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Scientific paper

Effects of the Extraction Conditions on Functional and Structural Characteristics of Proteins from Fenugreek Seeds

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Abstract

The study aims to optimize the extraction process and characterize the proteins found in fenugreek seeds. The water and oil holding capacities, coagulated protein content, foaming, and emulsification properties of the isolated proteins were investigated under all extraction conditions. Also, solubility, molecular weights, structural and thermal properties were determined. In the extraction processes carried out at different pH (pH 6.0–12.0) and solid:solvent ratios (20–60 g/L), it was determined that the highest extraction yield (94.3 ± 0.3%) was achieved when the pH was 11.47 and the solid-solvent ratio was 34.50 g/L. Three distinct bands (46, 59, and 80 kDa) in the range of 22–175 kDa were determined for the fenugreek seed protein isolate obtained under optimum extraction conditions. Protein secondary structures were determined using Fourier Transform Infrared (FT-IR) spectra and it was determined that β-sheet structures were highly present. In addition, denaturation temperature and denaturation enthalpy were calculated as ~119 °C and 28 mJ/g, respectively.

Keywords: Protein isolate, fenugreek seeds, extraction, secondary structure, emulsifying properties

1. Introduction

Proteins are crucial macronutrients in human nutrition, and historically, they have been primarily obtained from animal sources. However, with the increasing population in recent years, the availability of animal protein sources has been decreasing. As a result, there is now an increasing demand for alternative protein sources, such as plant-based proteins. Plant-based proteins are becoming more popular due to their health benefits and their ability to promote physical function.[1,2] Although animal sources contain high-quality proteins, they contain high levels of components such as cholesterol and saturated fatty acids, which cause diseases such as cardiovascular diseases and cancer when consumed frequently. Diets containing plant-based proteins are known to prevent cardiovascular diseases, hypertension, obesity, and some types of cancer.[3] In addition to increasing awareness of healthy nutrition, increasing sustainability concerns regarding food supply also increases consumers' tendency to prefer plant-based proteins. Furthermore, the fact that plant proteins, preferred by special consumer groups such as vegans and vegetarians, are cheaper and have a wide variety of sources, has made plants an alternative protein source for their use in food applications.[4,5]

Although plant-based proteins have many advantages, plant protein sources contain non-nutritive components (tannins, phytic acid, trypsin inhibitors, oligosaccharides, etc.), show weaker amino acid diversity than animal proteins, and their digestibility is not good. In addition, the fact that the functional properties of different protein isolates obtained from a wide variety of plant sources have not been well established limits their use in food formulations.[6] However, knowing the physico-chemical properties that affect the use of plant proteins in food formulations is very important in terms of improving the quality properties of the product. The physico-chemical properties of proteins are defined as the physical and chemical properties that affect the behavior of proteins in foods during production, storage, preparation, and consumption. Solubility, gelling, emulsification, foam formation, water and oil holding capacity, viscosity and film formation are some of the common physico-chemical properties of proteins. In addition to the structural properties of the proteins such as amino acid composition, surface hydrophobicity and hydrophobic/hydrophilic ratio, the extraction meth-

od and the parameters used in their production are also parameters that affect the physico-chemical properties of proteins.[7,8]

Extraction of plant-based proteins, like other proteins, is generally carried out by dissolving the material in a medium far from the isoelectric point and then precipitating the soluble proteins at the isoelectric point.[9] Alkaline extraction, which provides high protein yield, is generally used in the extraction of plant proteins. With the increase in the pH value of the solvent medium, acidic and neutral amino acids become ionized, and thus the solubility of proteins increases. More than 90% protein yield can be obtained with the alkaline extraction method.[10] Although high yields are obtained with alkaline extraction, the digestibility of the protein is affected because the structure of lysine and cysteine is disrupted, which negatively affects the overall quality of the protein.[11] Therefore, it is necessary to determine the alkaline conditions specific to that protein source that will improve or not affect the physicochemical properties of the protein. In addition, alkali concentration as well as other parameters such as solid:-solvent ratio, extraction time, and temperature should be optimized for maximum protein yield and preservation of physicochemical properties.[10] To identify new protein sources and gain application areas, it is necessary to characterize the obtained proteins. For this reason, in recent years, studies on the optimization of alkaline extraction conditions of plant-based proteins in terms of protein yield and physico-chemical properties of isolated proteins have been published in the literature.[2,12–16]

Fenugreek (*Trigonella foenum graecum*), known to have many health benefits, is an annual herbaceous plant in the legume family. Fenugreek, which has a widespread area in the world, differs from other legumes with its appearance and different smell. The protein content of fenugreek seeds has been reported to be in the range of 25–38%. The proteins in fenugreek seeds consist of albumin, globulin, glutelin, and prolamins. In a study where the flour obtained from fenugreek seeds was used in different proportions instead of wheat flour, it was reported that the protein content of products such as bread, biscuits, noodles, and pasta increased significantly, and there was an improvement in their sensory and rheological properties.[17] Therefore, fenugreek seeds, which have high nutritional value, are thought to be a potential protein source.

In this study, the effects of different solid:solvent ratios and pH levels on the extraction yield of the proteins in fenugreek seeds were determined, and the conditions that ensure the highest extraction yield were optimized using response surface methodology. The effects of the extraction conditions on the functional properties namely water holding capacity, oil holding capacity, coagulated protein content, foam capacity, foam stability, emulsion activity, emulsion stability, and emulsion capacity of the isolated proteins were investigated. Additionally, the structural and thermal properties and molecular weight patterns of fenugreek seed protein isolates obtained under optimum extraction conditions were determined.

