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Optimizing alkaline and enzymatic extraction of black bean proteins: a comparative study of kinetics, functionality, and nutritional properties

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This study aimed to elucidate the impact of fundamental extraction parameters on protein extraction yields, kinetics, functionality, and nutritional properties of black bean proteins generated by the aqueous (AEP) and enzyme-assisted extraction processes (EAEP). Extractions evaluating the interplay of different solids-to-liquid ratios (SLR) and protease concentrations revealed a 14% increase in total protein extractability (TPE) for more concentrated slurries (1 : 7.5 SLR), demonstrating lower water requirements for enzymatic extractions. Kinetic modeling revealed that aqueous extractions followed first order (R^2 = 0.94) and Peleg's (R^2 = 0.91) models while enzymatic extractions exhibited multi-step kinetics with a burst-drop initial phase (0–20 min) followed by an increase corresponding to first order (R^2 = 0.94) and Peleg's models (R^2 = 0.92). The optimized AEP (pH 9.0, 50 °C, 1 : 15 SLR, 30 min) and EAEP (pH 9.0, 50 °C, 1 : 7.5 SLR, 1.0% enzyme, 60 min) achieved 82 and 78% TPE, respectively. EAEP increased the degree of hydrolysis from 4.6 to 21.1% and shifted the protein isoelectric point from pH 3.4 to <2. EAEP proteins exhibited significantly higher solubility in acidic conditions and foaming capacity at pH 3.4 but were unable to form emulsions at pH 3.4 and 7.0. Proteolysis also increased *in vitro* protein digestibility from 34 to 61%, decreased trypsin inhibitor activity from 136 to 100 TUI per mg protein, and reduced hemagglutination activity from 640 to 320 HU per mg protein, demonstrating that enzyme addition is a useful strategy to not only reduce water usage in aqueous extractions, but also enhance the nutritional properties of black bean proteins.

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Sustainability spotlight

This study explores scalable, relatively low-energy strategies (aqueous and enzyme-assisted extractions) to produce black bean protein ingredients, with the goal of understanding the effects of key extraction parameters on protein yields, functionality, and nutritional properties. Reduced water usage, which was achieved through the enzyme-assisted extraction process described in this study, is critical in the pursuit of responsible consumption and production (SDG 12) and is also related to climate action (SDG 13). In addition, understanding impacts of extraction conditions on the functional and nutritional properties of the resulting proteins is vital for the successful incorporation of these proteins in food systems, contributing to SDG 2 (end hunger).

1. Introduction

As the demand for plant protein ingredients continues to grow, it is necessary to understand the protein extraction mechanisms that govern process efficiency and the quality of the resulting proteins. Pulses (*e.g.*, peas, beans, lentils, chickpeas) are low-cost and sustainable sources of plant proteins with promising applications in bakery goods, meat analogues, and beverages for nutritional and/or functional purposes.[1] Commercial methods for producing pulse proteins typically involve alkaline extraction (pH 8–11) with or without mild heating, followed by downstream protein recovery (isoelectric precipitation or membrane filtration) and spray drying.[2] Although dry fractionation strategies (*i.e.*, air classification) have been suggested as alternative solvent-free methods for producing protein ingredients, aqueous extraction processes (AEP) are recognized for achieving superior extraction yields and higher protein purity in the final product.[3] Fundamentally, the AEP relies on the solubility of proteins and the insolubility of starch and fiber in the aqueous media, allowing for the separation of a protein-rich aqueous extract from the starch/fiber-rich insoluble fraction. There are several strategies to facilitate extraction by improving protein solubilization in the extraction media and/or disrupting the integrity of the plant cell wall to release protein and oil bodies from the cell matrix. These strategies can be physical (*e.g.*, ultrasonication, microwave), chemical (*e.g.*, pH adjustment, salt addition), or biochemical (*e.g.*, enzymatic hydrolysis,

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fermentation).[4,5] Of these methods, the enzyme-assisted aqueous extraction process (EAEP) stands out as a scalable and environmentally-friendly strategy that can not only improve protein extractability from plant material, but also modulate the functional and nutritional properties of the final protein product through controlled hydrolysis.

AEP and EAEP have been widely studied with respect to protein and oil extraction from a variety of crops including soybean,[6–8] sunflower,[9] and almond.[10,11] Extraction conditions in the AEP (*e.g.*, temperature, solids-to-liquid ratio (SLR), time) can significantly impact protein yields due to fundamental differences in the extraction slurry (viscosity, diffusivity) that influence mass transfer and extraction kinetics.[7,8,12,13] The addition of enzyme in the EAEP also affects extractability by hydrolyzing proteins into smaller, more soluble protein subunits and peptides that can more easily diffuse into the aqueous phase, as well as exposing cell matrix-entrapped proteins to the extraction media.[11,14] We have demonstrated that the use of commercial proteases (EAEP) to assist black bean protein extraction (pH 9.0, 50 °C, 1 : 10 SLR, 0.5% w/w alkaline protease) was able to significantly enhance protein extractability from 75 to 81%, compared to the AEP (no enzyme).[15] However, an in-depth exploration of the effects of key extraction parameters, specifically SLR, amount of enzyme, and extraction time, has yet to be performed for black beans. To our knowledge, there has also been limited research on how proteolysis (*i.e.*, EAEP) affects protein extraction kinetics in aqueous systems. Protein extractability over time has been reported for protease-assisted extraction for chickpea, rapeseed, soybean, and algae, but with large time intervals that are insufficient for kinetic modeling.[13,16]

Beyond increases in extraction yields, EAEP inherently modifies protein structure (*e.g.*, molecular weight, surface charge, surface hydrophobicity), influencing several functional properties that may be desirable for applications in food products (*e.g.*, solubility, emulsifying capacity, foaming capacity/stability).[12,14,15,17] EAEP has also been shown to reduce proteinaceous antinutritional factors in soy including trypsin inhibitors and agglutinins.[6,18–20] The removal or inactivation of antinutritional factors is key for the incorporation of common bean-based protein ingredients in food products. To our knowledge, this aspect has not been studied for bean proteins produced by the EAEP. In addition, the *in vitro* protein digestibility (IVPD) of enzymatically extracted pulse proteins has been scarcely explored. Because EAEP proteins are already "pre-digested" due to the proteolysis that occurs during extraction, there are nuances in defining the percent digestibility of the hydrolysates in a physiologically relevant way that have yet to be clearly described.

The elucidation of the mechanisms and drivers of black bean protein extraction in the AEP and EAEP can help inform processing decisions, and the determination of the effects of proteolysis on the functional and nutritional properties of the extracted proteins is vital in evaluating whether the use of enzymes is worth the additional cost. Overall, this work aimed to select extraction parameters for the AEP and EAEP of black bean proteins by first understanding the effects of solids-to-liquid ratio and enzyme loading on protein extractability, then modeling protein extraction kinetics to determine the optimal time of extraction. Following the selection of the best conditions, the protein extracts were characterized for functionality, *in vitro* protein digestibility, and anti-nutritional properties to assess the impacts of proteolysis on factors of commercial relevance. The present work helps to reveal relationships between processing conditions, protein yields, and protein properties that can aid in the improvement of sustainable commercial extraction methods for common bean proteins and more widely, pulses in general.

2. Materials and methods

2.1. Preparation of black bean flour (BBF)

Whole black beans (Eclipse variety, 2020 crop) were provided by Inland Empire Foods (Riverside, CA, USA) and milled (WonderMill Electric Grain Mill, Pocatello, ID) into a black bean flour with 11% moisture, 20% protein, 2% oil, 4% ash, and 63% total carbohydrates (by difference) as previously reported.[15]

2.2. Understanding extraction mechanisms through scanning electron microscopy of insoluble fractions (extraction byproduct)

To generate insoluble fractions for scanning electron microscopy (SEM), AEP was performed by dispersing bean flour (50 g) in deionized (DI) water (500 g, preheated to 50 °C) in a 1 L beaker. Upon temperature and pH adjustment to the desired extraction conditions (50 °C, pH 9.0), extraction occurred for 1 h under constant stirring (120 rpm) and pH maintenance. For the EAEP, the same extraction conditions were used (pH 9.0, 50 °C), except by the addition of enzyme (0.5% w/w; weight of enzyme/weight of flour) following the initial pH adjustment of the slurry. FoodPro® alkaline protease (Danisco, Rochester, NY, USA), a food-grade subtilisin produced from *Bacillus licheniformis*, was used in the EAEP. After extraction, the slurry was centrifuged (3283×*g*, 30 min, 4 °C) to obtain a protein extract (supernatant) and insoluble fraction (precipitate). AEP and EAEP conditions were selected based on our previous work, achieving 75 and 81% total protein extractability (TPE) for the AEP and EAEP, respectively.[15]

For SEM, bean flour and freshly prepared AEP and EAEP insoluble fractions were immediately covered in fixative solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer). Samples were rinsed twice (0.1 M sodium phosphate buffer, 15 min), then sequentially dehydrated with ascending concentrations of ethanol (30%, 50%, 70%, 95%; 30 min each), followed by two final dehydrations with 100% ethanol (20 min each). Samples were then critical point dried (Tousimis 931 GL Super Critical Autosamdri), mounted onto aluminum stubs, and sputter coated with gold (Pelco Auto Sputter Coater SC-7). Imaging was performed using a Thermo Fisher Quattro S Environmental SEM (Waltham, MA, USA) at 5 kV. SEM imaging services were provided by the University of California, Davis Biological Electron Microscopy (BioEM) Facility.

2.3. Impact of solids-to-liquid ratio and enzyme loading on TPE

In view of the substantial water requirement to achieve high extraction yields in single-stage extractions and the potential to improve protein extractability with enzymatic extraction,[15,21] the integrated impact of solids-to-liquid ratio (SLR) and enzyme loading on TPE was evaluated, using the AEP and EAEP as described in Section 2.2. SLR was varied from 1 : 5 to 1 : 25, corresponding to the addition of 20–100 g bean flour to 500 g of DI water. For the EAEP, alkaline protease was added at concentrations of 0.25, 0.5, 0.75, or 1.0% w/w. Extraction slurries were centrifuged as described in Section 2.2, and the fractions obtained (extract and insoluble) were weighed and analyzed for subsequent mass balance calculations. All extractions were performed in triplicate. The protein content of the extracts and insoluble fractions were measured using the Dumas combustion method (Vario MAX Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany) with a nitrogen conversion factor of 6.25. TPE was calculated using eqn (1):

$$\text{TPE}\ (\%) = \left[1 - \left(\frac{\text{protein (g) in insoluble fraction}}{\text{protein (g) in bean flour}}\right)\right] \times 100 \quad (1)$$

2.4. Extraction kinetics, modeling, and validation of best conditions

2.4.1. Time-dependent extraction profiles and yields of AEP and EAEP proteins. To gain a better understanding of the protein extraction profile over time and characterize kinetic parameters (rate, maximum yield) of the AEP and EAEP, extractions (~5 L slurry volume) were performed in a 10 L jacketed chemical reactor (CG-1965-610M, Chemglass Life Sciences LLC, Vineland, NJ, USA) to allow for sampling in small time intervals without significantly changing the total volume of the extraction slurry. AEP (1 : 15 SLR, pH 9.0, 50 °C) and EAEP (1.0% w/w alkaline protease, 1 : 7.5 SLR, pH 9.0, 50 °C) were performed using SLR and enzyme concentrations selected from the lab-scale extractions (Section 2.3). The total time for kinetic modeling (2 h) was selected based on the work of Tan *et al.*,[22] who reported a decrease in pinto bean protein extractability with extraction times longer than 2 h.[22] In addition, longer extraction times are undesirable from an industrial perspective, as it is more energy-intensive, while being lower throughput.

