

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/332054081>

Molecular changes of phenolic–protein interactions in isolated proteins from flaxseed and soybean using Native–PAGE, SDS–PAGE, RP–HPLC, and ESI–MS analysis

Article in *Journal of Food Biochemistry* · May 2019

DOI: 10.1111/jfbc.12849

CITATIONS

8

READS

335

4 authors, including:



Muhammad H. Aludatt

Jordan University of Science and Technology

133 PUBLICATIONS 2,409 CITATIONS

[SEE PROFILE](#)



Taha Rababah

Jordan University of Science and Technology

140 PUBLICATIONS 3,921 CITATIONS

[SEE PROFILE](#)



Stan Kubow

McGill University

187 PUBLICATIONS 5,012 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Dietary Factors Associated with MetS [View project](#)



Biomarkers of Chronic Fatigue Syndrome [View project](#)

Molecular changes of phenolic–protein interactions in isolated proteins from flaxseed and soybean using Native-PAGE, SDS-PAGE, RP-HPLC, and ESI-MS analysis

Muhammad H. Alu'datt^{1,2}  | Taha Rababah¹ | Stan Kubow³ | Inteaz Alli²

¹Faculty of Agriculture, Department of Nutrition and Food Technology, Jordan University of Science and Technology, Irbid, Jordan

²Department of Food Science and Agricultural Chemistry, Macdonald Campus, McGill University, Ste-Anne-De-Bellevue, Quebec, Canada

³School of Dietetics and Human Nutrition, Macdonald Campus, McGill University, Ste-Anne-De-Bellevue, Quebec, Canada

Correspondence

Muhammad H. Alu'datt, Faculty of Agriculture, Department of Nutrition and Food Technology, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan.
Emails: Muhammad.alu'datt@mail.mcgill.ca; malodat@just.edu.jo

Funding information

Jordan University of Science and Technology; McGill University

Abstract

The effects of protein–phenolic interactions on the molecular characteristics of soybean and flaxseed proteins were investigated. Proteins were isolated from soybean and flaxseed using isoelectric precipitation, followed by extraction of free and bound phenolics. The effects of elimination of the phenolic compounds on molecular characteristics of the protein isolates were studied using reversed phase-high performance liquid chromatography (RP-HPLC), Native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and electron spray ionization-mass spectrometry (ESI-MS). The Native-PAGE fractions from isolated proteins from full-fat flaxseed and soybean revealed that protein migration was affected by removal of bound phenolics. SDS-PAGE from full-fat and defatted protein isolates of flaxseed and soybean revealed that the removal of bound phenolics affected the molecular characteristics of protein subunits. Soybean protein isolates had protein–phenolic interactions through acidic and basic subunits. RP-HPLC and ESI-MS showed that the removal of bound and free phenolics had only minor effects on the molecular characteristics of isolated proteins from defatted and full-fat soybean. With respect to isolated proteins from flaxseed, the removal of bound phenolics showed little effect on the electrophoretic behavior of the proteins or the protein subunits.

Practical applications

Phenolic–protein and phenolic–lipid–protein interactions may affect the nutritional, physicochemical, and functional properties of isolated proteins from food in flaxseed and soybean.

KEYWORDS

bound, electron spray ionization-mass spectrometry (ESI-MS), free, isolated protein, molecular characteristics, phenolics, phenolic–protein interactions

1 | INTRODUCTION

There has been a rapidly accumulating interest regarding the health promoting and functional attributes of phenolic compounds that are generally present as minor food components in a wide variety of plant foods, such as legumes, vegetables, and fruits (Alu'datt

et al., 2017, 2019; Alu'datt, Rababah, & Alli, 2014; Alu'datt, Rababah, Ereifej, Brewer, & Alli, 2013). Plant phenolics have been indicated to provide a variety of preventative and therapeutic benefits against disease because of their anti-inflammatory, anti-viral, hypoglycemic, anti-cancer, and hypolipidemic properties (Alu'datt et al., 2017, 2019; Bravo, 1998). The major oil bearing plants that have been

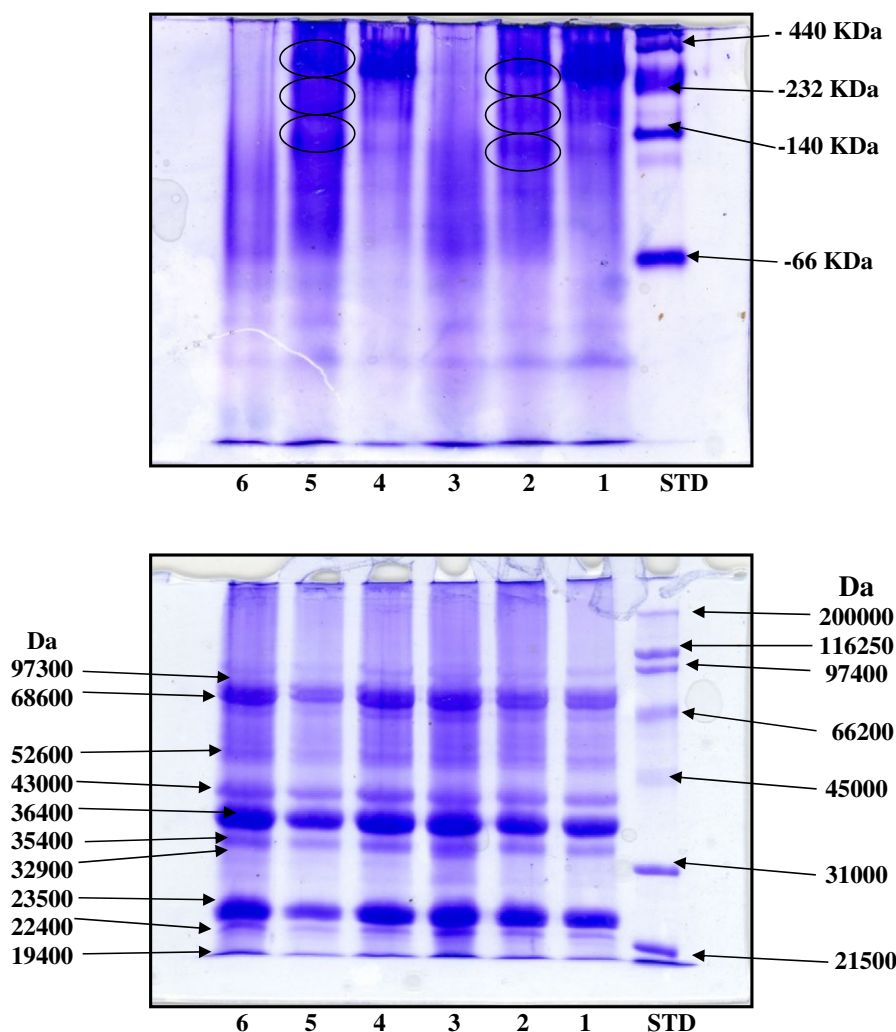


FIGURE 1 Native-PAGE and SDS-PAGE for protein isolate (I) from defatted (D) and full-fat (F) soybean (s); (STD) standard (1) Dsl; (2) Dsl after removal of free phenolic compounds; (3) Dsl after removal of free and bound phenolic compounds; (4) Fsl; (5) Fsl after removal of free phenolic compounds; (6) Fsl after removal of free and bound phenolic compounds

recognized as a vital food source of edible proteins and oils are flax and soy seeds (Alu'datt et al., 2017, 2019, 2014, 2013). The occurrence of phenolic compounds in such foods has been shown to affect their nutritional, functional and biological properties (Alu'datt et al., 2017, 2019, 2014, 2013). The nature of phenolics chemical structure permits them to bind naturally with proteins, minerals, carbohydrates and lipids because of the presence of aromatic, hydroxyl, and carboxylic acids (Bravo, 1998; Escarpa & Gonzalez, 2001; Lipworth, Martinez, Angell, Hsieh, & Trichopoulos, 1997; Toschi et al., 2000). Beauchamp and Maller (1977) reported that the interactions of phenolics with other food components may affect the palatability of foods. Bravo (1998) found that the tannin-protein and tannin-carbohydrate complexes occur frequently in food systems. Ratty and Das (1988) and Sabally (2006) demonstrated that the phenolic compounds interactions can also readily occur with carbohydrates and lipids. There has been relatively little information, however, reported about naturally phenolic interactions with proteins in food systems (Alu'datt et al., 2019, 2014, 2013).