2. Materials and Methods

2. 1. Material

After removing the foreign substances in the fenugreek seeds purchased from a local market, the seeds were powdered using a household grinder. The powdered seed samples were passed through a 630 μm sieve, and defatting was applied to the under-sieve samples using hexane. To remove the residual hexane, the samples were left to dry at 50 °C for 12 hours and the obtained defatted fenugreek seeds samples were used for protein extraction.

2. 2. Chemicals

H_2SO_4 (CAS#: 7664-93-9), HCl (CAS#: 7647-01-1), NaOH (CAS#: 1310-73-2), Brilliant Blue G-250 (CAS#: 6104-58-1) and Na_2HPO_4 dibasic dihydrate (CAS#: 10028-24-7) were obtained from Sigma-Aldrich, Germany. Boric acid (CAS#: 1043-35-3), methanol (CAS#: 67-56-1) and H_3PO_4 (CAS#: 7664-38-2) were obtained from Merck KGaA, Germany. Hexane (CAS#: 110-54-3) and citric acid monohydrate (CAS#: 5949-29-1) were provided by Tekkim Chemicals, Turkey. Kjeldahl tablets (Kjeltabs ST, AA 09) were obtained from Gerhardt, Germany. Tashiro indicator (CAS#: 64-17-5) was obtained from Riedel-de Haën™, Germany. Biuret Reagent (CB2145) was obtained from ChemBio, Turkey. Sodium phosphate dibasic (CAS#: 151-21-3) was obtained from BioBasic, Canada.

2. 3. Extraction Process and Isolation of the Proteins

Protein extraction from the defatted fenugreek seeds was carried out by mixing (at 750 rpm for 4 hours) the suspensions prepared in different solid:solvent ratios with distilled water as a solvent at different pH values. To optimize the extraction process, pH value (pH 6.0–12.0) and solid-solvent ratio (20–60 g/L) were chosen as independent variables, and a 'Central Composite Design' was carried out (Table 1). The samples were centrifuged at 6000 rpm for 15 minutes at the end of the extraction process. Extraction yield was calculated by proportioning the amount of protein in the supernatant phase (extract) to the protein amount of the initial powdered seed sample (Eq. 1). During the study, the protein contents of the samples were determined by Kjeldahl method[18] in all protein isolates and powder samples, and by Bradford method[19] in supernatant phases.

$$\text{Extraction yield (\%)} = [\text{Protein amount of extract (g)} / \text{Protein amount of fenugreek seeds (g)}] \times 100 \quad (1)$$

In the optimization process, the extraction yield was used as a response and the conditions providing the highest extraction yield were determined with a desirability function approach. The model used for regression analysis is given in Eq. 2.

$$\text{Extraction yield (\%)} = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \quad (2)$$

(k=1, 2)

where, β_0, β_i, β_{ii} and β_{ij} are the coefficients, X is the independent variable and k is the number of independent variables.

After the extraction process, the pH values of the extracts were adjusted to 4.0 and incubated at room temperature for 6 hours. After the incubation, the samples were centrifuged at 9000 rpm for 60 minutes, the supernatant was removed, and the precipitate was washed three times (5 minutes at 6000 rpm) using distilled water. The washed precipitates were then collected and lyophilized for 72 hours (Christ Alpha 1–4 LSC Plus, Germany). Powder protein isolates obtained as a result of the lyophilization were stored in sealed tubes at –18 °C until the analyses. By determining the amount of protein remaining in the supernatant phase at the end of precipitation, the average recovery in the precipitation process was calculated as 93.75 ± 0.55%.

2. 4. Characterization of the Protein Isolates

2. 4. 1. Coagulated Protein

The percentage of the coagulated protein in the samples was determined using the method described by Kramer and Kwee.[20] For this purpose, 0.2 g protein isolate was dissolved with 10 ml of citrate-phosphate solution (pH 7.0) at a concentration of 0.025 M and centrifuged. Biuret reagent was added to the supernatant phase and the solution was kept in the dark for 30 minutes. The solution was then incubated at 100 °C for 15 minutes and cooled to room temperature. After the cooling, the heating process was applied once again. The coagulated protein (%) was calculated using the absorbances of the samples before heating (A_1) and after heating (A_2) at 540 nm (Eq. 3).

$$\text{Coagulated protein (\%)} = [(A_1 - A_2) / (A_1)] \times 100 \quad (3)$$

2. 4. 2. Water and Oil Holding Capacity

Water holding capacity and oil holding capacity were determined by modifying the method of Vinayashree and Vasu.[10] After vortexing 250 mg of protein isolate with 15 ml of distilled water, it was kept at room temperature for 1 hour. Then, it was centrifuged at 3000 rpm for 20 minutes, the supernatant phase was removed, and the remaining sample was weighed. The water holding capacity is calculated in the g water/g sample. To determine the oil holding capacity, olive oil was used instead of water, and the oil holding capacity was expressed in g oil/g sample.

