of YE bean protein concentrates. The objective of this study was to evaluate how germination and dehulling pre-treatments individually and when combined influence protein extraction efficiency, physiochemical properties (surface hydrophobicity and intrinsic fluorescence), and the functionality (solubility, oil and water holding capacities) of salt and alkaline extracted protein concentrates. Compared to the salt extracted concentrates, the alkaline protein concentrates exhibited higher protein recovery yields (16–23% vs. 43–56%) respectively. Conversely, the salt extracted protein concentrates exhibited superior functional properties as observed by improved water holding capacities and less variation in their solubilities at different pH values (4 to 10). When the pre-treatments were combined, the salt extracted concentrates exhibited improved extraction efficiencies and improved hydrophobicity and intrinsic fluorescence, whereas the opposite trend was observed in the alkaline protein concentrates. These observations were attributed to differences in the protein content and composition of the salt vs. alkaline protein concentrates. Overall, these findings suggest that dehulling and germination are potential processing methods that may be used to improve the physiochemical characteristics of salt extracted protein concentrates from yellow eye beans. Future research may investigate the potential application of these ingredients in different food formulations.

**Keywords:** plant protein, germination, dehulling, protein extraction, protein concentrate
INTRODUCTION

Over the past decade there has been increased efforts to reduce animal-based proteins and replace them with plant-based proteins to form healthier, more affordable, and more sustainable food products (Vainio et al., 2016). Underutilized pulse varieties, such as Nova Scotian Yellow Eye (YE) beans (*Phaseolus vulgaris* L.), have a high protein content (26.5%) and can serve as alternative protein ingredients in food production, however, their efficacy must be shown (English et al., 2019a).

Proteins in the form of concentrates (65–90% protein dry basis) or isolates (>90% protein dry basis) can be separated from different plant or animal sources and used as food ingredients (Ma et al., 2022). Traditionally, the preferred protein sources have been from soy, wheat, or dairy products (such as whey), however, the presence of allergens and certain dietary restrictions are making pulse proteins a more attractive alternative for food formulations (Soller et al., 2015; Nwachukwu and Aluko, 2021). Although there are different ways to define protein functionality, this terms most often refers to the ability of proteins to form and stabilize networks in food systems such as gels, foams, and gels, and includes physiochemical properties such as solubility, and water and fat binding abilities (Foegeding and Davis, 2011; Vogelsang-O’Dwyer et al., 2021). Indeed, pulse proteins have shown similar functional properties compared to soy proteins (Burger and Zhang, 2019). For example, common bean proteins have shown promising emulsifying and gelling properties (Shevkani et al., 2015; Rahmati et al., 2018), whereas pea proteins have exhibited high solubility and foaming properties. Knowledge of the functional properties of proteins is therefore important in selecting the most ideal ingredients for successful food formulations (Vogelsang-O’Dwyer et al., 2021). However, in terms of their functional properties not all proteins are equal, differences in the functional properties can be attributed to inherent variations between cultivars or differences in protein content, composition, and conformation (Rui et al., 2011). The type of protein extraction methodology used can also contribute to differences in functional properties because of: (1) changes in the protein fraction extraction affinity, and (2) conformational changes achieved during the extraction process (Karaca et al., 2011).

Currently, the techniques used to extract plant proteins may be grouped into dry and wet processes (Fernando, 2021; Yang et al., 2021). Dry processes achieve protein separation based on differences in density whereas in wet processes, proteins are extracted based on their solubilities in alkaline or salt solutions, followed by isoelectric or micellar precipitation, respectively (Stone et al., 2015; Tanger et al., 2020). The work in the present study has focused on wet processes since these methods are commonly used with pulses (Cui et al., 2020). Among the wet extraction methods, the alkaline extraction process is more commonly used because of its simple and cost-effective nature (Zhang et al., 2014; Momen et al., 2021). Although this approach primarily targets globulins and produces higher protein concentrate yields, the alkaline and acidic environments necessary with this approach can result in partial denaturation of the proteins which in turn reduces protein quality and functionality (Yang et al., 2021). Conversely, protein extraction techniques using salt solutions have been shown to produce a more complete protein profile (Karaca et al., 2011). However, compared to alkaline methods, salt extraction procedures can be time-consuming because an additional desalting step is required (Hadjadev et al., 2017). Determining how these two extraction techniques impact the functionality of YE bean protein concentrates is critical to the successful development and application of these ingredients.

