Separation and Purification Technology 211 (2019) 242–251

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Impact of a high hydrostatic pressure pretreatment on the separation of bioactive peptides from flaxseed protein hydrolysates by electrodialysis with ultrafiltration membranes

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ARTICLE INFO

Keywords:
Flaxseed protein
High hydrostatic pressure
Electrodialysis with ultrafiltration membrane
Bioactive peptides

ABSTRACT

High hydrostatic pressure (HHP) and electrodialysis with ultrafiltration membranes (EDUF) are two efficient technologies used respectively to improve protein enzymatic hydrolysis and recovery of bioactive peptides, but they have never been tested together. Hence, in this study, HHP pre-treatment was performed on defatted flaxseed protein isolate prior to enzymatic hydrolysis and the resulting peptides were separated by EDUF. HHP pretreatment influenced particle size, protein conformation, and degree of hydrolysis. After EDUF separation, peptide fractions (generated after enzymatic hydrolysis of control and pressure-treated protein isolate) recovered in KCl fraction were enriched in arginine and associated with a decrease in systolic blood pressure (SBP) in spontaneously hypertensive rats. Additionally, the final EDUF hydrolysate generated from pretreated protein and the initial EDUF hydrolysate from native protein were also associated with lower SBP. However, only the control KCl fraction obtained from native protein hydrolysate was associated with anti-diabetic activity.

1. Introduction

Canada is the top producer and global exporter of flax (*Linum usitatissimum L.*), having produced 940 000 metric tons in 2015–2016 [1]. Various parts of flax, such as the seeds are exploited by the oleaginous industry. However, oil extraction performed via trituration of the seeds generates a large quantity of by-products, which in 2012 were estimated to be one million tons [2]. These by-products not only include oil residues but also proteins, which are used in animal feed [3]. The large quantity of generated by-products has been a driving force for the development of bioactive peptide extraction processes, especially in the last decade [4]. Bioactive peptides are defined as being specific fragments of proteins associated with various physiological functions once separated from the source protein [5]. The types of bioactive peptides which can be generated depend on the primary sequence of the source protein and the specificity of the enzymes used to generate the peptides [5]. Peptides from flaxseeds have been shown to possess anti-hypertensive, anti-diabetic [6], and antioxidant [7] properties. Indeed, low-molecular weight peptides rich in arginine from a flaxseed protein isolate have been associated with blood pressure-reducing effects, possibly due to the arginine-synthesized nitric oxide exhibiting a vasodilatory effect or possibly angiotensin converting enzyme (ACE) and renin inhibition by cationic peptides [3].

Currently, the extraction of bioactive peptides is hindered by process limitations such as: enzymatic hydrolysis duration, enzyme costs, industrialisation costs, low yield of chromatographic processes [8], and low membrane selectivity and fouling involving pressure-driven membrane processes [9]. Several recent studies have shown that enzymatic hydrolysis of a wide range of food proteins was accelerated by high hydrostatic pressure (HHP) [10]. More specifically, the effects of HHP pre-treatments were previously tested on chickpea protein isolates [11]. Moreover, HHP modified peptide profile and enhance bioactive peptide

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https://doi.org/10.1016/j.seppur.2018.09.063
Received 24 August 2018; Received in revised form 20 September 2018; Accepted 21 September 2018
Available online 22 September 2018
1383-5866/

generation [12]. Furthermore, recently, Perreault et al. [13] demonstrated that an HHP pretreatment could improve the antioxidant capacities of flaxseed protein hydrolysates. The improved enzymatic hydrolysis associated with HHP could then be paired with a separation technology such as electrodialysis with filtration membranes (EDUF) to improve the extraction bioactive peptides.

EDUF is an innovative membrane separation process driven by a difference in electrical potential, which consists of ion-exchange and ultrafiltration membranes stacked in the ED cell [14]. EDUF couples size exclusion capabilities of UF membranes with the charge selectivity of electrodialysis (ED). EDUF acts by attracting the molecules, according to their charge, through filtration membranes with the proper molecular weight cut-off (MWCO) range according to the fraction of interest. In addition, since no pressure is applied in the ED cell, only the charged molecules migrated under the effect of the electric field and the neutral molecules stay in the primary solution and do not reach or pass the filtration membrane [15]

Peptides and amino acids can be separated by conventional pressure-driven processes, but these processes are limited by their low selectivity for the separation of similar size biomolecules, the complexity of the hydrolysate and the tendency of membranes to foul [16]. EDUF can improve the separation of interesting components from a complex matrix according to its double charge/size selectivity to obtain more purified products [16,17]. EDUF technology was successfully applied to separate and concentrate bioactive peptides from various food hydrolysates including flaxseed [6,15].

Thus, the objectives of this work were to: (1) study the impact of an HHP pre-treatment on the structure and sensitivity to hydrolysis of flaxseed proteins, (2) study the effect of this pre-treatment on the EDUF separation of peptides and (3) identify the impact of an HHP pre-treatment on the anti-hypertensive and anti-diabetic properties of the various fractions obtained through EDUF separation.

2. Materials and methods

Defatted flaxseed meal was kindly provided by Bioriginal Food & Science Corporation (Saskatoon, SK, Canada). The meal contained 36.1 ± 0.1% proteins (Truspec, LECO Corporations, St-Joseph, MI, USA), 5.38 ± 0.02% ash (AOAC 938.08) [18] and 5.96 ± 0.07% moisture (AOAC 950.46) [19]. Both bovine pancreatic trypsin and cellulase from *Aspergillus niger* were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Pronase from *Streptomyces griseus* was purchased from Roche Diagnostics GmbH (Mannheim, Germany). NaOH pellets ACS, HCl Certified ACS Plus, KCl and Na_2SO_4 were purchased from BDH (VWR Analytical, Radnor, PA, USA) and Fisher Scientific (Oakville, ON, Canada), respectively.

2.1. Flaxseed protein isolate

The flaxseed protein isolate was prepared following the method described by Udenigwe et al. (2009) [20] and modified by Perreault et al. (2017) [13] using cellulase (2% w/V) during 4 h for fibre hydrolysis. After inhibition of the cellulase at pH 10, the mixture was centrifuged (8200*g*, 20 min, 21 °C) and the supernatant was adjusted at pH 4.2 for protein precipitation. The mixture was centrifuged once again (8200*g*, 20 min at 21 °C) and the resulting precipitate was collected and washed with acidified water (pH 4.2) and centrifuged (8200*g* for 10 min at 21 °C) three times. A small volume of water was added, and the pH was adjusted to 7.0 using 2 mol/L NaOH. The suspension was freeze-dried and stored in a vacuum desiccator at 4 °C. The resulting flaxseed protein isolate (FPI) comprised 82% protein, as determined by the Dumas combustion method (Truspec, LECO Corporation, St-Joseph, MI, USA) using a conversion factor of 6.25 [21]. The flowchart shown in Fig. 1a represents the treatments and analyses performed in the present study.