At each time point, 40 mL aliquots of the slurry were collected through the stopcock of the reactor, weighed, and immediately centrifuged (3283×*g*, 30 min, 4 °C). The supernatants were decanted, and the weights of the insoluble fractions were determined. Extractions were performed in triplicate. The protein contents of the extract and insoluble fractions at each time point were determined using the Dumas combustion method and TPE was calculated using a modified version of eqn (1) (eqn (2)):

$$\text{TPE at time } t\ (\%) = \left[1 - \left(\frac{\text{protein (g) in insoluble fraction at time } t}{\text{protein (g) in aliquot of extraction slurry}}\right)\right] \times 100 \quad (2)$$

2.4.2. Kinetic modeling. Protein extraction kinetics were modeled using the first-order kinetic model proposed by Aguilera & Garcia[23] for the aqueous extraction of lupin protein (eqn (3)):

$$\frac{C_\infty - C(t)}{C_\infty - C_w} = e^{-kt} \quad (3)$$

where C_∞ is the theoretical maximum TPE, $C(t)$ is the TPE at time t, C_w is the TPE at time 0, and k is the rate constant (min^{-1}). For the purposes of modeling, eqn (3) was transformed to eqn (4):

$$C(t) = C_\infty - [(C_\infty - C_w) \times e^{-kt}] \quad (4)$$

Protein extraction was also modeled using the Peleg model (eqn (5)), an empirical model for moisture sorption that has been widely applied to solid–liquid extraction:[24]

$$C(t) = C_0 + \frac{t}{k_1 + k_2 t} \quad (5)$$

where C_0 is the initial TPE at $t = 0$ (%), k_1 is Peleg's rate constant ($\text{min}\ \%^{-1}$), and k_2 is Peleg's capacity constant ($\%^{-1}$). Many studies consider $C_0 = 0$, as the extraction media is pure water or solvent;[25–27] however, due to the rapid extraction in small time scales and the unavoidable time required to mix the slurry and adjust pH before extraction begins, it would be inaccurate to negate the C_0 term from the expression. The estimated rate constants from the Peleg model can be further derived to calculate B_0 (% min^{-1}), the initial extraction rate at $t = 0$ (eqn (6)), and C_e (%), the maximum extraction yield at $t \to \infty$ (eqn (7)).[28]

$$B_0 = \frac{1}{k_1} \quad (6)$$

$$C_e = C_0 + \frac{1}{k_2} \quad (7)$$

The TPE over time data were fit to eqn (4) and (5) using MATLAB R2017a (MathWorks, Torrance, CA) with the "Trust Region" algorithm in the Curve Fitting Tool to estimate model parameters.

2.4.3. Validation of best AEP and EAEP conditions. Based on results from the kinetic modeling, optimal extraction times were selected for the AEP and EAEP. These conditions were validated by performing lab-scale extractions as described in Section 2.3, with the optimized conditions (AEP: 1 : 15 SLR, pH 9.0, 50 °C, 30 min; EAEP: 1 : 7.5 SLR, pH 9.0, 50 °C, 1% alkaline protease, 60 min). Extractions were performed in triplicate. Protein extracts were freeze-dried for subsequent characterization of functional and nutritional properties (FreeZone, Labconco, Kansas City, MO, USA).

2.5. Determination of isoelectric point of AEP and EAEP proteins using zeta potential

The zeta potential (ZP) values of the AEP and EAEP protein-rich extracts were determined under different pH conditions using a Zetasizer Nano with DTS1070 capillary cells (Malvern

Instruments Ltd, UK). Protein extracts were diluted in DI water (1 : 50 for AEP, 1 : 100 for EAEP), then adjusted to pH 2.0 through 9.0 with 0.5 M HCl or 0.5 M NaOH. Measurements were conducted in triplicate. The isoelectric point (pH at which ZP = 0) was estimated by linear interpolation.

2.6. Degree of hydrolysis

Degree of hydrolysis (DH) was determined using the *o*-phthaldialdehyde (OPA) method described by Nielsen *et al.*[29] with L-serine (100 μg mL^{-1}) as the standard. The reaction mixture consisted of 400 μL diluted protein extract (2% v/v) and 3 mL OPA reagent (containing *o*-phthaldialdehyde, disodium tetraborate decahydrate, sodium dodecyl sulfate, and dithiothreitol). Absorbance was measured at 340 nm following 2 min of incubation. DH (%) was calculated as:[29]

$$\mathrm{DH}\ (\%) = 100 \times \frac{h}{h_{\mathrm{tot}}} \tag{8}$$

where h (milliequivalents per g protein) is the number of peptide bonds cleaved and h_{tot} is 7.43 milliequivalents per g protein as previously determined for *Phaseolus vulgaris*.[30] Duplicate measurements of each extraction replicate were performed ($n = 6$).

2.7. Functional properties

Protein solubility and interfacial properties (emulsifying and foaming) were assessed for the AEP and EAEP extracts at several pH values of interest: pH 3.4 (pI of AEP proteins in the present work), 5.5 (approximate pH of meat and meat analogues), and 7.0 (pH of neutral foods).

2.7.1. Solubility. Solubility was determined according to the method of Rickert *et al.*[31] Briefly, 1% w/w dispersions of freeze-dried AEP or EAEP extracts were prepared in DI water. The dispersions were adjusted to pH 3.4, 5.5, and 7.0 and the pH was maintained for 1 h under constant stirring (25 °C, 400 rpm). The dispersions were centrifuged (10 000×*g*, 10 min, 20 °C) and the protein content of the supernatant was determined using the Dumas method ($n = 6$). Solubility was calculated as the percentage ratio of the protein content in the supernatant to the total protein content in the dispersion (calculated based on the protein content of the freeze-dried powder) (eqn (9)):

$$\mathrm{Solubility}\ (\%) = \left(\frac{\text{protein content in supernatant }(\%)}{\text{protein content in dispersion }(\%)}\right) \times 100 \tag{9}$$

2.7.2. Emulsifying capacity. Emulsifying capacity (EC) was determined according to Bian *et al.*[32] with modifications as described by Yang *et al.*[15] Briefly, 5 mL of 1% protein dispersions (*i.e.*, 0.05 g protein) were adjusted to pH 3.4, 5.5, or 7.0, and were homogenized at 10 000 rpm (Polytron PT 2500, Kinematica AG, Lucerne, Switzerland). During the homogenization, soybean oil with 4 ppm Sudan Red 7B was continuously added into the dispersion until the point of emulsion inversion or breakage was reached. EC was calculated as the ratio of the g of oil emulsified before phase inversion to the g of protein in the dispersion (eqn (10)):

$$\mathrm{EC}\ (\text{g oil per g protein}) = \frac{\text{g of oil for phase inversion}}{0.05\ \text{g protein}} \tag{10}$$

2.7.3. Foaming capacity and stability. Foaming capacity (FC) and stability (FS) were measured using the method of Sathe & Salunkhe[33] with modifications from Yang *et al.*[15] Foams were prepared by whipping 5 mL of a 1% protein dispersion (pH 3.4, 5.5, or 7.0) with a homogenizer at 20 000 rpm for 1 min (Polytron PT 2500, Kinematica AG, Lucerne, Switzerland). FC (eqn (11)) was reported as the percentage total increase in volume immediately after whipping (V_0) compared to the initial volume (5 mL). FS (eqn (12)) was reported as the percentage ratio of the volume of foam remaining 60 min after whipping ($V_{\mathrm{foam\ 60}}$) to the volume of foam present immediately after whipping (V_{foam}):

$$\mathrm{FC}\ (\%) = \left(\frac{V_0 - 5\ \mathrm{mL}}{5\ \mathrm{mL}}\right) \times 100 \tag{11}$$

$$\mathrm{FS}\ (\%) = \left(\frac{V_{\mathrm{foam\ 60}}}{V_{\mathrm{foam}}}\right) \times 100 \tag{12}$$

2.8. Nutritional properties

2.8.1. Amino acid profile. The amino acid profiles of the optimized AEP and EAEP extracts were measured using an L-8800a amino acid analyzer (Hitachi High-Tech America, Santa Clara, CA, USA) at the Molecular Structure Facility of the Proteomics Core Facility in the Genome Center at UC Davis. Samples were hydrolyzed with 6 M HCl (24 h, 110 °C) prior to analysis.[34,35] For cysteine and methionine quantification, samples were oxidized using performic acid to convert cysteine to cysteic acid and methionine to methionine sulfone prior to undergoing acid hydrolysis.[36] For tryptophan quantification, samples were prepared using the acid ninhydrin method as previously described.[37,38] Analyses were performed in triplicate.

2.8.2. *In vitro* protein digestibility (IVPD). Simulated *in vitro* protein digestions of AEP and EAEP extracts were performed according to the harmonized INFOGEST 2.0 method described by Brodkorb *et al.*[39] with slight modifications. Amylase and lipase were omitted from the simulated digestion due to the inherently low quantities of starch and oil in the extracts. Activities (U) of digestive enzymes (pepsin from porcine gastric mucosa, Sigma P6887, 3906 U per mg; pancreatin from porcine pancreas, Sigma P7545, 5.08 trypsin U per mg) and bile salt concentration (bile extract porcine, Sigma B8631, 1.62 mmol bile salts per g; assayed using Sigma MAK309 bile acid kit) were experimentally determined according to INFOGEST recommended methods.[39] Simulated salivary (SSF: 15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 M $(NH_4)_2CO_3$), gastric (SGF: 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2(H_2O)_6$, 0.5 M $(NH_4)_2CO_3$) and intestinal (SIF: 6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$) electrolyte fluids were prepared at 1.25×

concentration and stored at 4 °C until use. Pepsin and bile solutions were prepared on the day of digestion at the recommended final concentrations (2000 U per mL pepsin, 10 mM bile). However, pancreatin was prepared at concentrations of 100, 20, and 10 trypsin U per mL to investigate the effects of different pancreatin loadings on the calculated IVPD due to previous reports of autolysis and/or high enzyme blanks at high pancreatin concentrations.[40–42]

For the digestion protocol, 2.5 mL of AEP and EAEP extracts were added to 50 mL conical centrifuge tubes, and the exact weights of the samples were recorded. Enzyme blanks were also prepared with 2.5 mL reverse osmosis (RO) water instead of sample. For clarity, the concentrations herein refer to the concentration of each simulated fluid component before addition to the digestion tube. To each sample tube, salivary phase components were added (2 mL 1.25× SSF, 12.5 μL $CaCl_2$ (0.3 M), 488 μL RO water) and the tubes were incubated for 2 min in a shaking water bath (37 °C, 140 rpm). Next, the gastric phase components were added (4 mL 1.25× SGF, 2.5 μL of 0.3 M $CaCl_2$, 0.25 mL pepsin solution (80 000 U per mL)) and the pH was adjusted to 3.0 ± 0.2 using 1 M HCl, followed by the addition of RO water to reach a final volume of 10 mL. The tubes were incubated for 120 min in a shaking water bath (37 °C, 140 rpm). Lastly, the intestinal phase components were added (4.25 mL 1.25× SIF, 1.25 mL bile solution (0.16 mM in SIF), 20 μL of 0.3 M $CaCl_2$, 2.5 mL pancreatin solution (800, 160, or 80 trypsin U per mL in SIF)), and the pH was adjusted to 7.0 ± 0.2 using 1 M NaOH or HCl. RO water was added to each tube to achieve a final volume of 20 mL, and tubes were incubated for 120 min in a shaking water bath (37 °C, 140 rpm). Triplicate digestions of each sample were performed, with six replicates of the enzyme blank.

After digestion, 20 mL of TCA (24% w/v, in water) were added to each digestion tube, and the samples were incubated at 4 °C overnight to facilitate protein precipitation. The tubes were then centrifuged (3283×*g*, 30 min, 4 °C) and the supernatant (containing soluble "digested" protein) was decanted. As chloride ions present in TCA can damage the Dumas combustion nitrogen analyzer,[43] the pellet was washed using 10 mL of cold acetone (1 h incubation at −20 °C, followed by centrifugation at 3283×*g*, 30 min, 4 °C). The washed pellets were allowed to dry in the fume hood for ∼4 h, then weighed to determine the exact mass of the pellet (containing undigested proteins). The nitrogen contents (N%) of the pellets were determined by Dumas combustion (Vario MAX Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany).