Apart from the challenges related to protein extractions, the presence of undesirable components in pulses can impact the functionality and quality of pulse proteins. Antinutrients such as tannins have been linked with decreased protein bioavailability which in turn limits protein functionality (Samtiya et al., 2020). Various pre-treatments including germination and the subsequent enzymatic hydrolysis of indigestible proteins have helped to improve protein digestibility (Ali and Elozeiri, 2017). Dehulling (removal of the seed coat) and soaking have also been used to reduce anti-nutrients that impact protein adsorption (Wang, 2008). Many of the studies evaluating the effectiveness of pre-treatments have focused on flour samples with little emphasis on the protein isolates or concentrates. For example, Liu et al. (2018), demonstrated an increase in protein content and water absorption index in germinated mung bean flour over a 72-h period. In addition, GhavideL and Prakash (2007) used a combination of pre-treatments (dehulling and germination) on lentils, chickpeas, green gram, and cowpeas and observed an increase in protein and protein digestibility. Indeed, our own observations with other food model systems containing yellow eye beans have shown that blending pre-treatments (germination and soaking) were more effective strategies for improving the functional properties and the aroma profiles of these pulse ingredients (English et al., 2019b). Although the use of combined pre-treatments is not a new approach the improvement in protein functionality suggests that there may be merit in further investigating this strategy.

Accordingly, the main objective of the present study was to evaluate the impact of dehulling and germination individually and a combination of these pre-treatments on Nova Scotia Yellow-eye bean protein concentrates. Protein extractions were conducted using alkaline and salt solutions, and the physiochemical (hydrophobicity and intrinsic fluorescence) and functional properties (solubility and oil and water binding capacity) of the different concentrates were evaluated. It was hypothesized that improved protein functionality and physiochemical properties may be observed in protein concentrates exposed to combined pre-treatments, since blending pre-treatments has been shown to have a greater impact on protein functionality (English et al., 2019b). Moreover, because salt extraction techniques have been demonstrated to reduce conformational changes in the extracted protein isolates (Grover and Ryall, 2005), it was further hypothesized that the salt extraction protein concentrates would have improved physiochemical properties compared to their alkaline extracted counterparts. Understanding the impact of these processes on the physiochemical and functional properties of yellow eye bean protein concentrates is important for the production and development of new pulse ingredients of high quality.
METHODS

Materials
Yellow Eye beans (*Phaseolus vulgaris* L.) grown in Cambridge, Hants County, Nova Scotia, were purchased from a grocery store, Superstore in Antigonish, Nova Scotia and stored in a cool, dark cupboard until use. Bradford reagent, tris, and mini-PROTEAN electrophoresis gels were obtained from Bio-Rad (Mississauga, ON). Ethanol and sodium hypochlorite were purchased from VWR (Mississauga, ON). Sodium hydroxide, β-mercaptoethanol, and methanol were obtained from Fisher Scientific (Ottawa, ON). Sodium phosphate dibasic, sodium phosphate monobasic, and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Oakville, ON). Hydrochloric acid and sodium hydroxide were purchased from VWR (Mississauga, ON). Ethanol and sodium hypochlorite were obtained from Fisher Scientific (Ottawa, ON). All chemicals were reagent grade and did not require any additional purification.

Preparation of Bean Flours for Protein Extraction
A total of 5 bean variations were prepared for this study (Figure 1). Prior to flour preparation, beans were sorted and sanitized in a 0.07% (w/v) sodium hypochlorite solution for 30 min, then washed with distilled water until a pH of 7.0 was reached. Beans were soaked at a ratio of 1:4 of beans to distilled water at room temperature for 20 h. Once soaking was finished, the remaining distilled water was discarded and excess water was removed from the beans. Whole/soaked (WS) beans were freeze dried and ground using a Blendtec 51-601-BHM Kitchen Mill. Untreated, raw beans (RB) were used as a control for this study and were milled using a Blendtec 51-601-BHM Kitchen Mill. The dehulled/soaked (DS) beans were first manually dehulled, then dried, ground, and stored as previously described.

Germination
Variations described as whole/germinated (WG) and dehulled/germinated (DG) were germinated using a method adapted from Ma et al. (2018) and Xu et al. (2019). Beans were first sanitized and soaked following the method outlined in Section Preparation of Bean Flours for Protein Extraction. Twenty-two beans were placed into a sterile petri dish lined with Grade 1 Whatman® filter paper moistened with 1 mL of distilled water. The beans were covered with another piece of filter paper moistened with 500 µL of distilled water. Beans were germinated in a Memmert Humidity Chamber HCP (Büchenbach, Germany) at 20 °C and 95% relative humidity for a maximum of 72 h, with germinated beans being collected every 24 h. Beans were considered germinated when their radicles reached a length of over 2 mm. Beans that failed to germinate after the 72 h were discarded. Variation WG was then freeze dried and ground into flour, whereas variation DG was dehulled prior to freeze drying and grinding. All flours were stored in airtight containers at 4 °C until they were used.

Protein Content and Ash Content Analysis
Protein and ash analyses, and crude fat determination of all flours were determined at the Department of Agriculture and Food Operations Laboratory (Truro, Nova Scotia, Canada) using similar methods outlined in English et al. (2019a). Ash content was determined following the Association of Official Agricultural Chemists (AOAC) method 923.03 (AOAC, 2005). Protein content was determined using combustion analysis (Laboratory Services Analytical Laboratory, LSAL, Method 410) using a LECO CN828 macro combustion instrument (St. Joseph, KS, USA) using a protein to nitrogen conversion rate of 6.25 (Yang et al., 2021). Crude fat was calculated using a solvent extraction method. Carbohydrate content was determined by subtraction of the other chemical components from 100.

