

Effect of removal of phenolic compounds on structural and thermal properties of sunflower protein isolate

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Abstract The present study evaluated the effect of removal of polyphenols on the structural properties of protein isolates extracted from sunflower seed and kernel. The structural and thermal changes in protein upon phenolic interaction were studied using circular dichroism, differential scanning calorimetry, thermal gravimetric analysis, X-ray diffraction, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and Fourier Transform Infrared (FT-IR) spectroscopy. Presence of phenolic compounds in proteins decreased the ordered structure content with parallel increase in unordered structure content. Denaturation temperature was higher for protein isolates with phenolic compounds while, enthalpy decreased upon phenolic interaction. In the presence of phenolic compounds, higher mass loss was observed upon heating. Crystallinity and crystal size got increased after removal of phenolic compounds. Protein isolates from kernels had higher percentage of crystallinity and crystal size as compared to seed protein isolates. Higher molecular weights were observed for protein isolates with phenolic compounds. Presence of polyphenols reduced the hydrophobicity as well the sulfhydryl content and increased the particle size of proteins.

Keywords Sunflower · Protein isolate · Polyphenols · Structure · Thermal

Introduction

Sunflower is cultivated worldwide for extraction of oil. After oil extraction, the meal is generated as a by-product which is a rich source of protein. The protein content of sunflower seed is about 20 % whereas protein content of sunflower meal ranges from 30 to 50 % (Dorrell and Vick 1997). This high protein content of sunflower meal makes it an attractive source of proteins. The utilization of this meal as an unconventional source of protein for human consumption could be an alternative approach, which will result in complete use of this by-product. These proteins are low in anti-nutritional compounds and are devoid of toxic substances, which make it a valuable alternative food ingredient (González-Pérez and Vereijken 2007). The amino acid composition of sunflower proteins complies largely with the FAO (Food and Agriculture Organization) pattern with the exception of lysine (Gassmann 1983).

Currently sunflower meal is used as an animal feed in spite of having high protein content and structural similarities with the soy protein. The presence of phenolic compounds (1–4 %) mainly chlorogenic acid is the main reason for the underutilization of sunflower meal. The presence of these phenolic compounds significantly affects the quality of sunflower proteins e.g., digestibility is reduced, undesirable browning occurs, organoleptic properties are altered and structure is modified which have an adverse effect on functional properties of the proteins and their behaviour in various food systems (González-Pérez and Vereijken 2007). Phenol oxidase and alkaline conditions during the conventional protein extraction, results in

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rapid oxidation of phenolic compounds and their concomitant covalent binding to the protein side chains (Pawar et al. 2001). As a consequence of these reactions, nutritive value of sunflower protein is lowered. Thus, the removal of phenolic compounds from sunflower meal is important to enable its use in food applications to a greater extent and offer an alternative to more expensive protein sources like soy protein.

Various researchers have revealed the structural changes in proteins upon phenolic interaction (Ali et al. 2013; Rawel et al. 2002b; Jia et al. 2016). Modification of protein structure upon phenolic interaction has direct implication on the behaviour of protein during processing, preparation, consumption and storage in food systems. Prigent et al. (2003) reported that molecular weight of α -lactalbumin and lysozyme was increased by 680–690 Da after incubation with chlorogenic acid at pH 7.0. The interaction of phenolic compounds with proteins induces the cross linking of proteins and subsequently net charge in the protein molecule is altered. As the consequence of these reactions, the secondary and tertiary structure gets altered. Hasni et al. (2011) revealed that upon interaction of polyphenols with casein, there were conformational changes in casein with reduction in α -helix and β -sheet and increase in random coils indicating a destructive effect of polyphenols. Structural changes in protein upon phenolic interaction also results in reduction of surface hydrophobicity (Rawel et al. 2002a). The effect of naturally present polyphenols in sunflower meal on structural and thermal properties of protein isolates extracted during alkaline conditions has not been explored yet both at national and international level. Thus, the aim of this study was to evaluate the effect of polyphenol removal from sunflower meal on structural and thermal properties of protein isolates. A positive outcome of this study would provide the proteins with better structural and thermal properties, which would meet the industrial demands.

Materials and methods

Materials

PSH 996 variety of sunflower seed was collected from Punjab Agricultural University Ludhiana, Punjab, India. All the chemicals used were of analytical grade.

Methods

Meal preparation

Meal obtained after oil extraction from both seed and kernel was subjected to defatting for 8 h by soxhlet

extraction using hexane as solvent. Defatted meal was then dried overnight at 45 °C in a hot air oven to remove the residual solvent. Part of defatted meal of both seed and kernel was dephenolized with 60 % methanol at 1:10 ratio. Both defatting and dephenolizing were carried out using procedure of González-Pérez et al. (2002). Four portions of meals were obtained i.e. two from each seed and kernel. The four portions of meals obtained were denoted as seed meal with polyphenol (SMWP), seed meal free of polyphenols (SMFP), kernel meal with polyphenols (KMWP) and kernel meal free of polyphenols (KMFP).