2. 4. 3. Foaming Capacity and Foam Stability

The foaming capacity and foam stability of the protein isolates were determined by the method proposed by Timilsena *et al.*[21] Aqueous solutions of the protein isolate at a concentration of 20 g/L were homogenized with a homogenizer (Ultra-Turrax IKA T-18 Basic, USA) at 10000 rpm for 5 minutes. Total volumes before homogenization (V_0) and after homogenization (V_1) were measured, and foaming capacity (%) was calculated using Eq. 4.

$$\text{Foaming capacity (\%)} = [(V_1 - V_0) / (V_0)] \times 100 \quad (4)$$

The foam stability was calculated using Eq. 5 by determining the total volume (V_2) of the homogenized sample after it was kept at room temperature for 1 hour.

$$\text{Foam stability (\%)} = [(V_2 - V_0) / (V_1 - V_0)] \times 100 \quad (5)$$

2. 4. 4. Emulsifying Properties

Emulsion activity and emulsion stability of the protein isolates were determined using the turbidity method modified by Feyzi *et al.*[22] First, 22.5 mg of the sample was weighed into a 15 mL tube, 4.5 mL of phosphate buffer solution (pH 7.0) was added, and the sample was vortexed for 1 minute. Sunflower oil (1.5 mL) was added to this mixture and homogenized at 22000 rpm for 2 minutes. To determine the emulsion stability, immediately after the homogenization (t=0), 250 µL emulsion was mixed with 50 mL sodium dodecyl sulfate at a concentration of 1 g/L, and the absorbance of this mixture at 500 nm was recorded (A_0). Similarly, the same process was applied to the initial emulsion that was kept at room temperature for 15 minutes (t=15) and its absorbance was recorded (A_{15}). Emulsion stability (min) was calculated using Eq. 6.

$$\text{Emulsion stability (min)} = [A_0 / (A_0 - A_{15})] \times t \quad (6)$$

Emulsion activity (m^2/g) was determined using Eq. 7.

$$\text{Emulsion activity } (m^2/g) = (2T \times D) / (\Phi \times C) = (2 \times 2.303 \times A_0 \times D) / (\Phi \times C \times L) \quad (7)$$

where, T is the turbidity ($T = 2.303 \times A_0/L$), D is the dilution factor (200), Φ is the emulsion oil volume fraction (g oil/g sample), C is the protein concentration in the solution (0.005 g/ml), L is the cuvette path length (10^{-2} m).

The method given by Neto *et al.*[23] was modified to determine the emulsion capacity. First, an equal volume of sunflower oil was added to the protein isolate solutions

prepared at a concentration of 1.0 % (w/v), and an emulsion was formed by homogenizing with ultra-turrax (7200 rpm, 2 min). These emulsions were then centrifuged at 3250 rpm for 2 minutes. The total height of the emulsion in the tube before centrifugation was expressed as H_0 (cm), the height of the emulsified layer of the centrifuged emulsion was expressed as H_1 (cm), and the emulsion capacity was calculated using Eq. 8.

$$\text{Emulsion capacity (\%)} = [H_1 / H_0] \times 100 \quad (8)$$

2. 4. 5. Protein Solubility

The solubility of the protein isolates (g/L) obtained under optimum extraction conditions was determined by the method reported by Feyzi *et al.*[22] The pH values of the protein isolate solutions prepared with distilled water at a concentration of 15 g/L were adjusted to values in the range of 2.0–12.0 using HCl or NaOH. After agitating the samples for 30 minutes at room temperature, they were centrifuged at 6000 rpm for 15 minutes to determine the protein content in the supernatant phase.

2. 4. 6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE method[24] was used to determine the molecular weights of proteins obtained under optimum extraction conditions. In the study, 12% gel was used and 10 μg and 50 μg of samples were loaded. Samples were run under 200 V voltage for 50 min and the gel was stained with Comassie Brillant Blue R-250.

2. 4. 7. Fourier Transform Infrared (FT-IR) Spectroscopy

Structural properties of protein isolates obtained under optimum conditions were determined using a Fourier Transform Infrared (FT-IR) Spectrometer (Perkin Elmer 400, USA). Diamond ATR method was used in the analysis and measurements were made in the spectrum range of 4000–400 cm^{-1}. Considering the Amide I region (1600–1700 cm^{-1}) in the FT-IR spectra, the protein secondary structures of the protein isolates were determined by deconvolution of the peaks and curve fitting using the Peakfit v4.12 package program (Systat Software, USA).

2. 4. 8. Thermal Properties

Denaturation temperature (T_d, °C) and denaturation enthalpy (ΔH_d, mJ/g) of the protein isolates obtained under optimum conditions were determined using a Differential Scanning Calorimetry (DSC) (Perkin Elmer DSC 8000, USA). Analyses were carried out in a nitrogen environment, in the temperature range of 20–200 °C and at a heating rate of 5 °C/minute.

2. 5. Statistical Analysis

The one-sample t-test and 'Univariate Variance Analysis, Duncan post hoc' test were performed using the SPSS 21.0 software package. Regression analysis, contour plots, and optimization processes were performed using Design Expert 7.0 (Stat-Ease, Inc., USA) software to determine the effects of all process variables.

3. Results and Discussion

3. 1. Extraction Process

The experimental design used for the extraction process of the proteins found in fenugreek seeds and the extraction yields are given in Table 1. According to the results, the highest extraction yield (93.13 ± 1.36%) was obtained under the condition that the solid-solvent ratio was 20 g/L and the pH value was 12.0. The lowest extraction yield was determined when the solid-solvent ratio was 60 g/L and the pH value was 6.0 (Table 1). Lower extraction yields were observed at all pHs when the solid-solvent ratio was the highest (60 g/L). The decrease in protein extraction yields when the solid-solvent ratio is high can be explained by the fact that non-protein compounds (gum, mucilage, etc.) in the extraction medium make protein extraction difficult. It is thought that protein extraction yields increase by providing a more effective mixing process at low solid-solvent ratios (20–40 g/L) and increasing the solid-solvent contact surface. It was determined that the pH value chosen as another independent variable in the protein extraction process also affects the extraction yield. The extraction yields increased with increasing pH values in all solid-solvent ratios (Table 1). This situation is associated with the increased solubility of the proteins in fenugreek seeds at high pH values. Similarly, Feyzi *et al.*[25] reported that the solubility of fenugreek seed proteins increased in an alkaline environment (pH 9.25). Jarpa-Parra *et al.*[26] reported that the extraction yield and purity of the obtained proteins increased by using pH values ≥9.0 in protein extraction from lentils. Gao *et al.*[27] carried out protein extraction from yellow peas, which belong to the legume family as fenugreek seeds, and found that the protein extraction yield increased with increasing pH.