In this work, IVPD was defined as the percent ratio between digested nitrogen (soluble nitrogen in the supernatant after digestion), and the nitrogen present in the original undigested sample (2.5 mL of AEP or EAEP extract). IVPD was calculated as (eqn (13)):

$$\text{IVPD}\ (\%) = \left(\frac{N_{\text{undigested extract}}\ (\text{mg}) - \left[N_{\text{digested pellet}}\ (\text{mg}) - N_{\text{enzyme blank}}\ (\text{mg})\right]}{N_{\text{undigested extract}}\ (\text{mg})}\right) \times 100 \quad (13)$$

in which the undigested extract refers to the original AEP and EAEP samples (2.5 mL, before digestion), the digested pellet refers to the TCA-precipitated pellet after digestion, and the enzyme blank refers to the TCA-precipitated pellet of the enzyme blank tubes (2.5 mL of RO water instead of sample). The mass of N (mg) was calculated as the product of the mass of the extract or pellet and its corresponding N%.

Three different pancreatin loadings (100, 20, and 10 trypsin U per mL digest) were used in the simulated digestions of the AEP and EAEP extracts to determine the optimum amount for the samples in this study. To visualize the protein molecular weight distribution of the digests with different pancreatin loadings, sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions. To generate samples for the gel, digestions were performed as described above, but instead of TCA precipitation, the digests were frozen immediately to stop digestion. Undigested AEP and EAEP extracts, pepsin, and pancreatin solutions were also included for comparison, and were diluted to mimic their respective concentrations in the final digest (*e.g.*, 2.5 mL AEP or EAEP were diluted to a final volume of 20 mL with RO water). For the gels, all samples were diluted 1 : 1 with 2× Laemmli sample buffer containing 5% β-mercaptoethanol (Bio-Rad, Hercules, CA, USA). Samples were heated at 85 °C for 10 min and cooled to room temperature before loading 15 μL of samples into each well of a pre-cast 4–20% Criterion™ TGX Precast Midi Protein Gel (Bio-Rad, Hercules, CA, USA). The gel was stained using Bio-Safe™ Coomassie Blue (Bio-Rad, Hercules, CA, USA) and destained using DI water, and the gel image was taken using a Bio-Rad Gel Doc™ EZ Imager with a white light sample tray (Bio-Rad, Hercules, CA, USA).

2.8.3. Trypsin inhibitor activity. Trypsin inhibitor activity (TIA) was performed according to the method of Kakade *et al.*[44] with modifications from Hall & Moraru[45] and Liu[46] for a reduced volume assay (5 mL). Freeze-dried AEP and EAEP samples were dispersed in DI water for a final concentration of 40 μg mL^{-1} protein. The substrate solution containing *N*α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) (MilliporeSigma, Burlington, MA, USA) was prepared by dissolving 80 mg of BAPNA in 4 mL DMSO, then adding pre-heated (37 °C) Tris–HCl buffer (50 mM, pH 8.2, containing 20 mM $CaCl_2$) to a final volume of 200 mL. The enzyme solution was prepared by dissolving trypsin (from porcine pancreas, 13 000–20 000 BAEE U per mg protein, MilliporeSigma, Burlington, MA, USA) in 1 mM HCl to achieve a final concentration of 18 μg mL^{-1}, which yielded a reference reading of <0.450 absorbance units.[47]

The assay was performed by mixing 1 mL of sample (or water for the reference reading), 1 mL of trypsin solution, and 2.5 mL of substrate solution, followed by incubation for 10 min at 37 °C. The addition of 0.5 mL acetic acid (30% w/w) stopped the reaction, and the reaction mixture was centrifuged (1230×*g*, 5 min, 25 °C). The absorbance of the resulting supernatant was measured at 410 nm. Sample background measurements were determined by mixing 1 mL of sample, 1 mL of trypsin solution, and 0.5 mL of acetic acid solution, then adding 2.5 mL of substrate solution. Each extraction replicate was measured in duplicate ($n = 6$). TIA was expressed in trypsin units inhibited

per mg protein (TUI per mg protein), in which one trypsin unit corresponded to a 0.02 increase in A410 for the reduced volume assay.[46]

2.8.4. Hemagglutination activity. Hemagglutination activity (HA) was determined using the visual agglutination assay in a 96-well plate as described by He *et al.*[48] with modifications. A sample solution (2 mg mL^{-1} protein) was prepared by dissolving freeze-dried AEP and EAEP powder in phosphate-buffered saline (PBS, pH 7.2) and serially diluted to obtain dilution factors of $1:2^0$, $1:2^1$, $1:2^2$, $1:2^3$, $1:2^4$, $1:2^5$, $1:2^6$, $1:2^7$, $1:2^8$, $1:2^9$, $1:2^{10}$, and $1:2^{11}$. The assay was performed by mixing 50 μL of a 2% suspension of rabbit erythrocytes (Rockland Immunochemicals Inc., Pottstown, PA, USA) with 50 μL of serially diluted AEP and EAEP extracts in a U-shaped 96-well plate (Greiner Bio One, Monroe, NC, USA). The plate was gently shaken (5 s, 300 rpm) to ensure sufficient mixing, followed by incubation at 4 °C for 90 min. PBS was used as a negative control. HA (hemagglutination units per mg protein) was reported as (eqn (14)):

$$\text{HA (HU per mg protein)} = \frac{2^n}{\text{protein (mg)}} \quad (14)$$

where n is the highest numbered well with visible agglutination and the protein (mg) refers to the first well ($1:2^0$).

2.9. Statistical analyses

Statistical analyses were performed using Student's *t*-test (for the nutritional property experiments) and one-way and two-way analysis of variance (ANOVA) with Tukey's post hoc test (all other analyses) using JMP® 16.1 (Cary, NC, USA), with a significance level set at $p < 0.05$.

3. Results and discussion

3.1. Understanding AEP and EAEP extraction mechanisms through scanning electron microscopy images of bean flour and insoluble fraction

As a preliminary investigation of the microstructure of bean flour components (protein, starch, cell wall fragments) before and after aqueous extraction, scanning electron microscopy (SEM) was performed on the bean flour and insoluble fractions from the AEP (pH 9.0, 50 °C, 1 : 10 SLR, 1 h) and EAEP (same conditions as AEP with 0.5% w/w alkaline protease) (Fig. 1). Flour microstructure has been used to help elucidate the mechanisms of AEP and EAEP from soybean, particularly to visualize the distribution of protein and oil bodies in the cell matrix.[8,49] The SEM images of the bean flour (Fig. 1a) showed that milling thoroughly disrupted the black bean cell walls, leaving behind a protein/cell wall fragment matrix with embedded starch granules, as similarly reported by Berg *et al.*[50] No oil bodies were visible, which agrees with the low oil content (<3% w.b.) of the black bean flour.[15] In the SEM images of the AEP and EAEP insoluble fractions (after extraction) (Fig. 1b and c), the starch granules were no longer tightly embedded in the flour matrix. This suggests that during extraction, the surrounding protein and soluble carbohydrate fragments were washed and/or dissolved into the aqueous media, leaving exposed starch granules in the insoluble phase. These extraction conditions for the AEP and EAEP were previously demonstrated to achieve 75 and 81% TPE from black beans, respectively,[15] but there was no discernable difference between the AEP and EAEP insoluble fractions in SEM. This was similarly observed by Campbell & Glatz[51] for the insoluble residue from soybean protein extraction with and without protease.

Hoover & Sosulski[52] reported that starch from some bean varieties swelled at around 45 °C, with visible exudate from the granules. The intact, smooth starch granules observed in all the images of the black bean flour and insoluble fractions in this work suggest that either (1) the milling and extraction conditions did not damage the granules or induce gelatinization, or (2) damaged starch granules may have been extracted into the aqueous extract fraction.[53] Damaged starch caused by milling and/or high pH conditions (>pH 9.5) may be co-extracted along with proteins in aqueous extraction,[54] which ultimately decreases the purity of the protein extract.[55]

3.2. Effect of solids-to-liquid ratio (SLR) and amount of enzyme on protein extractability

Black bean proteins were extracted using the AEP and EAEP under various conditions to understand the impacts of SLR and the amount of enzyme on protein extractability. SLR and enzyme loading are key drivers of protein extractability in aqueous and enzymatic extractions. These factors have inherent effects on mass transfer and protein solubility, the latter being significantly influenced by proteolysis. Notably, pH is also an

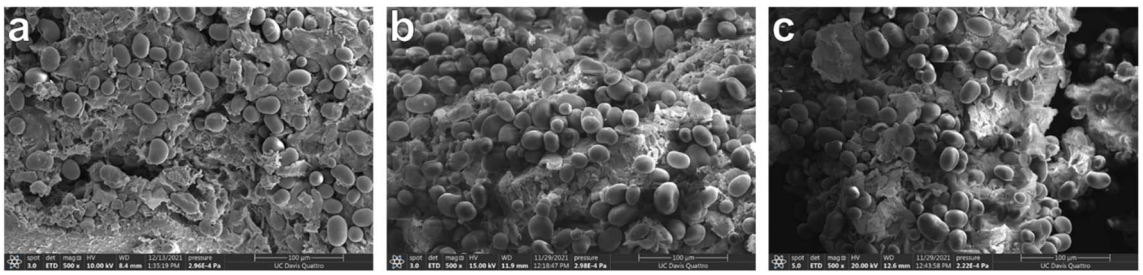

Fig. 1 Scanning electron microscopy (SEM) images (500× magnification) of (a) black bean flour and the insoluble fractions of (b) the aqueous extraction process (AEP, pH 9.0, 1 : 10 SLR, 50 °C, 1 h) and (c) the enzyme-assisted aqueous extraction process (EAEP, pH 9.0, 1 : 10 SLR, 50 °C, 1 h, 0.5% w/w alkaline protease).

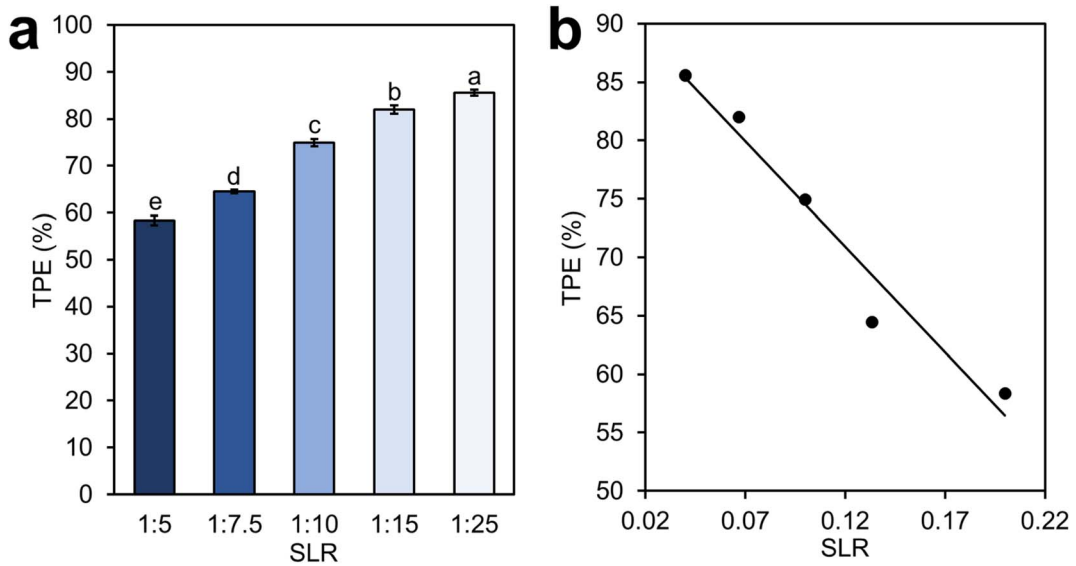

Fig. 2 Effect of solids-to-liquid ratio (SLR) on (a) total protein extraction (TPE, %) of the aqueous extraction process (AEP: pH 9.0, 50 °C, 1 h) (n = 3, ± SD) and (b) linear association between SLR and TPE (%). Different letters represent significant differences (p < 0.05) as determined by one-way ANOVA with Tukey's test.

important parameter that influences protein solubility and, therefore, extractability. However, due to the pH constraints of the enzyme (alkaline protease) used in the EAEP, the pH for all extractions in this study were fixed at pH 9.0.