2.2. High hydrostatic pressure treatment

The freeze-dried FPI was suspended in 2 L of deionized water (1.5% w/v), stirred overnight at 4 °C, and transferred to flexible plastic bottles. HHP treatments of FPI solutions were performed at an industrial scale using a discontinuous hydrostatic pressurization unit (Hiperbaric 135, Hiperbaric, Burgos, Spain) equipped with a 135 L vessel using water as the pressure transmitting fluid. The pressure level (400 MPa, for 20 min at 21 °C) was selected based on the work of Puppo et al. (2004) [22], which demonstrated that pressure between 400 and 600 MPa induced the denaturation of soybean proteins, as well the work of Perreault et al. (2017) [13]. The treatment duration was chosen to improve the results in terms of hydrolysis and bioactivities obtained in a previous work involving high pressure treatments of a flaxseed protein isolate [13]. The compression rate was approximately 130 MPa/min while decompression was instantaneous. Non pressure-

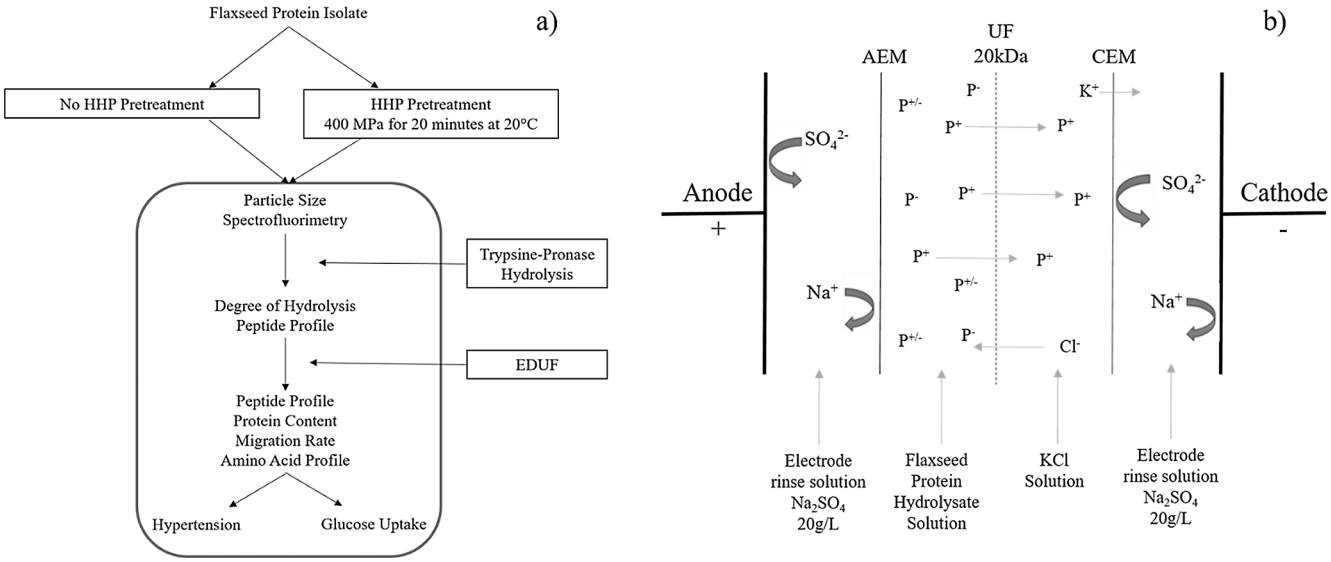

Fig. 1. (a) Flowchart of the experimental protocol and (b) EDUF cell configuration used for the separation of HHP pretreated and non-pretreated flaxseed protein hydrolysates (FPH). AEM: anion-exchange membrane (AMX-SB), CEM: cation-exchange membrane (CMX-SB), UFM: ultrafiltration membrane (PES), P^+: cationic peptide, FPI: flaxseed protein isolate.

treated FPI solution (0.1 MPa) was used as control. All experiments were repeated four times, for each treatment. Samples were pressure-treated around two hours before flaxseed protein hydrolysis and were kept on ice.

2.3. Flaxseed protein hydrolysis

Flaxseed protein hydrolysis was carried out at normal atmospheric pressure (0.1 MPa) on all flaxseed protein samples (pressure-treated or not) using trypsin and trypsin-pronase based on a modified version of the methods previously described by Udenigwe et al. (2012) [3] and modified by Perreault et al. (2017) [13]. The successive action of trypsin and pronase were chosen since this combination of enzymes allowed the production of bioactive peptides from flaxseed hydrolysates [3]. Briefly, a volume of 2 L of control and pressurized FPI solutions were adjusted to pH 7.0 using 2 mol/L NaOH at 37 °C. To initiate protein hydrolysis, trypsin was added at an E/S ratio of 2:100 (weight basis), while the pH and temperature conditions mentioned above were maintained for 2 h. To stop the reaction, pH was decreased to 4.0 using 0.5 mol/L HCl and the hydrolysate was then cooled to room temperature. Solutions were centrifuged (7000*g* for 45 min at 21 °C), the supernatants adjusted to pH 6.5 using 0.5 mol/L NaOH. Following the trypsin hydrolysis, the pronase hydrolysis of the supernatant was performed on each control and pressurized sample. The supernatant was adjusted to pH 7.4 using 2 mol/L NaOH at 40 °C and pronase added at an E/S ratio of 1:100 (weight basis). During hydrolysis, the reaction mixture was maintained at pH 7.4 for 2 h using 2 mol/L NaOH. To stop the reaction, 6 mol/L HCl was added until pH 4.0 was reached and hydrolysates were heated at 80 °C for 15 min to inactivate the enzyme. The solutions were then cooled to room temperature adjusted to pH 6.5 using 0.5 mol/L NaOH.

2.4. Electrodialysis and cell configuration

2.4.1. Cell and configuration

An MP type electrodialysis cell with a 100 cm^2 of effective surface area, manufactured by ElectroCell Systems AB Company (Täby, Sweden), was used. A dimensionally-stable anode (DSA) and the cathode (316 stainless steel electrode) were supplied with the MP cell. The anode/cathode voltage difference was supplied by a variable 0–100 V power source. The cell comprised one CMX-SB cationic membrane (Astom, Tokyo, Japan), one AMX-SB anionic membrane (Astom, Tokyo, Japan), and one polyethersulfone (PES) ultrafiltration membranes (UFM) with a molecular weight cut-off (MWCO) of 20 kDa (GE, Clifton, NJ, USA). The cell thickness was of 3.5 cm. As seen in Fig. 1b, the electrodialysis configuration was divided into 3 compartments. The solutions included a cationic peptide recovery solution of 2 g/L KCl, the flax protein hydrolysate (15 g/L), and the electrode rinse solutions of Na_2SO_4 20 g/L. The solutions were circulated using three centrifugal pumps and the flow rates were controlled with flow metres. The KCl and the feed solution flow rates were 3 L/minute while it was 4 L/minute for the Na_2SO_4 solution. The configuration was chosen to separate the anti-hypertensive and anti-diabetic fraction in the feed compartment as previously performed by Doyen et al. (2014) [6]. The experiments took place at temperatures between 15 °C and 18 °C to minimize bacterial contamination.

2.4.2. Electroseparation protocol

Six electrodialysis runs were performed, including three using the hydrolysate generated from pre-treated protein by HHP and three control hydrolysate samples (from native proteins). Each run used 2.4 L of hydrolysate at 15 g/L, a concentration previously demonstrated as being effective for the separation of bioactive peptides from flaxseed [6]. The separations were performed for 4 h using 4 V/cm electric field strength with an intensity of 1.1–2.2 A and a conductivity of 4.9–7.25 mS/cm. A pH 3.0 was maintained throughout the separation process (flaxseed peptides have a pI = 4.5) [23] to generate the cationic forms of bioactive peptides from flax. Cationic form of peptides was chosen because they demonstrated antihypertensive activity [6].

