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Utilization of tofu processing wastewater as a source of the bioactive peptide lunasin

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ABSTRACT

The goal of our study was to design a simple and feasible method to obtain lunasin, a naturally-occurring bioactive peptide, from tofu whey wastewater. A combination of alcoholic precipitation of high-molecular weight proteins from the whey, isoelectric precipitation of lunasin enriched material, and purification via gel filtration chromatography was selected as the best approach using tofu whey prepared at the laboratory scale. This process was applied to tofu whey produced by a local tofu factory and 773 mg of 80% purity lunasin was obtained per kg of dry tofu whey. Significant reduction of nitric oxide, and pro-inflammatory cytokines TNF-α and IL-6 over lipopolysaccharide activated murine macrophages demonstrate its biological activity. Our three-step process is not only simpler and faster than the previously reported methods to obtain lunasin but provides a sustainable approach for the valorization of a waste product, promoting the better utilization of soybean nutrients and active compounds.

1. Introduction

Tofu is a cheese-like product obtained from soybeans. Lactose intolerance, celiac disease, increased health awareness, and interest in vegetarian and vegan lifestyles have positioned tofu as a globally growing market (Grand View Research, 2019). Tofu production involves the grinding of hydrated soybeans to obtain soymilk, which is then coagulated to form the tofu curd. During the process, two waste by-products are obtained in large quantities: okara and tofu whey. Tofu whey is the liquid waste fraction that remains after the curd formation and pressing (Singh & Banerjee, 2018). About 9 kg of whey, containing up to 1% carbohydrates, 1% fats, 0.8% proteins, and 0.4% minerals, are produced per kg of soybeans used to make tofu (Chua & Liu, 2019). Transformation into functional and alcoholic beverages, recovery of nutrients and bioactive compounds, and utilization as a growth media for the propagation of biomass are the most commonly studied applications of this by-product (Chua & Liu, 2019; Singh & Banerjee, 2018). However, whey continues being mostly discarded into the sewage or soil, posing an environmental threat due to its high biological and chemical oxygen demand (BOD: 6000 – 8000 mg/L; COD: 7500 – 26,000 mg/L) (Chua & Liu, 2019).

Lunasin is a 5 kDa soy-derived peptide composed of 43 amino acids and has gained interest on its potential application as a dietary supplement for the prevention of several chronic and degenerative diseases. This is due to the increasing body of evidence that supports its chemopreventive, anti-inflammatory, antioxidant, anti-hypertensive, cholesterol-regulating, neuroprotective, and immunomodulatory properties (Fernández-Tomé & Hernández-Ledesma, 2019), suggesting its potential utilization against several chronic and degenerative diseases. The ability of lunasin to inhibit tumor growth and invasiveness of cancer cells has been demonstrated *in vitro* and *in vivo* for melanoma, breast, lung, and colorectal cancer (Wan et al., 2017), while the anti-inflammatory potential have been mostly studied *in vitro* and is associated with the reduction of pro-inflammatory molecules such as nitric oxide (NO), TNF-α, and PGE_2 (Hsieh, Martínez-Villaluenga, de Lumen, & Hernández-Ledesma, 2018). Most of these properties seem to be modulated at the epigenetic level by particular amino acid sequences present in the lunasin structure: a polyaspartic acid tail that can inhibit acetylation of

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core histones H3 and H4, an RGD domain that modulate integrin signaling and mediate cellular internalization, and a chromatin-binding region that provides immunomodulatory functions (Vuyyuri, Shidal, & Davis, 2018).

The high cost associated with the utilization of synthetic lunasin has led to the formulation of several approaches to isolate the peptide from natural sources. Krishnan and coworkers developed a simple procedure to isolate a protein fraction from soybeans highly enriched in lunasin, Bowman Birk Inhibitor, and Kunitz Trypsin Inhibitor via aqueous ethanol extraction and calcium precipitation (Krishnan & Wang, 2015). The three peptides combined account for 60% of the total protein content in the final product based on densitometric analysis, or up to 80% if anion-exchange chromatography is used for further purification. Seber *et al*. designed a six-step isolation process involving PBS extraction, ionic exchange chromatography (IEC), treatment with a reducing agent, ultrafiltration, reverse phase chromatography, and ultrafiltration, using defatted soy white flakes as starting material and yielding a product with >99% purity based on densitometric analysis (Seber et al., 2012). The purity reported for these isolation techniques indicate the relative abundance of the active peptide only as a ratio to other peptides and proteins that are revealed during the electrophoretic separation, while the presence of other non-peptide components in the final product was not considered. Dia *et al.* developed a four-steps process that involved aqueous extraction of defatted soy flour, IEC, ultrafiltration, and gel filtration chromatography achieving 80% purity of lunasin in the final product as assayed via HPLC (Dia, Wang, Oh, De Lumen, & De Mejia, 2009). These methods are complicated and costly, as they deal with the complete and complex protein composition of soybeans.

Lunasin has been found in several commercially available soybean products, including soymilk and tofu (Cavazos, Morales, Dia, & De Mejia, 2012; Hernández-Ledesma, Hsieh, & de Lumen, 2009b). Then it is reasonable to hypothesize that tofu by-products, may contain remaining fractions of lunasin and can serve as alternative sources of the bioactive peptide. So far, there have been no attempts to identify or isolate lunasin from soymilk or tofu waste by-products. Our objective was to design a simple and feasible method to isolate lunasin from tofu whey wastewater and to characterize the isolated lunasin-enriched material. In addition, the *in vitro* anti-inflammatory properties of lunasin were assessed to corroborate that the isolated lunasin maintain its bioactive properties.

2. Materials and methods

2.1. Materials

Non-GMO soybeans (Newest Crop) and food-grade calcium sulfate tofu coagulant (Terra Alba) were obtained from Soymerica. Industrially produced tofu whey was kindly provided by a local tofu manufacturer located in Summertown, TN, USA. Lunasin polyclonal antibody was raised in rabbits using the 15 amino acid sequence corresponding to the C-terminus of lunasin and was produced by ProteinTech Group (Chicago, IL). Lunasin standard was synthesized by LifeTein LLC (Somerset, NJ), soybean Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI) standards were from VWR International LLC (Atlanta, GA) and Sigma-Aldrich Corp. (St. Louis, MO), respectively. SDS-PAGE and Western blot gels, membrane transfer kits, reagents, and goat-antirabbit secondary antibody were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemical and biological substances were purchased from VWR International, Sigma-Aldrich, and Thermo Fisher Scientific unless otherwise specified.

2.2. Laboratory scale preparation of tofu whey

Tofu whey was prepared at the laboratory scale and used to design the isolation method. Briefly, 100 g of soybeans were soaked overnight. Excess water was drained, and fresh water added to a final ratio of 1:9 dry beans:water. The mixture was blended for 3 min and the solid fiber (okara) was separated from the liquid fraction (soymilk) using a cheesecloth. Soymilk was boiled for 10 min, allowed to cool down to 70 °C, and 3 g of calcium sulfate suspended in water were added to promote formation of tofu curd. Tofu was separated from the whey by applying a constant pressure of about 1360 Pa over the curd using a regular kitchen tofu press until dripping of the whey stopped.