To optimize the extraction process, the extraction yield was chosen as the response, and a second-order polynomial model was constructed. According to the ANOVA results given in Table 2, the developed model was found to be statistically significant ($p < 0.05$), and the lack of fit was found to be statistically insignificant ($p > 0.05$). The linear and quadratic effects of the solid-solvent ratio and pH on extraction yield were determined to be statistically significant ($p < 0.05$). On the other hand, it was observed that the solid-solvent ratio-pH interaction did not have a statistically significant effect on the extraction yield ($p >$

Table 1. Experimental design and extraction yields (%)

Experiment no	Solid:solvent ratio (g/L) (X_1)	pH (X_2)	Extraction yield (%)
1	40	9.0	84.72 (± 0.68)
2	20	6.0	58.62 (± 1.36)
3	60	9.0	68.30 (± 0.72)
4	40	9.0	83.76 (± 0.41)
5	40	9.0	85.96 (± 0.27)
6	20	12.0	93.13 (± 1.36)
7	60	12.0	80.12 (± 0.63)
8	40	12.0	91.71 (± 0.54)
9	40	9.0	85.00 (± 0.54)
10	40	9.0	84.43 (± 0.81)
11	40	6.0	54.52 (± 1.08)
12	20	9.0	85.46 (± 1.90)
13	60	6.0	41.14 (± 0.27)

0.05) (Table 2). The model equation, written in terms of the real values of the factors, is given in Eq. 9.

Table 2. ANOVA table and statistical parameters

Source	Degrees of freedom	Sum of squares	Mean square	F Value	p – Value
Model	5	3055.79	611.16	417.80	< 0.0001
X_1	1	378.55	378.55	258.79	< 0.0001
X_2	1	2041.64	2041.64	1395.72	< 0.0001
X_1X_2	1	5.00	5.00	3.42	0.1069
X_1^2	1	114.18	114.18	78.06	< 0.0001
X_2^2	1	286.98	286.98	196.19	< 0.0001
Residual	7	10.24	1.46		
Lack of Fit	3	7.62	2.54	3.88	0.1118
Pure Error	4	2.62	0.66		
Total	12	3066.03			

R^2: 0.9967, adj- R^2: 0.9943, adequate precision: 64.234, *PRESS*: 60.10, *C.V.* (%): 1.58

X_1: solid:solvent ratio (g/L), X_2: pH, adj- R^2: adjusted R^2, *PRESS:* predicted residual error sum of squares, *C.V.* (%): coefficient of variation

$$\text{Extraction yield (\%)} = -65.85+0.72X_1+25.79X_2-0.02X_1^2-1.13X_2^2 \quad (9)$$

The conditions at which maximum protein extraction yield was achieved were determined by the desirability (d) function approach. The scale of the desirability function ranges from the completely unacceptable response (d=0) to the response corresponding to the target value (d=1), and the value of d increases as the desirability of the dependent variable increases. The response surface contour plots of the predicted desirability values and the extraction yields (%) are shown in Fig. 1a and Fig. 1b, respectively. As seen in Fig. 1, the conditions where the maximum desirability value (d=1) was obtained (maximum extraction yield) were selected as the optimum extraction conditions. The solid:solvent ratio was 34.5 g/L and pH was 11.47, and the predicted extraction yield was 94.08% at the optimum conditions (Fig. 1b). For the experimental validation, the extraction process was performed in triplicate under the predicted optimum conditions, and no statistically significant difference (p>0.05) was determined between the experimental extraction yield (94.29 ± 0.26%) and the predicted one.

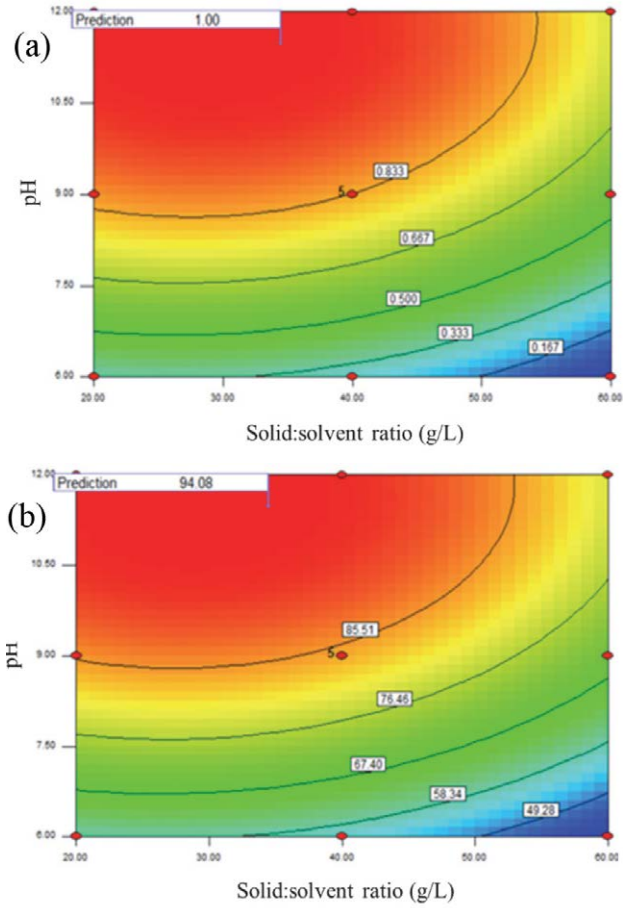

Fig 1. Counter plots of (a) desirability values and (b) predicted extraction yields (%)