Throughout the separation process, 1.5 mL samples were taken from the feed and recovery (KCl) compartments at 0, 30, 60, 120, 180 and 240 min to analyse the peptide content of the fractions. The final feed and KCl fraction for HHP-pretreated and control hydrolysates were also recovered and freeze-dried for further analyses. Afterwards, the KCl fractions were demineralized using a conventional ED process prior to *in vivo*-experiments and amino acid analysis.

2.5. Analyses

2.5.1. Particle size

Control and pressure-treated flaxseed proteins were first centrifuged (5800*g* for 5 min at 21°) and the supernatants were collected. Particle size distributions were measured in triplicate using a Mastersizer 3000 (Malvern Instruments Ltd, Malvern, UK) as performed previously by Perreault et al. (2017) [13]. The optical parameters used were the refractive indices of water and flax protein (1.33 and 1.47, respectively). Results are expressed in volume density as a function of the particle size population.

2.5.2. Spectrofluorimetry

Fluorescence analyses were performed using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, CA, USA). Control and pressure-treated flaxseed protein samples were first centrifuged (5800*g* for 5 min at 21 °C) to recover soluble protein in supernatants (same supernatant as in section 2.5.1). The intrinsic fluorescence was measured using the tryptophan excitation wavelength of 280 nm [24,25]. This hydrophobic residue was analyzed because the modifications of its fluorescence spectra indicate changes in protein structure and conformation [26,27]. The fluorescence emission spectra were analyzed between 300 and 400 nm. Average intensity values were recorded for each treatment (control; 400 MPa for 20 min at 21 °C).

2.5.3. Degree of hydrolysis

The degree of hydrolysis (DH) of flaxseed protein hydrolysates was determined according to the o-phtaldialdehyde (OPA) method of Churchet al. (1983) [28]. Briefly, a final OPA reagent volume of 200 mL was prepared with 100 mL of 100 mM sodium tetraborate, 10 mL of 20% SDS (w/w), 160 mg of OPA dissolved in 4 mL of methanol and 400 µL of β-mercaptoethanol. A 150 µL aliquot of diluted samples (1:13.5 for control and 1:20 for HHP treated hydrolysates) was added to the OPA reagent and incubated for 2 min at room temperature and then, the absorbance was measured at 340 nm with the Vision 32 software (Helios Alpha, Thermo Spectronic, England). DL-leucine was used as a standard.

2.5.4. Peptide migration rate and content

The peptide concentration in the sample feed and recovery (KCl) compartments were measured using a colorimetric microBCA™ kit (Pierce Biotechnology Inc., Rockford, IL, USA). Assays were conducted on a microplate by adding 150 µL of samples to 150 µL of the working reagent. The microplate was incubated at 37 °C for 2 h and the absorbance was read at 562 nm on a microplate reader (xMark™ Microplate Spectrophotometer, BioRad, Mississauga, ON, Canada). The concentration was determined using bovine serum albumin (BSA) as a standard. At the end of the process, the migration rate (g peptides/m^2 h) was calculated according to the following equation:

$$Migration\ rate(g/m^2.\ h) = \frac{Amount\ of\ peptides(g)}{Area(m^2) * Time(h)}$$

where the amount of peptides is determined with microBCA assay, the area is the effective membrane surface of 100 cm^2 and the time is the duration of the process.

2.5.5. Peptide profiles and molecular weights

Peptide characterization was performed using a 1290 Infinity II UPLC (Agilent Technologies, Santa Clara, CA, USA). Samples were injected into an Acquity UPLC CSH 130 1.7 µm C18 (2.1 mm X 150 mm, Waters Corporation, Milford, MA, USA) column. Sample peptide concentration was 1% (w/V). Solvent A (LC-MS water with 0.1% formic acid) and B (LC-MS grade acetonitrile with 0.1% formic acid) were used for elution, going from 2% to 45% in 40 min holding until 42 min, then back to initial conditions until 45 min. Each sample was run in triplicate for statistical evaluation of technical reproducibility.

As seen in Mikhaylin et al. (2017) [29], a hybrid ion mobility quadrupole TOF mass spectrometer (6560 high definition mass spectrometry (IM-Q-TOF), Agilent, Santa Clara, USA) was used to identify and quantify the relative abundances of the trypsin-pronase peptides. All LC-MS/MS experiments were acquired using Q-TOF. Signals were recorded in positive mode at Extended Dynamic Range, 2 Ghz, 3200 *m/z* with a scan range between 100 and 3200 *m/z*. Nitrogen was used as the drying gas at 13.0 L/min and 150 °C, and as nebulizer gas at 30 psig. The capillary voltage was set at 3500 V, the nozzle voltage at 300 V and the fragmentor at 400 V. The instrument was calibrated using an ESI-L low concentration tuning mix (G1969-85000, Agilent Technologies, Santa Clara, CA, USA). Data acquisition and analysis was done using the Agilent Mass Hunter Software package (LC/MS Data Acquisition, Version B.07.00 and Qualitative Analysis for IM-MS, Version B.07.00 with BioConfirm Software).

2.5.6. Amino acid analysis

Amino acid analysis was performed using an ACQUITY UPLC (Waters, Milford, MA, USA) system equipped with an AccQ-Tag Ultra C-18 (2.1 mm × 100 mm; 1.7 µm) column [30]. The method used was based on the AccQ-Tag amino acid analysis procedure used to determine amino acids resistant to acid hydrolysis, including taurine [31]. The AccQ-Tag method is a precolumn derivatization technique for amino acids in peptide and protein hydrolysates. The amino acids were separated by RP-HPLC (LC 18) and quantified by fluorescence detection [31]. The acid hydrolysis used was based on a classic acid hydrolysis using 6 N hydrochloric acid at 110 °C for 23 h [32]. Following the acid hydrolysis, the amino acids aspartic acid, glutamic acid, alanine, arginine, cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, taurine, threonine, tyrosine, and valine can be dosed. A basic hydrolysis based on a method using 4.2 M sodium hydroxide at 120 °C for 4 h before neutralization [33] was performed for the tryptophan analysis on the C-18 column.