When evaluating the effect of SLR in the AEP (no enzyme) on protein extraction yields, the results demonstrated that TPE increased as SLR decreased (more dilute system) (Fig. 2a). Specifically, a TPE of 58% was achieved using 1 : 5 SLR, while a TPE of 86% was achieved using 1 : 25 SLR. Increased extractability at lower SLR was expected and has also been reported for chickpea, lentil, and navy bean protein extractions.[56] A lower SLR corresponds to a steeper concentration gradient between the protein in the flour and extraction media, thus driving protein diffusion into the liquid phase.[49,57] A lower SLR also reduces the viscosity of the extraction slurry, which facilitates mass transfer between the protein in the flour particles and the aqueous media.[8,21] The linear regression shown in Fig. 2b (TPE = −181 × SLR + 92.7) shows that protein extraction in the AEP was linearly associated with SLR (R^2 = 0.95, p < 0.001).

Notably, extractions performed at a 1 : 5 SLR were too viscous to be well-mixed, and therefore, would not represent a practical option for commercial adoption. Although the 1 : 25 SLR showed the highest extraction yields, a 3% increase in TPE compared to the AEP performed using 1 : 15 SLR did not justify the 67% increase in water usage. In addition, a major disadvantage of selecting low SLRs like 1 : 25 is that the resulting

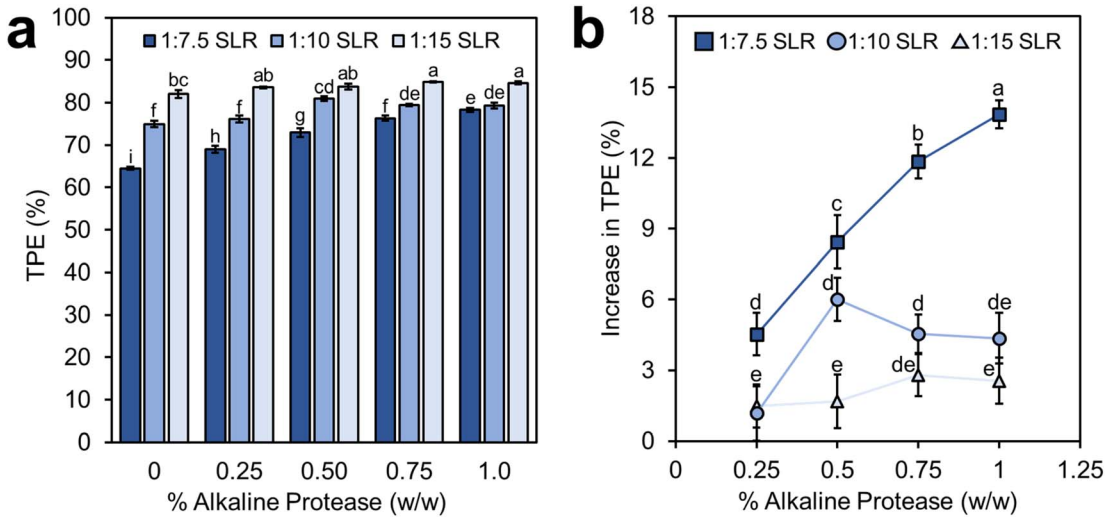

Fig. 3 Effect of amount of alkaline protease (% w/w) on (a) total protein extraction (TPE, %) of the enzyme-assisted aqueous extraction process (pH 9.0, 50 °C, 1 h) for 1 : 7.5, 1 : 10, and 1 : 15 SLR (n = 3, ±SD) and (b) the increase in TPE (%) compared to the AEP control (same conditions, without enzyme). Different letters represent significant differences (p < 0.05) as determined by two-way ANOVA with Tukey's test.

protein extract is very dilute, which may complicate downstream protein recovery (*i.e.*, high volumes of slurry for centrifugation, high volume of liquid "whey" byproduct following precipitation, or large permeate volumes upon filtration). Thus, the 1 : 7.5, 1 : 10, and 1 : 15 SLRs were selected for subsequent study of the effects of enzyme loading on the EAEP.

Fig. 3a shows the effects of the amount of enzyme (0 to 1% w/w; weight of enzyme/weight of flour) in the EAEP on TPE. A two-way analysis of variance (ANOVA) revealed that SLR, the amount of enzyme, and the interaction term (SLR × amount of enzyme) were all significant ($p < 0.001$) in explaining the variation in protein extractability. When comparing the impact of these two factors on TPE, the ANOVA suggests that SLR had a stronger influence ($F = 1187$, $p < 0.001$) compared to the amount of enzyme ($F = 185$, $p < 0.001$). Similar trends have been reported for green coffee proteins and carbohydrate-digested rice proteins.[12,58] Importantly, the results also showed that the addition of alkaline protease was more impactful for extractions with a higher SLR (more concentrated extraction slurry) (Fig. 3b). Although the addition of 1% enzyme in the EAEP with a 1 : 15 SLR provided a marginal increase in TPE (<3%), it led to a substantial 14% increase in TPE for the EAEP with a 1 : 7.5 SLR. While extraction in more concentrated systems inherently faces higher resistances to mass transfer (*i.e.*, higher viscosity and lower concentration gradient between solute in the matrix and extraction media), these results reveal that for some systems, enzyme action can sufficiently counteract these resistances, therefore achieving high extractability with lower water inputs. Thus, with respect to commercial-scale processing, enzyme addition may be a useful strategy to improve extraction efficiency for higher SLR extractions. Decreased water usage and slurry volumes (potentially fewer tanks and/or centrifugation cycles) may help to offset the cost of the enzymes. Future technoeconomic analyses are required to assess the economic feasibility of such processes on large scale more quantitatively.

From the results of the SLR and enzyme loading optimization, conditions were selected for the AEP (pH 9.0, 50 °C, 1 : 15 SLR) and EAEP (pH 9.0, 50 °C, 1 : 7.5 SLR, 1% w/w alkaline protease) to explore protein extraction kinetics. With an extraction time of 1 h, the AEP and EAEP with the selected conditions achieved 82 and 78% TPE, respectively. Although the EAEP under these selected conditions yielded slightly lower extractability than the AEP (statistically significant), we selected the most concentrated slurry (1 : 7.5 SLR) and highest enzyme loading (1% alkaline protease) to further investigate the remarkable increase in extractability (from 64% for AEP to 78%) achieved by the EAEP with high SLR (1 : 7.5 SLR) (Fig. 3b).

3.3. Extraction kinetics and modeling

Kinetic modeling of protein extraction was performed to understand how processing parameters in the AEP and EAEP affect the rate of extraction. This analysis was performed to provide insights about the temporal evolution of protein extractability within each processing strategy to further guide the selection of the total extraction time for both processes. Two kinetic models were explored in this study: (1) a first order kinetic model (eqn (3)), which was used by Aguilera & Garcia[23] to model lupin protein extraction (similar pulse matrix as the black beans in this study), and (2) the Peleg model (eqn (5)), which has been more generally applied to solid–liquid extraction for a variety of plant components including proteins and phenolics.[24,26,27]

For the AEP, TPE at 1 min of extraction was 85%, demonstrating that the extraction of black bean proteins occurred very quickly. TPE increased sharply from 0–30 min, then plateaued, achieving 87% TPE after 2 h (Fig. 4a). Conversely, for the EAEP, TPE followed a parabolic-like pattern in the first 20 min of extraction. At 1 min of extraction, TPE for the EAEP was 73%, followed by a sharp increase in TPE until 8 min (77% TPE), then TPE decreased back to 73% at 20 min. After this initial burst-drop period (phase I in Fig. 4b), TPE gradually increased to 80% at 2 h of extraction. Notably, the scaled-up extraction (5 L) performed for the kinetic studies achieved slightly higher TPE (87% for AEP and 79% for EAEP) compared to the lab-scale extractions (500 mL, 82% TPE for AEP and 78% for EAEP) at 60 min discussed in Section 3.2. This could be attributed to the different geometries of the reactor and impeller that could achieve more consistent mixing compared to the magnetic stir bar used in lab-scale extractions,[17] as well as the different centrifugation volumes and geometries that may have resulted in different separation efficiencies (500 mL flat-bottom centrifuge tube for lab-scale, 50 mL conical centrifuge tube for the kinetic study).

Model fitting of the AEP to eqn (3) (first order model) showed that protein extraction could be explained with first order kinetics (adjusted $R^2 = 0.94$), with the TPE plateauing after around 30 min of extraction (Fig. 4a). This plateau of TPE over time has been hypothesized to coincide with pH stabilization, as the solubilization of proteins typically occurs rapidly for finely milled flours like the bean flour used in this study.[49] Although the estimated parameters for the first order model cannot be directly compared to the values obtained by Aguilera & Garcia[23] due to inherent differences in sample and extraction conditions (lupin proteins, pH 8.0, 1 : 25 SLR), the rate constants calculated for the bean AEP ($k = 0.114$ min^{-1}) were in a similar range to those for lupin protein extraction ($k = 0.065$ to 0.141 min^{-1}).[23] The parameter C_w is the "concentration of washing", which represents the amount of protein extracted at $t = 0$; in other words, C_w reveals the amount of protein that can be readily extracted as soon as the bean flour comes in contact with the extraction media. The small increment in extractability (<3%) between C_w and C_∞ (theoretical maximum TPE) for the AEP signifies that most of the extractable proteins diffused into the aqueous phase very quickly, likely due to the steep concentration gradient between the protein in the bean flour and the alkaline media.[21] Overall, the first-order model suggests that for the AEP at 1 : 15 SLR, it is possible to achieve 99% of the C_∞ (86% TPE) after only 10 min of extraction, and 99.9% of the C_∞ (87% TPE) after 30 min of extraction.

In contrast, protein extraction in the EAEP only followed first-order kinetics from 20–120 min (phase II in Fig. 4b), after the initial parabolic-like change in TPE in the first 20 min of extraction. In comparing the first-order model parameters of

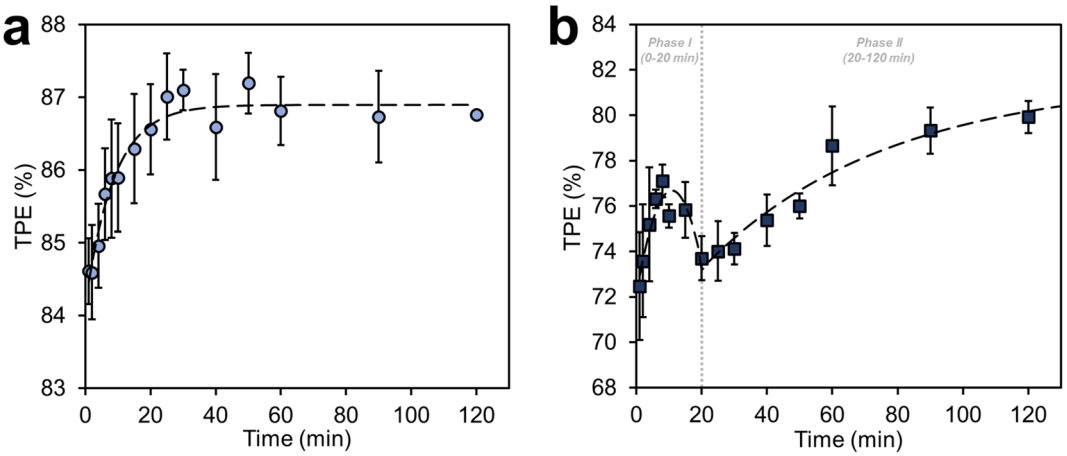

Fig. 4 Total protein extraction (TPE, %) as a function of extraction time (min) for the (a) AEP (pH 9.0, 50 °C, 1 : 15 SLR) and (b) EAEP (pH 9.0, 50 °C, 1 : 7.5 SLR, 1.0% w/w alkaline protease) ($n = 3$, ± SD). Dashed lines represent curves fit with nonlinear regression (first order kinetic model). SLR = solids-to-liquid ratio.

phase II to those obtained for the AEP (Table 1), all the fitted values for the EAEP were lower, indicating lower overall extractability and a slower rate of extraction. It is difficult to definitively attribute this trend to a single extraction parameter, as we demonstrated in Section 3.2 that SLR and the amount of enzyme added have individual and interactive effects on TPE. However, a plausible explanation for the lower C_∞, C_w, and k of the EAEP is the use of a higher SLR (1 : 7.5) compared to the AEP, where a much lower SLR (1 : 15) was used. As previously explained in Section 3.2, a high SLR can lead to higher resistances to mass transfer. As expected, the larger difference between C_w and C_∞ for the EAEP (12% TPE) and the lower rate constant demonstrates that for more concentrated slurries (higher SLR), longer extraction times are required to achieve high extractability (approaching theoretical maximum). Specifically, in the 2 h of extraction for the EAEP, the TPE did not plateau at the C_∞, compared to the AEP in which the TPE approached the C_∞ within 30 min. With 60 min of extraction for the EAEP, the first order model predicted a TPE of 77.5%, which was 95% of the theoretical maximum. Marginal increases in TPE (<2%) were observed for extraction times beyond 60 min.