Protein Isolation

Alkaline Extraction Method
A total of five protein concentrates were generated using an alkaline extraction protocol (Figure 1). A modified alkaline extraction procedure was developed using a combination of reports from de Evangelho et al. (2017), Du et al. (2018), and Karaca et al. (2011). Briefly, bean flours were suspended in distilled water (1:10 w/v) and the pH of the solutions were adjusted to 9.5 with a 4N NaOH solution, after which the solutions were left to stir for 1 h. The slurries were centrifuged at 5,000 x g for 40 min and the supernatants collected. The remaining pellets were resuspended in distilled water (1.5 w/v) and centrifuged at 5,000 x g for 30 min. The supernatants were pooled together, and the pH was adjusted to 4.5 using 1M HCl to precipitate the protein. The samples were then centrifuged at 3,500 x g for 15 min and the pellets of precipitated protein was kept and adjusted to a pH of 7.0.

Salt Extraction Method
Five protein concentrates were prepared using a salt extraction protocol as shown in Figure 1. The salt extraction method described by Mundi and Aluko (2012) was followed with some modifications. Bean flours were suspended in 0.1 M Phosphate buffer (pH 8.0, 1:10 w/v) and stirred constantly for 2 h at 4 °C. The slurries were then centrifuged at 4,260 x g for 45 min, after which the supernatants were collected. Ammonium sulfate was added to the supernatants until 40% saturation had been achieved. The mixtures were stirred for an additional 2 h at 4 °C and centrifuged following the previously described conditions. The supernatants were collected once again, and ammonium sulfate added until 80% saturation was reached. The slurries were centrifuged one more time, after which the pellets were collected. The pH values of the extracted pellets were adjusted to 7.0 and diafiltered with deionized water through a Vivaflow 200 polysulfone membrane with a molecular weight cut-off of 10 kDa at a volume concentration ratio (VCR) = 10.

Ultrafiltration
Both protein extracts were ultrafiltered using a Vivaflow 200 polysulfone membrane with a molecular weight cut-off of 10 kDa. The ultrafiltration was repeated twice at VCR = 5. The collected protein solutions were freeze-dried and weighed to calculate the protein yield, and the protein recovery yields were calculated as a percentage of the isolate weight of the protein content found in the associated flour (Equations 1 and 2). Protein purity of each
extracted protein concentrate (Equation 3) was determined by the Bradford protein assay (Bradford, 1976).

\[ \text{Extraction yield (\%)} = \frac{\text{weight of protein concentrate (g)}}{\text{total weight of flour (g)}} \times 100 \quad (1) \]

\[ \text{Protein recovery yield (\%)} = \frac{\text{weight of protein concentrate (g)}}{\text{Protein composition of flour (g)}} \times 100 \quad (2) \]

\[ \text{Protein purity (\%)} = \frac{\text{protein concentration (mg)}}{\text{Total weight of freeze – dried concentrate (mg)}} \times 100 \quad (3) \]

**Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis**

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) samples were prepared follow a method adapted from Aluko and McIntosh (2004) and English et al. (2019b). Concentrations of 10 mg/mL of each PI were prepared in Tris/HCl buffer (pH 8.0). Three point five microlitres of filtered protein extract was mixed with 10 µL of distilled water and 5 µL of protein sample buffer (pH 6.8, 1 M Tris-HCl, 5% SDS, 50% glycerol, 0.5% Bromphenol blue, and 10% β-mercaptoethanol) and heated in a boiling water bath for 10 min.

Fifteen microlitres of each sample were loaded onto 12% Mini-PROTEAN® TGXTM gels obtained from BioRad (Mississauga, ON). A pre-stained protein ladder (New England BioLabs, P77066, 10-250 kDa) was loaded in a 5 µL aliquot to estimate the molecular weights of proteins in the samples. Gels were loaded into a Mini-PROTEAN® Tetra Cell (Bio-Rad, Mississauga, ON) and the system was run for 1 hour at 170 V and 30 mA. Gels were stained for 30 min with a 0.1% Coomassie Brilliant Blue (R-250) staining solution and then de-stained overnight. A pre-stained protein marker (New England BioLabs, P77066, 10-250 kDa) was used to estimate the molecular weights of proteins. Images of the gels were captured using a BioRad Chemic DocTM MP imaging system.

**Intrinsic Fluorescence Measurement**

The intrinsic fluorescence spectrums of the protein concentrates were measured following a modified method from He et al. (2020) and Yang et al. (2021). Concentrations of 0.2 mg/mL of protein solutions were made in 10 mM phosphate buffer (pH 7.2). The intrinsic fluorescence spectra of the protein solutions were measured spectrophotometrically using a QuantaMasterTM spectrofluorometer from Photon Technology International Inc. (London, ON). The excitation wavelength was set to 280 nm, and the emission spectra was recorded from 290–400 nm at a rate of 1 nm/s and a slit width of 2 nm.