The presence of functional groups in the backbone structure of proteins and phenolics may allow them to interact via hydrophobic, ionic, covalent, and hydrogen interactions (Alu'datt et al., 2019; Hagerman & Butler, 1978; Loomis & Battaile, 1966; Mason, 1955; Rubino, Arntfield, Nadon, & Bernatsky, 1996). Such interactions may affect the functional, physicochemical, and nutritional properties of proteins in food systems. Alu'datt et al. (2013) demonstrated the presence of phenolic-protein interactions in isolated soybean proteins as well as phenolic-lipid-protein interactions in isolated flaxseed proteins. Alu'datt et al. (2014) reported that the presence of phenolic-protein interactions in proteins isolated from defatted soybean increased the glycinin thermal stability. They noted that in full-fat soybean the presence of phenolic-protein interactions decreased the water holding capacity of glycinin. The presence of phenolic-protein interactions in food systems may affect the biological, antioxidant activity, and functional properties (Alu'datt et al., 2019, 2014, 2013). The phenolic-protein interactions in isolated flaxseed proteins were associated with a decline in water holding

capacity, thermal stability, and viscoelastic properties (Alu'datt et al., 2019, 2014). The significant objective of this investigation was to evaluate the molecular changes of phenolic-protein interactions in flaxseed and soybean protein isolates. The specific objectives of this study were to: (a) study the effect of removal of phenolics (free and bound) from isolated proteins of defatted and full-fat soybean and flaxseed on reversed phase (RP)-high performance liquid chromatography (HPLC) fractions; (b) evaluate the effect of removal of phenolics (free and bound) on the molecular characterization of RP-HPLC fractions of isolated proteins from soybean and flaxseed by sodium dodecyl sulfate (SDS) and Native polyacrylamide gel electrophoresis (PAGE); and (c) investigate the molecular changes in the RP-HPLC fractions of protein isolates from soybean and flaxseed by electrospray ionization (ESI)-mass spectrometry (MS).

2 | MATERIALS AND METHODS

2.1 | Plant materials

Soybean seeds (Cultivar SB, 210; protein content $32.7\% \pm 1.4$) were obtained from Great Lakes Organic Inc. (Ontario, Canada).

Flaxseed seeds were obtained from La Meunerie Milanaise Inc. (Milan, PQ, Canada; protein content $19.2\% \pm 1.3$). Grain seeds were ground by coffee grinder and then kept in a container in a refrigerator at 4°C for chemical analysis. Samples were defatted with petroleum ether for 10 hr using a Soxhlet apparatus (AOAC, 1990).

2.2 | Isolation of proteins from flaxseed and soybean seeds

Isoelectric protein isolation from flaxseed and soybean proteins was used as described by the methods of Dev, Quensel, and Hansen (1986), Krase, Schultz, and Dudek (2002) and Alu'datt et al. (2014). Ten grams of defatted and full-fat flaxseed and soybean seed were mixed with 100 ml of diluted NaOH (2.0 M). The solution was stirred at 23°C for 60 min and then adjusted at pH 11.0 followed by centrifugation at $10,000 \times g$ for 30 min. The protein extracts were filtered by cheese cloth to remove insoluble residues. The extracted proteins were precipitated by adjusting pH to 4.6 using dilute HCl (0.1 M). The isolated proteins were collected by centrifugation at $10,000 \times g$ for 15 min, followed by lyophilization.

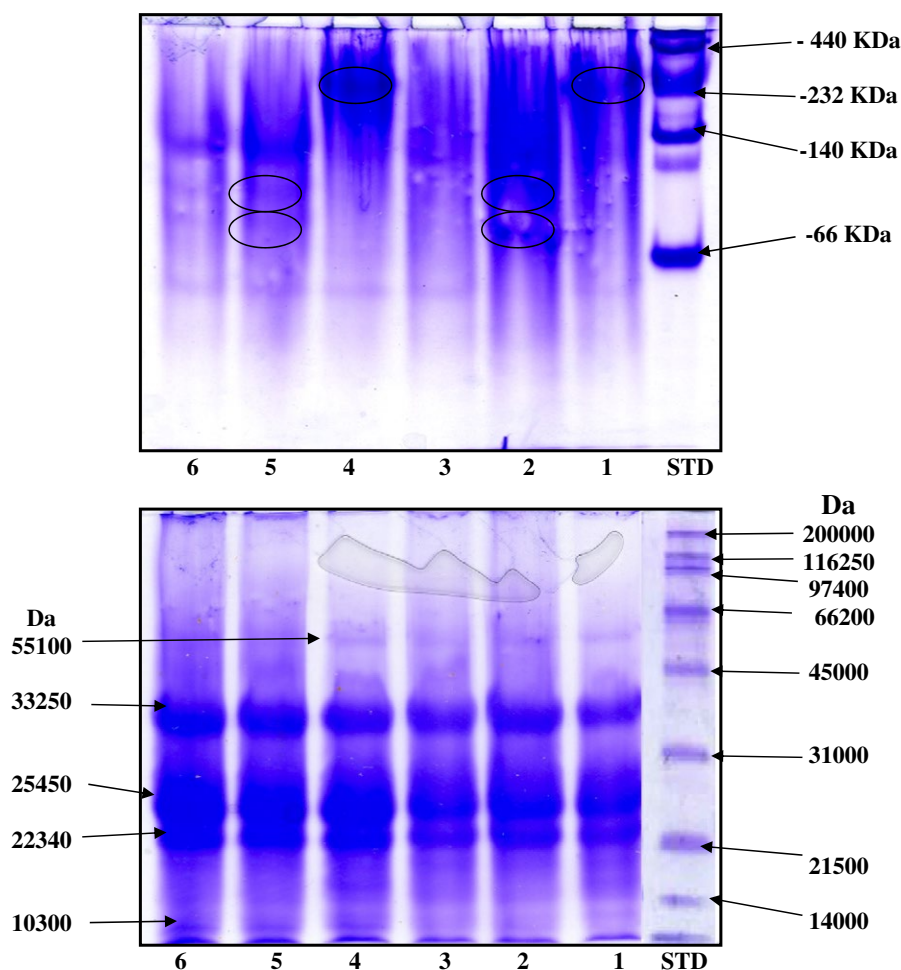


FIGURE 2 Native-PAGE and SDS-PAGE for protein isolate (I) from defatted (D) and full-fat (F) for flaxseed (f); (STD) standard (1) DfI; (2) DfI after removal of free phenolic compounds; (3) DfI after removal of free and bound phenolic compounds; (4) FfI; (5) FfI after removal of free phenolic compounds; (6) FfI after removal of free and bound phenolic compounds

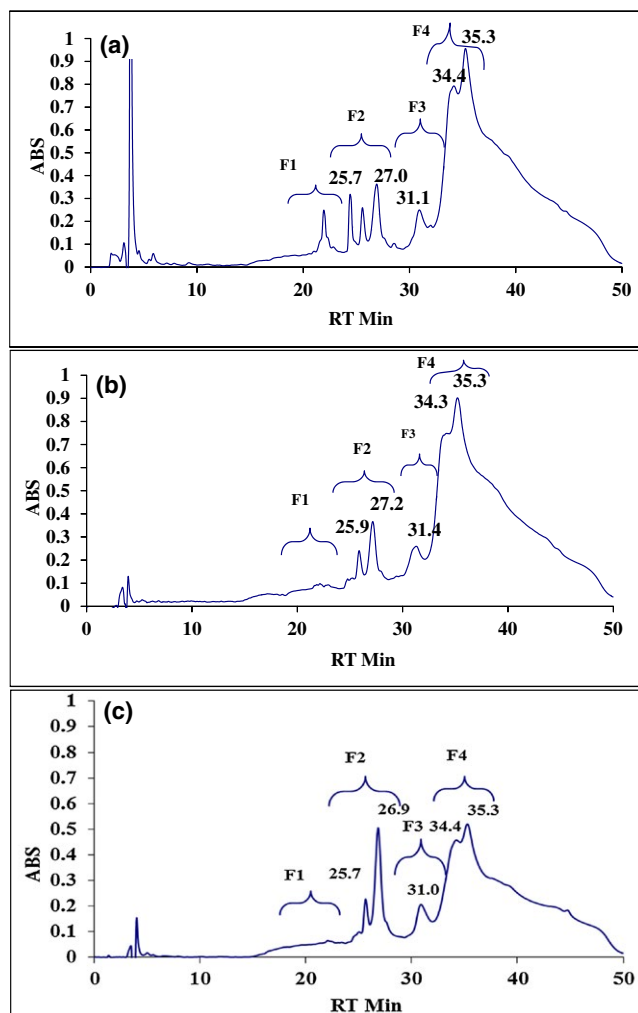


FIGURE 3 RP-HPLC separation for protein isolate (I) from full-fat (F) soybean (s); (a) Fsl; (b) Fsl after removal of free phenolic compounds; (c) Fsl after removal of free and bound phenolic compounds

2.3 | Removal of “free” phenolic compounds

The free phenolics were removed from isolated proteins of flax-seed and soybean according to the method reported by Alu'datt et al. (2014). One gram of isolated protein sample was subjected to methanolic extraction (25 ml; 100%) at 24°C for 1 hr followed by centrifugation at $10,000 \times g$ for 10 min. The phenolic extracts were separated by a cheese cloth followed by a discard of the free phenolics. The residues remaining after removal of methanolic extraction at 24°C for 1 hr were subjected to methanolic extracts at 60°C for 1 hr (25 ml, 100%) in order to complete removal phenolics and then centrifugation was performed at $10,000 \times g$ for 10 min, which was followed application of the protocol of heat-mediated phenolic extractions as reported by Johnsson, Kamal-Eldin, Lundgren, and Aaman (2000). The residues of isolated proteins remaining after removal free phenolics were stored at 4°C for further analysis.