2.3. Lunasin enrichment

Different volumes of ethanol were added to the whey to reach a final concentration of 20, 30, 40, or 50% ethanol v/v. Samples were stirred for 10 min, centrifuged at 15,800*g* for 15 min, and precipitates and supernatants were used to identify the effect of increasing concentrations of ethanol in the protein profile and the presence of lunasin in the soluble and non-soluble fractions. Treatment of the whey using a final concentration of 30% v/v ethanol was chosen for further analysis. To induce the precipitation of soluble proteins, the alcoholic supernatant, after centrifugation, was adjusted to pH 4.5, 5.5, or calcium chloride was added to a final concentration of 20 mM. Samples were centrifuged and the precipitates were freeze dried, pulverized using mortar and pestle, and used to identify the effect of the three precipitation techniques over the protein profile and the presence of lunasin. Bioactivity of the three final pre-enriched products was assessed by evaluating anti-inflammatory properties.

2.4. Adjustment of preparative gel filtration chromatography parameters

Preparative gel filtration chromatography was used for final purification of lunasin. Evaluation of the chromatographic parameters was performed using an ÄKTA pure protein purification system (GE, USA) equipped with sample pump and UV-detector (280 nm). The following chromatographic parameters were evaluated: i) composition of the mobile phase, 20 mM Tris-HCl buffer + 0.15 M NaCl, pH 7.6 vs. 100 mM ammonium bicarbonate (NH_4HCO_3), pH 7.8, ii) porosity of the packed bed in the chromatographic column, also referred as preparation grade (pg), pg 30 vs. pg 75, and iii) volume of sample injected, 0.5 mL vs. 3 mL. A HiLoad 16/600 Superdex column containing the indicated packed bed (30 or 75 pg) was equilibrated with 3.0 column volumes (CV) of the indicated mobile phase (Tris-HCl or NH_4HCO_3). Laboratory prepared whey was freeze dried and resuspended in the appropriate mobile phase at a concentration of 120 mg/mL. The indicated sample volume (0.5 or 3 mL) was injected in the column. Elution was performed using 3.0 CV of the appropriate mobile phase at 1 mL/min. Ten-mL fractions were collected during the elution phase and analyzed for protein, lunasin and total carbohydrate contents, and optical density at 400 nm. Base conditions were established performing a chromatographic run using NH_4HCO_3 mobile phase, 30 pg column, and 0.5 mL of sample injection.

2.5. Isolation of lunasin from industrially produced tofu whey

Industrially produced tofu whey was collected and frozen at −80 °C until use. After thawing at room temperature, suspended solids were removed using a cloth. The whey was divided into three fractions: the first fraction was freeze-dried, the second was spray-dried using a Buchi-B290 Mini Spray dryer (BÜCHI Corporation, Switzerland) at 40% pump flow rate and 170 °C inlet temperature, and the third fraction was treated with our designed lunasin enrichment method described in Section 2.3. Briefly, ethanol was added to the whey to a final concentration of 30% v/v, followed by stirring and centrifugation as described above. The supernatant was adjusted to pH 4.5 and after centrifugation, the precipitate was freeze-dried overnight. Approximately 2.0 mg ± 0.1 of dry lunasin enriched material was obtained per mL of industrial whey. Spray-dried whey (SDW), freeze-dried whey (FDW) and lunasin enriched material were used for chromatographic purification. This approach allowed the comparison of our designed lunasin enrichment

method with conventional hot (spray drying) and cold (freeze-drying) drying mechanisms. To inject samples with equivalent protein content 120 mg/mL of SDW or FDW, or 60 mg/mL of lunasin enriched material were resuspended in 100 mM NH_4HCO_3 by vortexing horizontally for 15 min. After centrifugation at 30,000*g*, the supernatant was filtered and 3 mL were injected in a HiLoad 16/600 75 pg Superdex column. Elution was performed as described above using 100 mM NH_4HCO_3. Aliquots of the collected fractions were analyzed for lunasin, optical density at 400 nm, and carbohydrates content. Fractions containing a significant amount of lunasin were placed in a shaking water bath, brought to 40 °C for 5 min to remove any remaining NH_4HCO_3 and then freeze-dried.

2.6. Analytical methods for the characterization of lunasin

2.6.1. SDS-PAGE and immunoblotting

Samples were denatured and reduced in the presence of sodium dodecyl sulfate and β-mercaptoethanol in boiling water for 5 min and electrophoretic separation was performed at 400 mA for 35 min in a Mini-PROTEAN tetra vertical electrophoresis cell (Bio-Rad, CA). The gel was stained with Coomassie Blue G-250 for 1 h, destained with water, and imaged using a ChemiDoc Touch Imaging System (Bio-Rad). For immunoblotting, non-fixed gels after SDS-PAGE were transferred to PVDF-membranes using a Trans-Blot Turbo transfer system (Bio-Rad), blocked with 5% non-fat dry milk in 0.01% Tween 20 Tris-buffered saline (TBST) for 1 h at room temperature. The membrane was incubated with 1:1000 lunasin, soybean KTI or BBI (Gillman, Kim, & Krishnan, 2015) primary antibodies raised in rabbits for 1.5 h at room temperature. After three 10-min washing with TBST, goat-antirabbit secondary antibody was added and incubated for 2 h at room temperature, followed by another cycle of TBST washings. Immunoreactive bands were visualized via chemiluminescence in a C-Digit Blot scanner (LI-COR, NE) using Image Studio™ software.

2.6.2. Enzyme-linked immunosorbent assay (ELISA)

Lunasin content in gel filtration liquid fractions and enriched precipitates were estimated via ELISA as previously described (Dia et al., 2009) with slight modifications: the plate was blocked with 5% bovine serum albumin in 0.5% Tween 20 phosphate-buffered saline, and incubation times with primary and secondary antibodies was 1 h each.

2.6.3. High performance liquid chromatography

Molecular weight profile via size exclusion chromatography was analyzed as previously described (Price, Pangloli, Krishnan, & Dia, 2016). For determination of lunasin in purified fractions, 20 μL of the sample was injected into an Agilent (Folsom, CA) 1200 HPLC system equipped with an Eclipse Plus C18 column (4.26 × 250 mm, 5 μm), and diode array detector (215 nm). Elution was performed with acetonitrile (buffer A) and 0.1% trifluoroacetic acid in water (buffer B) as follows: step elution starting at 10% buffer A, with 10% increments up to 50%, holding each condition for 5 min, followed by equilibration of the column with 10% buffer A for 3 min. Quantification of isoflavones was performed according to previously reported protocol (Dia et al., 2012) by injecting 20 μL of the sample in the same column and equipment used for lunasin.

2.6.4. Total carbohydrates, protein content, and trypsin inhibitory activity

Total carbohydrates were measured in gel filtration chromatography fractions via phenol–sulfuric acid method as described previously (Masuko et al., 2005); glucose (0 – 0.5 mg/ml) was used as standard. For gel filtration chromatography fractions, the relative protein content was estimated in μg/mL as A_{280nm}/ε, where ε is the mass attenuation coefficient for the whey, calculated as 0.018 by measuring the absorbance of different dilutions of tofu whey from a stock solution with known protein content. Protein content by Bradford assay and trypsin inhibitory activity using N-α-benzoyl-D, L-arginine 4-nitroanilide substrate were measured as previously described (Price et al., 2016).