**Surface Hydrophobicity**

A modified method by He et al. (2020) and Joshi et al. (2012), using 8 mM ANS prepared in 10 mM phosphate buffer (pH 7.2) as a probe, was followed. A concentration curve of 0.0–0.05% (w/v) and protein solutions were prepared using 10 mM phosphate buffer (pH 7.2). Briefly, 50 µL of ANS probe was added to 4 mL of each protein concentration, and then incubated in the dark for 5 min. The relative fluorescence intensity (RFI) of each dilution was then measured using a QuantaMaster™ spectrofluorometer from Photon Technology International Inc.
RESULTS AND DISCUSSION

Protein and Ash Content of Flour Samples From Treated and Untreated YE Beans

Protein content in the raw YE bean flour was 26.5%, and the highest value was observed in the flour from the dehulled/soaked beans (27.9%). Similar protein yields (20–30%) have been reported in the literature for other beans (Mundi and Aluko, 2012). The flour from the raw and soaked samples registered the highest ash content, 4.1 and 4.2%, respectively, whereas the lowest ash value of 3.6% was registered in flour samples from dehulled/soaked and dehulled/germinated seeds (Table 1).

Characterization of Alkaline and Salt Extracted Bean Protein Concentrates

Protein Extraction Efficiency

Extraction yields and protein recovery yields for alkaline extracted protein concentrates (APCs) ranged from 12.2 ± 0.5%–14.9 ± 0.28% and 43–56%, respectively (Table 2). Concentrate yields and protein recovery for salt extracted protein concentrates (SPCs) were much lower, ranging from 4.2–6.4% and 16–23% respectively. Ammonium sulfate because of its high solubility in water and negligible effects on protein has been the preferred salt used to extract protein fractions from other pulses including mungbeans, Mendoza et al., 2001, and cowpea (Rangel et al., 2003). However, compared to alkaline extraction methods, protein yields from salt concentrates are often lower. Karaca et al. (2011) reported lower protein yields in SPCs for chickpeas and lentils. In addition, Yang et al. (2021) also reported lower protein yields in extracts from yellow peas, and these observations were attributed to greater carbohydrate solubility in salt solutions. Sathe (2002) emphasized that carbohydrates are the major non-protein components in beans; and their complete removal is required for adequate protein separation. Other factors including high salt concentrations as well as the presence of lipids have been shown to impact protein solubilization which in turn impacts the protein yield (Aluko, 2004; Deak et al., 2006). Table 2 shows the carbohydrate (59.3–64%) and lipid (0.8 to 1.4%) values reported in the present study which are similar to published results (Karaca et al., 2011). The low lipid contents in the flour samples, suggests that in this instance, lipids may not have a significant impact on the protein extraction yields, however, the interfering effect of carbohydrates cannot be ruled out.

Both germination and dehulling pre-treatments impacted the yields of the protein concentrates generated from the alkaline and salt extractions. Compared to the raw beans used for alkaline extractions (RBA), germination decreased the yields in concentrates from beans that were germinated then alkaline extracted (WGA). Yields were also decreased in samples that were dehulled/germinated before undergoing a similar extraction process (DGA) (Table 2). The concentrate yield and recovery yield of the whole germinated seeds that underwent salt extractions (WGS) were also significantly decreased when compared to concentrates obtained from beans that were soaked followed by protein extraction using salts (WSS). These observations are opposite to the results of Sofi et al. (2020),
TABLE 1 | Ash and protein content of flour samples generated from treated and untreated yellow eye (YE) beans.

| Flour samples | Raw beans | Soaked | Whole germinated | Dehulled/soaked | Dehulled/germinated |
|---------------|-----------|--------|------------------|-----------------|---------------------|
| Protein content (%) | 26.5 | 26.8 | 25.7 | 27.9 | 27.7 |
| Ash content (%) | 4.1 | 4.2 | 3.7 | 3.6 | 3.6 |
| Carbohydrate (%) | 59.3 | 62.8 | 65.1 | 63.1 | 64 |
| Total Lipids (%) | 0.8 | 1.2 | 1.4 | 1.31 | 1.4 |

All analyses were carried out at the Department of Agriculture and Food Operations Laboratory (Truro, NS). Replicate data were not obtained therefore, no additional data analysis could be determined.

TABLE 2 | Protein extraction efficiency of untreated and pre-treated Yellow Eye bean protein concentrates prepared by alkaline and salt-extraction methods.