2.4 | Removal of “bound” phenolic compounds

Basic hydrolysis methodology was utilized to remove bound phenolics as reported by Krygier, Sosulski, and Hoggie (1982), Eliasson, Kamal-Eldin, Andersson, and Aman (2003) and Alu'datt et al. (2014). The residues remaining from removal of free methanolics after the heat treatments described in above section were subjected to hydrolysis with 25 ml of alkaline solution (0.1 M NaOH; pH 12.0) at 24°C/12 hr and then subjected to centrifugation for 10 min at $10,000 \times g$. The liberated bound phenolics in the supernatants were filtered with a cheese cloth and then lyophilized by freeze drying. The liberated bound phenolic compounds of the lyophilized supernatants were re-extracted with methanol for 1 hr at 23°C (25 ml; 100%) and centrifuged for 10 min at $10,000 \times g$. The bound methanolic extracts were filtered using cheese cloth and then discarded.

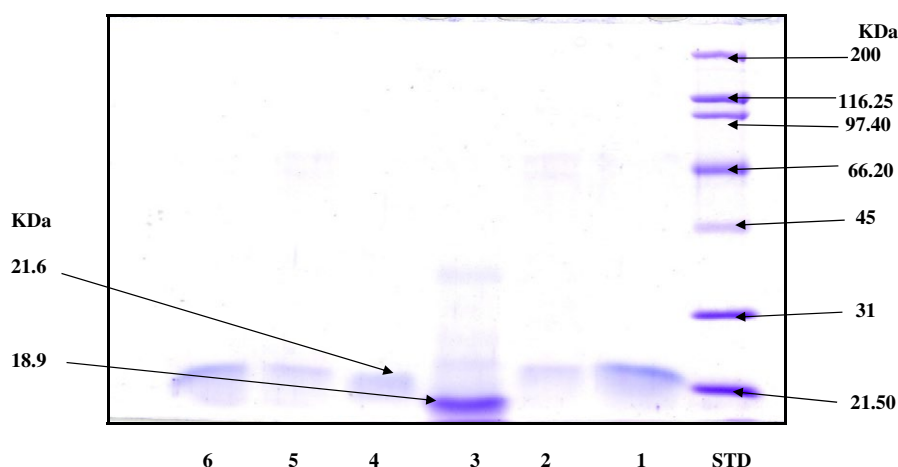
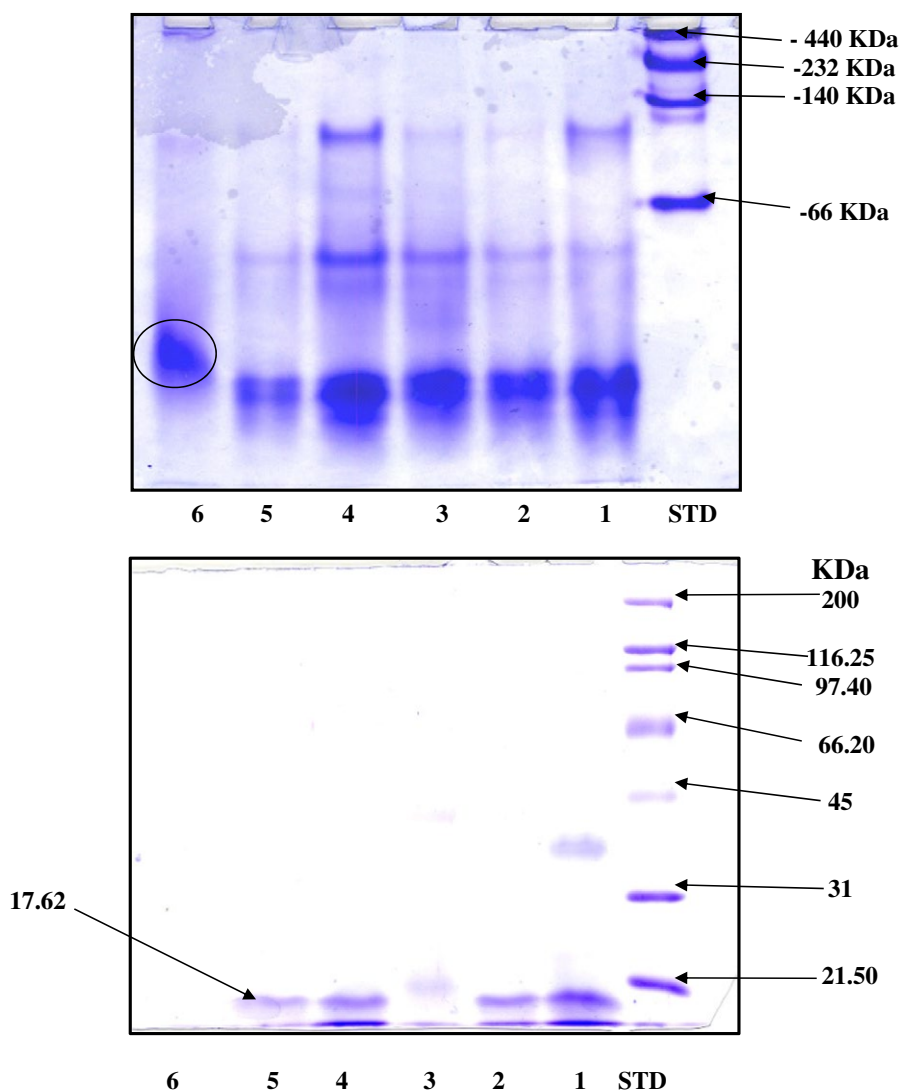


FIGURE 4 SDS-PAGE for F1 form protein isolate (I) of defatted (D) and full-fat (F) soybean (s); (STD) standard (1) Dsl; (2) Dsl after removal of free phenolic compounds; (3) Dsl after removal of free and bound phenolic compounds; (4) Fsl; (5) Fsl after removal of free phenolic compounds; (6) Fsl after removal of free and bound phenolic compounds

TABLE 1 Molecular weights (KDa) from SDS of F1, F2, F3, and F4 before and after removal of free and bound phenolic compounds separated by RP-HPLC from protein isolate (I) for defatted (D) and full fat (F) soybean (s)

Treatment	Subunits		Molecular Weight of Subunits	
	F1	F2	F3	F4
Dsl before removal of phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1, 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 41.3, 37.6, 32.5, 29.19, 23.8, 18.2, 14.8
Dsl after removal of free phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1, 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 37.6, 32.5, 29.19, 23.8, 18.2, 14.8
Dsl after removal of both free and bound phenolic compounds	18.9	ND	41.9, 38.0, 29.1	87.0, 52.9, 41.3, 37.6, 32.5, 29.19, 23.8, 18.2
Fsl before removal of phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1, 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 41.3, 37.6, 32.5, 23.8, 18.2
Fsl after removal of free phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1, 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 41.3, 37.6, 32.5, 23.8, 18.2
Fsl after removal of both free and bound phenolic compounds	21.6	ND	41.9, 38.0, 29.1	87.0, 52.9, 41.3, 37.6, 32.5, 23.8, 18.2

Note. ND: not detected.

**FIGURE 5** Native-PAGE and SDS-PAGE for F2 from protein isolate (I) of defatted (D) and full-fat (F) soybean (s); (STD) standard (1) Dsl; (2) Dsl after removal of free phenolic compounds; (3) Dsl after removal of free and bound phenolic compounds; (4) Fsl; (5) Fsl after removal of free phenolic compounds; (6) Fsl after removal of free and bound phenolic compounds