Table 1 Model parameters of the AEP and EAEP (phase II, 20–120 min only) using the first order model and the Peleg model

	AEP	EAEP (phase II)
First order model		
C_∞ (%)	86.9 ± 0.2	81.6 ± 5.1
C_w (%)	84.2 ± 0.4	69.7 ± 4.7
k (min^{-1})	0.114 ± 0.041	0.018 ± 0.022
Adjusted R^2	0.9435	0.9235
RMSE	0.2092	0.7042
Peleg's model		
C_0 (%)	83.8 ± 0.9	69.3 ± 7.5
k_1 (min $\%^{-1}$)	1.59 ± 1.59	3.83 ± 8.26
k_2 ($\%^{-1}$)	0.298 ± 0.071	0.0590 ± 0.0240
B_0 (min^{-1})	0.631	0.261
C_e (%)	87.2	86.2
Adjusted R^2	0.9071	0.9173
RMSE	0.2683	0.7322

For the Peleg model, the AEP and phase II of the EAEP fit the model well as shown by the high adjusted R^2 values (Table 1). The estimated C_0 for the AEP and EAEP were similar to C_w in the first order model, which was expected since they provide similar estimates of the initial protein extractability at $t = 0$. The values for B_0, the initial rate of extraction, also followed the same trend as the k values for the first order model, as the AEP had a faster initial rate compared to the EAEP. The C_e term, which describes the maximum predicted increase in TPE at $t = \infty$, yielded similar values as C_∞ for the first order model of the AEP (86.9% for first order *vs.* 87.2% for Peleg's). Conversely, for the EAEP, the Peleg model predicted a higher theoretical maximum of 86.2% TPE (*c.f.*, 81.6% for first order model). Both kinetic models predicted nearly identical curves for the EAEP in the time range of 20–120 min, but the first-order model achieved a better fit for the AEP compared to the Peleg model based on the adjusted R^2 and RMSE; therefore, only the first-order fitting curves were presented in Fig. 4. Over longer time scales beyond the experimental range, however (*e.g.*, 300 min), significant differences in the models were observed, as the Peleg model estimated a continual increase in TPE, while the first order model plateaued (data not shown). Overall, both models were able to explain the increase in protein extraction over time in the AEP and phase II of the EAEP.

With respect to the initial "burst-drop"-like stage of extraction (0 to 20 min) in the EAEP that did not fit the first order or the Peleg model, we hypothesize that proteolysis likely caused structural modifications that may have decreased the separation efficiency of the extracted proteins during the centrifugation step. Previous studies of common bean protein hydrolysates reported an initial increase in surface hydrophobicity (H_0) followed by a gradual decrease over time for hydrolysis using pepsin and papain.[59,60] This change, which has also been documented for rice endosperm protein and corn

glutelin,[61,62] has been attributed to the reburying of newly exposed hydrophobic residues as a result of proteolysis. In the initial stages of proteolysis, the cleavage of peptide bonds could have exposed hydrophobic groups that were formerly buried, therefore increasing H_0. While these proteins may have been extracted into the aqueous phase, the separation efficiency during centrifugation could have been hindered by the exposure of hydrophobic sites, which could favor interaction amongst protein molecules and result in the precipitation of these proteins in the insoluble phase. As proteolysis continued, however, these hydrophobic regions may have been cleaved, or the protein fragments may have refolded to bury the hydrophobic groups, causing H_0 to decrease and therefore resume the expected first-order increase in protein extractability over time. A significant decrease in the H_0 of black bean proteins extracted with alkaline protease (1 : 10 SLR 0.5% w/w, 60 min) was reported in our previous work compared to the AEP control (same conditions without enzyme),[15] demonstrating the effect of proteolysis on H_0 on a longer time scale. Future in-depth study of the relationship between proteolysis and surface properties is required to better explain the transient extraction behavior observed in phase I of the EAEP.

While the kinetics of the aqueous extraction can be described by the diffusion of proteins from the solid phase to the liquid phase, the time-dependent changes in protein size and surface properties in enzymatic extractions will dynamically affect the apparent diffusion coefficient, the viscosity of the slurry, and overall mass transfer. We acknowledge that the kinetic modeling performed in the present work provides a generalized view of the many complex reactions and phase transitions that occur, particularly in the EAEP. However, the results presented herein can enhance our understanding of bean protein extraction kinetics and inform commercial-scale processing decisions. A major conclusion from modeling is that for the AEP (1 : 15 SLR, pH 9.0, 50 °C), 30 min of extraction was sufficient to achieve maximum TPE (predicted 87% TPE), while for the EAEP (1 : 7.5 SLR, pH 9.0, 50 °C, 1% w/w alkaline protease), 60 min of extraction would be required to achieve the predicted 78% TPE.

Extractions using the selected conditions were performed at the 500 mL scale (same scale as extractions discussed in Section 3.2) and yielded 81.7 ± 0.7% and 78.3 ± 0.5% TPE for the AEP and EAEP, respectively. While for the AEP, extraction yields were slightly lower than the predicted values from the first-order model (87% TPE), this could be attributed to differences in reactor geometry and the agitation mechanism as aforementioned. These extracts were subsequently characterized with respect to their physicochemical, functional, and nutritional properties.

3.4. Isoelectric point of AEP and EAEP proteins

Extraction methods and conditions can impact the surface charge of proteins, consequently altering the functionality and stability of proteins in dispersions that can affect the quality and consistency of food products. Therefore, the zeta potential of the proteins in the AEP and EAEP extracts was determined from pH 2.0 to 9.0 to define the isoelectric point (pI) of the proteins and to elucidate the effect of pH on the surface charge of the black bean proteins (Fig. 5a). The pI of a protein is often the point of least solubility and is critical information if proteins will be recovered using isoelectric precipitation. The AEP pI was pH 3.4, while the EAEP pI was <pH 2. Similar results were reported for chickpea protein isolates, in which the pI of the unhydrolyzed protein isolate was pH 4.1, but the pIs of the hydrolysates (degree of hydrolysis, DH, of 4 to 14.67%) were all <pH 2.[63] This observation has been ascribed to the increased presence of ionizable groups as the result of proteolysis, therefore increasing the overall protein surface charge.[17] In previous work, the pI of common bean protein isolates has been reported to be around pH 4.6.[64] However, water-soluble albumins are often lost as a byproduct in the "whey" (supernatant) following IEP; therefore, the unique composition of the AEP and EAEP extracts (mixture of albumins and globulins) could explain the lower pI observed in this work.[15] The DH of AEP and EAEP proteins were 4.6 and 21.1%, respectively, demonstrating that the alkaline protease was very effective in hydrolyzing bean proteins (Fig. 5a).

3.5. Functional properties

3.5.1. Solubility. Solubility is often considered the most important and most studied functional property and is dependent on the intrinsic composition and structure of the proteins, which may be affected by processing (proteolysis, heating, drying, *etc.*), as well as environmental factors (temperature, pH, ionic strength, *etc.*).[65,66] The solubility of the proteins in the AEP and EAEP extracts were assessed at pH 3.4 (isoelectric point of AEP proteins; to simulate acidic foods), pH 5.5 (to simulate the pH of meat and/or plant-based meat/egg analogues), and pH 7.0 (to simulate neutral foods) (Fig. 5b). EAEP proteins were significantly more soluble under acidic conditions, achieving 43 and 40% higher solubility than the AEP proteins at pH 3.4 and 5.5, respectively. These results align with the zeta potential results (Fig. 5a) which showed that EAEP proteins had much higher surface charge at pH 3–4 compared to AEP proteins. Increased surface charge is often associated with higher protein solubility due to the repulsive forces of the charged particles that resist protein–protein interaction and aggregation.[67] There was no significant difference in solubility for AEP and EAEP proteins under neutral conditions (96–99%), which agrees with previous reports that the effects of proteolysis on protein solubility are often more prominent near or at the protein isoelectric point.[15,68]

3.5.2. Emulsification. The emulsifying capacity (EC) of the AEP proteins was not significantly different among the pH values tested (338–376 g oil per g protein) (Fig. 5c). Interestingly, the EAEP proteins were not able to form stable emulsions at pH 3.4 and 7.0, but at pH 5.5, they achieved similar EC as the AEP proteins. From our previous work, we observed that there was no significant difference in the EC of the AEP and EAEP (0.5% alkaline protease) proteins.[15] However, the present study employed a higher enzyme loading (1.0 *vs.* 0.5% w/w), resulting in a high level of proteolysis (DH = 21%). A possible explanation

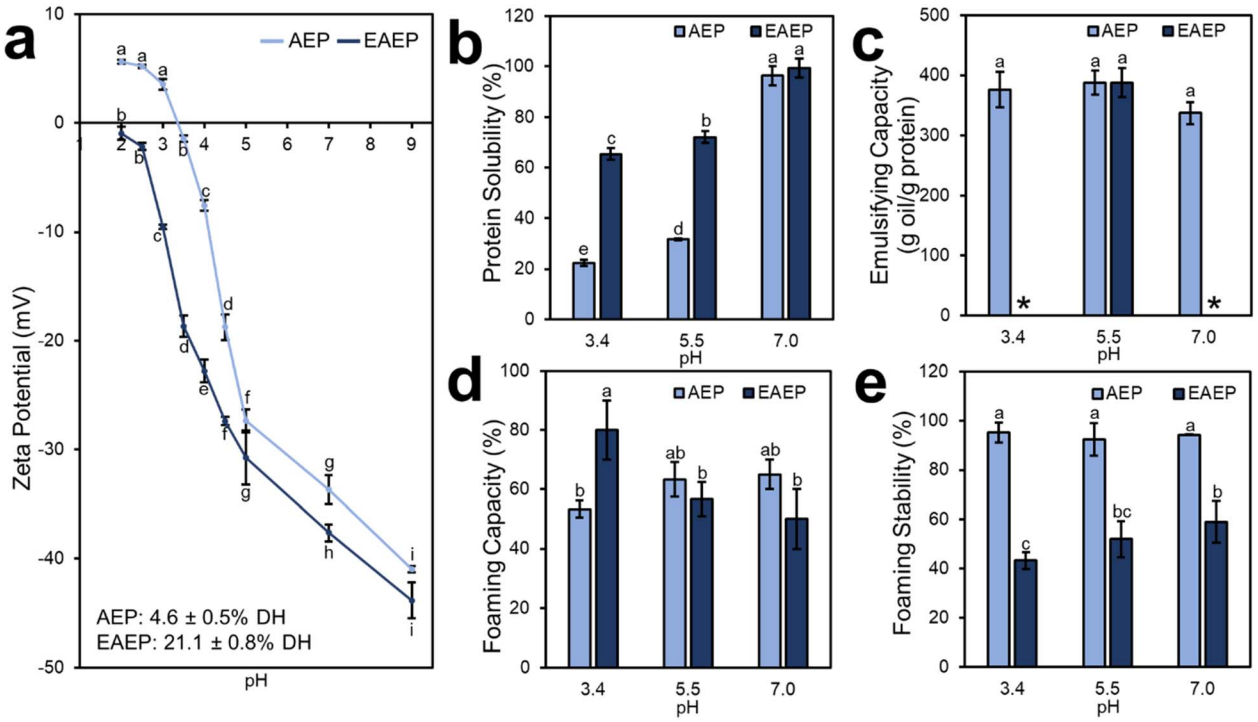

Fig. 5 (a) Zeta potential (mV), (b) protein solubility (%), (c) emulsifying capacity (g oil per g protein) where (*) indicates no emulsion formed, (d) foaming capacity (%), and (e) foaming stability (%) of AEP (pH 9.0, 50 °C, 1 : 15 SLR, 30 min) and EAEP (pH 9.0, 50 °C, 1 : 7.5 SLR, 60 min, 1% alkaline protease) proteins at pH 3.4, 5.5, and 7.0 ($n = 3$, ± SD). Different letters represent significant differences ($p < 0.05$) as determined by two-way ANOVA with Tukey's test.