3. 2. Characterization of the Protein Isolates

The functional properties of the protein isolates obtained under different extraction conditions are given in Table 3. The foaming properties of the fenugreek seeds protein isolates were determined by measuring the foaming capacity and foam stability. While foaming capacity is defined by the increase in the volume of the solution in the foaming process, foam stability is defined as the ability to keep the air in the foams formed.[28] The foaming capacity of fenugreek seed protein isolates was determined between 10.67 and 18.00%, and the foam stability was determined between 51.92 and 69.67%. As a result of extractions per-

formed at pH 12.0, it was determined that the highest foaming capacity and foam stability values were obtained ($p < 0.05$). Also, it was determined that the highest values were obtained in the extractions performed at a medium level (40 g/L) solid:solvent ratio (Table 3). Differences in the extraction conditions applied when obtaining the protein isolate caused the formation of different protein structures and fractions, affecting the foaming properties.[29] The foaming capacity of the protein isolate obtained under optimum conditions was determined as 19.00 ± 1.00%, and the foam stability was determined as 74.13 ± 2.16%.

The water and oil holding capacities are defined as the amount of water or oil absorbed per unit of protein, and the leakage of substances such as water or oil from the products can be prevented because of these properties of proteins during storage of the food. In addition, the oil holding capacity is important in terms of keeping the oil-soluble flavor substances and the texture of the product. The water and/or oil holding capacity of protein isolates is related to the number of polar or nonpolar amino acids in the structure, surface hydrophobicity and conformation of the proteins.[28] The water holding capacities of fenugreek seed protein isolates varied between 2.04 and 2.73 g/g. It was determined that the highest water holding capacity values belonged to the samples extracted at pH 12.0 (Table 3). In other studies, the water holding capacity value for fenugreek seed protein concentrate was 1.56 g/g[17] and for fenugreek seed protein isolate 2.70 g/g [25]. Liu *et al.*[30] characterized the flaxseed protein isolates and reached water holding capacity values in the range of 0.83–1.05 g/g. Kaur and Ghosal[31] reported the water holding capacity of protein isolate obtained from defatted sunflower meal as 2.00 g/g, and Yancheshmeh *et al.*[32] reported the water retention capacity of the protein isolate obtained from vetch seed as 2.01 g/g. When compared to the studies conducted in the literature, it was observed that the determined water holding capacity value of 2.64 ± 0.04 g/g of fenugreek seed protein isolate obtained under optimum extraction conditions was higher than many plant-derived protein isolates. It was determined that the oil holding capacity values of fenugreek seed protein isolates varied between 1.46 and 2.10 g/g. It was observed that the highest oil holding capacity values were obtained in the protein isolates produced as a result of extractions performed at pH 12.0 ($p<0.05$) (Table 3). The oil holding capacity value obtained under optimum conditions was determined to be 2.00 ± 0.01 g/g. El Nesri and El Tinay[17] determined the oil holding capacity value of fenugreek seed protein concentrate as 1.56 g/g. Feyzi *et al.*[25] determined the oil holding capacity value of fenugreek seed protein isolate as 6.06 ± 0.28 g/g. It is thought that different oil retention capacity values may be related to the extraction conditions of the seeds and the climate in which they are grown.[25]

The coagulated protein (%) refers to the protein percentage of the total soluble protein that will coagulate when heated to 100 °C. Since the uncoagulated protein is in soluble form, it can leak out of the system, which is undesirable. However, non-coagulating proteins are advantageous in forming viscous systems and increasing nutritional value in liquid systems (e.g., in breakfast drinks). It was determined that the coagulated protein values of fenugreek seed protein isolates varied between 3.01 and 4.96%. A decrease in the coagulated protein values was observed at increasing pH values during extraction ($p < 0.05$) (Table 3). Feyzi *et al.*[25] determined the coagulated protein value as 3.17% and emphasized that the proteins can be used in the production of beverages with high nutritional value and protein-added fruit juices due to their low coagulated protein percentages.

Proteins can prevent agglomeration and creaming by forming a layer around oil droplets at the water-oil interface, that are immiscible and thermodynamically unstable due to their amphiphilic structure. The emulsification properties of plant-derived proteins are of great importance for their use in the food industry. Emulsion properties of the proteins are affected by internal factors such as surface charge, hydrophobicity, solubility, molecular size, flexibility of the film formed, and external factors such as presence of other substances in the environment, pH, ionic strength, temperature, protein extraction methods and protein concentration.[33] Emulsion capacity is defined as the maximum amount of oil that can be emulsified by a certain amount of protein and is expressed as a percentage.[22] It was observed