2.6.5. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

The 5 kDa protein band was excised from Coomassie-stained SDS-PAGE gel for the identification of lunasin via MALDI-TOF-MS as previously described (Krishnan, Oehrle, & Natarajan, 2009).

2.7. Biological activity of the isolated lunasin

RAW 264.7 murine macrophages (ATCC, Manassas VA) were cultured, treated and analyzed as previously described (Nieto-Veloza, Wang, Zhong, Krishnan, & Dia, 2019). Cells were seeded at 5×10^3 cells/well in a 96-well plate, allowed to attach overnight, and then treated with lunasin at the indicated concentrations, in the presence of lipopolysaccharide (LPS), for 24 h. Supernatants were collected for analysis of pro-inflammatory cytokines IL-6 and TNF-α by ELISA (BioLegend, San Diego, CA) and nitric oxide (NO) production by Griess reagent assay. MTS assay (Promega, Madison, WI) was used to test cell viability, according to the manufacturer's protocol.

2.8. Statistical analysis

Results are reported as mean ± standard deviation for at least three replicates. Data were analyzed for significant differences (p-value < 0.05) via ANOVA followed by Tukey posthoc test, using Statgraphics Centurion ® software (Statgraphics Inc, The Plains, VA, USA).

3. Results and discussion

The yield of soymilk, tofu, okara, and whey for the laboratory-produced tofu are presented in Supplementary Table 1. In okara, the presence of soluble proteins is derived from the fractions of soymilk retained in the solid fiber which can considered as a potential source of the peptide. On the other hand, upon addition of calcium sulfate to soymilk, high molecular weight proteins belonging to the 11S (glycinin) and 7S (β-conglycinin) fractions aggregate forming the curd, while the low molecular weight proteins (<16 kDa) remain soluble in the whey (Kao, Su, & Lee, 2003). It could then be expected that the 5 kDa lunasin is present in the whey. Preliminary experiments using the whey and an alkaline extract from okara indicated that the amount of lunasin available in the whey is about 45 times higher than in okara (Supplementary Table 2), making it a better candidate for further exploration as a source of lunasin.

3.1. Lunasin enrichment from tofu whey

3.1.1. Alcoholic precipitation of high-molecular weight proteins

The purification of proteins via precipitation with alcohols has been reported and it has been shown that 4 M methanol and 3 M ethanol were capable of producing precipitates with retained enzymatic activity (Lim et al., 2020; Schubert & Finn, 1981). Ethanol has been successfully used to induce the aggregation of particular protein fractions from soybean preparations with bioactive properties (Krishnan & Wang, 2015; Peng, Xu, Li, & Tang, 2020). We evaluated the effect of different concentrations of ethanol (20, 30, 40, and 50% v/v) on the proteins present in tofu whey. Electrophoretic separation (Fig. 1A) indicated that upon increasing concentrations of ethanol, proteins with higher molecular weights were preferentially precipitated. At 20% v/v there was no apparent variation in the distribution of soluble proteins, while at 30% most of proteins higher than 30 kDa were almost depleted. At 40 and 50%, proteins smaller than 25 kDa remained in the supernatant. Fig. 1B presents the size exclusion chromatographic profile of the supernatants. Retention times and molecular weight of chromatographic standards are presented in Supplementary Table 3. As it can be observed, proteins with retention time lower than 6 min, corresponding to molecular weights higher than 6.5 kDa, were a predominant group in the whey. Variations in the profile corroborated the preferential impact of ethanol over this

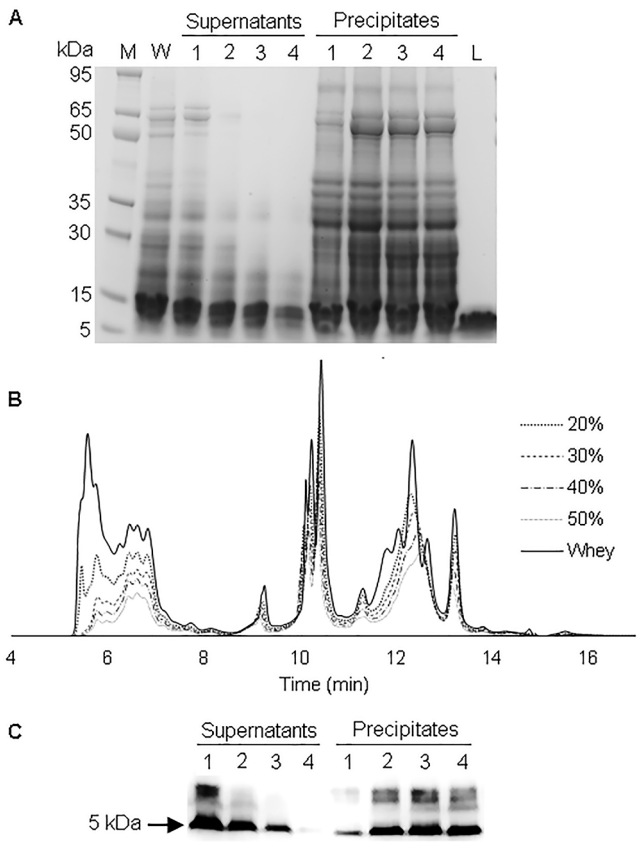

Fig. 1. Ethanolic precipitation of high molecular weight proteins. SDS-PAGE (A), molecular weight distribution profile (B) and lunasin immunoblotting (C) of supernatants (A, B, and C) and precipitates (A and C) after treatment of tofu whey with 20%, 30%, 40% or 50% v/v of ethanol (lines 1, 2, 3 and 4 respectively). Molecular weight marker (M), Non-treated tofu whey (W), and lunasin standard (L) are included as references.

subset of proteins in the whey. At a concentration of 20% the height of peaks associated with high molecular weight proteins was reduced and became comparable to the height of peaks associated with peptides with retention times between 6 and 8 min, corresponding to molecular weights between 1 and 6.5 kDa. At higher concentration of alcohol, the peptides fraction became the predominant group in the supernatant. It is known that the addition of miscible organic solvents increases intra- and intermolecular electrostatic forces that lead to variations in the folding of the protein molecules (Damodaran, 2008). It is feasible that in complex proteins, with higher molecular weight, augmented repulsive intramolecular interactions lead to the exposure of buried ionic and polar groups. These available groups could participate in attractive protein–protein interactions with the exposed oppositely charged groups resulting in aggregation.