for the emulsifying ability of the EAEP proteins at pH 5.5 could be related to the surface charge of the proteins. As pH increased (pH 3.4 to 5.5 to 7.0), the absolute zeta potential increased, suggesting higher electrostatic repulsion. Electrostatic repulsive forces are responsible for preventing oil droplet flocculation in protein-stabilized emulsions, as charged proteins form the interfacial membrane around oil droplets.[69,70] Perhaps at pH 3.4, electrostatic repulsion was insufficient to form a cohesive interfacial membrane, but at pH 7.0, the hydrolyzed proteins may have been too charged and hydrophilic (*i.e.*, excessive electrostatic repulsion) to form an interfacial layer strong enough to resist oil droplet coalescence.[71,72] In addition, the excessive migration of hydrolysates to the interface has been demonstrated to cause an overcrowding effect, preventing the required molecular rearrangement at the interface for emulsion formation.[63] The conditions at pH 5.5 may have achieved an ideal balance of surface charges to successfully form a cohesive protein film around the oil droplets.

Existing literature demonstrates the varying effects of hydrolysis on emulsification properties, with some studies showing that limited hydrolysis improved EC,[63,73] and others reporting that even very low levels of hydrolysis (<4–5% DH) were deleterious towards EC.[71,74] These findings emphasize the importance of the pH of the aqueous phase in dictating the EC of bean proteins, which is critical when considering practical applications in acidic or neutral food emulsions.

3.5.3. Foamability. The foaming capacity (FC) of the AEP and EAEP proteins were similar at pH 5.5 and 7.0 (50–65%) (Fig. 5d). However, at pH 3.4 (pI of AEP proteins), the FC of the EAEP samples was significantly higher (53% for AEP, 80% for EAEP). While improvements in protein solubility due to proteolysis may have contributed to the increased FC at pH 3.4, there was no significant difference in FC at pH 5.5 for the AEP and EAEP proteins despite their very different solubilities. This demonstrates that while solubility may be an important contributor to foaming properties close to the protein pI, there are many other factors involving foam formation that may have affected FC. As previously discussed for emulsifying properties, the effects of proteolysis on foaming properties are also highly variable and dependent on matrix, type of enzyme, enzyme loading, and degree of hydrolysis.[75,76] Some studies have found that enzymatic hydrolysis improved foaming capacity,[77–79] while others reported the opposite trend,[80] or no significant difference.[73]

The EAEP proteins exhibited lower foaming stability compared to the AEP proteins at all pHs values (Fig. 5e). Lower FS as a result of proteolysis agrees with the results in our previous study,[15] as well as with other findings for pea and lentil protein hydrolysates.[78,81] The stability of foams is related to the thickness of the protein film that surrounds air bubbles. Hydrolysates, especially with high DH like the EAEP proteins in this study, may form thinner films that collapse at a faster rate.[78] Los *et al.*[73] reported that foaming stability improved for bromelain-hydrolyzed carioca bean proteins compared to the unhydrolyzed control; however, the hydrolysis was limited to DH of 6 and 9%, which is much lower than for the EAEP proteins in the present work.

3.6. Nutritional properties

3.6.1. Amino acid composition. Table 2 shows the amino acid composition of the AEP and EAEP extracts. The most abundant amino acids were Asx (Asn/Asp) and Glx (Gln/Glu), followed by Leu, Lys, and Phe, which aligns with previously reported amino acid profiles for common bean (*Phaseolus vulgaris*) proteins.[82] The limiting amino acids were the sulfur-containing amino acids Cys and Met, which is characteristic for pulses.[83] Out of all the amino acids, the only significant differences between the two extraction methods were for Lys (6.4% higher in EAEP), Tyr (3.9% higher in EAEP), Gly (2.3% higher in AEP), and Phe (1.8% higher in AEP). Pulses (deficient in Cys and Met) are often blended with cereal proteins (deficient in Lys).[84] Therefore, a high-Lys EAEP protein extract may be of interest for applications in pulse/cereal blends to achieve a complete protein ingredient with a balanced amino acid profile. There were no significant differences in the amino acid classes (essential, sulfur-containing, aromatic, or hydrophobic) between the AEP and EAEP extracts. This indicates that for black beans, except for slight changes in the contents of a few amino acids, proteolysis by alkaline protease did not substantially affect the types of proteins that were extracted. Similar results showing no significant change in amino acid composition for the AEP and EAEP were found by de Moura *et al.*[6] for soy protein extracted with and without protease.

Table 2 Amino acid composition (% w/w of protein) of the AEP (pH 9.0, 50 °C, 1 : 15 SLR, 30 min) and EAEP (pH 9.0, 50 °C, 1 : 7.5 SLR, 60 min, 1% alkaline protease) proteins ($n = 3 \pm$ SD)[a]

	AEP (%)	EAEP (%)
Asx[b]	12.87 ± 0.01^{B}	12.92 ± 0.1^{B}
Thr	4.71 ± 0.04^{I}	4.71 ± 0.03^{FG}
Ser	5.78 ± 0.01^{G}	5.79 ± 0.03^{E}
Glx[c]	16.69 ± 0.07^{A}	16.66 ± 0.15^{A}
Pro	4.56 ± 0.04^{J}	4.20 ± 0.69^{GH}
Gly*	3.57 ± 0.02^{M}	3.49 ± 0.02^{I}
Ala	4.2 ± 0.04^{K}	4.26 ± 0.02^{GH}
Val	5.00 ± 0.03^{H}	5.02 ± 0.05^{F}
Ile	4.63 ± 0.02^{IJ}	4.62 ± 0.04^{FG}
Leu	8.42 ± 0.02^{C}	8.38 ± 0.07^{C}
Tyr*	3.77 ± 0.02^{L}	3.92 ± 0.05^{HI}
Phe*	6.27 ± 0.02^{E}	6.16 ± 0.05^{E}
His	2.98 ± 0.01^{N}	2.93 ± 0.03^{J}
Lys*	6.54 ± 0.05^{D}	6.95 ± 0.05^{D}
Arg	6.12 ± 0.02^{F}	6.07 ± 0.07^{E}
Cys	1.10 ± 0.01^{Q}	1.10 ± 0.01^{K}
Met	1.26 ± 0.00^{P}	1.26 ± 0.03^{K}
Trp	1.54 ± 0.06^{O}	1.57 ± 0.04^{K}
[d]EAA	41.36 ± 0.30	41.61 ± 0.32
[e]SCAA	2.36 ± 0.04	2.36 ± 0.04
[f]AAA	11.59 ± 0.07	11.65 ± 0.07
[g]HAA	39.46 ± 0.46	38.96 ± 0.55

[a] AA marked with (*) signify statistically different amino acid content between the AEP and EAEP using Student's *t*-test (rows, $p < 0.05$). Different capital letters signify statistically different amino acid contents within the AEP or EAEP using one-way ANOVA (columns, $p < 0.05$). [b] Asx: asparagine/aspartic acid. [c] Glx: glutamine/glutamic acid. [d] EAA: essential amino acids. [e] SCAA: sulfur-containing amino acids. [f] AAA: aromatic amino acids. [g] HAA: hydrophobic amino acids.

3.6.2. *In vitro* protein digestibility: method modifications and impacts of enzymatic extraction. Although beans are widely considered as protein-rich foods, bean proteins are relatively resistant to digestive enzymes and possess several antinutritional factors (*e.g.*, trypsin inhibitors, polyphenols) that may inhibit digestion or cause other undesirable side effects.[85,86] In protease-assisted extraction, the proteolysis that occurs during extraction has the potential to improve protein digestibility as it (1) serves as a primary digestion step and/or (2) may inactivate certain antinutritional proteins (*i.e.*, trypsin inhibitors). Interestingly, previous reports suggest that proteolysis has minimal or even deleterious effects on IVPD, which could be partly related to the method of calculation employed. For example, Betancur-Ancona *et al.*[80] reported that *Phaseolus lunatus* protein isolate had higher *in vitro* digestibility than its flavourzyme- and alcalase-derived hydrolysates; however, IVPD was calculated based on the change in pH during trypsin/chymotrypsin digestion, which may underestimate IVPD due to the presence of fewer hydrolysis sites at the beginning of the simulated digestion. This aligns with the observation by Ribéreau *et al.*,[87] that pre-digested pea proteins (bromelain, papain, or trypsin) experienced very limited hydrolysis in subsequent *in vitro* digestion processes due to the fewer available digestion sites. Similarly, de Souza *et al.*[88] reported that almond proteins produced using EAEP had slightly lower IVPD than the AEP proteins; in that study, only proteinaceous nitrogen was considered as the "protein in the sample before digestion", meaning the soluble peptides present in the EAEP extract were disregarded in the IVPD calculation.[88] These underestimations emphasize the importance of carefully defining the starting point of *in vitro* digestion for pre-hydrolyzed samples, as this could significantly affect the calculated IVPD.

For the simulated digestion of the bean samples, the INFOGEST 2.0 method was followed,[39] with modifications in pancreatin concentration as similarly described by other authors.[41,42,89] According to the INFOGEST method, pancreatin should be added in the intestinal phase to achieve 100 trypsin U per mL, which corresponds to the addition of 394 mg of pancreatin per digestion tube containing 2.5 mL sample. High amounts of digestive enzymes, in this case nearing enzyme/substrate ratios (E : S; weight pancreatin/weight protein in sample) of 13 : 1 for the AEP and 7 : 1 for the EAEP, result in high nitrogen enzyme blanks that could reduce the sensitivity of the final calculations. In addition, from a practical standpoint, preparing pancreatin solutions of such high concentrations results in a highly viscous slurry, therefore complicating accurate solution preparation and pipetting of the enzyme solution into the digestion tubes. Another problem with high pancreatin loadings is the susceptibility of pancreatin to autolysis when used in high concentrations.[40,90,91] The extent of autolysis may vary between the enzyme blank (water) and the samples due to the different protein contents (*i.e.*, amounts of available protein substrates) in the digests,[91] thereby obscuring the measured value of the nitrogen content of the enzyme blank. Previous studies have demonstrated that a 10-fold reduction in pancreatin (10 trypsin U per mL) minimally impacted *in vitro*

digestibility for various animal and plant proteins.[42,89] Beaubier *et al.*[92] also commented that for more pure foods like protein isolates, lower E : S ratios are more suitable; in their study, the pepsin and pancreatin activities were 14 and 100 times less, respectively, than the recommended INFOGEST concentrations.