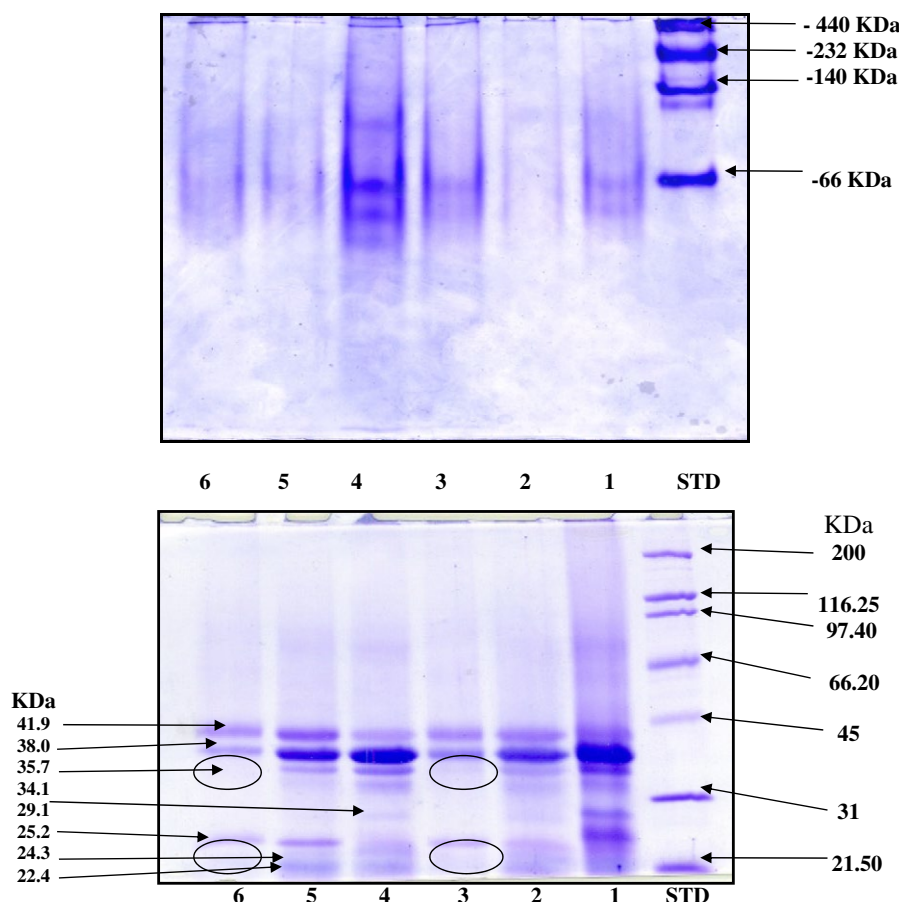


FIGURE 6 Native-PAGE and SDS-PAGE for F3 from protein isolate (I) of defatted (D) and full-fat (F) soybean (s); (STD) standard (1) Dsl; (2) Dsl after removal of free phenolic compounds; (3) Dsl after removal of free and bound phenolic compounds; (4) Fsl; (5) Fsl after removal of free phenolic compounds; (6) Fsl after removal of free and bound phenolic compounds

The residues remaining from extracted bound phenolics after alkaline extraction as described in the above section were subjected to acid hydrolysis with 25 ml of diluted HCl solution at 24°C/12 hr (0.1 M; pH 2.0), which follows the methodology using dilute acid to remove bound phenolics as described by several authors (Eliasson et al., 2003). The liberated bound phenolics in supernatants were filtered with cheese cloth and then lyophilized using a freeze dryer. The liberated bound phenolics of the lyophilized supernatant were re-extracted with methanol for 1 hr at 23°C (25 ml; 100%) and centrifuged for 10 min at 10,000 × g. The residues of isolated proteins remaining after removal bound phenolics were stored at 4°C for molecular structural studies.

2.5 | Molecular characterization using polyacrylamide gel electrophoresis (Native-PAGE)

Native-PAGE of the protein isolates before and after removal of free and bound phenolics from flaxseed and soybean described in the above section was performed using an electrophoresis cell unit (mini-protean II; Davis, 1964). Sample solutions (25 Lμ) of the freeze-dried protein precipitate were dissolved in sample

buffer (0.1% bromophenol blue, 2% glycerol, and 1.5 M Tris-HCl). Electrophoresis migration was carried out for 2–3 hr at 10 mA/gel in 10% running buffer using buffer of Tris-glycine (pH 8.3). Protein units of gels were subjected to stain with 0.1% Coomassie Brilliant Blue (R-250, 10% acetic acid, 20% methanol, and 70% water) and then washed with destaining solution (10% acetic acid, 20% methanol, and 70% water).

2.6 | SDS-PAGE

SDS-PAGE for the protein isolates obtained above was performed according to method described by Laemmli (1970). Sample solutions (25 Lμ) of freeze-dried protein precipitate were dissolved in sample buffer (0.1% bromophenol blue, 0.5 M β-mercaptoethanol, 10% SDS, 2% glycerol and 0.5 M Tris-HCl), followed by heating for 10 min at 95°C. A 12% of the acrylamide separation gel was prepared. Electrophoresis was carried out for 1.5–2 hr at 15 mA/gel. The protein sub-units were subjected to stain with 0.1% Coomassie Brilliant Blue (R-250, 10% acetic acid, 20% methanol, and 70% water) and then washed with destaining solution (10% acetic acid, 20% methanol, and 70% water).

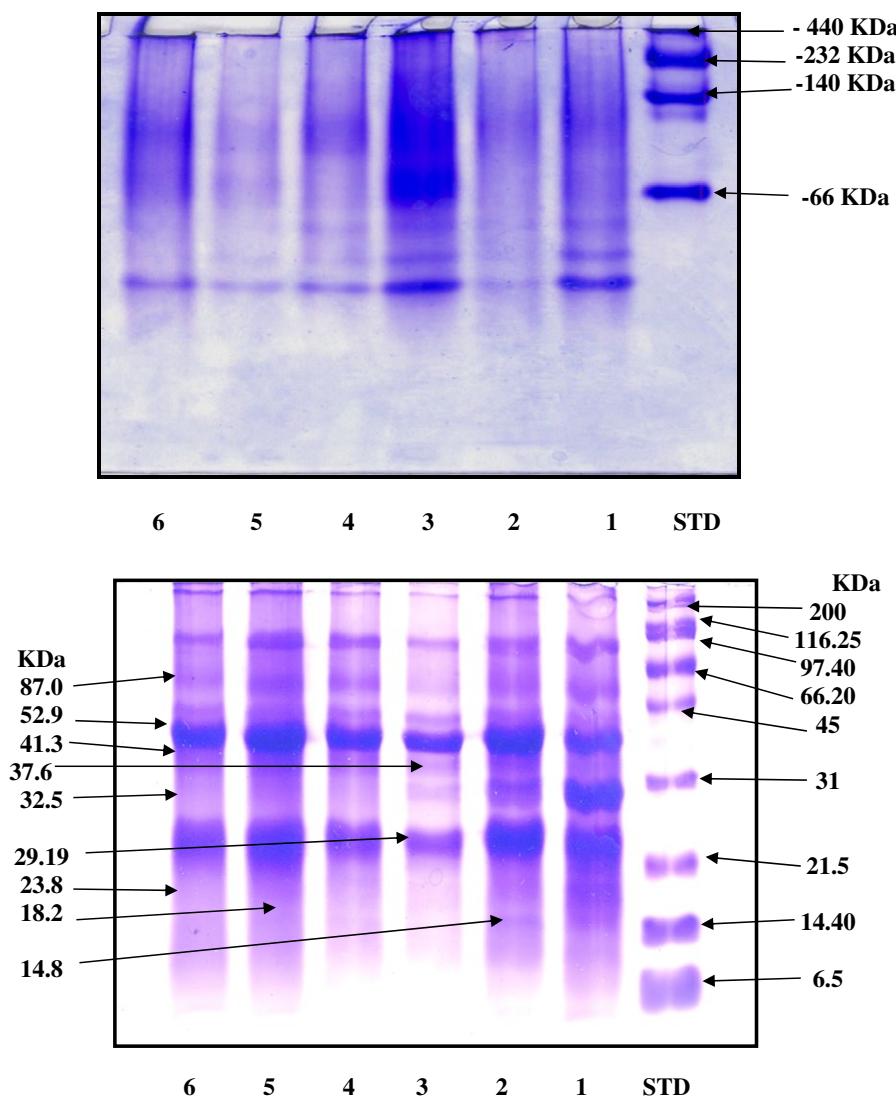


FIGURE 7 Native-PAGE and SDS-PAGE for F4 from protein isolate (I) of defatted (D) and full-fat (F) soybean (s); (STD) standard (1) Dsl; (2) Dsl after removal of free phenolic compounds; (3) Dsl after removal of free and bound phenolic compounds; (4) Fsl; (5) Fsl after removal of free phenolic compounds; (6) Fsl after removal of free and bound phenolic compounds

2.7 | Characterization and analysis of isolated proteins using liquid chromatography (LC)

2.7.1 | Reversed phase-high performance liquid chromatography

The 40 mg of protein isolate sample that was obtained as described above was solubilized in 1 ml of 0.1 trifluoroacetic acid in water and centrifuged for 10 min at 5,000 × g. The slurry was filtered using a 0.45 µm Millipore™ membrane and then injected into an HPLC. RP-HPLC analysis was performed as per the method described by El-Ramahi (2003) with a Beckman liquid chromatographic system (CA, USA) equipped with a programmable detector module (Model 166), a Beckman injector with a 100 µL loop and a solvent module system for high-pressure solvent delivery. The Gold System (version

V810) was used to analyze the chromatographic and spectral data. The data were translated into PRN format for Microsoft Excel manipulation.