2.6. Peptide bioactivity

2.6.1. Evaluation of antihypertensive activity in spontaneously hypertensive rats

Peptide bioactivity was evaluated by performing *in vivo* experiments which were conducted following the Canadian Council on Animal Care Ethics guidelines using a protocol approved by the University of Manitoba Animal Care Commitee. Six-week old male spontaneously hypertensive rats (SHR) were purchased from Charles River (Montreal, QC, Canada) and housed under a 12 h day/night cycle at 21 °C with regular chow feed and tap water provided *ad libitum*. The experiment comprised four rats per treatment group: initial hydrolysate from native protein (IHWO; 72.2% protein), initial hydrolysate generated from pressure-treated protein (IHW; 70.2% protein), final hydrolysate (after EDUF separation) from native protein (FHWO; 64.4% protein), final hydrolysate (after EDUF separation) from HHP pretreated protein (FHW; 61.2% protein), KCl compartment for hydrolysate from HHP pretreated protein (KW; 3.9% protein), saline, and 20 mg/mL Captopril). The samples were orally administered by gavage (100 mg/kg rat body weight dissolved 1.0 mL phosphate-buffered solution) using a disposable plastic syringe. Blood pressure measurements were recorded continuously for 24 h by telemetry. The surgical implantation of the telemetry sensors was performed under sterile conditions following a one-week acclimation period as previously described [34]. The rats recovered for 2 weeks, on a regular chow and water diet following the surgical implantation of the telemetry sensors, before the oral gavage took place. Blood pressure measurements were performed in a quiet room with each rat cage placed on top of one Model RPC-1 receiver (DSI instruments, MN, USA). Real time experimental data (including heart rates) were continuously recorded using the Ponemah 6.1 data acquisition software (DSI instruments, MN, USA). An APR-1 atmospheric pressure monitor (DSI instruments, MN, USA) was attached to the system, which normalized the transmitted pressure values so that recorded blood pressure signals were independent of atmospheric pressure changes. Data for systolic and diastolic blood pressure are presented as the changes in systolic and diastolic blood pressure values (values at time zero are subtracted from values obtained at 2, 4, 6, 8, 12, and 24 h).

2.6.2. Glucose uptake

2.6.2.1. Cell incubation. The cell culture protocol was adapted from Tremblay & Marette (2001) [35]. L6 myoblasts derived from neonatal rat thigh skeletal muscle were provided by Dr. A Klip (Hospital for Sick Children, Toronto, ON, Canada). Cells were grown and maintained in a monolayer culture using 10% FBS-αMEM (or GBS- αMEM) at 37 °C/5% CO_2. After 48 h, the L6 myoblasts were plated into 24-well plates (6×10^5 cells/plate) in 2% FBS-αMEM (or GBS- αMEM) and used following complete differentiation to myotubes (7 days post-plating).

2.6.2.2. Glucose-Transport assay. The glucose uptake assay has previously been described in Tremblay & Marette (2001) [35]. Briefly, myotubes were serum deprived for 3 h, then treated or not with the various treatments (1 ng/mL or 1 µg/mL) for 2 h without (basal) or with 10 nM insulin treatment for the final 45 min. The L6 cells were rinsed once with HEPES-buffered solution (20 mM HEPES pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, and 1 mM $CaCl_2$) and subsequently incubated for 8 min in transport medium (HEPES-buffered solution containing 10 µM unlabelled 2-deoxyglucose and 0.3 µCi/mL $_D$-2-deoxy-[^{3}H] glucose). After incubation in transport medium, cells were rinsed three times with ice-cold 0.9% NaCl solution and lysed by adding 500 µL of a 50 mM NaOH. Cell-incorporated radioactivity was determined by scintillation counting. Protein concentration was determined by the bicinchonic acid (BCA) method (Pierce®). The results are expressed in pmol of glucose/min per mg of protein, calculated using the following equation :

$$\frac{DPM\,(sample)}{CXDPM\,(2DG) \times t}$$

where DPM (sample) is the number of disintegrations per minute (DPM) measured for the tested sample, C is the concentration of protein (mg), DPM (2DG) is the number of DPM measured for the solution of radioactive 2-deoxy-$_D$-[^{3}H] glucose for 1 pmol, and t is the incubation duration with 2-deoxy- $_D$-[^{3}H] glucose.

2.7. Statistical analyses

Data obtained are reported as mean ± standard deviation unless stated otherwise. Student's *t*-tests were used to analyze the size, spectrofluorimetry, degree of hydrolysis, peptide concentration, HPLC-MS, and the *in vitro* data. Amino acid composition as well as *in vivo* experiments were analyzed using a one-way ANOVA with a parametric Tukey's test (when the Normality and Equal Variance tests passed) or a non-parametric Kruskal-Wallis test (when the Normality and Equal Variance tests failed). The Sigmaplot Software (Systat Software, San Jose, CA) was used to carry out analyses. A p-value < 0.05 was considered statistically significant.

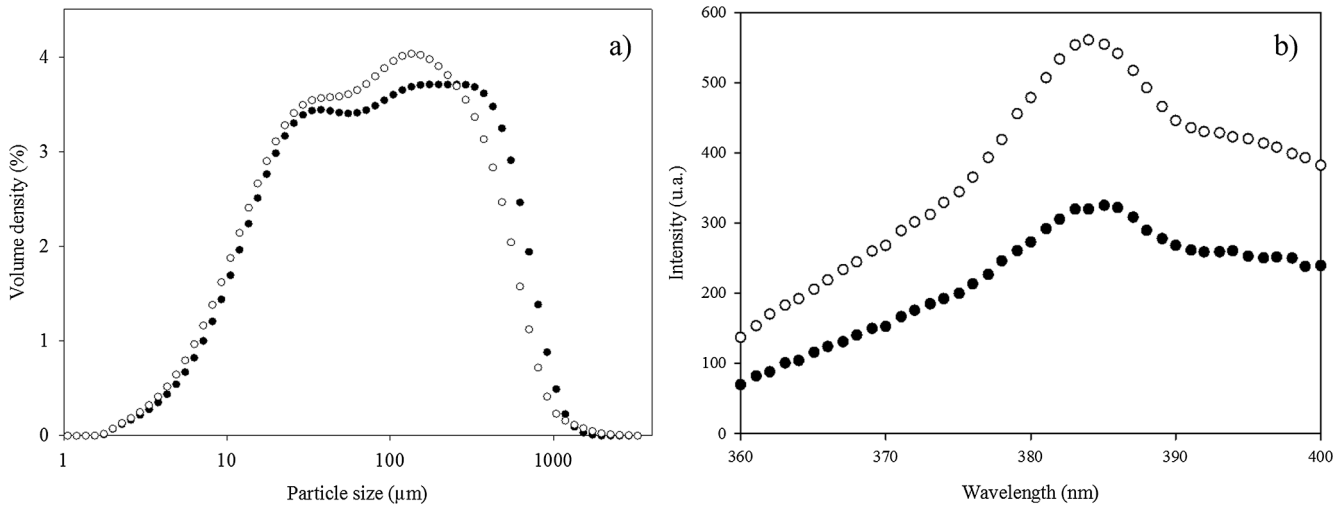

Fig. 2. (a) Particle size distribution of control (0.1 MPa, black circle) and pressure-treated (400 MPa, 20 min, white circle) flaxseed protein isolates. b) Tryptophan fluorescence intensity of control (0.1 MPa, black circle) and pressure-treated (400 MPa, 20 min, white circle) flaxseed protein isolates.

3. Results and discussion

3.1. Impact of HHP on protein particle size, structure and hydrolysis

Particle size analysis revealed the presence of two major populations in the flaxseed protein samples, as presented in Fig. 2a. The two main populations in control fraction (without HHP treatment) consisted of 33 ± 0.25 µm and 135 ± 0.16 µm while the values were 33 ± 0 and 256 ± 0 µm for the samples with HHP pretreatment. The pressure-treated protein samples had an observable increased particle size associated with a decreased volume density. Previous experiments performed by Perreault et al. (2017) [13] demonstrated that HHP pretreatment (600 MPa for 5 and 20 min) of a flaxseed protein isolate had an impact on particle size distribution. Indeed, it has previously been determined that high pressure treatments over 200 MPa can alter protein tertiary and quaternary structures by breaking intrinsic non-covalent bonds such as hydrophobic and electrostatic interactions [11,36]. Conformational changes could also result in hydrophobic and ionic groups to become exposed leading to protein aggregation [37]. Moreover, the aggregation of unfolded proteins through hydrophobic interactions leading to the formation of disulfide bonds has previously been observed in soy protein isolates [38]. Additionally, Yin et al. (2008) [37] suggested that disulfide bonds may be involved in the formation of soluble aggregates following a high-pressure treatment of a red kidney bean protein isolate.