To address this issue, digestions were performed with 100 trypsin U per mL (as recommended by INFOGEST) and with 20 and 10 trypsin U per mL. The molecular weight distribution of the enzyme blank, AEP, and EAEP digests at the three pancreatin loadings were visualized using SDS-PAGE, along with the undigested AEP and EAEP samples (Fig. 6a), and individual enzymes for comparison (Fig. 6b). The pattern of gel bands clearly shows that the proteins in the 100 trypsin U per mL digests were predominately from pancreatin. No visible differences were observed between the 20 and 10 U per mL enzyme blanks and digests. With respect to the measured IVPD, the results reveal the significance of pancreatin loading on the apparent digestibility (Fig. 7). For the AEP, similar values were obtained using 100 and 20 trypsin U per mL pancreatin (34% IVPD). However, with 10 trypsin U per mL, the IVPD of the AEP was significantly lower (21%), suggesting that perhaps more enzyme was necessary to achieve adequate hydrolysis. For the EAEP, the 20 and 10 trypsin U per mL pancreatin loadings achieved similar results (60–61% IVPD), which is plausible due to the pre-digestion of the EAEP proteins during the extraction step that would not require much protease to further digest the sample. However, with 100 trypsin U per mL pancreatin, the IVPD of the EAEP was significantly lower (42%). A potential explanation could be related to autolysis of the pancreatin, as the EAEP with 100 trypsin U per mL pancreatin had the highest E : S of all the conditions tested. With few digestion sites available due to the pre-hydrolysis of the EAEP proteins (*i.e.*, low substrate concentration), the pancreatin present in excess may have undergone autolysis, therefore reducing its digestive activity. Based on these findings, 20 trypsin U per mL could be used as a suitable pancreatin concentration for simulated digestions of hydrolyzed (~20% DH) and unhydrolyzed pulse protein extracts with protein contents ranging from 1.2–2.3% protein (w/v). Using this modified INFOGEST digestion, proteolysis in the EAEP improved IVPD from 34 to 61% compared to the AEP. This demonstrates that enzyme-assisted extraction is a feasible strategy to improve the *in vitro* digestibility of bean proteins compared to conventional aqueous extraction methods. However, there are still knowledge gaps regarding the extent of autolysis in the enzyme blanks and samples that should be explored in future studies.

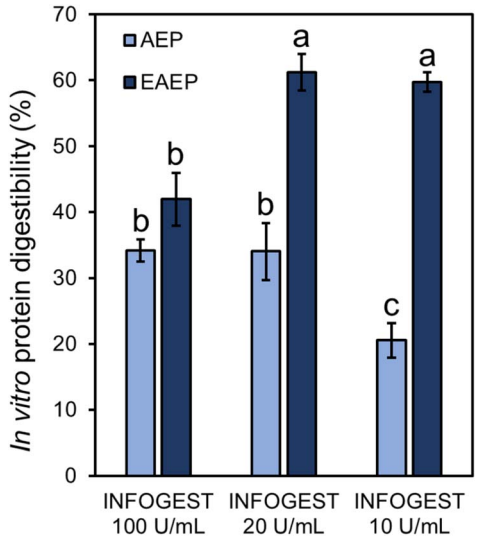

Fig. 7 *In vitro* protein digestibility (%) of AEP and EAEP proteins using pancreatin concentrations of 100, 20, and 10 trypsin U per mL digest. Different letters represent significant differences ($p < 0.05$) as determined by two-way ANOVA with Tukey's test.

3.6.3. Trypsin inhibitor activity (TIA). Common beans contain disulfide bond-rich Bowman–Birk trypsin inhibitors that possess two independent binding sites for trypsin and chymotrypsin.[93,94] When ingested, active trypsin inhibitors can hinder protein digestion and have also been linked to

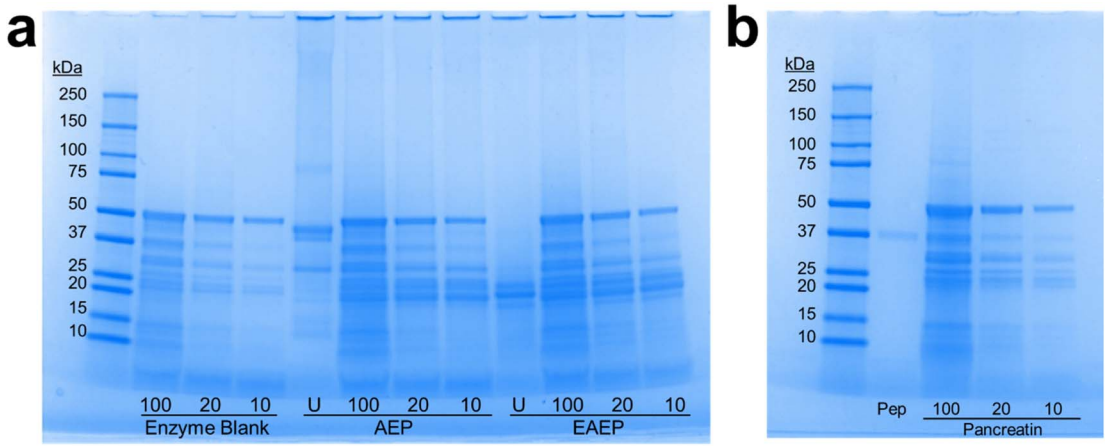

Fig. 6 (a) SDS-PAGE in reducing conditions of digested enzyme blanks, undigested (U) and digested AEP, and undigested (U) and digested EAEP samples, and (b) individual enzymes with pancreatin loadings of 100, 20, and 10 trypsin U per mL of digest. Undigested (U) AEP and EAEP and individual enzymes were diluted to match the concentration in the final digest using RO water.

overstimulation of pancreatic enzymes, therefore causing hypertrophy (enlargement of the pancreas).[94,95] The TIAs of the AEP and EAEP proteins were 135.6 ± 8.8 and 99.6 ± 6.6 TUI per mg protein, respectively, which demonstrates that the addition of enzyme conferred statistically significant ($p < 0.05$) reduction in trypsin inhibitors in the bean protein extract. While some trypsin inhibitors can be inactivated through heat treatments, the common bean Bowman–Birk inhibitor is known to be thermostable up to 90 °C and stable from pH 0 to 14.[96] In considering potential food applications, thermal treatments are typically not favorable due to protein denaturation and subsequent loss of functionality. Therefore, these results are promising in that the EAEP may improve the nutritional properties of black bean proteins, which is also reflected by the higher IVPD as previously discussed. However, as there was some residual trypsin inhibitor activity in the EAEP sample, there may be future opportunities to combine enzymatic extraction with other strategies to achieve enhanced inactivation.

3.6.4. Hemagglutination activity. Lectins are glycoproteins that can bind carbohydrates with great specificity. Phytohemagglutinins are a type of lectin in beans that are known antinutritional factors as they can cause cell agglutination, leading to growth retardation, poor nutrient absorption, and transient gastrointestinal issues.[95,97] The EAEP extracts in this study exhibited lower hemagglutination activity (HA) compared to the AEP extracts (640 HU per mg protein for AEP *vs.* 320 HU per mg protein for EAEP), suggesting that alkaline protease was able to partially degrade the lectins in the bean extracts. This is corroborated by our previous work in which the lack of ~31 kDa phytohemagglutinin band in the SDS-PAGE suggested degradation in the EAEP with alkaline protease, although notably, the extractions in the previous work were performed at slightly different SLR and enzyme loadings (1 : 10 SLR, 0.5% protease).[15] Ma & Wang[18] performed single- and multiple-enzyme hydrolyses of soybean agglutinin with trypsin, chymotrypsin, and thermolysin and similarly found that hydrolysis reduced HA on thermally treated soy proteins. The results herein demonstrate that the EAEP is an effective strategy to decrease the HA of bean proteins. Future work could explore the utilization of EAEP in conjunction with other strategies to achieve a more complete inactivation of lectins in bean protein extracts.

4. Conclusions

This study demonstrated that fundamental processing parameters (SLR, amount of enzyme, and extraction time) have significant effects on protein extractability and extraction kinetics from black bean flour using aqueous and enzyme-assisted processes. While the use of alkaline protease considerably improved extraction yields for the extractions with higher SLR (1 : 7.5 SLR), enzyme addition had a minor effect on total protein extraction for more dilute systems (1 : 15 SLR). This demonstrates that enzymatic extraction can be a useful water-saving strategy to enhance process sustainability and facilitate downstream water removal. Alkaline protease in the EAEP extensively hydrolyzed the proteins, improving solubility and foaming capacity at the isoelectric point, while generally hindering emulsion formation and foaming stability. These findings suggest that the high amount of enzyme (1%) led to excessive hydrolysis that specifically diminished the interfacial properties of the EAEP proteins and underscore the importance of tailoring the enzyme loadings to yield a suitable DH for the desired functional application. This work also explored challenges in defining and measuring the digestibility of protein hydrolysates and demonstrated the significant impact of the pancreatin loading used in the INFOGEST protocol on the calculated IVPD. Overall, the optimization of black bean protein extraction and characterization of the functional and nutritional properties of the protein extracts provides an important framework for future processing optimization that utilize more holistic strategies considering not only extractability, but also functionality, nutritional properties, and economic feasibility.

Data availability

Data is available from the authors upon request.

Author contributions

Jasmin S. Yang: methodology, data curation, formal analysis, writing – original draft preparation, writing – reviewing and editing; Fernanda F. G. Dias: methodology, data curation, formal analysis, writing – reviewing and editing; Juliana M. L. N. de Moura Bell: supervision, project administration, resources, writing – reviewing and editing.

Conflicts of interest

The authors declare no conflict of interest.

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References

1 L. Malcolmson and J. Han, in *Health Benefits of Pulses*, ed. W. J. Dahl, Springer Nature, 2019, pp. 129–149.
2 J. I. Boye, F. Zare and A. Pletch, *Food Res. Int.*, 2010, **43**, 414–431.
3 M. A. I. Schutyser and A. J. van der Goot, *Trends Food Sci. Technol.*, 2011, **22**, 154–164.
4 M. d. M. Contreras, A. Lama-Muñoz, J. Manuel Gutiérrez-Pérez, F. Espínola, M. Moya and E. Castro, *Bioresour. Technol.*, 2019, **280**, 459–477.
5 B. Byanju and B. Lamsal, *Food Rev. Int.*, 2021, **39**, 3314–3343.