Isolation of proteins

Proteins were isolated from four portions of sunflower meal using the method of Hagenmaier (1974) modified by Song et al. (2013) with slight modification. Meal was mixed with distilled water (1:10); pH of mixture was adjusted to 10.5 and stirred for 1 h at 45 °C. Then mixture was centrifuged at 10,000 g for 25 min at room temperature, and supernatant was collected and sediment was extracted again. The supernatant collected from two extractions was adjusted to pH 4.5 using 2 N HCL and allowed to stand for 30 min for complete precipitation of proteins. The mixture was centrifuged (10,000 g for 25 min at room temperature), and precipitated proteins were collected, washed with distilled water and subsequently neutralized and freeze dried. The four protein isolates obtained from respective meals were named as sunflower protein isolate with polyphenols (SPIWP), seed protein isolate free of polyphenols (SPIFP), kernel protein isolate with polyphenols (KPIWP) and kernel protein isolate free of polyphenols (KPIFP).

Circular dichroism spectroscopy

Circular dichroism spectroscopy of sunflower protein isolates were carried out using a Jasco J-815 Spectropolarimeter (Jasco Corporation, Japan) in the range of 190–260 nm using the method adopted by Chandrapala et al. (2011). The protein solutions for measurement were prepared at final concentration of 0.5 mg/mL in 100 mM phosphate buffer at pH 7.0. The conditions for the measurement were 25 °C temperature, 0.1 cm path length quartz cell, 10 nm/min scan rate and 0.2 nm band width. Three scans were taken and spectra were taken average of these three scans. 100 mM phosphate buffer was used as a blank and correct spectrum of each sample was obtained by subtracting the spectrum of blank from the average three spectrum of each sample. The percentage of α -helix, β -sheet, turn and unordered structures in the protein mixtures were calculated with the CDPro analysis software and the SELCON3 program which are part of the Jasco software.

Differential scanning calorimetry

Thermal properties of sunflower protein isolates were determined using Differential Scanning Calorimetry (DSC Q100 V9.8 Build 296 New Castle, Del., USA) following the method adopted by Salgado et al. (2011). Hermetically sealed aluminium pans containing 10–15 mg sunflower protein solution (20 % w/v) were scanned over a range of 20–120 °C at a rate of 10 °C/min. Peak temperature (T_d) and denaturation enthalpy (ΔH) was taken from the corresponding thermograms (Universal Analysis V4.2E, TA Instruments, New Castle, DE, USA).

Thermal gravimetric analysis

The thermal gravimetric analysis of sunflower protein isolates were carried out using EXSTAR 6300 TGA (Japan) according to the procedure of Yu et al. (2015) and thermogram was obtained in the temperature range 30–600 °C. The heating rate was 10 °C/min and nitrogen was used as a cooling gas at the rate of 20 mL/min.

X-Ray diffraction

X-ray diffraction (XRD) pattern of the sunflower protein isolate powders was measured using Panalytical- X'pert PRO MRD (Almelo, Netherlands) X-ray diffractometer with $\text{CoK}\alpha < 1$ radiation. Diffractograms were taken between 0° and 40° (2 θ) at a rate of 1.20°/min (2 θ) and with a step size of 0.05° (2 θ). The data collected by X'pert data collector was processed using X'pert HighScore software for crystallinity measurement.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS-PAGE of sunflower protein isolates were performed using EPS301 electrophoresis apparatus (GE Healthcare Laboratories, Richmond, CA, USA) and Mini VE electrophoresis tank (Amersham Biosciences Company, USA) using method of Laemmli (1970). 12 % polyacrylamide was used as resolving gel and 5 % acrylamide as stacking gel. Samples were prepared by mixing 10 mg of protein isolates with 100 μL of sample buffer (0.0625 M Tris–HCl, 10 % glycerin, 2 % SDS, and 5 % 2- β -mercaptoethanol, 0.0025 % bromophenol blue). The samples were then immersed in a boiling water bath for about 5 min to denature all the proteins. Then samples were cooled immediately using ice. An aliquot of 10 μL of sample were loaded into the wells and initially run at 80 V. The voltage was increased to 120 V when samples were within the resolving gel and was allowed to run until tracking dye reached the bottom of the gel. The gel was removed and

stained using Coomassie Brilliant Blue R-250 stain solution (GE Healthcare Laboratories) for about 2 h. After staining, the gel was destained using 20 % methanol and 10 % acetic acid solution, until a clear background was achieved. 3B prestained protein ladder was used as a standard consisting of proteins in the range of 3.5–245 kDa, to determine the molecular weight of each band. AlphaEase FC gel imaging system (Alpha Inc., USA) was used to scan the SDS-PAGE gel.