Concerning spectrofluorimetric analyses, it can be observed in Fig. 2b that maximum emission wavelength of control and pressure-treated protein samples occurred at 385 nm. However, pressurized samples had a fluorescence intensity of 1.7 times higher than control samples indicating an increased exposure of tryptophan residues to the solvent [37]. Previously, high hydrostatic pressure treatments were shown to increase the fluorescence intensity of red kidney bean protein isolates due to the increased exposure of tryptophan residues to the solvent [37]. The unfolding of proteins due to the HHP pretreatment may have resulted in an increased exposure of tryptophan to its medium [39]. Thus, these results confirm the protein denaturing effects of the HHP pretreatments.

The degree of hydrolysis for the hydrolysate generated from pressure-treated proteins was 38.49 ± 4.38 and 30.72 ± 1.52 for the hydrolysate from native protein ($p = 0.01$). This effect may be due to the increase in accessible cleavage sites associated with the protein being unfolded by HHP pretreatment [11]. Previously, Zhang et al. (2012) [11] found that an HHP treatment of chickpea protein isolates was associated with a higher degree of hydrolysis. The increase in the degree of hydrolysis associated with an HHP pretreatment is consistent with the spectrofluorometric results indicating changes in protein conformation. In addition, Quirós et al. (2007) [10] demonstrated that the hydrolysis of ovalbumin, tested with three different enzymes, was accelerated when performed under high pressure conditions.

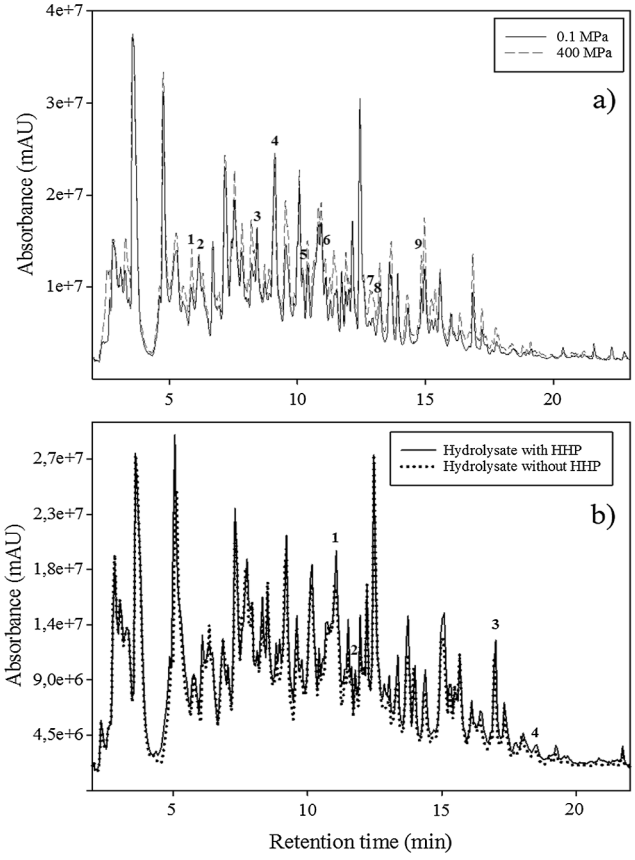

Fig. 3. (a) Peptide profile of the initial (a) and final (b) flaxseed protein hydrolysate generated from conventional and pressure-treated flaxseed protein isolate. Numbers represents the peaks with significant differences between treatments.

3.2. Impact of HHP on the concentration, migration rate, peptide and amino acid profiles

3.2.1. Peptide concentration and migration rate

Peptide concentration increased over time in the KCl fractions generated from conventional and pressure-treated flaxseed proteins, changing from 5.98 ± 0.97 to 28.5 ± 3.45 μg/mL and 6.41 ± 0.47 to 30.2 ± 2.65 μg/mL, respectively (see supplementary material). However, there was no significant difference between the peptide concentrations of control and HHP pretreated samples ($p = 0.874$). The results suggest that pressurization did not have an impact on the separation and concentration of peptides in the recovered fractions, probably because HHP treatment did not affect the charge and the size of the extent of hydrolysates following pressurization of proteins. The mean migration rate of both EDUF steps was going from 1.43 g/m^2·h (native proteins) to 1.35 g/m^2·h (pressure-treated proteins), which is considered an acceptable value compared to the general migration rates reported in the literature and ranging from 0.5 to 14 g/m^2·h.

3.2.2. Peptide profile

Profiles of peptide fractions generated from control and pressure-treated flaxseed protein were presented in Fig. 3a and Table 1. The chromatograms of the initial hydrolysate with and without HHP pretreatment were quite similar, as seen in Fig. 3a. However, significant differences in terms of area under the curve for 9 peaks were detected. More specifically, the peaks numbered 2 to 6 were all significantly ($P < 0.05$) more abundant in the initial hydrolysate from native protein. Conversely, the peak areas related to the peaks 1 and 7 to 9 were significantly more abundant in the initial ($P < 0.05$ for all four peaks) hydrolysate generated after enzymatic hydrolysis of pressure-treated proteins.

Similar results were obtained when comparing the chromatograms of the final EDUF hydrolysate generated after enzymatic hydrolysis of control and pressure-treated proteins (Fig. 3b and Table 1). Three peaks numbered from 1 to 3 were significantly more abundant in the control hydrolysate sample than with HHP pretreatment ($P < 0.05$ for all three peaks). Conversely, peak number 4 was significantly more

Table 1
UPLC IM-Q-TOF results for peaks with significant differences in peak areas for the initial hydrolysates, final hydrolysates after EDUF and KCl fractions with and without the HHP (Control) pretreatment.