Western blot analysis (Fig. 1C) revealed that fractions of lunasin progressively precipitated as concentration of ethanol increased. Although variations in intramolecular electrostatic interactions may not have a considerable effect over the intrinsically disordered structure of lunasin (Aleksis, Jaudzems, Muceniece, & Liepinsh, 2016), the dense negative charge of the polyaspartic acid tail could participate in peptide-protein interactions with the exposed positively charged side chains from the partially unfolded structures of larger proteins. This could lead to partial co-precipitation of lunasin with high molecular weight proteins. At 20%, in addition to the 5 kDa lunasin, an immunoreactive band located around 14 kDa is present. Seber and coworkers reported a 14 kDa complex originated between lunasin and a 9 kDa peptide via disulfide bridges (Seber et al., 2012). However, the reducing nature of our electrophoretic separation, mediated by β-mercaptoethanol, and side test using dithiothreitol (data not shown) suggested that the complex present in the whey is not formed by disulfide bonds. The 30% ethanol was the lower concentration at which precipitation of most high molecular weight proteins occurred, including the 14 kDa complex, with a minor effect over the solubility of lunasin. Hence, this concentration of ethanol was selected for the pre-enrichment process.

The characterization of the precipitate induced by ethanol treatment indicated that this by-product contained between three and seven times more protein, nine to fourteen times more lunasin, and comparable biological activity to some commercially available lunasin enriched dietary supplements (Supplementary Table 4 and Supplementary Fig. 1), suggesting that it could be explored as a product with potential market value.

3.1.2. *Precipitation of lunasin enriched material*

Isoelectric precipitation at pH 4.5, 5.5, or precipitation mediated by calcium were tested as potential mechanisms to recover lunasin from the alcoholic whey after removal of high-molecular weight proteins. Table 1 presents the characterization of the obtained lunasin enriched fractions. Calcium has been previously used as a tool for the fractionated precipitation of seed storage proteins present in soybeans, peanut, beans, peas, and alfalfa (Krishnan et al., 2009). In aqueous solutions, calcium forms strong complexes with the carboxylic groups of aspartic acid and glutamic acid (Tang & Skibsted, 2016). As lunasin contains nine consecutive aspartates at the carboxylic end, and two glutamic acids present in the structure (Vuyyuri et al., 2018), we expected that addition of calcium would lead to the formation of complexes with lunasin mediated by the interaction of the divalent cation mostly with the polyaspartic acid tail. However, the significantly lower lunasin content in calcium precipitate in contrast to the isoelectric precipitates, indicated a low efficiency of lunasin complexation. Electrophoretic profile (Fig. 2A) shows strong bands in the calcium precipitate that could correspond to the acid and basic fractions of glycinin (32 and 20 kDa respectively) and to the β and α subunits of β-conglycinin (52 and 68 kDa respectively). It has been shown that glycinin and β-conglycinin precipitate at a minimum calcium concentration of 0.4 mM and 1.4 mM, respectively (Saio, Watanabe, & Kaji, 1973); then it is possible that calcium favored the precipitation of these two fractions, over lunasin complexation. The presence of peaks at retention times later than 10 min (Fig. 2B), suggested the presence of compounds with molecular weight smaller than 75 Da, which may correspond to organic salts probably formed with the divalent cation. In addition, the significantly lower protein content corroborated that calcium preferentially induced precipitation of non-protein compounds, present mostly in the precipitate.

Isoelectric precipitation is one of the most used mechanisms to isolate proteins. While precipitation at pH 4.5 has been shown to provide the highest yields during the production of soy protein isolates (Honig, Wolf, & Rackis, 1984), two-dimensional electrophoresis of an extracted fraction, from defatted soy flakes, enriched in lunasin exhibited a 5 kDa band at pH of 5.5 (Galbas, Porzucek, Woźniak, Słomski, & Selwet, 2013),

Table 1
Characteristics of calcium and isoelectric precipitates from ethanolic whey.

Parameter	pH 4.5	pH 5.5	Ca
mg solid/ml whey	1.2 ± 0.1^{ab}	1.0 ± 0.1^{b}	1.4 ± 0.1^{a}
μg protein/mg solid	339 ± 90^{ab}	423 ± 55^{a}	247 ± 28^{b}
μg lunasin/mg solid*	59 ± 7^{a}	70 ± 2^{a}	39 ± 10^{b}
% lunasin in solid	5.9 ± 0.7^{a}	7.0 ± 0.2^{a}	3.9 ± 1.0^{b}
Daidzin (μg/mg)	3.7 ± 0.1^{a}	3.8 ± 0.4^{a}	2.9 ± 0.1^{b}
Genistin (ng/mg)	294 ± 3^{a}	295 ± 3^{a}	215 ± 1^{b}
Glycitin (μg/mg)	ND	ND	ND
Daidzein (μg/mg)	ND	ND	ND
Genistein (μg/mg)	ND	ND	ND
Glycitein (μg/mg)	ND	ND	ND
Trypsin Inhibitory Activity (TIA)	ND	ND	ND

ND: Non-detectable, *Estimated via ELISA. Different letters in the same row indicate significant differences among treatments ($p < 0.05$)