Fourier transform infrared (FT-IR) spectroscopy

Infrared spectra of all sunflower protein isolates were recorded using NXR FT-IR module (Thermo Electron Corporation, USA) followed the procedure adopted by Kumar et al. (2014). Moisture free protein isolates were mixed with potassium bromide in a ratio of 1:100 (w/w). The mixtures were pressed by vacuum hydraulic to obtain pellet. The FT-IR spectrum of pellets was recorded in the 4000–400 cm^{-1} region at room temperature.

Surface hydrophobicity

Surface hydrophobicity (H_0) of protein dispersions was determined according to the method of Hayakawa and Nakai (1985) using the fluorescence probe 1-anilino-8-naphthalenesulfonate (ANS). ANS is much utilized hydrophobic probe for determining the surface hydrophobicity of proteins, due to its affinity to bind to the hydrophobic amino acids containing an aromatic ring such as tryptophan and phenylalanine (Benjakul et al. 1997). Protein isolate dispersions were diluted with phosphate buffer (0.1 M, pH 7) to a concentration range of 0.05–0.4 mg mL^{-1} . 4 mL of diluted protein solution was mixed with 20 μL of ANS (8.0 mM in phosphate buffer 0.01 M, pH 7) solution, vortexed and kept in the dark for 15 min. Fluorescence spectrometer (RF-5301pc Shimadzo) at 390 nm (excitation wavelength, slit 2.5 nm) and 468 nm (emission wavelength, slit 2.5 nm), with a scanning speed of 5 nm s^{-1} was used for relative fluorescence intensity (RFI) measurement. The relative fluorescence intensity (RFI) of both buffer blank and each protein solution (from lowest to highest concentration) was measured. The net RFI was obtained by subtracting RFI of each dilution blank from the corresponding protein solution with ANS. The initial slope of the plot of standardised net RFI values versus % protein concentration was expressed as surface hydrophobicity.

Thiol group determination

Ellman's reagent (5, 5-dithiobis (2-nitrobenzoic acid), DTNB) (Sigma Chemical Co., St. Louis, MO, USA), was used to determine the concentration of surface and total free sulfhydryl (SH) groups of sunflower protein isolate

solutions according to the Ellman's procedure (1959) with the modifications of Shimada and Cheftel (1988). Sulfhydryl groups of proteins reacts with 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and produces measurable yellow coloured compound namely 2-nitro-5-thiobenzoic acid (TNB). For free sulfhydryl group determination, protein solutions (0.2 %) were prepared in pH 8.0 standard buffer (0.086 M Tris 0.09 M glycine 4 mM Na₂EDTA) and were incubated at 25 °C for 24 h in a shaking water bath. The mixture was then centrifuged at 12,857 g for 20 min, at 20 °C. The supernatants were used for determination. 0.03 mL of Ellman's reagent solution (4 mg DTNB/mL buffer) was added to 3 mL aliquot of supernatant and was rapidly mixed and allowed to stand for 15 min. The solution was then read at 412 nm on a UV-Vis spectrophotometer (Coleparmer S2100, Germany). The same technique was used for the determination of total SH groups content, but denaturing buffer was used in place of standard buffer. Denaturing buffer consists of the standard buffer plus 8 M urea and 0.5 % w/v sodium dodecyl sulphate (SDS). Buffer was prepared for both free and total thiol determination by mixing 3 mL of respective buffer with 0.03 mL of Ellman's reagent. A molar extinction coefficient of $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate micromoles of SH/g of protein.

Particle size analysis

Laser light diffraction particle size analyzer (Shimadzu SALD-2300, M/s Shimadzu Corporation, Kyoto, Japan) was used to determine the particle size distribution of sunflower protein isolates. The protein dispersion was added to cuvette drop wise till the refractive index reached between 20 and 40 %. The percentage number of particles with different sizes was evaluated and particle diameter of 50 % of normalized particle amount was noted as average particle diameter.

Statistical analysis

A one-way analysis of variance test (ANOVA) was carried out using the software Statistica 7.0 to examine the effect of polyphenols on denaturation temperature, enthalpy, sulfhydryl content, surface hydrophobicity and particle size of protein isolates. Significant differences between means were determined by Duncan's Test at the 5 % significance level. Data reported is mean of triplicate observation.

Results and discussion

Circular dichroism (CD) spectroscopy

The secondary structure of sunflower protein isolate dispersions was determined using CD spectroscopy and the

proportions of α -helix, β -sheet, β -turn and random coil are shown in Table 1. Circularly polarized light have two polarized light beams, right and left, which differ in speed and wavelength as well as the extent to which they are absorbed in an optically active medium. Two polarized light differs in their absorption pattern and CD spectrum is obtained by measuring this wavelength dependent difference in absorption. Depending on whether left circularly polarized light is absorbed more than right or vice versa, the circular dichroism signal can be positive or negative (Chandrapala et al. 2011).