Table 3. Functional properties of the protein isolates

pH	Solid:solvent ratio (g/L)	Foaming capacity (%)	Foam stability (%)	Coagulated protein (%)	Water holding) capacity (g/g	Oil holding capacity (g/g)
6	20	11.50 ± 0.71^{c}	52.80 ± 2.45^{d}	4.81 ± 0.26^{a}	2.13 ± 0.03^{d}	1.57 ± 0.02^{d}
	40	11.50 ± 0.71^{c}	68.06 ± 1.20^{ab}	4.98 ± 0.26^{a}	2.04 ± 0.01^{e}	1.52 ± 0.03^{e}
	60	10.67 ± 0.58^{c}	51.92 ± 2.72^{d}	5.06 ± 0.13^{a}	2.04 ± 0.03^{e}	1.46 ± 0.04^{f}
9	20	14.00 ± 1.00^{b}	60.66 ± 2.60^{c}	3.69 ± 0.13^{c}	2.18 ± 0.02^{c}	1.91 ± 0.02^{c}
	40	15.00 ± 1.00^{b}	63.33 ± 4.71^{bc}	4.02 ± 0.28^{bc}	2.18 ± 0.03^{c}	1.98 ± 0.02^{b}
	60	14.00 ± 0.00^{b}	61.25 ± 1.77^{c}	4.15 ± 0.18^{b}	2.19 ± 0.01^{c}	1.89 ± 0.01^{c}
12	20	17.00 ± 0.00^{a}	64.29 ± 0.00^{abc}	3.07 ± 0.27^{d}	2.66 ± 0.02^{b}	2.07 ± 0.04^{a}
	40	18.00 ± 0.00^{a}	69.67 ± 1.30^{a}	3.08 ± 0.21^{d}	2.65 ± 0.04^{b}	2.09 ± 0.03^{a}
	60	17.00 ± 0.00^{a}	69.44 ± 3.93^{a}	3.16 ± 0.18^{d}	2.73 ± 0.02^{a}	2.10 ± 0.02^{a}

$^{a–g}$ Mean values given different letters in the same column are statistically different from each other ($p < 0.05$).

that the emulsion capacities of the fenugreek seed protein isolates varied between 18.30 and 26.00% (Fig. 2a). It was determined that the emulsion capacities of the obtained fenugreek seed protein isolates were higher when extracted at higher pH values ($p < 0.05$) (Fig. 2a). The emulsion capacity of fenugreek seed protein isolates obtained under optimum conditions was determined as 26.52 ± 0.26%. Emulsion activity is defined as the maximum emulsion surface area per unit protein measured spectrophotometrically based on turbidity.[34] It was determined that the emulsion activities of fenugreek seed protein isolates ranged between 75.82 and 80.95 m^2/g (Fig 2b). Furthermore, the emulsion activity of fenugreek seed protein isolates obtained under optimum conditions was determined as 78.21 ± 0.28 m^2/g. Emulsion stability was determined based on the change in turbidity over time. The emulsion stability of fenugreek seed protein was observed to vary between 23.65 and 28.06 minutes (Fig. 2c). The emulsion stability of fenugreek seed protein isolates obtained under optimum conditions was determined as 28.73 ± 0.35 minutes.

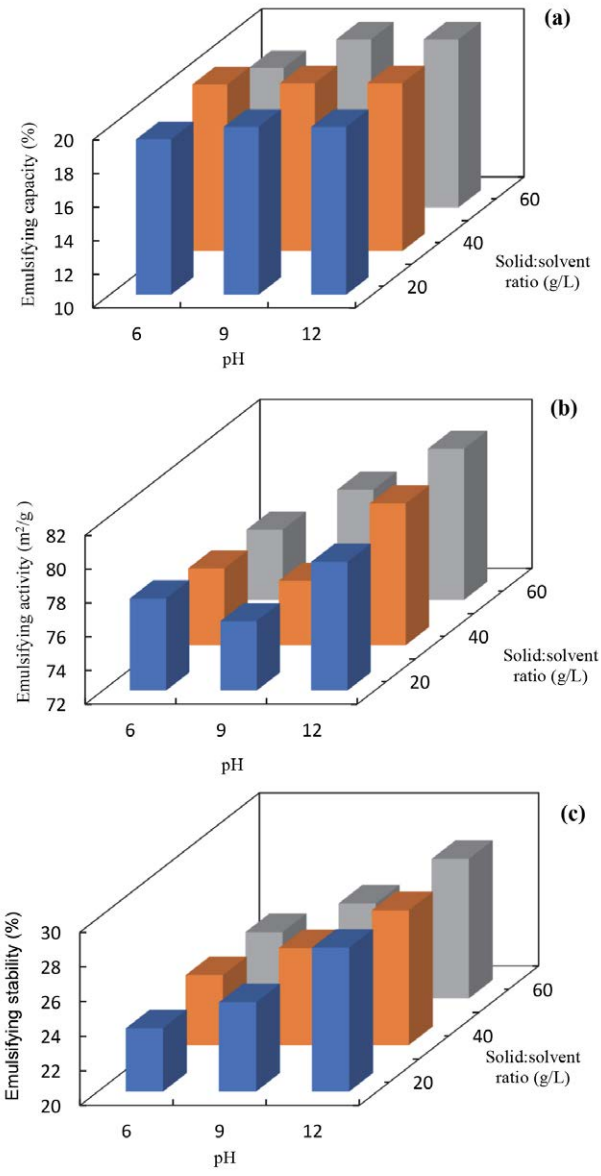

Fig 2. Emulsifying properties of fenugreek protein isolates (a) emulsifying capacity, (b) emulsifying activity, (c) emulsifying stability