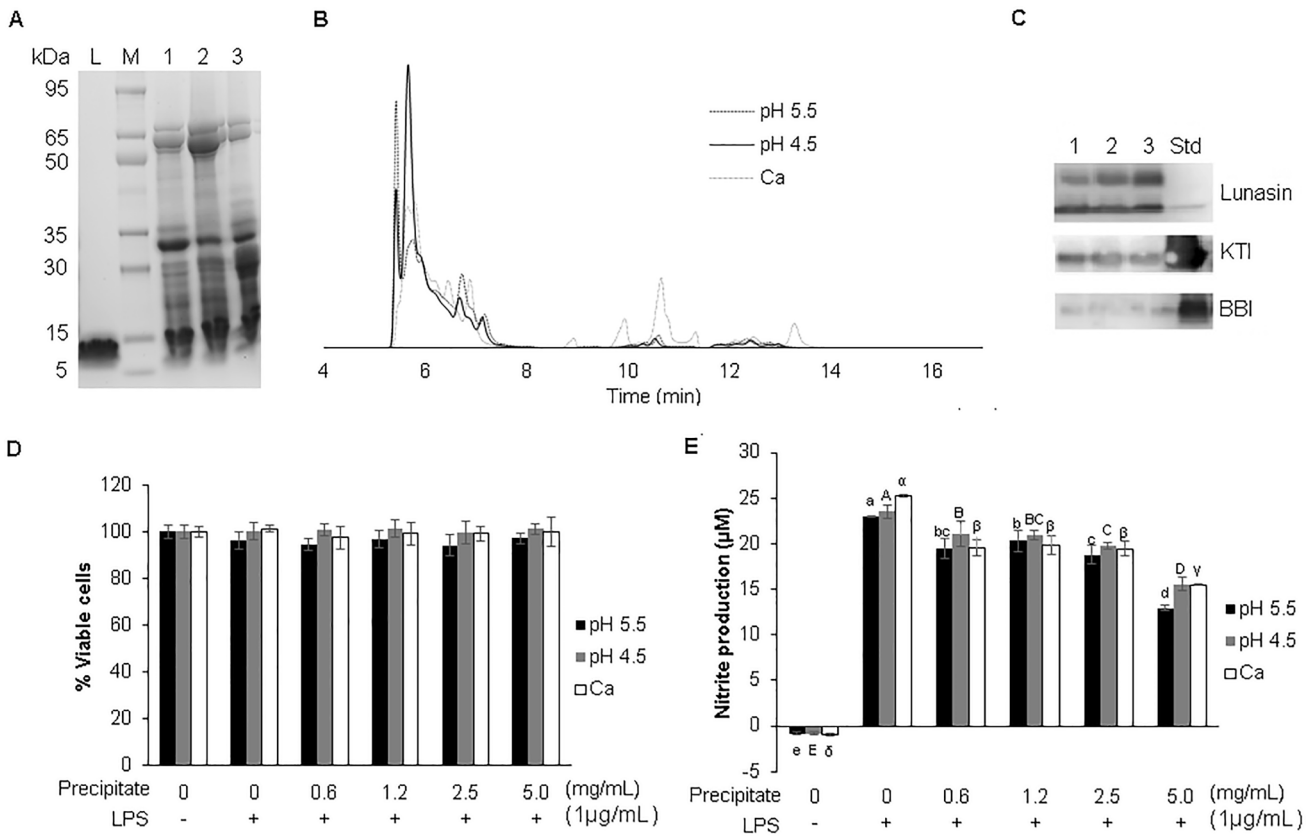

Fig. 2. Precipitation of soluble proteins from ethanolic whey. After treatment of the whey with 30% ethanol, lunasin enriched fractions were produced by precipitating remaining soluble proteins at pH 4.5 (line 1), pH 5.5 (line 2), or with calcium chloride (line 3). Protein precipitates were characterized via SDS-PAGE (A), size exclusion chromatography (B), and Western blot (C). Lunasin (line L), Kunitz trypsin inhibitor (KTI), and Bowman-Birk inhibitor (BBI) standards (line Std) were included for reference. Cell viability (D) and release of nitric oxide (E) were measured as indicators of the biological activity of the lunasin enriched fractions. LPS-activated murine macrophages were treated with increasing concentrations of lunasin enriched fractions. Non-treated non-activated cells (Control), and non-treated LPS-activated cells (LPS) were used as controls. Different letters indicate significant differences between the different concentrations of the same sample ($p < 0.05$).

suggesting this particular pH as a more specific isoelectric point for the peptide. No significant differences were found for any of the parameters quantified for the two isoelectric precipitates. A stronger band at 32 kDa in the pH 4.5 precipitate indicated a higher impact over the acid glycinin fraction, while pH 5.5 had a higher effect over the β subunit of β-conglycinin (52 kDa) (Fig. 2A). These observations agreed with the molecular weight distribution exhibited in the chromatographic profiles. Immunoblotting revealed that not only lunasin, but the 14 kDa complex, as well as Kunitz (KTI) and Bowman-Birk (BBI) inhibitors, were present in all the precipitates. No detectable trypsin inhibitory activity in any of the three precipitates indicated that the protease inhibitors were present as inactive molecules. The boiling of the soymilk before the curd coagulation led to the irreversible denaturation of the active sites required for the inhibitory activity.

Soybeans are known to contain several biologically active compounds. Among them, isoflavones have gained great recognition due to their ability to have a preventive effect over several diseases such as type II diabetes, osteoporosis, cardiovascular diseases, and hormone-associated cancers (Pabich & Materska, 2019). As they have been reported to be present in tofu whey (Zhao, Kim, & Eun, 2020), we tested for the presence of the major soy isoflavones in the precipitates. As expected, none of the aglycone forms was detectable while the more water-soluble glucosides daidzin and genistin were both present (Table 1).

To ensure that the biological activity was conserved, the anti-inflammatory properties of the precipitates was assessed by measuring their ability to reduce the level of nitric oxide produced by activated murine macrophages. A significant dose-dependent decrease of nitric oxide released into the media was observed (Fig. 2E), with no effect over the proliferation of the immune cells (Fig. 2D), suggesting that the biological activity was still present.

During storage at 23 °C, 51% relative humidity, the pH 5.5 precipitate turned brownish color and highly hygroscopic, while pH 4.5 powder maintained its original characteristics as a whitish homogeneous dry powder. Considering that lunasin enriched material at pH 4.5 has higher lunasin and protein content than calcium precipitate, and apparent better storage stability than the product at pH 5.5, isoelectric precipitation of the alcoholic whey at pH 4.5 was selected for the preparation of the lunaisn enriched material.

3.2. Adjustment of preparative gel filtration chromatography parameters

Since the presence of proteins of different sizes continued to be an interference in the lunasin enriched material, a size-based separation approach can be used for lunasin purification. Gel filtration chromatography has been widely applied for the fractionation and purification of protein, peptides, enzymes and DNA based on their size and geometry while maintaining their biological activity (Naqvi, Khan, & Saleemuddin, 2010; Singh, Walia, & Kennedy, 2019). To establish the most appropriate chromatographic conditions for the separation of lunasin from tofu whey, the effects of mobile phase composition, volume of sample injected, and the porosity of the packed bed (preparation grade-pg) were evaluated., The baseline was established by injecting 0.5 mL of sample, prepared as described in the experimental section, in a 30 pg column using 20 mM Tris-HCl + 0.15 M NaCl pH 7.6 as mobile phase.

Modifications in the characteristic profiles were evaluated using alternative conditions (Fig. 3): 100 mM NH_4HCO_3 pH 7.8 as mobile phase, a 75 pg column, or injecting 3 mL of the sample.

3.2.1. Effect of mobile phase (non-volatile vs. volatile)

During preliminary experiments using the base conditions, we observed that buffering salts in the mobile phase became the major component of the final product after freeze drying, making the content of lunasin negligible. Desalting trials using macrosep advance centrifugal device 1 K MWCO allowed removing 98% of the salts present, though, the remaining salt continued to be 10 times higher than lunasin in the final dry product. So far, only one report has clearly stated the performance of concentration and buffer exchange steps, via ultrafiltration, after the final chromatographic purification (Seber et al., 2012). To address this issue, we proposed the use of a volatile buffer as mobile phase. Upon mild heating, NH_4HCO_3 easily decomposes into the volatile compounds ammonia and carbon dioxide, (Ivanchenko, Shapoval, Makarov, & Borsch, 2020). Preliminary tests indicated that after heating 100 mM NH_4HCO_3 at 40 °C for 5 min no solid residue remained. Then we performed the gel filtration chromatography using 100 mM NH_4HCO_3 pH 7.8 as mobile phase. Under base conditions, using Tris-HCl buffer, lunasin eluted at 60 min and the resolution of the peaks (Fig. 3A) and carbohydrates profile (Fig. 3B) suggested a good level of separation of the peptide from other compounds present in the whey. Fig. 3A and C show that the chromatographic profile, as well as retention times for lunasin and carbohydrates using ammonium bicarbonate, resemble those obtained with Tris-HCl buffer. The fact that no shift in the retention time of any peaks was observed, indicated that nonspecific ionic interactions between tofu whey components and the packed bed were negligible, and the presence of strong ionic salts in the mobile phase, such as NaCl, was not required. Then we concluded that NH_4HCO_3 was a more suitable mobile phase than Tris-HCl for the chromatographic separation and recovery of lunasin from tofu whey.