CD spectroscopy is the valuable method to study the structure of proteins and examining the extent of structural changes upon interaction with phenolic compounds. In this study interaction of polyphenols with sunflower proteins showed considerable changes in the secondary structure of proteins. Protein isolates (SPIWP and KPIWP) with phenolic compounds were found possessing higher unordered structure and lesser structured elements. It was observed that phenolic interaction induced the conformational changes in protein molecules which resulted in loss of structure elements. Since secondary structure of protein depends not only on the sequence of amino acids but also on the interaction between different parts of the molecule, and change in secondary structure is due to the disruption of these interactions by phenolic compounds. Upon interaction with phenolic compounds, the protein become more unordered which is accompanied by the corresponding loss of structured elements (α -helix and β -strand) and an increase in random coils. Similar results were reported by Hasni et al. (2011) on casein protein and it was revealed that conformation was changed by phenolic compound with major reduction of α -helix and β -sheet and increase of random coil. Ali et al. (2013) used coffee specific phenolic to modify the whey proteins. They reported that there was a reduction in structured elements and increase in unordered structure upon incubation of protein with coffee specific phenolic compounds. Rawel et al. (2002b) reported that upon interaction of chlorogenic acid with bovine serum albumin (BSA), there was a reduction in α helix with a parallel increase in remaining structures, indicating destructing effect on BSA. Contrary to these results Kanakis et al. (2011) reported different results for β lactoglobulin upon interaction with polyphenols. They reported that in the presence of polyphenols, conformation of β lactoglobulin was changed with an increase in β -sheet and α -helix, suggesting stronger structural stabilization of proteins. The contradictory results may be due to the different samples been analysed.

Differential scanning calorimetry

All the four protein isolates showed a single endothermic peak at around 101 °C, which is related to the denaturation temperature (T_d). Single endotherm peaks were also

Table 1 Secondary structure, denaturation temperature (T_d), enthalpy (ΔH), sulfhydryl content (free and total) and surface hydrophobicity of sunflower protein isolates

Type of protein isolates	α -Helix (%)	β -Sheet (%)	β -Turn (%)	Random coils (%)	T_d ($^{\circ}\text{C}$)	ΔH (J/g)	Free SH ($\mu\text{mol/g}$)	Total SH ($\mu\text{mol/g}$)	Surface hydrophobicity (H_o)
SPIWP	01.3	30.0	23.1	45.6	102.1 \pm 0.1 ^b	1.3 \pm 0.1 ^c	4.8 \pm 0.1 ^d	36.6 \pm 0.3 ^d	108.7 \pm 1.3 ^d
SPIFP	05.5	49.6	17.0	27.9	100.4 \pm 0.1 ^d	1.8 \pm 0.1 ^b	6.9 \pm 0.1 ^c	44.6 \pm 0.3 ^c	114.7 \pm 1.2 ^c
KPIWP	04.6	24.4	28.1	42.9	102.9 \pm 0.2 ^a	1.9 \pm 0.1 ^b	7.1 \pm 0.1 ^b	59.3 \pm 0.2 ^b	119.8 \pm 1.0 ^b
KPIFP	10.6	47.6	16.8	25.0	101.4 \pm 0.1 ^c	3.1 \pm 0.1 ^a	7.6 \pm 0.1 ^a	78.8 \pm 0.2 ^a	129.5 \pm 1.2 ^a

Values are mean \pm SD. Means with similar superscript in a column did not differ significantly ($P > 0.05$)

SPIWP seed protein isolate with polyphenol, SPIFP seed protein isolate free of polyphenols, KPIWP kernel protein isolate with polyphenol, KPIFP kernel protein isolate free of polyphenols, T_d denaturation temperature, ΔH enthalpy, SH sulfhydryl

reported for kidney bean and field pea protein isolates at 94.7 and 85.5 $^{\circ}\text{C}$ respectively (Shevkani and Singh 2015). T_d is measurement of thermal stability of protein, at which transition occurs. Denaturation is endothermic process as it results in dissociation of intra-molecular bonds (non-covalent and in some cases, covalent (disulphide) bonds). In the present study endothermic reaction dominates within the temperature range studied, even though both exothermic and endothermic reactions would be expected under these conditions. T_d and ΔH are two important parameters measured by DSC, which are providing information about thermal properties of proteins. Thermal stability is reflected by T_d and varied from 100.4 to 102.9 $^{\circ}\text{C}$ for all sunflower protein isolates (Table 1). Proteins isolates with polyphenols showed higher T_d , compared with the protein isolates free of polyphenols. The increase in thermal stability with the interaction of polyphenols was also reported by Prigent et al. (2003) upon interaction of chlorogenic acid with bovine serum albumin (BSA), α -lactalbumin and lysozyme. They found that denaturation temperature of BSA and α -lactalbumin was increased by 5 and 1 $^{\circ}\text{C}$ respectively, while that of lysozyme it decreased by less than 1 $^{\circ}\text{C}$. Enthalpy values decreased upon phenolic interaction and could be correlated with decrease in content of ordered structure and decrease in the proportion of undenatured proteins. Protein isolates with higher percentage of proteins (KPIFP and KPIWP) showed higher values of enthalpy than the protein isolates with lower protein percentage. A similar observation was reported by Murray et al. (1985) on fababean, field pea and canalo preparation, they reported that ΔH values increased as the phenolic level decreased and protein level increased. Higher ΔH values reflect the more ordered structure as was evident in the XRD and CD results. Similar results were reported by Ali et al. (2013) on whey protein upon modification with coffee specific phenolics.