Peak	Treatment	Liquid Chromatography		Mass Spectrometry
		Retention time (min)	Peak Area (a.u.) (10^7)	Molecular Weight (Da)
Initial Hydrolysate Before EDUF (Trypsin-Pronase)				
1	Control	5.8	2.59 ± 0.31	316.1288; 334.1393; 343.1972; 460.2394; 480.2084; 690.3298; 802.4284
	with HHP		3.57 ± 0.45	
2	Control	6.1	3.63 ± 0.27	316.1500; 428.2497; 493.2284; 500.2707; 602.3132; 744.3140; 1021.1124; 1062.1137
	with HHP		2.46 ± 0.51	
3	Control	8.4	2.74 ± 0.21	330.2384; 397.6896; 521.2041; 617.3128; 756.4245; 757.4273; 758.4305; 759.4322; 944.4311; 959.9640
	with HHP		1.29 ± 0.54	
4	Control	9.1	14.21 ± 1.44	279.1702; 369.1779; 437.2024; 584.3399; 699.4037; 700.4055; 701.4090; 702.4106; 786.3990
	with HHP		10.32 ± 0.79	
5	Control	10.2	1.19 ± 0.08	242.1496; 373.2441; 481.2651; 668.2760; 1062.1138
	with HHP		0.75 ± 0.21	
6	Control	11.1	1.48 ± 0.15	319.1472; 433.2081; 599.3393; 600.3423; 601.3450; 602.3470; 660.3555; 791.3574; 1062.1137
	with HHP		1.07 ± 0.17	
7	Control	12.9	1.55 ± 0.39	227.1754; 358.2697; 415.2334; 484.7794; 552.2120; 744.3772; 850.3936; 1062.1138
	with HHP		3.00 ± 0.79	
8	Control	13.2	3.11 ± 0.28	211.1442; 300.1550; 465.2336; 652.2815; 1021.1129
	with HHP		3.77 ± 0.23	
9	Control	14.9	1.35 ± 0.14	316.4770; 483.2161; 633.3237; 927.4771; 928.4805; 929.4836; 930.4851; 1068.3884
	with HHP		1.71 ± 0.08	
Final Hydrolysate After EDUF				
1	Control	11.0	19.99 ± 1.60	407.2290; 419.1927; 431.2291; 439.2090; 585.3242; 599.3397; 660.3562
	with HHP		16.37 ± 0.37	
2	Control	11.8	1.76 ± 0.26	332.6237; 334.1165; 522.2554; 626.3867; 648.3681
	with HHP		1.16 ± 0.14	
3	Control	15.1	10.66 ± 0.86	506.2973; 575.2823; 927.4775
	with HHP		9.14 ± 0.27	
4	Control	18.5	0.75 ± 0.05	471.3539; 591.4515; 698.3706; 813.4345; 1062.1138
	with HHP		0.87 ± 0.04	
KCl After EDUF				
1	Control	5.7	0.92 ± 0.79	343.1985; 349.1510; 352.1502; 377.1821; 387.2712; 399.2596; 439.2179; 456.2808; 474.2554; 486.2549
	with HHP		2.81 ± 0.27	
2	Control	6.1	7.50 ± 0.44	265.1550; 316.1292; 334.1396; 460.2402
	with HHP		5.94 ± 0.73	
3	Control	9.2	3.84 ± 0.15	279.1655; 366.2027; 527.3181; 699.4028
	with HHP		2.46 ± 0.16	
4	Control	10.2	9.73 ± 0.41	227.1759; 344.2544; 408.2492; 463.2552; 481.2650
	with HHP		7.07 ± 1.47	

Table 2
Amino acid profile of the initial hydrolysates, final hydrolysates after EDUF, and KCl fractions with and without the HHP pretreatment.

	Without HHP Pretreatment			With HHP Pretreatment		
	Initial Hydrolysate	Final Hydrolysate	KCl	Initial Hydrolysate	Final Hydrolysate*	KCl
Aspartic acid	9.28 ± 0.11^{a}	8.99 ± 0.16^{a}	4.16 ± 0.03^{a}	10.68 ± 0.61^{a}	10.61^{a}	4.40 ± 0.08^{a}
Serine	4.33 ± 0.26^{bd}	4.16 ± 0.28^{bd}	2.86 ± 0.12^{acd}	4.85 ± 0.53^{b}	4.34^{bc}	2.72 ± 0.28^{a}
Glutamic acid	20.02 ± 0.83^{a}	19.11 ± 0.44^{a}	7.31 ± 0.47^{a}	22.48 ± 1.06^{a}	21.98^{a}	7.68 ± 0.72^{a}
Glycine	4.74 ± 0.37^{c}	4.81 ± 0.31^{c}	3.49 ± 0.08^{ab}	5.71 ± 0.05^{c}	4.55^{bc}	3.32 ± 0.10^{a}
Histidine	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00 ± 0.00
Taurine	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00 ± 0.00
Arginine	9.46 ± 0.29^{a}	8.74 ± 0.63^{a}	15.90 ± 0.46^{c}	10.72 ± 0.34^{a}	8.83^{a}	13.17 ± 0.87^{b}
Threonine	2.89 ± 0.13^{b}	2.69 ± 0.17^{b}	1.82 ± 0.01^{a}	3.23 ± 0.34^{b}	2.92^{b}	1.87 ± 0.06^{a}
Alanine	3.84 ± 0.12^{a}	3.76 ± 0.06^{a}	4.45 ± 0.09^{a}	4.57 ± 0.36^{a}	4.26^{a}	4.53 ± 0.14^{a}
Proline	3.24 ± 0.20^{c}	3.22 ± 0.19^{c}	1.03 ± 0.03^{a}	3.84 ± 0.04^{b}	3.31^{bc}	1.07 ± 0.02^{a}
Cystein	0.30 ± 0.17^{a}	0.22 ± 0.02^{a}	0.00 ± 0.00^{a}	0.27 ± 0.17^{a}	0.51^{a}	0.00 ± 0.00^{a}
Tyrosine	2.39 ± 0.22^{a}	2.52 ± 0.13^{a}	5.47 ± 0.17^{c}	3.08 ± 0.02^{b}	2.09^{a}	5.21 ± 0.10^{c}
Valine	2.04 ± 2.83^{a}	3.67 ± 0.30^{a}	5.84 ± 0.07^{a}	4.84 ± 0.53^{a}	4.08^{a}	6.15 ± 0.14^{a}
Methionine	1.40 ± 0.19^{a}	1.30 ± 0.16^{a}	2.43 ± 0.02^{b}	1.60 ± 0.04^{a}	1.21^{a}	2.35 ± 0.05^{b}
Lysine	2.57 ± 0.12^{a}	2.34 ± 0.05^{a}	2.73 ± 0.09^{a}	2.93 ± 0.22^{a}	3.04^{a}	2.62 ± 0.08^{a}
Isoleucine	3.54 ± 0.14^{ab}	3.10 ± 0.26^{a}	4.90 ± 0.03^{c}	4.07 ± 0.43^{bc}	3.43^{ab}	5.02 ± 0.15^{c}
Leucine	4.56 ± 0.18^{a}	4.36 ± 0.19^{a}	8.47 ± 0.06^{c}	5.38 ± 0.06^{c}	4.59^{a}	8.34 ± 0.20^{c}
Phenylalanine	4.90 ± 0.41^{a}	4.89 ± 0.34^{a}	9.97 ± 0.05^{b}	5.62 ± 0.37^{a}	4.07^{a}	9.36 ± 0.32^{b}
Tryptophan	1.13 ± 0.04^{a}	0.99 ± 0.23^{a}	1.81 ± 0.14^{a}	1.36 ± 0.03^{a}	1.18 ± 0.00^{a}	1.10 ± 0.06^{a}

Mean values in the same line followed with different letters are significantly different (Kruskal-Wallis or Tukey) at $P < 0.05$.
* Only one repetition was analysed.

abundant in the final hydrolysate after EDUF with than without the HHP pretreatment ($P < 0.05$).

The peptide profiles for the recovery (KCl) fractions of the samples with and without an HHP pretreatment were also shown to be quite similar. Peaks numbered 2 to 4 were significantly more abundant in the fractions without HHP pretreatment. Conversely, peak number 1 was significantly more abundant in the fraction with HHP pretreatment ($P < 0.05$). Thus, EDUF separation was able to concentrate specific peptide fractions in the KCl recovery compartment.