3.2.2. Column preparation grade (30 pg vs. 75 pg)

Preparation grade (pg) refers to the grade of fractionation or separation that the column can achieve in relation to the relative molecular mass (Mr) of the compounds present in the sample (GE, 2000). In the 30 pg column (Mr < 10,000) probably all proteins in the range of 10 to 75 kDa traveled externally to the packing beads, leaving the column as a whole group represented by a wide peak with retention times between 40 and 50 min (Fig. 3A). In the 75 pg column (3000 < Mr < 70,000), a better resolution of the proteins and peptides was observed, accompanied by a shift in the retention time of lunasin peak to 88 min, 28 min later than the 30 pg column. Analysis of the relative amounts of lunasin and proteins (Fig. 3D) showed that most of the compounds absorbing at 280 nm eluted at around 108 min, 20 min later than lunasin, while lunasin containing fractions exhibited a minimum content of proteins. The better resolution of the peaks achieved by the 75 pg column made it more suitable than the 30 pg column for purification of lunasin

3.2.3. Injection volume of the sample (0.5 vs. 3 mL)

Sample volume plays a decisive role in the resolution of chromatogram peaks, as larger volumes result in peak broadening; thus a sample volume no>4% CV is recommended for high resolution fractionation (GE, 2000). For a 75 pg XK16 column with CV = 120 mL, 0.5 mL sample volume (0.4% of the CV) showed a good resolution for lunasin peak (Fig. 3A); however, the amount of dry lunasin recovered per run was negligible. Fig. 3A and E show the impact of a 6-fold increase in the sample injection volume (3 mL, equivalent to 2.5% of the CV). As expected, lunasin recovered in the fractions increased proportionally, accompanied by a remarkable widening of the peak, which now goes from 58 to 88 min. The consequent decrease in the resolution led to the overlapping of lunasin to adjacent peaks associated with larger and smaller molecules.

The four fractions exhibiting relevant content of lunasin, corresponding to retention times 58, 68, 78, and 88 min were further analyzed. Electrophoretic separation (Fig. 3F) showed the progressive and significant reduction of high-molecular weight proteins in the fractions: eluate collected at 58 min contained proteins between 15 and about 60 kDa while eluate at 88 min was free from proteins above 15 kDa. Immunoblotting (Fig. 3G) indicated that while the two earlier fractions contained only the 14 kDa complex, fractions at 78 and 88 min contained mostly the 5 kDa peptide. Molecular weight distribution (Fig. 3H) revealed that the 88 min fraction contained the main peak at 7.2 min, corresponding to 5 kDa lunasin, while for fraction at 78 min this peak was combined with a larger one at 7.0 min, probably associated with the 14 kDa complex. The difference in retention times during HPLC size exclusion chromatography profiling observed for the isolated lunasin (7.2 min) and the synthetic lunasin standard (6.6 min) could be a result of differences in the folding and geometry of the synthetic peptide in contrast to the native state.

We selected 3 mL injection volume, a 75 pg column, and 100 mM NH_4HCO_3 pH 7.8 as operating conditions for purification of lunasin via preparative gel filtration chromatography.

3.3. Application of adjusted enrichment and purification processes to industrially produced tofu whey

We used industrially produced whey to contrast our designed enrichment process using ethanol and pH 4.5 (EtOHpH4.5) with the more commonly used hot and cold drying alternatives to obtain solid fractions from liquid streams: spray drying (SDW), and freeze-drying (FDW). The three samples were purified via gel filtration chromatography as described in Section 2.5. Chromatographic profile (Fig. 4A) clearly showed that the ethanolic-isoelectric concentration process drastically switched the protein profile in the whey, making more prevalent the peak associated with lunasin (retention time 88 min). Peaks for lunasin (Fig. 4B), compounds responsible for yellow coloration (Fig. 4C), and carbohydrates (Fig. 4D) were present in the three samples at the same retention times, suggesting that no evident conformational changes associated with size and geometry occurred to these set of compounds by exposure to the drying temperature and conditions, or to the enrichment process. However, in our lunasin enriched material the concentration of lunasin was considerably higher, while the presence of other compounds was almost negligible in comparison to the whole whey. This demonstrates that ethanolic – isoelectric precipitation not only removed an important quantity of undesired proteins, but other non- protein compounds present in the whey. In addition, it favors the completion of the chromatographic purification in a shorter time.

Electrophoretic and size exclusion chromatographic profile (Supplementary Fig. 3) of purified fractions collected at 78 and 88 min (F78 and F88 respectively), from the sample treated with our enrichment method, showed that F78 still contained significant amount of the 14 kDa complex and other proteins of similar size, while F88 mostly correspond to the 5 kDa lunasin.

Approximately 193 mg of F78 and 34 mg of F88 were obtained per liter of industrial tofu whey, containing 46% and 80% w/w lunasin, respectively (Supplementary Table 5). MALDI-TOF-MS analysis of the 5 kDa band in F78 and F88 (Table 2) indicated a 34% and 56% coverage of the 2S albumin precursor, respectively, and 100% coverage of lunasin sequence, corroborating the isolation of the peptide.

A six-step isolation process involving PBS extraction, ionic exchange chromatography (IEC), treatment with a reducing agent, ultrafiltration, reverse phase chromatography, and ultrafiltration, reported a yield of 442 mg lunasin/kg defatted soy flour with > 99% purity (Seber et al., 2012). This report based the estimation of purity on the visualization of a unique electrophoretic band at 5 kDa in the final product, leaving behind the presence of other compounds of different nature, such as carbohydrates, that are soluble in aqueous solutions and are present in the soybeans in abundant quantities but cannot be visualized via SDS-PAGE. Another four-step process that involved aqueous extraction,