Thermal gravimetric analysis

Thermal gravimetric analysis is used to study the changes in a material sample during temperature rise. These changes include evaporation of water, loss of crystalliferous water, release of low molecular volatile matter and oxidative decomposition of material (Yao et al. 2008). All the protein isolates studied showed similar TGA curves (Fig. 1). TGA curves showed that the degradation of sunflower protein take place in two steps. In the first step from 24 to 225 $^{\circ}\text{C}$, the weight loss was minimal and was attributed mainly to the evaporation of water. The second step from 225 to 425 $^{\circ}\text{C}$ was observed with maximum weight loss. The second weight loss may be due to the thermal decomposition of proteins and subsequent volatilization with loss of maximum weight at the melting point. The weight loss of proteins occurred with further increase in temperature and is mainly attributed to the degradation of major protein components. Kernel protein isolates (KPIFP and KPIWP) showed higher loss in weight compared to that of seed protein isolates (SPIFP and SPIWP) in both first and second phase. The presence of higher ash content in seed protein isolates can be attributed to less loss of weight, because mineral content of protein do not degrade during heating. The weight loss in protein isolates with phenolic compounds was higher than protein isolates free of polyphenols. The weight loss in KPIFP was 10.77 and 55.54 %, respectively during first and second phase of TGA while for SPIFE it was 8.98 and 45.55 % respectively. Whereas, KPIWP showed 16.55 and 58.75 % weight during first and second phase of TGA and for SPIWP it was 9.77 and 48.99 % respectively. This indicates that phenolic groups broke easily from the sunflower protein molecular chain with increase in temperature and thus protein isolates with phenolic compounds (KPIWP and SPIWP) decomposes at lower temperatures than

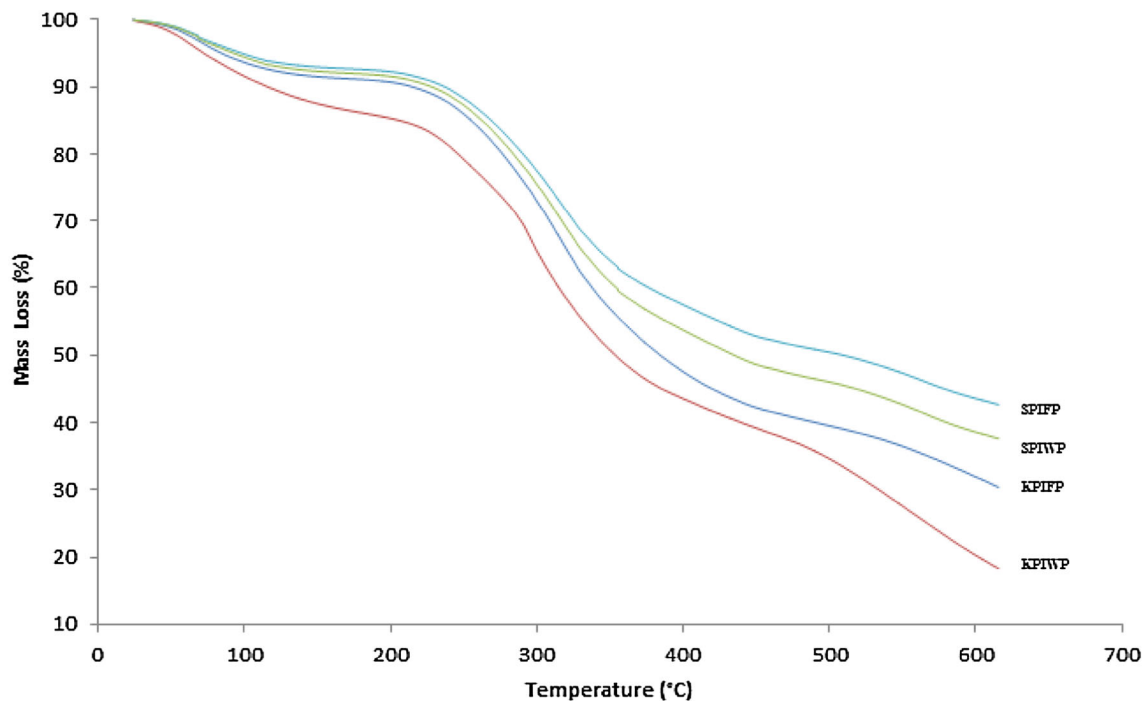


Fig. 1 TGA thermogram of sunflower protein isolates. *SPIWP* seed protein isolate with polyphenol, *SPIFP* seed protein isolate free of polyphenols, *KPIWP* kernel protein isolate with polyphenol, *KPIFP* kernel protein isolate free of polyphenols

protein isolates (KPIFP and SPIFP) which are free of polyphenols.