For chromatographic separation, 100 µL samples were injected into a reversed phase column (C₁₈, 5 µm, 250 × 4.6 mm, J.T Baker Inc., USA). The elution flow rate of the samples was 1 ml/min with gradient two-buffer system including: solvent A; 30% of 0.1% TFA in water and 70% of acetonitrile; solvent B, water (0.1% TFA), initially for 10 min at 10% solvent A, and increasing linearly over 30 min from 10 to 90%. The starting settings over 10 min were then re-established. The eluted protein samples were monitored by calibrated UV detector at wavelength of 210 nm. Eluted fractions of samples from the reversed phase column were pooled, collected and concentrated, and then lyophilized for analysis by SDS-PAGE, Native-PAGE, and ESI-MS analysis. Each experiment was done in triplicate.

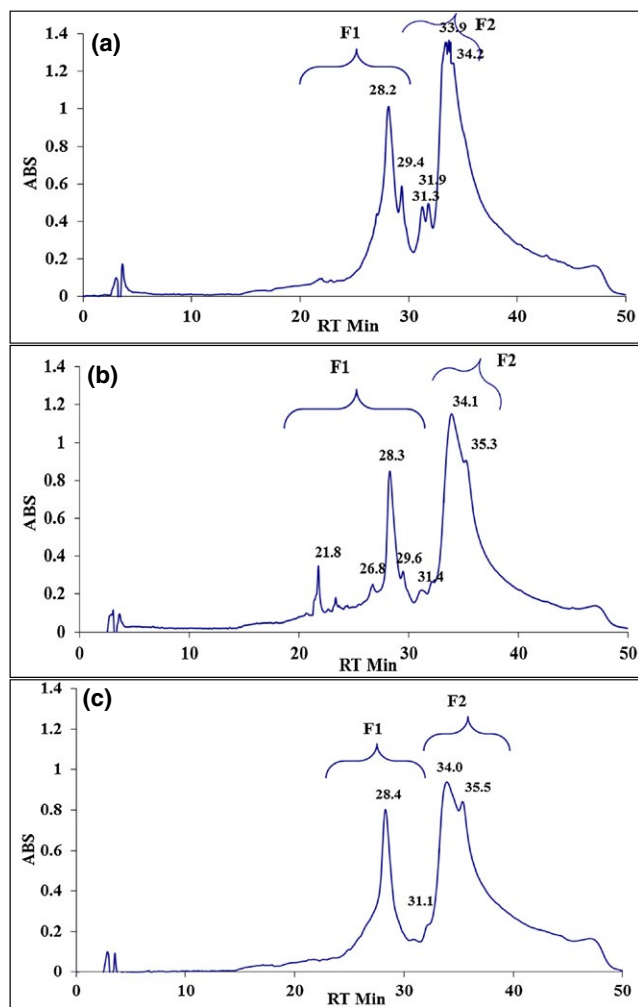


FIGURE 8 RP-HPLC separation for protein isolate (I) from full-fat (f) flaxseed (f); (a) FfI; (b) FfI after removal of free phenolic compounds; (c) FfI after removal of free and bound phenolic compounds

2.7.2 | Molecular analysis by electrospray ionization mass spectrometry (ESI-MS/MS)

To determine the effect of removal free and bound phenolics from isolated proteins of flaxseed and soybean on the molecular weight of fractionated proteins collected by RP-HPLC, samples were dissolved in 10% acetic acid. The samples were mixed extensively by vortex and then centrifuged for 10 min at $5,000 \times g$. The sample was subjected to filtration by $0.45 \mu\text{m}$ Millipore™ membrane and analyzed by ESI-MS using a ESI-MS hybrid mass spectrometer (Waters Micromass QTOF Ultima Global Micromass; Manchester, UK) equipped with a nanoflow electrospray source. The MS was operated at 3.80 kV in positive (+ESI) ionization mode with 80°C and 150°C source and de-solvation temperatures, respectively. The TOF was worked at collision energy, cone voltage, and acceleration voltage of 10 eV, 100 V, and 9.1 kV, respectively. The mass range (m/z) of MS survey was 400–1990, and scanned continuously over the chromatographic run. The mass spectrometer was calibrated and modified with

Glu-Fibrinopeptide B (Sigma Chemical Co., Louis, MO). Data analysis and instrument control was performed by MassLynx V4.0 software (Waters Corporation, 2005). Each experiment was done in triplicate.

3 | RESULTS AND DISCUSSION

3.1 | Polyacrylamide gel electrophoresis

Figures 1 and 2 show the Native-PAGE and SDS-PAGE of soybean and flaxseed protein isolates before and after removal of bound and free phenolic compounds. For the isolated proteins from defatted and full-fat of both soybean and flaxseed, Native-PAGE revealed that the circled major bands before removal of phenolic compounds were major bands as compared to the isolated protein after removal of free phenolic compounds (Figures 1 and 2). The latter effect became more pronounced after removal of both bound and free phenolic compounds. This latter finding could be the result of the basic and acidic hydrolysis used for removal of bound phenolic compounds. The results suggest that the removal of bound and free phenolic compounds reduces the molecular weight of proteins in soybean and flaxseed. SDS-PAGE (Figures 1 and 2) showed that removal of bound and free phenolic compounds from isolated proteins of defatted and full-fat soybean and flaxseed had little effect on the subunit structure of the proteins.

3.2 | RP-HPLC and PAGE

Figure 3a–c show the RP-HPLC chromatograms from isolated proteins for full-fat soybean before and after removal of bound and free phenolic compounds. The chromatograms for isolated proteins from full-fat soybean were similar to those from the defatted soybean (data not shown). The chromatograms showed four major fractions; F1 (retention time 20.0–25.0 min), F2 (retention time 25.0–29.0 min), F3 (retention time 29.0–32.5 min), and F4 (retention time 32.5–40.0 min). Different numbers of protein fractions of soy protein isolates have been reported to be separated by RP-HPLC. Mujoo, Trinh, and Ng (2003) showed that RP-HPLC of soybean protein isolates led to the separation of ten fractions. Proteins extracted from soybean protein isolates have also been reported to be separated by RP-HPLC into 17 fractions (Oomah, Voldeng, & Fregeau-Reid, 1994), 11 fractions (Wolf, Peterson, & Schaer, 1992), and 9 fractions (Castro-Rubio, Castro-Rubio, Garcia, & Marina, 2005). The present RP-HPLC results showed little effect of removal of bound and free phenolics on retention time and the polarity of protein isolates from soybean, which is likely due to the low content and molecular weight of the phenolics.

The 12% acrylamide separation gel of SDS-PAGE (Figure 4 and Table 1) showed the presence of subunits with an apparent molecular weight 18.9 kDa in F1 from the protein isolate of defatted soybean (lane 1). For the isolated protein from the full-fat soybean, the F1 fraction did not show the presence of the 18.9 kDa subunit (lane 4). The SDS-PAGE for F2 from the isolated proteins from defatted and full-fat soybean showed that the subunit with an apparent

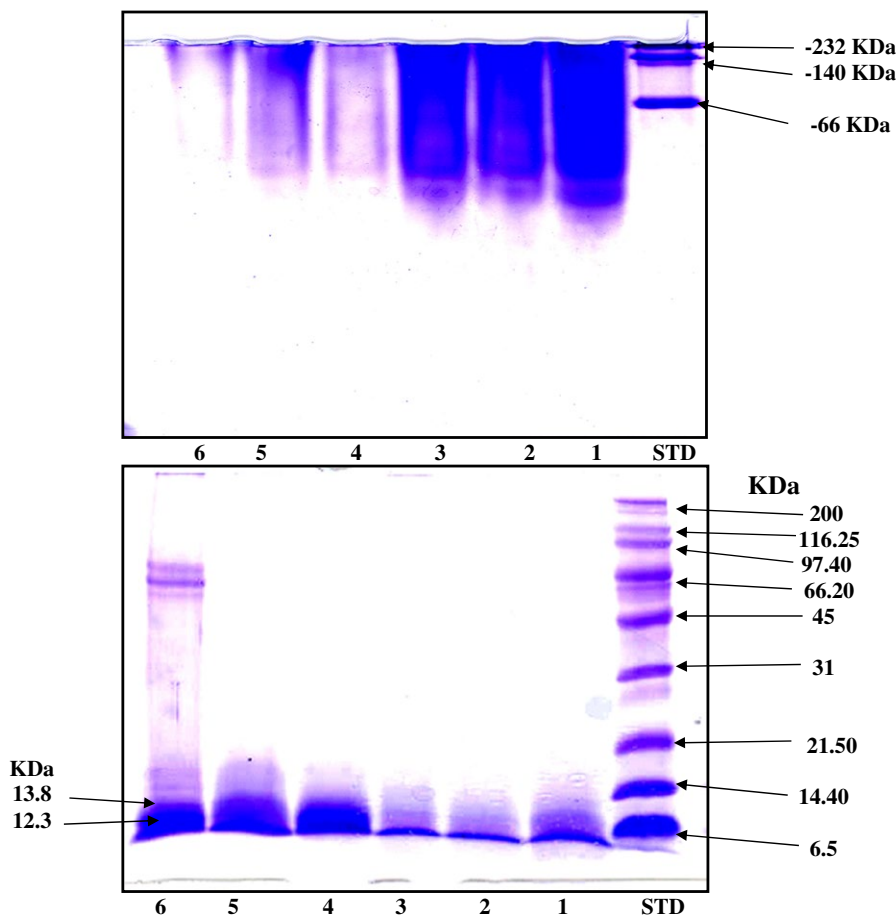


FIGURE 9 SDS and Native PAGE for F1 from protein isolate (I) of defatted (D) and full-fat (F) flaxseed (f); (1) DfI; (2) DfI after removal of free phenolic compounds; (3) DfI after removal of free and bound phenolic compounds; (4) FfI; (5) FfI after removal of free phenolic compounds; (6) FfI after removal of free and bound phenolic compounds

molecular weight 17.62 KDa (lanes 1 and 4); however, this subunit was not detected in the isolated proteins after removal of phenolic compounds (lanes 2, 3, 5, and 6) (Figure 5 and Table 1).