| Sample | Extraction yield (%) | Protein recovery yield (%) | Protein purity (% protein) |
|--------|----------------------|---------------------------|--------------------------|
| (i) Alkaline extracted protein concentrates | | | |
| Raw bean | 14.9 ± 0.30a | 56.1 ± 1.10a | 93.3 ± 1.50a |
| Whole soaked | 13.4 ± 0.70ab | 50.1 ± 2.61b | 98.6 ± 0.94b |
| Whole germinated | 12.2 ± 0.50d | 47.5 ± 1.90d | 98.7 ± 0.41b |
| Dehulled soaked | 13.4 ± 0.71ab | 48.0 ± 2.54b | 98.0 ± 0.60b |
| Dehulled germinated | 12.2 ± 0.71ab | 43.8 ± 2.60b | 91.3 ± 2.91a |
| (ii) Salt extracted protein concentrates | | | |
| Raw bean | 4.26 ± 0.31c | 16.1 ± 1.20c | 76.8 ± 0.41c |
| Whole soaked | 6.19 ± 0.22d | 23.1 ± 0.82d | 75.5 ± 0.94c |
| Whole germinated | 4.31 ± 0.40c | 16.8 ± 1.20c | 86.2 ± 0.90c |
| Dehulled soaked | 5.87 ± 0.52d | 21.1 ± 1.90d | 89.3 ± 0.91d |
| Dehulled germinated | 6.34 ± 0.63d | 22.9 ± 2.30d | 90.0 ± 1.50d |

1. Extraction yield, recovery yield and protein content values shown are the mean ± the standard deviation (n = 3). Values with different letters in each column represent significant differences (p < 0.05) by Tukey’s test. 2. The extraction yields (%) represent the weight of protein concentrate relative to the total weight of bean flour used for the extraction. 3. The protein recovery yields (%) represents the weight of protein concentrated relative to the protein composition of the beans. 4. The protein purity (%) represents the concentration of protein (determined by the Bradford assay) relative to the total weight of freeze-dried protein concentrate.

who found a slight increase in yields from alkaline extracted protein concentrates generated from germinated chickpeas. This is potentially due to differences in germination time, as Sofi et al. (2020) used a shorter time of 48 h, whereas the beans in the present study were left to germinate for up to 72 h. Indeed, a study by Rumiya and Jayasena (2012) highlighted a decrease in protein concentrate yield in dehulled/germination Australian sweet lupin APIs starting at 72 h of germination, and a continued decrease in concentrate yield from 17.6 ± 3.8% on day 3, to 4.7 ± 0.4% on day 9 of germination. Thus, it is likely that by 72 h, protein was being used as an energy source of germination in common beans (Ali and Elozeiri, 2017), which might offer a possible explanation for the lower extraction yields reported in the present study. For example, using the alkali method, the extraction yield registered for the germinated samples was 12.2 ± 0.49 % compared to 14.9± 0.28% for the raw YE bean (Table 2). Lower yields were observed in the salt extracted concentrates, 4.26 ± 0.31% for the raw beans vs. 4.31 ± 0.38% for the germinated samples. It is well known that beans are composed of different protein fractions, however, differences in the solubility of these fractions may have contributed to the lower yields obtained in the composite protein concentrates. Boyle et al. (2018) also highlighted that extraction procedures that combine more than one extraction solvent, can maximize solubilization of different protein fractions, which in turn improves extraction yields.

Dehulling did not improve the protein yields in the alkaline extracted concentrates, however, the dehulled salt concentrates showed a significant increase in yield compared to the concentrates generated from raw beans whose proteins were extracted with salt solutions (RBS). Seed coats primarily consist of carbohydrates and only contained 2–8% protein (Zhong et al., 2018). Therefore, removing the seed coat can increase the amount of protein to non-protein components, thus increasing concentrate yield.

Combined pre-treatments of dehulling and germination also improved protein extraction efficiency in the salt extracted samples (DGS). However, this was not observed for similar pre-treated beans that underwent alkaline extractions (DGA), in these samples, concentrate and protein recovery yields both decreased compared to concentrates from untreated beans (RBA) as well as those from beans that were soaked prior to alkali extractions, the WSA concentrates. These differences may be due to the presence of condensed tannins and protein-tannin interactions, which can form tannin-protein complexes and reduce protein solubility under alkaline conditions (Tajoddin et al., 2014; Shen et al., 2020). Because the seed coats were removed after germination in the present study, it is possible that these condensed tannins diffused into the cotyledon during the germination process (Chagas and Santoro, 1997). Condensed tannins can be extracted through alkaline methods and can form tannin-protein interactions which have been found to limit extraction yields at pH 8.0 (Brouwer et al., 2019). Moreover, the addition of salt solutions to proteins has been linked with decreased protein-tannin affinity (Kilmister et al., 2016).

Physicochemical Characteristics of Protein Concentrates

Significant variations were observed in the physicochemical properties of the untreated and pre-treated concentrates obtained from salt and alkaline extractions. As hypothesized, the measured physicochemical properties of the salt extracted concentrates exhibited reduced conformational changes when compared to...
the alkaline extracted concentrates, which showed signs of protein denaturation. In addition, the pre-treatments resulted in changes to the tertiary structure of the protein concentrates and the exposure of more hydrophobic surface regions in the alkaline extracted concentrates, as indicated by the higher surface hydrophobicity values ranging from 398 ± 0.7–537 ± 9.6, (Figure 2). The opposite was observed in concentrates from salt extraction solutions where hydrophobicity values ranged from 77.6 ± 5.7 to 121.4 ± 5.3. Similar results were also reported by Yang et al. (2021) who recently showed that pea protein isolates extracted by an alkaline method exhibited a greater effect on protein conformation compared to protein extracted using salt solutions; higher surface hydrophobicity values (847.9 ± 32.9) were also reported for the alkaline extracted pea concentrates compared to the salt extracted counterparts (732.2 ± 42.8).