X-ray diffraction (XRD)

The crystal structure analysis of protein is mainly carried out using XRD. The crystal size is determined from diffraction intensity and diffraction angle (2θ). Lower the diffraction intensity and the bigger the diffraction angles, smaller is the size of the crystal (Yu et al. 2015). XRD of sunflower seed and kernel protein isolate is given as supplementary figure (Fig. S1). Two peaks were observed at $2\theta = 10^\circ$ (Low intensity) and $2\theta = 20^\circ$ (High intensity) for KPIFP, KPIWP, SPIFP and SPIWP. In addition to these two peaks, a small peak at around $2\theta = 32^\circ$ was also observed. Cao and Tan (2005) reported that $2\theta = 10^\circ$ is called crystalline region I and $2\theta = 20^\circ$ is crystalline region II. Joshi et al. (2011) on lentil protein isolate reported two characteristic XRD peaks at 10° and 24° . Wang et al. (2006) reported three diffraction peaks (8.5° , 19.5° and 24.5°) for soy protein isolate. The intensity of peaks for protein isolates free from polyphenols (KPIFP and SPIFP) was higher compared with their corresponding protein isolates which contain phenolic components (KPIWP and SPIWP). Crystal size of protein was decreased upon interaction with phenolic compounds. This may be due to the fact that new chemical bonds were formed in protein molecule upon phenolic interaction

which effect the regular arrangement of protein molecule and led to reduction of rigidity, with the result that the crystal size becomes smaller. This was further validated by the reduction in percentage crystallinity of protein isolate upon phenolic interaction. These results were in accordance with Yu et al. (2015) on pea nut protein. They reported that upon modification of pea nut proteins by phosphate, the crystal size got reduced. KPIFP showed highest crystallinity of 24 % which was reduced upon phenolic interaction. The crystallinity of seed proteins showed lower values than kernel proteins. The crystallinity of SPIFP was 17.49 % which reduced to 15.72 % upon phenolic interaction. This may be due to lower protein percentage in seed protein isolates compared with kernel protein isolate.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The interaction of polyphenols with sunflower proteins showed considerable effect on the polypeptide composition of proteins. Protein isolates (SPIWP and KPIWP) with phenolic compounds did not showed any band in SDS–PAGE (Fig. 2). This may be due the fact that the protein–phenolic interactions increase the molecular weight of proteins. These higher molecular weight proteins could not enter the resolving gel and hence leaving the band empty. Prigent et al. (2003) reported that molecular weight of α -

lactalbumin and lysozyme increased after incubation with chlorogenic acid at pH 7. Another possible reason is that the interaction of proteins with polyphenols may mask their recognition by commonly used stains such as Coomassie. Depending upon the degree of interaction, the protein bands will either appear as series of closely located bands or will be blocked completely and hence, preventing binding of stain (Alvarez 2014).

Fourier transform infrared spectroscopy

FTIR spectroscopy provides information about the secondary structure (α -helix and β -sheet) of proteins. Amide I and amide II are the characteristic bands found in the infrared spectra of proteins, arising from amide bonds. In amide I band, absorption leads to stretching vibration of C=O bond while as, in amide II band absorption leads primarily to bending vibration of N–H bond (Shevkani et al. 2014). The secondary structure of protein is very sensitive to the location of both amide I and amide II band, because hydrogen bonding between the different elements of secondary structure involves the both C=O and N–H bonds. Band associated with various peptide conformation are also found below 1400 cm^{-1} (amide III) (Adochitei and Drochioiu 2011). The spectra of all the four protein isolates did not show a major difference. The FT-IR spectra of all sunflower protein isolates is shown as supplementary figure (Fig. S2). Infra-red spectrum of protein isolates is indicated by the presence of broad band of 3408.7 cm^{-1} for KPIFP, 3404.0 cm^{-1} for KPIWP, 3311.9 cm^{-1} for SPIFP