Proteins exhibit maximum solubility in highly acidic or basic conditions far from the isoelectric point. The results obtained in the study showed that the solubility properties of the fenugreek seed protein isolate obtained under optimum extraction conditions comply with this phenomenon. As seen in Fig. 3a, solubility values follow a characteristic U-shaped curve in the pH range of 2–12. While the solubility values of the samples ranged between 0.27 and 8.46 g/L, the lowest solubility was observed at pH 4.0. This can be explained by the fact that fenugreek seed proteins have an isoelectric point in the pH range of 4.0–4.5.[22] Since an equilibrium occurs between negatively and positively charged ions at the isoelectric point, the net charge becomes zero. Thus, as the electrostatic repulsion forces decrease, proteins lose their solubility and collapse as a result of the hydrophobic interactions. On the other hand, the electrostatic repulsion force that occurs between the charged ions in acidic and alkaline conditions far from the isoelectric point, which may be different for each protein, ensures the dissolution of the proteins.[35] When the solubilities at high pH values were examined, it was seen that the highest values were obtained at pH 11.0 and pH 12.0 (Fig. 3a). The better solubility of the fenugreek seed proteins at high pH values can be explained by the inhibition of the formation of protein aggregates by the repulsive force of a larger number of negatively charged ions.[25] Similar results for some plant-derived proteins in the literature have been obtained for soy protein isolate, Moringa oleifera seed protein isolate, bitter melon protein isolate, flaxseed protein isolate, and chickpea protein isolate.[36–40] The molecular weight distribution of the fenugreek seed protein isolate was determined to be between ~175 kDa and ~22 kDa, and 10 bands with molecular weights of approximately 175, 159, 80, 59, 46, 38, 31, 27, 23, and 22 kDa were detected. However, 3 distinct bands were observed. These three most prominent bands were detected as ~80, 59, and 46 kDa (Fig. 3b). The bands between 22 and 70 kDa are found to be associated with globulins, specifically legumins and vicilins, which constitute the primary protein constituents in legumes.[41] These proteins were further fractionated into distinct subunits: β-legumin was observed at approximately 22 kDa, while α-legumin was observed at around 40 kDa.[42] Hence, the bands obtained at ~38 and 46 kDa could be associated with α-legumin for the fenugreek seed protein isolate. Moreover, the visible bands at ~22 and 23 kDa could be associated with the presence of β-legumin. The bands ranging from 50 to 80 kDa have been attributed to vicilin and covicilin. Two of the predominant bands observed at ~59 and 80 kDa could be ascribed to the polypeptide constituents of vicilin and convicilin.[43,44]

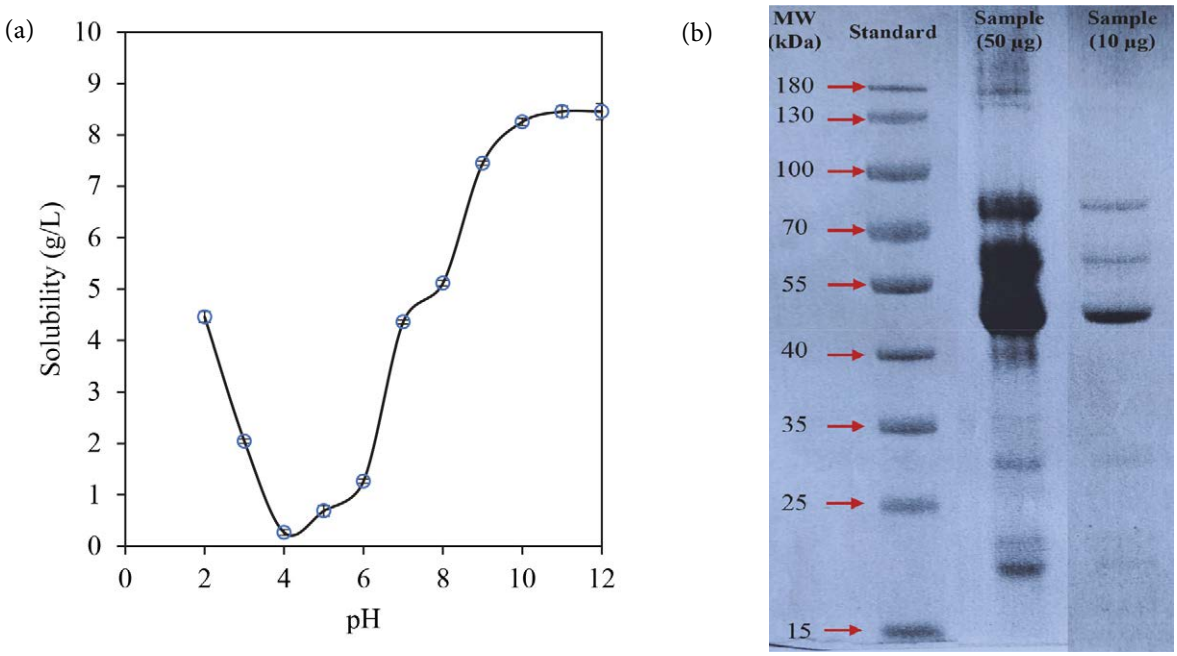

Fig 3. Characteristics of the protein isolates produced at optimum extraction conditions (a) solubility, (b) SDS-PAGE image

Fig. 4 shows the FT-IR spectra of the fenugreek seed protein isolates obtained under optimum conditions. As seen in Fig 4, the Amide I band is observed at the wave number of 1600–1700 cm^{-1}. The region between wave numbers of 1480–1585 cm^{-1} is defined as the Amide II region, and around 40–60% N–H bending vibration and around 18–40% C–N stretching vibration are observed in this region. It was observed that the peaks obtained at the