Variations in the physiochemical characteristics of the concentrates may be attributed to: (1) the types of proteins extracted and isolated during each extraction process and, (2) the conformation and structural changes to the proteins that occurred during the extraction process (Stone et al., 2015; Yang et al., 2021). Differences in the physiochemical properties may also occur because of the types of proteins extracted using salt vs. alkaline techniques. For example, salt extraction procedures typically isolate both globulins and albumins, whereas alkaline extraction methods primarily extract and precipitate the globulin fractions due to the pH of the precipitation step (Karaca et al., 2011; Tanger et al., 2020). Differences in protein fractions were also observed in the SDS-PAGE data, as the alkaline extracted concentrates had thicker bands because of the globulin subunits (40 and 45 kDa) whereas a smaller albumin band (27 kDa) was observed in the salt extracted concentrates (Figure 3). This alteration of the globulin/albumin ratio can influence the physiochemical characteristics of the protein. Importantly, since globulins tend to be more hydrophobic than albumins (Mundi and Aluko, 2012), this property can explain the higher surface hydrophobicity (H0-ANS) observed in alkaline concentrates compared to those from the salt extraction (Figure 3). In addition, globulins can also dissociate into their 11S legumin and 7S vicilin subunits under acidic and alkaline conditions, thus further exposing more hydrophobic side chains which in turn increases surface hydrophobicity (Papalamprou et al., 2010).

The fluorescence spectra of the aromatic amino acids, phenylalanine, tryptophan, and tyrosine can be used as a tool to observe changes in the tertiary structure of proteins caused by solvents used for protein extraction as well as pre-treatments used before extraction (Johnson, 2006). Since bean protein isolates have been reported to contain ∼ 6.0% phenylalanine, and 3.4% tyrosine, (and tryptophan not determined) (Fernández-Quintela et al., 1997; Boye et al., 2010), this intrinsic fluorescence variability may be used to detect differences in the treated and untreated protein concentrates. Similar approaches have been used by other researchers to evaluate structural changes in pea protein isolates (Yang et al., 2021) and black turtle bean protein isolates (He et al., 2020). The maximum fluorescence emission registered for alkaline extracted concentrates in the present study was much lower than that observed for the salt extracted concentrates (Figures 4A,B), indicating a decrease of exposed aromatic amino acid residues (Yang et al., 2021). Indeed, a negative correlation was observed between hydrophobicity and intrinsic fluorescence (r = −0.8725). This negative correlation may result from the formation of less compact structures which are induced by partial protein unfolding that is promoted by the alkaline conditions (Jiang et al., 2009; Yang et al., 2021). Decreased maximum fluorescence may also be a result of inter- and intramolecular hydrophobic interactions of previously exposed tryptophan chromophores, thus promoting aggregation (Shen and Tang, 2012).

In addition, a shift toward higher wavelengths (red shift) was observed in the pre-treated concentrates obtained by alkaline extraction, and the maximum emission wavelength of the soaked, WSA (328 nm), germinated, WGA (329 nm), and dehulled concentrates, DGA (328 nm) increased compared to the alkaline concentrates from raw beans, RBA (327 nm). Conversely, the maximum emission wavelength (327 nm) of the alkaline protein concentrates from dehulled beans, DSA, remained the same (Figure 4A). The fluorescence intensity of the germinated alkaline protein concentrates also increased compared to the alkaline protein concentrates from raw beans, RBA. This observation indicates that germination and the required soaking process had created a more hydrophobic environment for the tryptophan residues, thereby increasing the surface availability of hydrophobic amino acids (Ghavidel and Prakash, 2007). Indeed, the surface hydrophobicity of all the pre-treated samples also increased compared to the alkaline protein concentrates from raw beans, RBA (Figure 2). All these measurements can be linked to the conformational changes of the extracted proteins (He et al., 2020). Specifically, alkaline protein concentrates from germinated beans, WGA as well as alkaline protein concentrates from dehulled and germinated beans, DGA, had significantly higher surface hydrophobicity.
FIGURE 3 | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of Yellow Eye bean proteins extracted using salt- (A) and alkaline-extraction (B) techniques. Molecular distribution was compared to a New England BioLabs Precision Plus pre-stained protein marker (P77066, 10–250 kDa) in lane 1. The samples were separated using a 12% mini-Protean precast gel stained with Coomassie Brilliant Blue (0.1%).

FIGURE 4 | (A) Intrinsic fluorescence measurement of alkaline-extracted protein concentrates and (B) salt-extracted protein concentrates prepared from untreated and pre-treated Yellow Eye beans (0.2 mg/mL in 10 mM phosphate buffer, pH 7.2) (n = 3).

compared to their ungerminated counterparts. This indicates an increase in exposed hydrophobic sites of amino acids. Enzymatic protein degradation is also one of the mechanisms initiated during germination which can alter protein conformation and/or denaturation and expose hydrophobic areas of the protein, thus increasing surface hydrophobicity (Ghavidel and Prakash, 2007; Zahir et al., 2021).