and 3314.6 cm^{-1} for SPIWP are attributed to the –OH stretching vibrations. This is due to the presence of phenols and carboxylic group in the protein isolate. These peaks also revealed the presence of primary amine (N–H stretching) in protein isolate. Presence of phenolic compounds was also validated by the presence of peaks in the range of 1410 cm^{-1} – 1310 cm^{-1} which is due to the O–H bending. The peak in the amide I band at 1630.7 cm^{-1} for KPIFP, 1630.8 cm^{-1} for KPIWP, 1631.0 cm^{-1} for SPIFP and 1630.5 cm^{-1} for SPIWP is attributed to the functional compounds of protein amide groups (CONH), which are attributed to the carbonyl (C=O) stretch vibration. These peaks were correlated with the β -sheet content of the protein isolate. Shevkani et al. (2015) reported that in kidney bean and field pea protein isolates peaks around 1638, 1654, 1663 and 1680 cm^{-1} corresponding to β -sheet, α -helix, β turn and anti-parallel β -sheets (β -A) conformations, respectively. The presence of peak at 1596.7 cm^{-1} for KPIFP, 1595.8 cm^{-1} for KPIWP, 1591.7 cm^{-1} for SPIFP and 1595.5 cm^{-1} for SPIWP corresponds to the N–H stretching which falls in the amide II band. These peaks validate the presence of primary and secondary amine functional group in sunflower proteins isolate. The peaks at around 1245 cm^{-1} (aryl O-stretch), 1100 cm^{-1} (C–O stretching) in all the protein isolates confirm the presence of ether functional group. The peaks corresponding to the alkanes were also found in spectra in the range of 3000 cm^{-1} – 2850 cm^{-1} , which is due to the C–H stretching. The aldehyde functional group were detected in the frequency range of 2830 cm^{-1} – 2690 cm^{-1} .

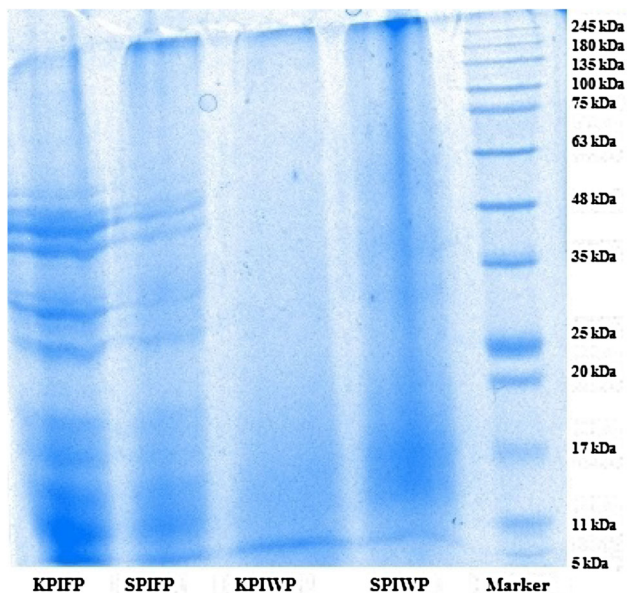


Fig. 2 SDS-PAGE pattern of sunflower protein isolates. *KPIFP* kernel protein isolate free of polyphenols, *SPIFP* seed protein isolate free of polyphenols, *KPIWP* kernel protein isolate with polyphenol, *SPIWP* seed protein isolate with polyphenol

Surface hydrophobicity

Surface-hydrophobicity is an important protein characteristic, which determines their functional properties such as foaming and emulsion properties. The change in the surface hydrophobicity of sunflower protein isolate upon interaction with phenolic compounds is shown in Table 1. Protein isolates (KPIWP and SPIWP) with phenolic compound showed a decrease in surface hydrophobicity compared with protein isolates (KPIFP and SPIFP) free of phenolic compounds. Similar results were reported by Rawel et al. (2002a) on soy protein upon interaction with phenolic compounds. The decrease in surface hydrophobicity of sunflower protein isolates with phenolic compounds was possibly caused by hydroxyl and carboxyl groups of phenolic compounds attached to protein isolate. The decrease in the surface hydrophobicity of protein isolates indicates that the phenolic compounds bound with proteins contributed the increase in hydrophilicity of sunflower protein isolates. Rawel et al. (2002a) revealed that the decrease in surface hydrophobicity of protein isolates upon phenolic interaction may due to combined effect of

Table 2 Particle size distribution and particle volume of sunflower protein isolates

Type of protein isolates	<25 % (μm)	<50 % (μm)	<75 % (μm)	Volume (μm^3)
SPIWP	42.4 \pm 2.9 ^c	88.9 \pm 4.6 ^c	163.3 \pm 7.3 ^b	80.7 \pm 4.2 ^c
SPIFP	36.6 \pm 1.6 ^c	78.9 \pm 3.4 ^d	150.6 \pm 5.1 ^b	72.7 \pm 2.5 ^d
KPIWP	84.4 \pm 4.2 ^a	182.6 \pm 6.9 ^a	340.1 \pm 8.9 ^a	154.4 \pm 1.6 ^a
KPIFP	55.3 \pm 4.0 ^b	104.1 \pm 4.5 ^b	161.9 \pm 1.5 ^b	87.8 \pm 3.4 ^b