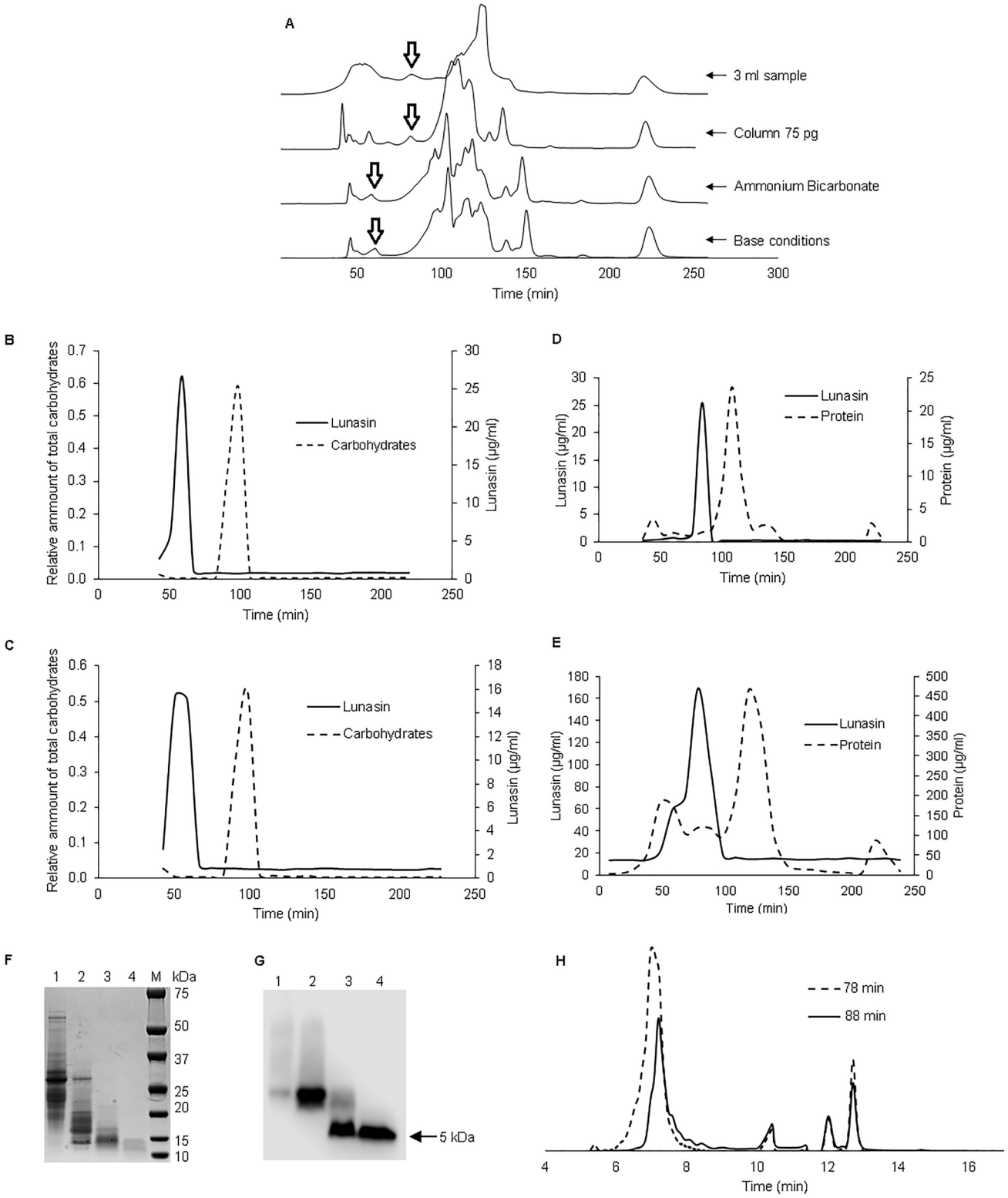

Fig. 3. Adjustment of preparative gel filtration chromatography parameters. The effect of the mobile phase (Tris-HCl or ammonium bicarbonate), preparation grade of the chromatographic column (30 or 75 pg) and sample volume injection (0.5 or 3 mL) were evaluated. Gel filtration chromatographic profile (A), lunasin, carbohydrates, and protein profiles (B-E) under the different separation conditions. Base conditions: 30 pg column, Tris- HCl mobile phase and 0.5 mL injection volume (B), NH_4HCO_3 as mobile phase (C, D and E), 75 pg column (D and E), 3 mL injection volume (E). Open arrows indicate lunasin peak. As indicated in section 2.4, 3 mL of resuspended whey were purified using NH_4HCO_3 mobile phase and 75 pg column. Consecutive fractions of eluent were collected every 10 min. Fractions with a significant content of lunasin corresponding to average retention times of 58, 68, 78, and 88 min (lines 1 to 4 respectively) were characterized via SDS-PAGE (F), western blot against lunasin antibody (G) and via HPLC for molecular weight distribution profile (H).