Figure 4B shows the impact of the various pre-treatments on the fluorescence patterns observed for the protein concentrates generated from salt extractions. A spectral shift toward lower wavelength (blue shift) was observed in the pre-treated, salt extracted protein concentrates, with decreased maximum emission wavelengths for the soaked, WSS (324 nm), germinated, WGS (325 nm), dehulled and germinated, DGS (325 nm) protein concentrates compared to those of the raw, RBS (327 nm) and dehulled salt extracted protein concentrates, DSS (327 nm) (Figure 5). In addition, the fluorescence intensity of the pre-treated salt extracted protein concentrates decreased compared to fluorescence intensity from the salt extracted concentrates from raw beans, RBS. This indicated an increase of tryptophan residues exposed to a hydrophilic environment, thus decreasing the surface availability of hydrophobic amino acids (He et al., 2020).
In particular, the surface hydrophobicity of the soaked (WSS) and dehulled, (DSS) salt extracted protein concentrates decreased compared to the hydrophobicity of the salt extracted protein concentrates from raw beans, RBS, which is likely due to the leaching of insoluble proteins into the soaking water (Barak et al., 2014). However, it should be noted that the surface hydrophobicity registered for the salt extracted protein concentrates from the germinated, beans (WGS) was significantly higher when compared to the soaked samples (WSS). These changes can be linked to the degradation of nutrients used for energy during germination and the increased exposure of hydrophobic amino acids (Xu et al., 2020; Zahir et al., 2021).

### Protein Functionality

The conformation of a protein has long been established as an important property that can impact protein functionality (Boye et al., 2010). Contrary to the initial hypothesis, salt extracted protein concentrates exhibited decreased functionality when compared to the alkaline extracted concentrates, particularly relating to protein solubility. The solubilities of the alkaline extracted protein concentrates were significantly impacted by pH, as the concentrates precipitated at pH 4.0, and then solubility steadily increased as the pH of the concentrates also increased (Figure 5A). Interestingly, the impact of pH on salt extracted concentrates was less severe (Figure 5B). This can once again be attributed to the type of protein fractions extracted.

The isoelectric point of globulins is pH 4.5, which explains the insolubility of alkaline extracted protein concentrates at pH 4.0 (Tanger et al., 2020). The combination of albumin and globulin protein fractions extracted in the salt extracted protein concentrates would explain the lack of precipitation at pH 4.0, as albumin has been found to be more soluble than globulin at

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**FIGURE 5** | (A) Protein solubility (% protein dissolved, 10 mg/mL in 0.1 M Phosphate buffer, pH values 4 to 10) of untreated and pre-treated Yellow Eye bean protein concentrates prepared using alkaline extraction; (B) and salt solutions (n = 3).

**FIGURE 6** | (A) Water holding capacity of alkaline- and salt-extracted Yellow Eye bean protein; (B) Oil holding capacity of alkaline- and salt-extracted Yellow Eye bean protein concentrates. Different letters (a to e) represent significant differences by Tukey’s multiple comparison test (p < 0.05), (n = 3).
pH 4.0 (Makeri et al., 2017). It should be noted that the solubility of both alkaline and salt extracted protein concentrates in this study are typically lower than other pulse protein concentrates, particularly at more alkaline pH values (Mundi and Aluko, 2012; Shevkani et al., 2015; Ge et al., 2021). Paraman et al. (2007) attributed high insolubility to be a result of higher rates of interpeptide hydrophobic interactions or sulphydryl-disulfide interactions. These changes can prevent protein-water interactions and thus reduce solubility. A better understanding of the bonds and linkages present in these bean protein concentrates would further elucidate these interactions.

Although the salt extracted concentrates had lower surface hydrophobicity values, these samples demonstrated lower solubility curves compared to raw beans extracted in salt solutions (RBS). Several factors including protein structure, surface charge, and the degree of aggregation can influence protein solubility (Hayakawa and Nakai, 1985). The lower solubility of the pre-treated salt extracted protein concentrates may be a result of intermolecular aggregation of its β-sheets (Yang et al., 2021). On the other hand, the pre-treated alkaline extracted protein concentrates, particularly the germinated (WGA) and dehulled/germinated (DGA) samples, had increased solubility curves compared to the alkaline extracted protein concentrates from raw beans (RBA). This can be attributed once again to protein hydrolysis of globulins and the exposure of hydrophilic proteins for protein-water interactions (Cao et al., 2010).