Figure 6 and Table 1 show SDS-PAGE for F3 of the isolated proteins from defatted and full-fat soybean before and after removal of free and bound phenolics. Eight major subunits were obtained for the protein isolate before and after removal of free phenolic compounds (lanes 1, 2, 4, and 5) with apparent molecular weights 41.9, 38.0, 35.7, 34.1, 29.1, 25.2, 24.3, and 22.4 KDa. For the isolated proteins from defatted and full-fat soybean after removal of free and bound phenolic compounds (lanes 3 and 6), only three major subunits were obtained with apparent molecular weights 41.9, 38.0, and 29.1 KDa, whereas subunits with the apparent molecular weights 35.7, 34.1, 25.2, 24.3, and 22.4 were absent after removal of free and bound phenolics (lanes 3 and 6).

Figure 7 and Table 1 show SDS-PAGE of F4 from the isolated proteins from defatted and full-fat soybean before and after removal of free and bound phenolic compounds. A total of nine major subunits were obtained for F4 from the protein isolate from defatted soybean (lane 1) with apparent molecular weights 87.0, 52.9, 41.3, 37.6, 32.5, 29.2, 23.8, 18.2, and 14.8 KDa. For F4 from the isolated protein from defatted soybean after removal of free and bound phenolic

compounds (lanes 2 or 3), eight major subunits were obtained with the absence of only the subunit with the apparent molecular weight 14.8 KDa. No differences were observed in the appearance of subunits of the F4 fraction from the isolated proteins from full-fat soybean after removal of free and bound phenolic compounds (lanes 4, 5, and 6) (Figure 7 and Table 1).

Figure 8 shows the RP-HPLC chromatograms for the isolated proteins from full-fat flaxseed. Chromatograms for isolated proteins from full-fat flaxseed were similar to those for the isolated proteins from defatted flaxseed. The chromatograms show two major fractions: F1 (retention time 20.0–30.0 min) and F2 (retention time 30.0–40.0 min). El-Ramahi (2003) previously reported the presence of three major fractions following RP-HPLC of the protein extracts from flaxseed.

Figures 9 and 10 show the SDS-PAGE and Native-PAGE of F1 and F2 for isolated proteins from defatted and full-fat flaxseed before and after removal of phenolic compounds. The identified subunits were: (a) two subunits for F1 with the apparent molecular weights of 13.3 and 9.5 KDa for isolated protein from defatted flaxseed and 13.8 and 12.3 KDa for isolated protein from full-fat flaxseed (Figure 9); and (b) six subunits for F2 with apparent molecular weights of 37.5, 29.2, 26.2, 24.5, 16.4, and 13.5 KDa for the protein

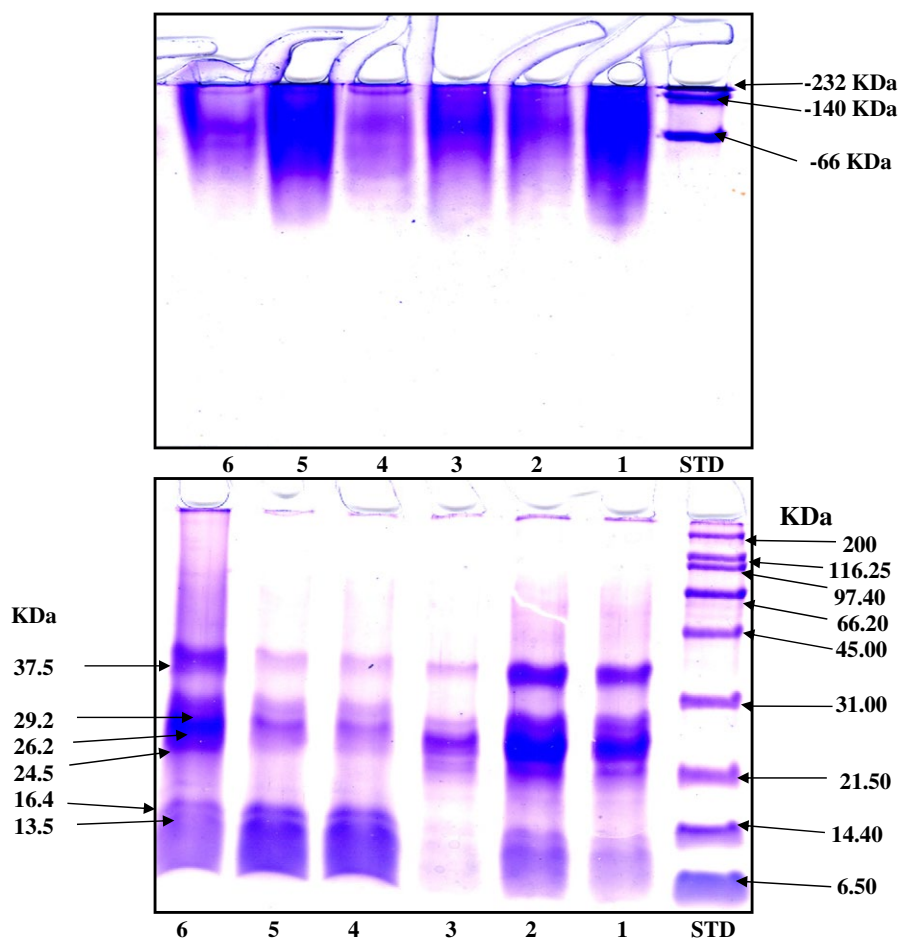


FIGURE 10 SDS and Native PAGE for F2 from protein isolate (I) of defatted (D) and full-fat (F) flaxseed (f); (1) Dfl; (2) Dfl after removal of free phenolic compounds; (3) Dfl after removal of free and bound phenolic compounds; (4) Ffl; (5) Ffl after removal of free phenolic compounds; (6) Ffl after removal of free and bound phenolic compounds

TABLE 2 Molecular weight MW (Da) of protein subunits before and after removal of free and bound phenolic compounds for fraction F4 from protein isolate (I) from defatted (D) and full-fat (f) soybean (s) from ESI-MS

	Observed MW before removal of phenolic compounds		Observed MW after removal of free phenolic compounds			Observed MW after removal of free and bound phenolic compounds	
	Dsl	Fsl	Literature values	Dsl	Fsl	Dsl	Fsl
A ₅	13,025	13,639	10,000–11,750 ^a	12,711	12,124	11,878	11,370
KTI	16,770	15,880	18,000 ^b	16,771	15,879	16,425	15,059
B _{1a}	19,956	20,205	22,000–24,000 ^c	18,628	19,493	17,892	18,577
B _{1b}	21,324	21,067	22,000–24,000 ^c	20,028	21,067	18,692	19,495
B ₂	21,360	22,278	22,000–24,000 ^c	20,206	22,277	18,728	21,068
B ₃	23,294	22,950	22,000–24,000 ^c	22,207	22,950	21,101	22,208
B ₄	23,530	24,540	22,000–24,000 ^c	22,359	24,539	21,212	22,952
A ₁	35,510	37,161	35,000–38,000 ^d	35,119	37,075	33,053	35,510
A ₂	36,647	37,535	35,000–38,000 ^d	35,510	37,532	34,326	37,077
A ₃	36,734	39,178	35,000–38,000 ^d	35,558	39,175	35,507	38,033
A ₄	37,742	39,799	35,000–38,000 ^d	36,906	39,796	35,623	39,621

Note. Results based on Supplementary material (Figures S1 and S2).

^aSathe, Lilley, Mason, and Weaver (1987) and Fontes, Pacheco, Moreira, Davies, and Nielsen (1984).