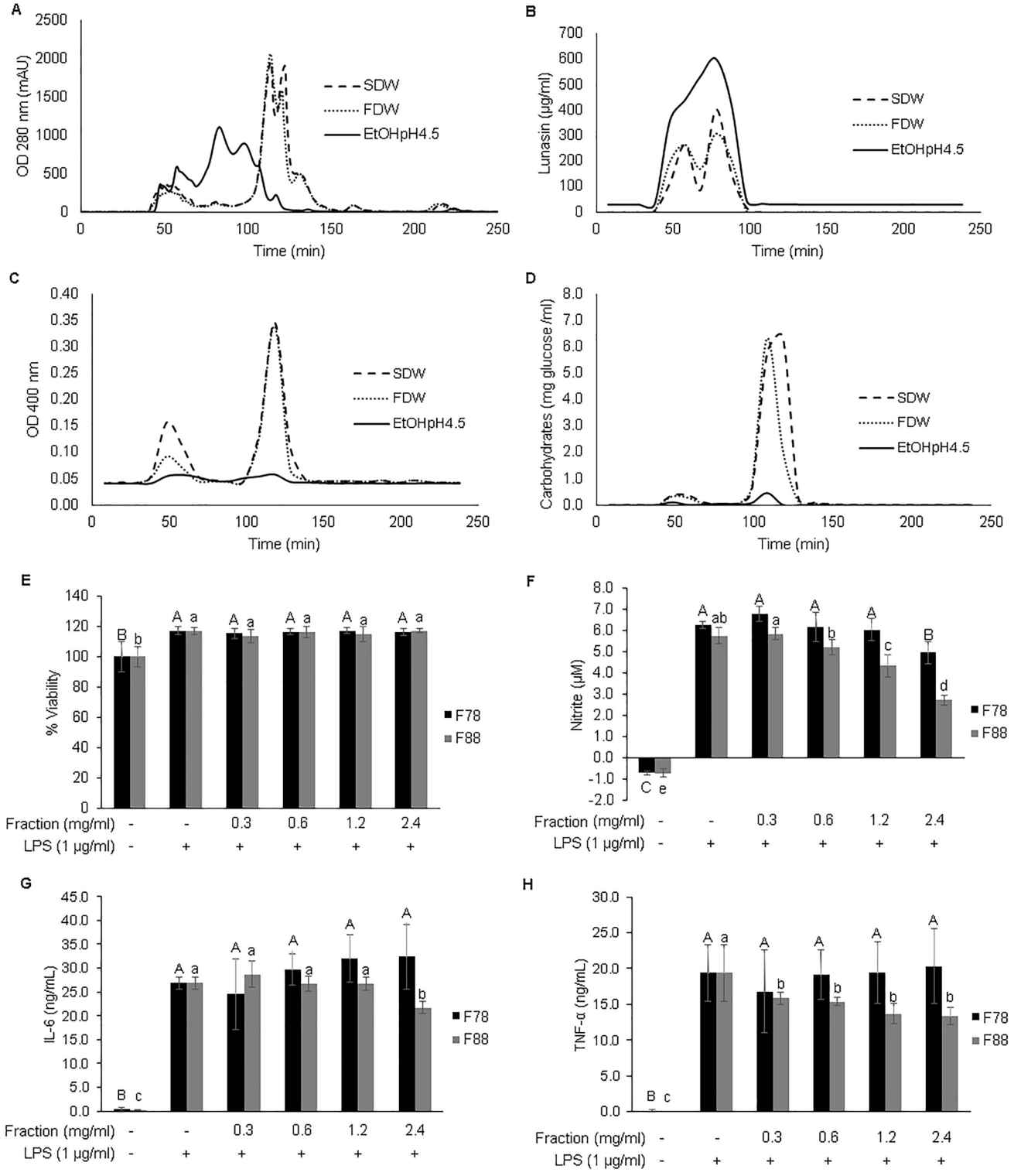

Fig. 4. Isolation of lunasin from industrially produced tofu whey. Industrially produced tofu whey was spray dried (SDW), freeze-dried (FDW), or concentrated using 30% ethanol and pH 4.5 (EtOHpH4.5). Each sample was then purified via preparative gel filtration chromatography as described in section 2.5. Consecutive fractions of eluent were collected every 10 min and characterized for protein content (A), lunasin content (B), yellow color intensity (C), and total carbohydrates content (D). Fractions with a significant content of lunasin, corresponding to average retention times of 78 min (F78) and 88 min (F88) were tested for bioactivity. Viability (A), production of nitric oxide (B), and release of pro-inflammatory cytokines IL-6 (C) and TNF-α (D) were evaluated in LPS-activated murine macrophages in the presence or absence of different concentrations of fractions F78 and F88.

Table 2
MALDI-TOF-MS identification of the 5 kDa lunasin immunoreactive bands in fractions obtained via gel filtration chromatography of ethanolic-isoelectric enriched material from industrially produced tofu whey.

Fraction	Protein identified (NCBInr accession)	MOWSE (20 ppm)	Sequence coverage (%)	Peptides matched
F88	2S albumin precursor Gm-c1019 *Glycine max* (AW186094) 1 LLFCIAHTCS ASKWQHQQDS CRKQLQGVNL TPCEKHIMEK IQGRGDDDDD 51 DDDDNHILRI MRGKINYITR NEGKDEDEEE EGHMQKCCTE MSELRSPKCQ 101 CKALQKIMEN QSEELEEKQK KKMEKELINL ATMCRFGPMI QCDLSSDD_E 151 VKSNVVTCTY _HMM__FM	462	34	HIMEKIQGR WQHQQDSCRK KWQHQQDSCR QLQGVNLTPCEK SKWQHQQDSCR FGPMIQCDLSSDD KQLQGVNLTPCEK IQGRGDDDDDDDD IQGRGDDDDDDDDN QLQGVNLTPCEKHIMEK
F78	2S albumin precursor Gm-c1055 *Glycine max* (BG042158) 1 GKMTKFTILL ISLLFCIAHT CSASKWQHQQ DSCRKQLQGV NLTPCEKHIM 51 EKIQGRGDDD DDDDDDNHIL RTMRGRINYI RRNEGKDEHE EEEGHMQKCC 101 TEMSELRSPK CQCKALHKIM ENQSEELEEK QKKKMENELI NLATMCRFGP 151 MIQCDLSSDD	841	56	HIMEK CCTEMSELR ELINLATMCR WQHQQDSCR KWQHQQDSCR WQHQQDSCRK KWQHQQDSCR QLQGVNLTPCEK SKWQHQQDSCR IMENQSEELEEK FGPMIQCDLSSDD KQLQGVNLTPCEK IQGRGDDDDDDDD IMENQSEELEEKQ IQGRGDDDDDDDDN IMENQSEELEEKQK

Matched peptides are shown in red and the underlined segment corresponds to lunasin peptide amino acid sequence (SKW…DDD).

IEC, ultrafiltration, and gel filtration chromatography, based on the data presented, was able to obtain 1.3 g lunasin/kg defatted soy flour, with 80% purity as assayed via HPLC (Dia et al., 2009). Our three-step process that involves ethanolic precipitation, isoelectric precipitation, and gel filtration chromatography yielded 772 mg/kg of dry tofu whey with at least 80% purity as analyzed via HPLC, with the remaining fraction being proteins of slightly higher molecular weight (Supplementary Fig. 2 and Supplementary Table 5). This process is simpler, faster and valorizes a by-product largely produced by the tofu industry. Hence, our method can be used to obtain the naturally occurring bioactive peptide lunasin in a more economical, and environmentally friendly process leading to reduce wastage and increase utilization of tofu whey.

3.4. Biological activity of isolated lunasin

Oxidative stress and inflammation have been recognized as two of the principal factors contributing to the onset and development of non-communicable and infectious diseases (Nediani & Giovannelli, 2020). The antioxidant and anti-inflammatory properties of lunasin have attracted attention to this peptide as a promising alternative for the prevention and treatment of some of these diseases (de Mejia & Dia, 2009; Dia et al., 2009; Hernández-Ledesma, Hsieh, & de Lumen, 2009a). Upon infection, components of pathogens, including the LPS outer membrane of gram-negative bacteria, are recognized by innate immune cells such as macrophages, triggering signaling cascades that lead to the expression and release of pro-inflammatory cytokines and nitric oxide (Mogensen, 2009; Tripathi, Tripathi, Kashyap, & Singh, 2007). However, chronic inflammation derived from the inappropriate regulation of this process can produce damage to the host cells and lead to inflammation-related diseases (Furman et al., 2019).

To verify that the isolated lunasin maintained its biological activity, we tested the ability of the two purified fractions, F78 and F88, obtained from industrially produced whey treated with our designed enrichment method, to reduce pro-inflammatory response in LPS-activated murine macrophages. No detrimental effect over the viability of the immune cells (Fig. 4E) was detected upon treatment with increasing concentrations of the two fractions. No effect was observed in most of the cases for fractions F78, while significant reduction of the pro-inflammatory markers was observed for fraction F88 (Fig. 4 F-H), corroborating that our isolated lunasin is biologically active, and that a considerably high level of purity is required to exert the bioactive properties. The 30% reduction of TNF-α achieved by our isolated material at 2.4 mg/mL (190

μM lunasin) is comparable to the 23% reduction of TNF-α reported using 200 μM of synthetic lunasin under similar conditions of treatment of the same cell line (Hernández-Ledesma et al., 2009a)

4. Conclusions

A simple and feasible approach combining ethanolic-isoelectric precipitation and gel filtration chromatography was developed for the isolation of bioactive lunasin from tofu whey. In contrast to current isolation methods that waste valuable nutrients, from the defatted soy flour or whole soybeans, removed during the isolation process, the implementation of our designed approach would favor the generation of three valuable products from the soybeans: tofu, an intermediate product resulting from the ethanolic treatment of the whey during the enrichment process with potential as a protein dietary supplement, and lunasin. Our three-step process is competitive with current alternatives in terms of the quantity and purity of the final product, but faster and more sustainable. It promotes the valorization of a by-product largely produced by the tofu industry and at the same time facilitate a better utilization of soybean nutrients for the benefit and well-being of humans.

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CRediT authorship contribution statement

Andrea Nieto-Veloza: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Qixin Zhong:** Writing - review & editing, Funding acquisition. **Won-Seok Kim:** Investigation, Writing - review & editing. **Doris D'Souza:** Writing - review & editing. **Hari B. Krishnan:** Writing - review & editing. **Vermont P. Dia:** Conceptualization, Resources, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130220.

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