6 J. M. L. N. de Moura, K. Campbell, A. Mahfuz, S. Jung, C. E. Glatz and L. Johnson, *J. Am. Oil Chem. Soc.*, 2008, **85**, 985–995.
7 A. Rosenthal, D. L. Pyle, K. Niranjan, S. Gilmour and L. Trinca, *Enzyme Microb. Technol.*, 2001, **28**, 499–509.
8 K. A. Campbell and C. E. Glatz, *J. Agric. Food Chem.*, 2009, **57**, 10904–10912.
9 K. A. Campbell, G. Vaca-Medina, C. E. Glatz and P. Y. Pontalier, *Food Chem.*, 2016, **208**, 245–251.
10 N. M. Almeida, F. F. G. Dias, M. I. Rodrigues and J. M. L. N. de Moura Bell, *Processes*, 2019, **7**, 844.
11 F. F. G. Dias, A. Y. Taha and L. N. d. M. Bell, *Future Foods*, 2022, **5**, 100151.
12 F. S. Almeida, F. F. G. Dias, A. C. K. Sato and J. M. L. N. de Moura Bell, *Food Bioprod. Process.*, 2021, **129**, 144–156.
13 K. Machida, Y.-P. Huang, F. F. G. Dias, D. Barile and J. M. Leite Nobrega de Moura Bell, *Food Bioprocess Technol.*, 2022, **15**, 1760–1777.
14 F. F. G. Dias and J. M. L. N. de Moura Bell, *Food Hydrocolloids*, 2022, **127**, 107534.
15 J. S. Yang, F. F. G. Dias, T. T. K. Pham, D. Barile and J. M. L. N. de Moura Bell, *Food Hydrocolloids*, 2024, **146**, 109250.
16 Y. W. Sari, M. E. Bruins and J. P. M. Sanders, *Ind. Crops Prod.*, 2013, **43**, 78–83.
17 T. S. P. de Souza, F. F. G. Dias, M. G. B. Koblitz and J. M. L. N. de Moura Bell, *Food Bioprod. Process.*, 2020, **122**, 280–290.
18 Y. Ma and T. Wang, *J. Agric. Food Chem.*, 2010, **58**, 11413–11419.
19 Q. Zhang, Y. Li, Z. Wang, B. Qi, X. Sui and L. Jiang, *Food Sci. Nutr.*, 2019, **7**, 858–868.
20 V. P. Dia, T. Gomez, G. Vernaza, M. Berhow, Y. K. Chang and E. G. De Mejia, *J. Agric. Food Chem.*, 2012, **60**, 7886–7894.
21 K. Machida, F. F. G. Dias, Z. Fan and J. M. L. N. De Moura Bell, *Processes*, 2022, **10**, 2349.
22 E. Tan, Y. Ngoh and C. Gan, *Food Chem.*, 2014, **152**, 447–455.
23 J. M. Aguilera and H. D. Garcia, *Int. J. Food Sci. Technol.*, 1989, **24**, 17–27.
24 M. Peleg, *J. Food Sci.*, 1988, **53**, 1216–1217.
25 A. Oreopoulou, G. Goussias, D. Tsimogiannis and V. Oreopoulou, *Food Bioprod. Process.*, 2020, **123**, 378–389.
26 M. Naik, V. Natarajan, N. Modupalli, S. Thangaraj and A. Rawson, *LWT–Food Sci. Technol.*, 2022, **155**, 112997.
27 A. Bucić-Kojić, M. Planinić, S. Tomas, M. Bilić and D. Velić, *J. Food Eng.*, 2007, **81**, 236–242.
28 N. Milićević, P. Kojić, M. Sakač, A. Mišan, J. Kojić, C. Perussello, V. Banjac, M. Pojić and B. Tiwari, *Ultrason. Sonochem.*, 2021, **79**, 105761.
29 P. M. Nielsen, D. Petersen and C. Dambmann, *J. Food Sci.*, 2001, **66**, 642–646.
30 D. Tagliazucchi, S. Martini, A. Bellesia and A. Conte, *Int. J. Food Sci. Nutr.*, 2015, **66**, 774–782.
31 D. A. Rickert, L. A. Johnson and P. A. Murphy, *J. Food Sci.*, 2004, **69**, 303–311.
32 Y. Bian, D. J. Myers, K. Dias, M. A. Lihono, S. Wu and P. A. Murphy, *J. Am. Oil Chem. Soc.*, 2003, **80**, 545–549.
33 S. K. Sathe and D. K. Salunkhe, *J. Food Sci.*, 1981, **46**, 71–81.
34 C. Cooper, N. Packer and K. Williams, in *Methods in Molecular Biology*, Humana Press, Totowa, NJ, 2000, vol. 159.
35 J. Ozols, in *Guide to Protein Purification*, Methods in Enzymology, ed. M. P. Deutscher, Academic Press, San Diego, CA, 1990, vol. 182, pp. 587–601.
36 C. H. W. Hirs, *Methods Enzymol.*, 1967, **11**, 59–62.
37 I. Molnar-Perl and M. Pinter-Szakacs, *Anal. Biochem.*, 1989, **177**, 16–19.
38 B. Penke, R. Ferenczi and K. Kovacs, *Anal. Biochem.*, 1974, **60**, 45–50.
39 A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carrière, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C. Martins, S. Marze, D. J. McClements, O. Ménard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and I. Recio, *Nat. Protoc.*, 2019, **14**, 991–1014.
40 N. Atallah, B. Deracinois, A. Boulier, A. Baniel, D. J. R. Bouveresse, R. Ravallec, C. Flahaut and B. Cudennec, *Foods*, 2020, **9**, 1580.
41 R. Wang, T. Sar, A. Mahboubi, R. Fristedt, M. J. Taherzadeh and I. Undeland, *Food Biosci.*, 2023, **54**, 102862.
42 R. M. C. Ariëns, S. Bastiaan-Net, D. B. P. M. van de Berg-Somhorst, K. El Bachrioui, A. Boudewijn, R. T. M. van den Dool, G. A. H. de Jong, H. J. Wichers and J. J. Mes, *J. Funct. Foods*, 2021, **87**, 104748.
43 Y. Komatsu, M. Tsuda, Y. Wada, T. Shibasaki, H. Nakamura and K. Miyaji, *J. Agric. Food Chem.*, 2023, **71**, 2503–2513.
44 M. L. Kakade, J. J. Rackis, J. E. McGhee and G. Puski, *Cereal Chem.*, 1974, **51**, 376–382.
45 A. E. Hall and C. I. Moraru, *LWT–Food Sci. Technol.*, 2021, **152**, 112342.
46 K. Liu, *J. Am. Oil Chem. Soc.*, 2019, **96**, 635–645.
47 C. Smith, W. van Megen, L. Twaalfhoven and C. Hitchcock, *J. Sci. Food Agric.*, 1980, **31**, 341–350.
48 S. He, J. Shi, Y. Ma, S. J. Xue, H. Zhang and S. Zhao, *J. Food Eng.*, 2014, **142**, 132–137.
49 A. Rosenthal, D. L. Pyle and K. Niranjan, *Food Bioprod. Process.*, 1998, **76**, 224–230.
50 T. Berg, J. Singh, A. Hardacre and M. J. Boland, *Carbohydr. Polym.*, 2012, **87**, 1678–1688.
51 K. A. Campbell and C. E. Glatz, *Biotechnol. Prog.*, 2010, **26**, 488–495.
52 B. R. Hoover and F. Sosulski, *Starch*, 1985, **6**, 181–191.
53 P. Colonna, D. Gallant and C. Mercier, *J. Food Sci.*, 1980, **45**, 1629–1636.
54 H. C. Lee, A. K. Htoon, S. Uthayakumaran and J. L. Paterson, *Food Chem.*, 2007, **102**, 1199–1207.
55 M. Jarpa-Parra, F. Bamdad, Y. Wang, Z. Tian, F. Temelli, J. Han and L. Chen, *LWT–Food Sci. Technol.*, 2014, **57**, 461–469.
56 F. A. Higa, L. Boyd, E. Sopiwnyk and M. T. Nickerson, *Cereal Chem.*, 2022, **99**, 1049–1062.

57 M. A. A. Meireles, *Extracting Bioactive Compounds for Food Products*, CRC Press, Boca Raton, FL, 2008.
58 S. Braspaiboon, S. Osiriphun, P. Peepathum and W. Jirarattanarangsri, *Heliyon*, 2020, **6**, e05403.
59 Y. Zhang and H. M. Romero, *Int. J. Biol. Macromol.*, 2020, **162**, 1516–1525.
60 J. A. do Evangelho, N. L. Vanier, V. Z. Pinto, J. J. De Berrios, A. R. G. Dias and E. da Rosa Zavareze, *Food Chem.*, 2017, **214**, 460–467.
61 X. Q. Zheng, J. T. Wang, X. L. Liu, Y. Sun, Y. J. Zheng, X. J. Wang and Y. Liu, *Food Chem.*, 2015, **172**, 407–415.
62 I. Paraman, N. S. Hettiarachchy, C. Schaefer and M. I. Beck, *Cereal Chem.*, 2007, **84**, 343–349.
63 A. M. Ghribi, I. M. Gafsi, A. Sila, C. Blecker, S. Danthine, H. Attia, A. Bougatef and S. Besbes, *Food Chem.*, 2015, **187**, 322–330.
64 Y. H. Zhang, C. H. Tang, Q. B. Wen, X. Q. Yang, L. Li and W. L. Deng, *Food Hydrocolloids*, 2010, **24**, 266–274.
65 J. E. Kinsella, *Crit. Rev. Food Sci. Nutr.*, 1976, **7**, 219–280.
66 L. Grossmann and D. J. McClements, *Food Hydrocolloids*, 2023, **137**, 108416.
67 A. Gerzhova, M. Mondor, M. Benali and M. Aider, *Food Chem.*, 2016, **201**, 243–252.
68 A. G. B. Wouters, I. Rombouts, E. Fierens, K. Brijs and J. A. Delcour, *Compr. Rev. Food Sci. Food Saf.*, 2016, **15**, 786–800.
69 S. Damodaran, *J. Food Sci.*, 2005, **70**, 54–66.
70 D. J. McClements, *Curr. Opin. Colloid Interface Sci.*, 2004, **9**, 305–313.
71 N. A. Avramenko, N. H. Low and M. T. Nickerson, *Food Res. Int.*, 2013, **51**, 162–169.
72 S. Severin and W. S. Xia, *J. Food Biochem.*, 2006, **30**, 77–97.
73 F. G. B. Los, I. M. Demiate, R. C. Prestes Dornelles and B. Lamsal, *LWT–Food Sci. Technol.*, 2020, **125**, 109191.
74 B. P. Lamsal, C. Reitmeier, P. A. Murphy and L. A. Johnson, *J. Am. Oil Chem. Soc.*, 2006, **83**, 731–737.
75 D. Betancur-Ancona, T. Sosa-Espinoza, J. Ruiz-Ruiz, M. Segura-Campos and L. Chel-Guerrero, *Int. J. Food Sci. Technol.*, 2014, **49**, 2–8.
76 S. Jung, P. A. Murphy and L. A. Johnson, *J. Food Sci.*, 2005, **70**, 180–187.
77 E. Eckert, J. Han, K. Swallow, Z. Tian, M. Jarpa-Parra and L. Chen, *Cereal Chem.*, 2019, **96**, 725–741.
78 M. Barac, S. Cabrilo, S. Stanojevic, M. Pesic, M. Pavlicevic, B. Zlatkovic and M. Jankovic, *Int. J. Food Sci. Technol.*, 2012, **47**, 1457–1467.
79 V. García Arteaga, M. Apéstegui Guardia, I. Muranyi, P. Eisner and U. Schweiggert-Weisz, *Innovative Food Sci. Emerging Technol.*, 2020, **65**, 102449.
80 D. Betancur-Ancona, R. Martínez-Rosado, A. Corona-Cruz, A. Castellanos-Ruelas, M. E. Jaramillo-Flores and L. Chel-Guerrero, *Int. J. Food Sci. Technol.*, 2009, **44**, 128–137.
81 J. Ahmed, M. Mulla, N. Al-Ruwaih and Y. A. Arfat, *Legume Sci.*, 2019, **1**, e10.
82 M. P. Hojilla-Evangelista, N. Sutivisedsak, R. L. Evangelista, H. N. Cheng and A. Biswas, *J. Am. Oil Chem. Soc.*, 2018, **95**, 1001–1012.
83 S. K. Sathe, *Crit. Rev. Biotechnol.*, 2002, **22**, 175–223.
84 M. G. Nosworthy and J. D. House, *Cereal Chem.*, 2017, **94**, 49–57.
85 G. S. Gilani, K. A. Cockell and E. Sepehr, *J. AOAC Int.*, 2005, **88**, 967–987.
86 S. S. Deshpande and S. S. Nielsen, *J. Food Sci.*, 1987, **52**, 1326–1329.
87 S. Ribéreau, A. N. A. Aryee, S. Tanvier, J. Han and J. I. Boye, *J. Food Process. Preserv.*, 2018, **42**, e13375.
88 T. S. P. de Souza, F. F. G. Dias, J. P. S. Oliveira, J. M. L. N. de Moura Bell and M. G. B. Koblitz, *Sci. Rep.*, 2020, **10**, 10873.
89 E. Lander, B. Kirkhus, D. Lindberg and T. Raastad, *J. Funct. Foods*, 2023, **102**, 105452.
90 R. J. C. Stewart, H. Morton, J. Coad and K. C. Pedley, *Int. J. Food Sci. Nutr.*, 2019, **70**, 71–77.
91 O. Ménard, L. Chauvet, G. Henry, D. Dupont, C. Gaudichon, J. Calvez and A. Deglaire, *Food Res. Int.*, 2023, **173**, 113242.
92 S. Beaubier, C. Pineda-Vadillo, O. Mesieres, X. Framboisier, O. Galet and R. Kapel, *Food Chem.*, 2023, **407**, 135132.
93 H. D. Belitz and J. K. P. Weder, *Food Rev. Int.*, 1990, **6**, 151–211.
94 I. E. Liener, *Crit. Rev. Food Sci. Nutr.*, 1994, **34**, 31–67.
95 S. Kumar, A. K. Verma, M. Das, S. K. Jain and P. D. Dwivedi, *Nutrition*, 2013, **29**, 821–827.
96 Y. S. Chan, Y. Zhang and T. B. Ng, *Appl. Biochem. Biotechnol.*, 2013, **169**, 1306–1314.
97 S. K. Sathe and D. K. Salunkhe, *Crit. Rev. Food Sci. Nutr.*, 1984, **21**, 263–287.