^bSathe, Mason, and Weaver (1989). ^cSathe et al. (1987), Moreira, Hermodson, Larkins, and Nielsen (1979) and Fontes et al. (1984). ^dSathe et al. (1987), Fontes et al. (1984), Moreira et al. (1979) and Utsumi and Kinsella (1985).

isolate from full-fat flaxseed and 36.4, 28.6, 23.6, 22.0, 13.6, and 10.5 KDa for the protein isolate from defatted flaxseed (Figure 10). Oomah and Mazza (1998) reported the presence of four subunits in flaxseed with apparent molecular weights of 14, 24, 25, and 34 KDa. Marcone, Kakuda, and Yada (1998a, 1998b) reported five major subunits with apparent molecular weights of 14.4, 24.6, 30, 35.2, and 50.9 KDa. Marcone, Kakuda, and Yada (1998a, 1998b) found that the protein fractions in flaxseed contain subunits with apparent molecular weights of 20–27 KDa and 30–39 KDa. Madhusudhan and Singh (1983) reported five major subunits in flaxseed with apparent molecular weights of 11.0, 18.0, 29.0, 42.0, and 61.0 KDa. El-Ramahi (2003) reported that flaxseed protein isolate fractionated by RP-HPLC into three subunits had apparent molecular weights of 10.3, 25.6, and 36 KDa. The removal of bound and free phenolic compounds showed little effect on the electrophoretic behavior as detected by Native-PAGE and SDS-PAGE of the flaxseed proteins and their protein subunits (Figures 9 and 10).

3.3 | Electrospray ionization mass spectrometry

The identification of protein subunits in each RP-HPLC fraction for isolated soybean and flaxseed proteins for all samples was based on SDS-PAGE, which was followed by RP-HPLC and LC-MS/MS analysis to identify the effect of removal of either free or both free and bound phenolic compounds on molecular weights of the protein subunits. Table 2 shows the obtained data from the mass spectra of Supplementary Materials (Figures S1 and S2) of fraction F4 for isolated proteins from defatted and full-fat soybean before removal of phenolics and after removal of free or both free and bound phenolic compounds. Before removal phenolics from defatted soybean protein isolate, mass spectra for the F4 fraction gave 11 subunits with molecular weight of 13,025, 16,700, 19,956, 21,324, 21,360, 23,294, 23,530, 35,510, 36,647, 36,734, and 37,742 Da. The obtained results revealed that the removal of free phenolic compounds

from the isolated proteins of defatted soybean resulted in a decrease in molecular weight for all of the protein subunits in fraction F4. Furthermore, removal of both free and bound phenolic compounds from isolated protein of defatted soybean showed lower molecular weights of all the protein subunits in fraction F4 with values of 11,878, 16,425, 17,892, 18,692, 18,728, 21,101, 21,212, 33,053, 34,326, 35,507, and 35,623 Da.

For the isolated protein from full fat soybean, the mass spectra revealed 11 subunits in fraction F4 with molecular weights of 13,639, 15,880, 20,205, 21,067, 22,278, 22,950, 24,540, 37,161, 37,535, 39,178, and 39,799 Da. In contrast, removal of free phenolics from the isolated proteins of full-fat soybean showed that the mass spectra for fraction F4 had similar molecular weights of the protein subunits in fraction F4 except for subunits of 13,639 and 20,205 Da that decreased to 11,370 and 18,577, respectively after removal of free phenolic compounds. Additionally, removal of both free and bound phenolics from isolated protein of full-fat soybean revealed lower molecular weights of all protein subunits in fraction F4 with values of 11,370, 15,059, 18,577, 19,495, 21,068, 22,208, 22,952, 35,510, 37,077, 38,033, and 39,621 Da (Table 2).

Table 3 shows the obtained data from mass spectra of Supplementary Materials (Figures S3 and S4) for fraction F2 from isolated proteins from defatted and full-fat flaxseed. The molecular weights of protein subunits in fraction F2 were 23,179, 26,745, 33,339, 38,437, and 40,807 Da for isolated proteins from defatted flaxseed and 15,605, 23,986, 26,613, 30,544, 37,502, and 39,074 Da for isolated proteins from full-fat flaxseed. Removal of free phenolics from isolated proteins of defatted flaxseed was associated with lower molecular weights of all the protein subunits in fraction F2. On the other hand, the molecular weights of protein subunits in the fraction F2 for isolated protein from full-fat flaxseed after removal of free phenolic compounds were unchanged.

For fraction F2, removal of both bound and free phenolic compounds gave molecular weight of 15,231, 22,540, 25,535, 30,500,

Observed MW before removal of phenolic compounds			Observed MW after removal of free phenolic compounds		Observed MW after removal of free and bound phenolic compounds	
Dfl	Ffl	Literature values	Dfl	Ffl	Dfl	Ffl
ND	15,605	14,000–15,000 ^a	ND	15,605	15,231	15,581
23,179	23,986	20,000–29,000 ^b	22,923	23,985	22,540	23,567
26,745	26,613	20,000–29,000 ^b	26,249	26,613	25,535	25,312
33,339	30,544	30,000–39,000 ^c	31,215	30,544	30,500	30,000
38,437	37,502	30,000–39,000 ^c	37,527	37,503	34,607	36,309
40,807	39,074	40,000–42,000 ^d	38,436	39,074	38,246	38,771

Note. ND: not detected; Results based on Supplementary material (Figures S3 and S4).

^aMarcone, Kakuda, and Yada (1998a, 1998b) and Oomah and Mazza (1998). ^bEl-Ramahi (2003), Madhusudhan and Singh (1983), Chung, Lei, and Li-Chan (2005), Marcone, Kakuda, and Yada (1998a, 1998b) and Oomah and Mazza (1998). ^cEl-Ramahi (2003), Chung et al. (2005), Marcone, Kakuda, and Yada (1998a, 1998b) and Oomah and Mazza (1998). ^dMadhusudhan and Singh (1983) and Chung et al. (2005).

TABLE 3 Molecular weight MW (Da) of protein subunits before and after removal of free and bound phenolic compounds for fraction F2 from protein isolate (I) from defatted (D) and full-fat (f) flaxseed (f) using ESI-MS

34,607, and 38,246 Da in the protein isolate from defatted flaxseed, and 15,581, 23,567, 25,312, 30,000, 36,309, and 38,771 Da in full-fat flaxseed protein isolate (Table 3). The removal of both bound and free phenolic compounds for both defatted and full-fat protein isolates showed a decrease in the molecular weight of all the protein subunits. The presence of phenolic-protein interactions in isolated proteins from flaxseed and soybean may affect the functional, nutritional and biological properties of these phenolic-protein complexes. Although these effects are known, the proposed mechanism of naturally phenolic-protein interactions in isolated proteins isolate is not well established. The removal of bound and free phenolics led to molecular changes of the proteins due to changes in protein conformation in terms of the secondary, tertiary, and quaternary structures of the proteins.

4 | CONCLUSIONS

An extensive investigation was carried out using Native-PAGE, SDS-PAGE, RP-HPLC, and ESI-MS to examine the effects of bound and free phenolic compounds on the molecular characteristics of isolated proteins from defatted and full-fat flaxseed and soybean. Although removal of bound phenolic compounds from the isolated proteins of soybean and flaxseed markedly affected protein migration assessed via Native-PAGE, the SDS-PAGE findings showed that the removal of bound and free phenolic compounds from isolated proteins of soybean and flaxseed had little effect on the subunit structure of the proteins. In terms of removal of free phenolic compounds from the isolated soy proteins, fraction F4 showed a decrease in molecular weight of all protein subunits in defatted soybean while a decrease in molecular weights of protein subunits of 13,639 and 20,205 Da to 11,370 and 18,577 was seen in fraction F4 of full-fat soybeans. The removal of both free and bound phenolic compounds from the isolated soy proteins in full-fat and defatted soybean led to all protein subunits in fraction F4 showing a decrease in molecular weight. Removal of free phenolics from isolated flaxseed proteins was associated with lower molecular weights of all the protein subunits in fraction F2 for defatted flaxseed but no effect on the molecular weight of all subunits in fraction F2 for full-fat flaxseed. The removal of both bound and free phenolic compounds for both defatted and full-fat protein isolates showed a decrease in the molecular weight of all the protein subunits. Overall, the removal of bound and free phenolic compounds showed little effect on the electrophoretic behavior and molecular characteristics of the soybean and flaxseed proteins.

ACKNOWLEDGMENTS

The authors would like to acknowledge McGill University and Jordan University of Science and Technology for funding.

CONFLICTS OF INTEREST

The authors declared that they have no conflict of interest.