Due to an increase in hydrophobic surface regions, the alkaline protein concentrates exhibited more hydrophobic functional characteristics, including increased OHC and decreased WHC when compared to the salt extracted concentrates. The higher OHC and lower WHC of alkaline protein concentrates compared to salt extracted concentrates can be explained by variations in protein conformation (Figures 6A,B). The exposure of hydrophobic side chains produced through the alkaline extraction process may be responsible for the higher OHC and the relatively low solubility of alkaline proteins (Figures 5A, 6A) compared to the literature (Karaca et al., 2011). In theory, an increase in hydrophobicity would result in a decrease of hydrophilic groups on the surface of the protein, thus, limiting the potential for protein-water interactions and decreasing WHC (Stone et al., 2015; Mohan and Mellem, 2020). Indeed, our own observations showed that surface hydrophobicity was positively correlated with OHC (r = 0.877) and negatively correlated with WHC (r = −0.618).

Variation in pre-treatment effects on OHC, WHC, and protein solubility were also observed. In general, pre-treated alkaline extracted protein concentrates had decreased WHC compared to similar concentrates from raw beans, RBA. This can be attributed to the increase in surface hydrophobicity properties. In addition, the removal of water-binding matrix components including starch and fiber have been found to decrease WHC in yellow field pea alkaline extracted protein concentrates (Agboola et al., 2010). It is also possible that the extracted protein was denatured during the extraction process, which can also correlate with decreased WHC (Mohan and Mellem, 2020). These phenomena are most likely responsible for the decreased WHC of all other pre-treated alkaline extracted protein concentrates compared to similar extracted concentrates from raw beans (RBA). Conversely, the germinated salt extracted protein concentrates (WGS) and the germinated/dehulled salt extracted concentrates (DGS) had significantly higher WHCs compared to salt extracted concentrates from raw (RBS) and soaked (WSS) beans. This can be explained by the increase in soluble proteins generated during proteolysis (Sofi et al., 2020).

Despite increases in hydrophobic properties in the alkaline extracted protein concentrates, no significant differences in OHC were established between the concentrates from raw beans (RBA) and other alkaline extracted concentrates. However, the OHC of dehulled, alkaline extracted concentrates DSA was significantly lower than that of the soaked, WSA, germinated, WGA and dehulled DGA, alkaline extracted protein concentrates. Indeed, the surface hydrophilicity of the dehulled concentrates (DSA) was significantly lower than the dehulled/germinated (DGA) alkaline extracted concentrates, and there was no red shift in maximum emission wavelength. These changes can be attributed to a decrease in exposed hydrophobic amino acids, potentially due to tannin-protein interactions (Pal et al., 2017). Interestingly, despite the lower surface hydrophobicity of the dehulled sample (DSS), its OHC was significantly higher than that of the raw beans (RBS). The solubility curve of the salt extracted dehulled concentrate (DSS) was also decreased compared to concentrates from raw beans (RBS). Conformational changes that occurred during the extraction process may have attributed to these changes (Yang et al., 2021).

Although the salt extracted concentrates had lower surface hydrophobicity values, these samples demonstrated lower solubility curves compared to raw beans extracted in salt solutions (RBS). Several factors including protein structure, surface charge, and the degree of aggregation can influence protein solubility (Hayakawa and Nakai, 1985). The lower solubility of the pre-treated salt extracted protein concentrates may be a result of intermolecular aggregation of its β-sheets (Yang et al., 2021). On the other hand, the pre-treated alkaline extracted protein concentrates, particularly the germinated (WGA) and dehulled/germinated (DGA) samples, had increased solubility curves compared to the alkaline extracted protein concentrates from raw beans (RBA). This can be attributed once again to protein hydrolysis of globulins and the exposure of hydrophilic proteins for protein-water interactions (Cao et al., 2010).

**CONCLUSIONS**

This study assessed the impact of dehulling and germination and a combination of these pre-treatments on the protein extraction efficiency, physiochemical properties, protein solubility and the water/oil holding capacities of alkaline, and salt extracted protein concentrates from YE beans. In general, alkaline extracted protein concentrates had a higher protein extraction efficiency compared to salt extraction samples, however the former was more susceptible to conformational changes as indicated by the increase in surface hydrophobicity. This increase in hydrophobic surface regions, observed in the alkaline extracted protein concentrates resulted in an increase in OHC and a decrease in the WHC when compared to the salt extracted concentrates. When applied individually, dehulling of beans resulted in protein concentrates with increased yields in the salt extraction solutions.
compared to the protein concentrates generated from raw beans when proteins were extracted in salt solutions. Conversely, beans that were germinated prior to protein extraction registered greater surface hydrophobicity when compared to the soaked samples. Moreover, the combination of dehulling and germination pre-treatment decreased protein extraction efficiency and functionality when compared to the untreated and soaked alkaline extracted protein concentrates. The combined pre-treatments also resulted in improved protein extraction yields, physiochemical properties, and the functionality of the salt extracted concentrates. The beneficial effect observed from the combined pre-treatment (dehulling/germination) on the salt extracted protein concentrates suggests that these protein samples may be good potential candidates for further studies that evaluate their application in different food formulations.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

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**AUTHOR CONTRIBUTIONS**

LV co-designed the study, collected all data, interpreted results, drafted the manuscript, and approved the submission version. ME co-designed the study, supervision, and resources, edited, and proofread the manuscript, and approved the submission version. All authors contributed to the article and approved the submitted version.

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