Mass spectrometry analysis revealed that the same peptide molecular weights were detected in the peaks for the initial, final and recovery fractions of the hydrolysate produced from HHP pretreated protein and their corresponding fractions from native protein (Table 1). For instance, the molecular weights found in the final hydrolysate after EDUF with HHP pretreatment were the same as those found for the final hydrolysate after EDUF without HHP pretreatment. However, for some specific peptides at different concentrations, HHP would have an impact on EDUF separation. Thus, the HHP pretreatment may have been responsible for the changes in peak areas and consequently peptide concentration, as previously observed by Perreault et al. (2017) [13].

3.2.3. Amino acid profile

The abundance of total amino acids in the initial hydrolysate, final hydrolysate after EDUF, and KCl fractions with and without HHP pretreatments are presented in Table 2. Results show that among all the analyzed amino acids, His and Tau were the only ones not to be detected in any of the fractions. Regarding the acidic amino acids Asp and Glu, concentrations were similar between the initial and final hydrolysates after EDUF with and without HHP pretreatment. However, the abundance of the acidic amino acids was much lower in the KCl fractions for Asp and Glu in fractions with and without HHP pretreatment indicating that the EDUF separation was effective in retaining peptides containing these residues possibly due to their low electric charge or their size exceeding that of the molecular weight cut-off of the UFM [6]. This trend was also present for Ser. The abundance of the positively charged amino acid Lys appeared to be similar when comparing the initial, final, and KCl fractions with and without HHP pretreatment. Conversely, Arg had a similar abundance in both initial and final hydrolysate after EDUF fractions while the abundance was higher in the KCl fractions for both with and without HHP pretreatment indicating effective concentration. These results are in accordance with those found by Doyen et al. (2014) [6] and Udenigwe et al. (2012) [3] who also observed a higher abundance of Arg in the KCl fraction. The concentration of Arg is of particular interest as L-Arg has been shown to reduce systemic blood pressure in some forms of experimental hypertension [40]. Additionally, the KCl fraction of hydrolysate without HHP pretreatment have a significantly higher amount of Arg when compared to the KCl fraction of hydrolysate with HHP pretreatment ($p = 0.022$). Interestingly, the higher degree of hydrolysis, which could lead to increased Arg in the KCl fraction, was higher in the sample with HHP pretreatment. Thus, it is possible that the peptides released by this higher degree of hydrolysis (peaks 1–4; Fig. 3b) and containing Arg have a lower mass-to-charge ratio compared to the peptides which are not as extensively hydrolyzed resulting in decreased mobility and consequently decreased migration. Moreover, the peak areas which were higher for the KCl fraction without HHP pretreatment (peaks 2–4; Table 1) suggest they could contain Arg or higher levels of Arg and migrate more easily due to their mass-to-charge ratio.

Other amino acids having observable trends were Gly, Thr, and Pro which appeared to have a lower abundance in the KCl fractions than the initial and final hydrolysates after EDUF for samples with and without HHP pretreatment. Thus, it is possible that peptides containing these amino acids may not have been completely separated due to an overall net zero charge, exceeding the molecular weight cut-off of the UFM, or the EDUF conditions used [3]. Conversely, Tyr, Val, Meth, Iso, Leu, and Phe have a higher abundance in the KCl fractions than the initial and final hydrolysates after EDUF for the fractions with and without HHP pretreatment indicating a successful separation of peptides containing these residues.

3.3. Peptide bioactivities

The bioactivity of the initial and final hydrolysates after EDUF as well as the KCl fractions with and without an HHP pretreatment was evaluated using both *in vivo* (anti-hypertensive) and *in vitro* (glucose uptake) models.

3.3.1. Changes in blood pressure

Measures of hypertension were taken following the oral administration of the various fractions in spontaneously hypertensive rats (SHR). As predicted, Captopril was associated with decreased systolic blood pressure (SBP) values while saline was associated with increased