Values are mean \pm SD. Means with similar superscript in a column did not differ significantly ($P > 0.05$)

SPIWP seed protein isolate with polyphenol, *SPIFP* seed protein isolate free of polyphenols, *KPIWP* kernel protein isolate with polyphenol, *KPIFP* kernel protein isolate free of polyphenols

several change occurred in the protein structure. These include the conformational changes in the protein that increased exposure of some previously buried hydrophilic regions, covalently blocking the hydrophobic residues e.g. tryptophan, introduction of hydrophilic carboxylic residues (phenolic acid) and due to the incorporation of higher number of hydroxyl groups. However Aewsiri et al. (2009) reported that phenolic compounds also cause the blocking of hydrophilic groups like amino and thiol groups. On one hand, covalent attachment of phenolic compounds with proteins causes the blocking of hydrophilic groups like thiol and amino and on other side, there is an increase in the amount of apolar groups (benzene ring) and introduction of polar groups (hydroxyl and carboxyl groups as in case of the phenolic acid). All these changes affect the hydrophilic/hydrophobic properties of protein. Rawel et al. (2002a) have revealed that the effect of phenolic interaction of protein on their hydrophilic/hydrophobic character depends upon different factors like concentration of phenolic compound, pH, configuration of protein and/or phenolic compound. In order to increase the biological and technological utilization of protein a thorough knowledge of different interrelated parameters that influence their nutritional and functional properties is required.

Thiol content

Table 1 shows the quantity of sulfhydryl groups present in all the four sunflower protein isolates. Protein isolates from kernel showed higher quantity of sulfhydryl group than the seed protein isolates. This may be due the higher percentage of protein in kernel protein isolates. The presence of polyphenols in the protein isolates showed considerable variation in the quantity of sulfhydryl content. Both the free and total sulfhydryl content was decreased upon interaction of polyphenol with proteins. The reaction of oxidized phenolic compounds with proteins results in binding and blocking of reaction sites for DTNB. This leads to a corresponding decrease in amount of free and total sulfhydryl groups in sunflower protein isolates (Kroll et al. 2003). Possibility of conversion of sulfhydryl groups into the disulphide bridges is ruled out, since analysis was

performed under reducing conditions and in presence of SDS, and hence sulfhydryl groups were reacted with the phenolic compounds. Rohn et al. (2005) reported that upon interaction of bromelain with polyphenols there was reduction in thiol content of the proteins. Ali et al. (2013) modified the milk whey protein with plant phenolic compound. They reported that the sulfhydryl content was reduced from 42.9 nmol/mg of unmodified β lactoglobulin to 12.8 nmol/mg of modified β lactoglobulin. A similar experiment was carried out by Rohn et al. (2005) on derivatization of lysozyme with phenolic compound, they reported that there was a decrease in the number of thiol groups and consequently in its activity.

Particle size analysis

Table 2 shows the particle size distribution and volume of all sunflower protein isolates. Kernel protein isolate was found to have wider particle size distribution and higher average particle size compared to the seed protein isolate. Protein isolates with phenolic compounds both from seed and kernel showed wider particle size distribution compared with protein isolate free of phenolic compound. For KPIWP and SPIWP 75 % of the particles size was found below 340.96 and 163.31 μm respectively. The average particle size was also found higher for protein isolates with phenolic compounds compared to the protein isolates free of phenolic compounds. The average particle size of kernel protein isolate was 104.09 μm which increased to 182.60 μm upon phenolic interaction. The average volume of protein particle was found higher for proteins with phenolic compound compared with proteins free of phenolic compounds. The increase in particle size distribution, average size and average volume upon phenolic interaction can be attributed to phenol mediated aggregation of protein particles.

Conclusion

Protein isolates obtained from polyphenols free meal had better structural properties. Polyphenols present in meal interacted with proteins during extraction process at

alkaline pH, which resulted in destabilization of protein structure. Proteins become more unordered upon phenolic interaction with corresponding loss in structured elements (α -helix and β -strand) and an increase in random coils. Thermal stability was observed higher for protein isolates with polyphenols compared with the protein isolates free of polyphenols. Enthalpy values decreased upon phenolic interaction and could be correlated with decrease in content of ordered structure. Kernel protein isolates had higher weight loss upon heating than seed protein isolate. The interaction of polyphenols with protein creates some new chemical bonds which effect the regular arrangement of protein molecules and hence crystal size and crystallinity got decreased. The polyphenols interaction with proteins had also masked their detection on SDS-PAGE. The attachment of polyphenols to protein resulted in conformational changes that involves series of reactions and consequently resulted in hydrophobic reduction along with decrease in sulfhydryl content. Polyphenols free proteins isolates showed lower particle size distribution compared with protein isolate with of phenolic compound. It was concluded that among all the samples proteins isolated from polyphenol free kernel meal had better structural properties.

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