ORCID

Muhammad H. Alu'datt  <https://orcid.org/0000-0002-8976-5004>

REFERENCES

- Alu'datt, M. H., Rababah, T., Alhamad, M. N., Al-Mahasneh, M., Almajwal, A., Gammoh, S., ... Alli, I. (2017). A review of phenolic compounds in oil-bearing plants: Distribution, identification and occurrence of phenolic compounds. *Food Chemistry*, 218, 99–106. <https://doi.org/10.1016/j.foodchem.2016.09.057>
- Alu'datt, M. H., Rababah, T., Alhamad, M. N., Al-Rabadi, G. J., Tranchant, C. C., Almajwal, A., ... Alli, I. (2019). Occurrence, types, properties interactions of phenolic compounds with other food constituents in oil-bearing plants. *Critical Review in Food Science and Nutrition*, 58, 3209–3218. <https://doi.org/10.1080/10408398.2017.1391169>
- Alu'datt, M. H., Rababah, T., & Alli, A. (2014). Effect of phenolic compound removal on rheological, thermal and physico-chemical properties of soybean and flaxseed proteins. *Food Chemistry*, 146, 608–613. <https://doi.org/10.1016/j.foodchem.2013.09.104>
- Alu'datt, M. H., Rababah, T., Ereifej, K., Brewer, S., & Alli, A. (2013). Phenolic-protein interactions in oilseed protein isolates. *Food Research International*, 52, 178–184. <https://doi.org/10.1016/j.foodres.2013.03.010>
- AOAC. (1990). *Official methods of analysis* (15th ed.). Washington, DC: Author.
- Beauchamp, G. K., & Maller, O. (1977). The development of flavor preferences in humans. A review. In M. K. Kare & O. Mailer (Eds.), *The chemical senses and nutrition* (pp. 291–331). New York: Academic Press.
- Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Review*, 56, 317–333.
- Castro-Rubio, A., Castro-Rubio, F., Garcia, M. C., & Marina, M. L. (2005). Development of a perfusion reversed-phase HPLC method for the separation of soybean and cereal (wheat, corn, and rice) proteins in binary mixtures. Application to the detection of soybean proteins in commercial bakery products. *Journal of Separation Science*, 28, 996–1004.
- Chung, M. W. Y., Lei, B., & Li-Chan, E. C. Y. (2005). Isolation and structural characterization of the major protein fraction from NorMan flaxseed (*Linum usitatissimum* L.). *Food Chemistry*, 90, 271–279. <https://doi.org/10.1016/j.foodchem.2003.07.038>
- Davis, B. J. (1964). Disc electrophoresis-II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences*, 121, 404–427.
- Dev, D. K., Quensel, E., & Hansen, R. (1986). Nitrogen extractability and buffer capacity of defatted linseed (*Linum usitatissimum* L.) flour. *Journal of the Science of Food and Agriculture*, 37, 199–205. <https://doi.org/10.1016/j.ympev.2016.07.013>
- Eliasson, C., Kamal-Eldin, A., Andersson, R., & Aman, P. (2003). High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. *Journal of Chromatography*, 1012, 151–159. [https://doi.org/10.1016/S0021-9673\(03\)01136-1](https://doi.org/10.1016/S0021-9673(03)01136-1)
- El-Ramahi, R. (2003). *Isolation and characterization of proteins from defatted flaxseed* (M.Sc. thesis). McGill University, Montreal, Canada.
- Escarpa, A., & Gonzalez, M. (2001). Total extractable phenolic chromatographic index: An overview of the phenolic class contents from different sources of foods. *European Food Research and Technology*, 212, 439–444.
- Fontes, E., Pacheco, B., Moreira, M. A., Davies, C. S., & Nielsen, N. C. (1984). Urea-elicited changes in relative electrophoretic mobility of certain glycinin and beta-conglycinin subunits. *Plant Physiology*, 76, 840–842. <https://doi.org/10.1104/pp.76.3.840>

- Hagerman, A. E., & Butler, L. G. (1978). Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry*, 26, 809–812.
- Johnsson, P., Kamal-Eldin, A., Lundgren, L. N., & Aaman, P. (2000). HPLC method for analysis of secoisolariciresinol diglucoside SDG in flaxseeds. *Journal of Agricultural and Food Chemistry*, 48, 5216–5219.
- Krase, J., Schultz, M., & Dudek, S. (2002). Effect of extraction conditions on composition, surface activity and rheological properties of protein isolates from flaxseed (*Linum usitatissimum* L.). *Journal of the Science of Food and Agriculture*, 82, 970–976.
- Krygier, K., Sosulski, F., & Hoggie, I. (1982). Free, esterified, and insoluble-bound phenolic acids. Composition of phenolic acids in rapeseed flour and hulls. *Journal of Agricultural and Food Chemistry*, 30, 334–336.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685. <https://doi.org/10.1007/s00300-014-1623-6>
- Lipworth, L., Martinez, M. E., Angell, J., Hsieh, C. C., & Trichopoulos, D. (1997). Olive oil and human cancer: An assessment of the evidence. *Preventive Medicine*, 26, 181–190. <https://doi.org/10.1006/pmed.1996.9977>
- Loomis, W. D., & Battaile, J. (1966). Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry*, 5, 423–438.
- Madhusudhan, K. T., & Singh, N. (1983). Studies on linseed proteins. *Journal of Agricultural and Food Chemistry*, 31, 959–963. <https://doi.org/10.1021/jf00119a010>
- Marcone, M. F., Kakuda, Y., & Yada, R. Y. (1998a). Salt-soluble seed globulins of various dicotyledonous and monocotyledonous plants-I. Isolation/purification and Characterization. *Food Chemistry*, 62, 27–47.
- Marcone, M. F., Kakuda, Y., & Yada, R. Y. (1998b). Salt-soluble seed globulins of dicotyledonous and monocotyledonous plants-II. Structural Characterization. *Food Chemistry*, 63, 265–274.
- Mason, H. S. (1955). Reactions between quinines and proteins. *Nature*, 175, 771–772.
- Moreira, M., Hermodson, M. A., Larkins, B. A., & Nielsen, N. C. (1979). Partial characterization of the acidic and basic polypeptides of glycinin. *Journal of Biological Chemistry*, 19, 9921–9926.
- Mujoo, R., Trinh, D. T., & Ng, P. K. W. (2003). Characterization of storage proteins in different soybean varieties and their relationship to tofu yield and texture. *Food Chemistry*, 82, 265–273.
- Oomah, B. D., & Mazza, G. (1998). Flaxseed products for disease prevention. Chapter 4 in G. Mazza (Ed.), *Functional foods: Biochemical and processing aspects* (pp. 91–138). Lancaster, PA: Technomic Pub Co Inc.
- Oomah, B. D., Voldeng, H., & Fregeau-Reid, J. A. (1994). Characterization of soybean proteins by HPLC. *Plant Foods for Human Nutrition*, 45, 251–263. <https://doi.org/10.1007/BF01094094>
- Ratty, A., & Das, N. (1988). Effects of flavonoids on nonenzymic lipid peroxidation: Structure-activity relationship. *Biochemical Medicine and Metabolic Biology*, 39, 69–79.
- Rubino, M. I., Arntfield, S. D., Nadon, C. A., & Bernatsky, A. (1996). Phenolic protein interactions in relation to the gelation properties of canola protein. *Food Research International*, 29, 653–659. <https://doi.org/10.1073/pnas.1115169109>
- Sabally, K. (2006). *Lipase-catalyzed synthesis of selected phenolic lipid organic solvent media* (PhD thesis). McGill University, Montreal, Canada.
- Sathe, S. K., Lilley, G. G., Mason, A. C., & Weaver, C. M. (1987). High-resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis of soybean (*Glycine max* L.) seed proteins. *Cereal Chemistry*, 64, 380–384.
- Sathe, S. K., Mason, A. C., & Weaver, C. M. (1989). Thermal aggregation of soybean (*Glycine max* L.) sulfur-rich protein. *Journal of Food Science*, 54, 319–323. <https://doi.org/10.1186/s12862-015-0362-9>
- Toschi, G., Bordoni, A., Hrelia, S., Bendini, A., Lercker, G., & Biagi, P. L. (2000). The protective role of different green tea extracts after oxidative damage is related to their catechin composition. *Journal of Agricultural and Food Chemistry*, 48, 3973–3988.
- Utsumi, S., & Kinsella, J. E. (1985). Structure-function relationships in food proteins: Subunit interactions in heat-induced gelation of 7S, 11S, and soy isolate proteins. *Journal of Agricultural and Food Chemistry*, 33, 297–303.
- Wolf, W. J., Peterson, R. E., & Schaer, M. L. (1992). Preparative reversed-phase high-performance liquid chromatography of soybean proteins and characterization of fractions by gel electrophoresis. *Journal of Agricultural and Food Chemistry*, 40, 1809–1816. <https://doi.org/10.1021/jf00022a016>

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Alu'datt MH, Rababah T, Kubow S, Alli I. Molecular changes of phenolic-protein interactions in isolated proteins from flaxseed and soybean using Native-PAGE, SDS-PAGE, RP-HPLC, and ESI-MS analysis. *J Food Biochem*. 2019;e12849. <https://doi.org/10.1111/jfbc.12849>