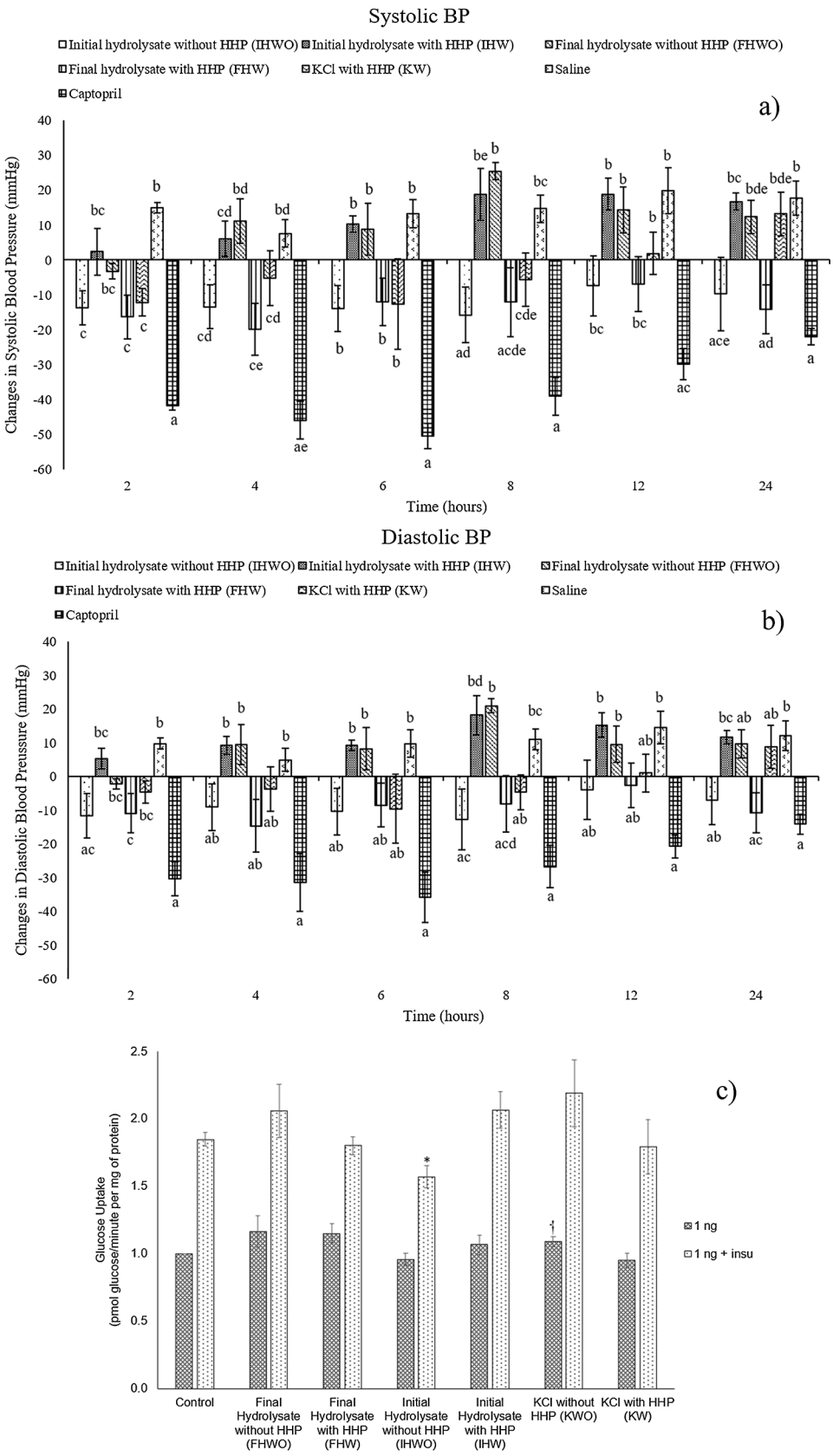

Fig. 4. Changes in systolic blood pressure (SBP) (a) and diastolic blood pressure (DBP) (b) in SHR resulting from one of the following treatment: initial hydrolysate without HHP-pretreatment (IHWO), initial hydrolysate with HHP-pre-treatment (IHW), final hydrolysate without HHP pretreatment (FHWO), final hydrolysate with HHP pretreatment (FHW), KCl with HHP pretreatment (KW), KCl without HHP pretreatment (KWO), saline and Captopril. Data is presented as mean ± SEM. (c) Glucose uptake (in pmol of glucose/minute per mg of protein) associated with the different treatments with and without insulin. *demonstrates a significant difference when compared to insulin alone while †demonstrates a significant difference when compared to control.

SBP values at all measured times (Fig. 4a). Generally, both IHWO and KWO were associated with decreased SBP with a maximum effect occurring at t = 8 h (−15.8 mmHg and −35.4 mmHg, respectively). The FHW treatment was also associated with a decreased SBP with a maximum effect (−19.8 mmHg) occurring at t = 4 h. KW also seemed to induce a decrease in SBP, however the maximum effect appeared to fluctuate at the various time points. Interestingly, IHW and FHWO were associated with increases in SBP values for almost all measured times.

Additionally, IHWO and FHW were associated with a diastolic blood pressure (DBP)-lowering effect, as seen in Fig. 4b. KW also appeared to lower DBP, however that effect was attenuated as of t = 12 h. Mean arterial pressure (MAP) measurements were also recorded (data not shown). The only significant differences between groups occurred 2 h following the initial gavage. MAP associated with the saline treatment was significantly higher when compared to that of FHWO and Captopril treatments ($p = 0.003$ and $p = 0.040$, respectively). Heart rate measurements were also taken and similarly to MAP values, only differed significantly two hours following the initial gavage. HR values associated with both IHWO and FHWO were significantly lower ($p < 0.05$) when compared to the Captopril treatment (data not shown).

The hypotensive effect of the final and KCl fractions obtained through EDUF separation has previously been observed [6]. Hypotensive effects associated with the recovered fraction of an EDUF separation of FPH have previously been attributed to the concentration of a bioactive peptide or a group of peptides with synergistic effects having migrated during the EDUF process [6]. In addition, the recovery (KCl) fractions with and without HHP pretreatment had a higher abundance of Arg, which may also be responsible for the hypotensive effects as the hypotensive effects of FPH have previously been associated with the presence of low-molecular weight peptides rich in Arg [3,6]. As expected, it does not appear that an HHP pretreatment influenced the changes in blood pressure of the peptides found in the KCl fractions, since very low differences were observed previously between the peptide profiles of the hydrolysate before EDUF.

The IHWO and FHW fractions were also generally associated with a decrease in SBP. Previously, Doyen et al. (2014) [6] observed a hypotensive effect associated with the final fraction of a FPH reportedly due to the concentration of antihypertensive peptide (s) present in the initial fraction following the peptide migration to the KCl compartment. At this time, it remains unclear why a hypotensive effect was associated with the IHWO fraction and why none was found to be associated with FHWO. It is possible that these effects may be due to the flaxseed cultivar used as the protein content and consequently the amino acid content may vary in different cultivars due to their genotype and environment [23] resulting in changes in the peptide sequences recovered after hydrolysis.

3.3.2. Glucose uptake

Various samples were tested *in vitro* to determine their impact on glucose uptake at concentrations of 1 μg/mL or 1 ng/mL. These samples included the initial and final hydrolysate after EDUF as well as the KCl fractions, all with and without HHP pre-treatment. Generally, there were no significant differences between any of the samples when compared to control or insulin when samples were tested at 1 μg/mL (data not shown). However, as seen in Fig. 4c when tested at concentrations of 1 ng/mL, the initial hydrolysate without HHP (IHWO) pre-treatment in combination with insulin had a significantly lower ($p = 0.016$) glucose uptake (pmol of glucose/minute per mg of protein) when compared to insulin alone and was not significantly different when compared to the control. This result suggests that IHWO may have diminished the ability of the cells to uptake glucose. It is possible that inhibitory peptides may be present in the IHWO fraction, exerting effects greater than the stimulatory peptides on the glucose uptake. In addition, the KCl fraction without HHP (KWO; and without the addition of insulin) was shown to have a significantly higher ($p = 0.043$) ability to transport glucose when compared to the control. Previous studies have demonstrated that recovered KCl fractions, following the EDUF treatment of flaxseed protein hydrolysates and soy protein hydrolysates, had the potential to increase glucose uptake in skeletal muscles [6]. Interestingly, the initial hydrolysate and the KCl fraction not having undergone the HHP pretreatment appeared to have opposite effects. Bioactive peptides are said to be inactive when part of the source protein [5], thus it is possible that the fractionation of the bioactive peptide (s) responsible for the observed effect do not have occurred in the initial hydrolysate without HHP pretreatment. Samples having undergone an HHP pretreatment did not significantly differ when compared to control or insulin only samples. Thus, it appears that samples with HHP pretreatment did not have a significant positive effect on the glucose uptake of skeletal muscle cells in contrast to non-treated samples.

4. Conclusion

This study demonstrated that a 400 MPa HHP pretreatment for 20 min at 21 °C induced flaxseed protein denaturation which increased the degree of hydrolysis of this isolate. Furthermore, this study showed that following an EDUF separation, the HHP pretreatment did not appear to modify peptide profiles of the various fractions. The EDUF separation was successful in concentrating peptides containing Arg residues as it was found to be more abundant in the KCl fractions of both HHP pretreated and non-pretreated samples. Certain peptides were found to be more abundant in the HHP pretreated fractions such as peaks 1 and 7–9 in the initial fraction, peak 4 in the final fraction after EDUF, and peak 1 in the KCl fraction. Moreover, the KCl fractions recovered after EDUF fractionation of control and hydrolysates generated after enzymatic hydrolysis of pressure-treated protein, as well as the FHW, exhibited blood pressure-lowering effects. Furthermore, the IHWO fraction was associated with a decreased glucose uptake while KWO was shown to increase glucose uptake in skeletal muscle cells. Additional studies are required to identify the peptides having demonstrated anti-hypertensive and anti-diabetic activities. Finally, EDUF and HHP are interesting technologies since the cost associated to EDUF is 0.3 to 0.5$/g (CND) of bioactive peptides for a surface membrane estimated to 10 m^2 [41] while HHP technology has a processing cost estimated to be of 0.117 €/kg of treated product at 600 MPa for 3 min [42].

Acknowledgments

This project was financially supported by The Fonds de Recherche du Québec – Nature et Technologies (FRQNT – QC, Canada). The authors would like to thank Diane Gagnon and Jacinthe Thibodeau from Department of Food Sciences, Université Laval for their technical assistance.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2018.09.063.

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