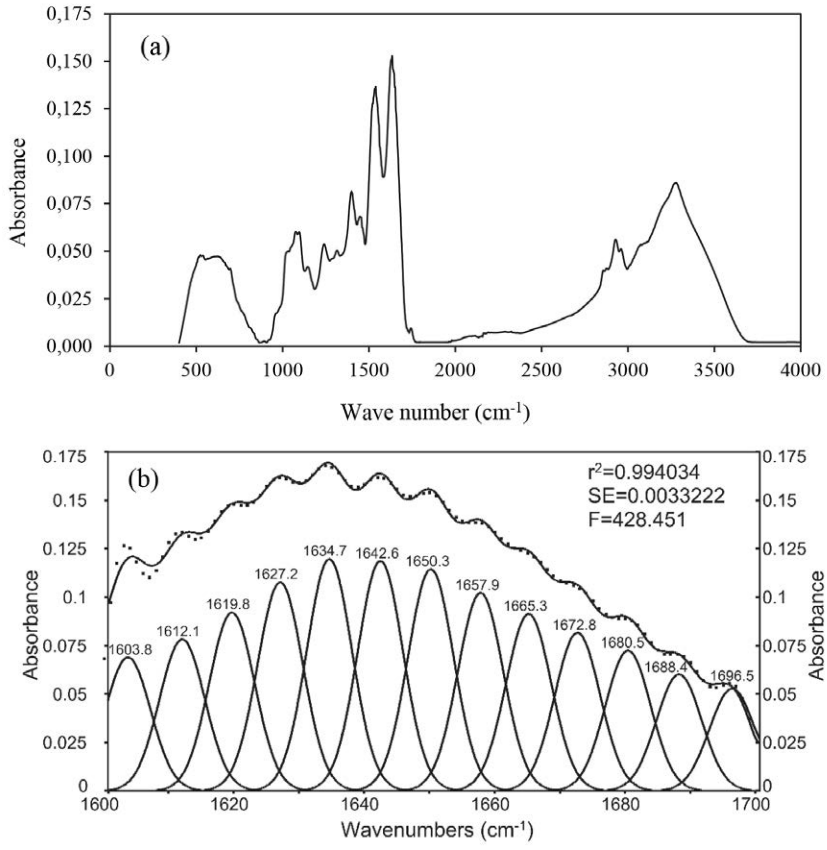

Fig 4. FT-IR spectra of the fenugreek seed protein isolate a) original spectrum, b) deconvolution in the Amide I region

wave numbers of 1447, 1515, and 1532 cm^{-1} for the obtained protein isolate were located in the Amide II region (Fig. 4a). The detection of Amide I and Amide II bands is considered an absolute indicator of the presence of protein structure.[45] The Amide III region is observed at the wave numbers between 1200–1400 cm^{-1} and indicates the existence of interactions between protein and other macromolecules such as carbohydrates. The presence of this region in proteins occurs depending on the side ring structure. C–N stretching vibrations and N–H bending vibrations are observed in this region.[46] It was determined that the peaks obtained at the wave numbers of 1240–1394 cm^{-1} for the fenugreek seed protein isolates were in the Amide III region (Fig. 4a). Secondary structures of the fenugreek seed protein isolates were determined using the peaks in the Amide I region (1600–1700 cm^{-1}) in the FT-IR spectrum. There are α-helix, β-sheet, random coil, or β-turn conformations in the Amide I band of proteins.[47] It has been reported that the β-sheet structure was observed in the wave numbers 1612–1640 cm^{-1} and 1689–1695 cm^{-1}.[48] Furthermore, the α-helix was observed at 1651–1660 cm^{-1} [49,50], the random coil conformation was observed at 1641–1650 cm^{-1} [49] and the β-turn was observed at 1661–1688 cm^{-1} [50]. In the FT-IR spectra, 13 peaks were observed in the Amide I region, and the ratio of the fractions in the protein secondary structure was determined by deconvolution of the peaks in the Amide I region (Fig. 4b). As a result of the analysis, 38.69% of the secondary structure is β-sheet, 18.96% is α-helix, 10.39% is random coil, 26.76% is β-turn and 5.20% is side ring. The high presence of the β-sheet structures indicates that protein isolates have high thermal stability.[50] To determine the thermal properties of the fenugreek seed protein isolates, denaturation temperatures (T_d) and denaturation enthalpies (ΔH_d) were determined using Differential Scanning Calorimetry (DSC). An endothermic peak was observed indicating that energy was required for denaturation to occur, and the denaturation temperature of the fenugreek seed protein isolate was 118.85 °C. In the literature, denaturation temperatures of 91 °C for cowpea protein isolate[51], 95 °C for flaxseed protein isolate[52], 103 °C for quince seed protein isolate[53] and 105 °C for fenugreek seed protein isolate have been reported[25]. The denaturation enthalpy value (ΔH_d) of the obtained protein isolate was also calculated and determined as 28 mJ/g.

4. Conclusion

The study aimed to extract proteins from fenugreek seeds using the alkaline extraction process at different pH values (pH 6.0–12.0) and solid:solvent ratios (20–60 g/L), and to determine the optimum conditions for the highest extraction yield. The optimum extraction conditions were determined as pH 11.47 and solid:solvent ratio 34.5 g/L, and an extraction yield of 94.3% was achieved under these conditions. The protein isolates obtained under different extraction conditions have various properties, including water holding capacity of ~2.0–2.7 g/g, oil holding capacity of ~1.5–2.1 g/g, coagulated protein content of ~3.0–5.0%, foam capacity of ~11.0–18.0%, foam stability of ~%52.0–70.0, emulsion stability of ~24.0–28.0 minutes, emulsion activity of ~76.0–81.0 m^2/g, and emulsion capacity of ~18.3–26.0%. Solubility properties showed that the fenugreek seeds protein isolate was soluble both acidic and basic conditions, which makes it a good candidate for both types of drinks. The study also included secondary structure analysis and thermal property determination, which revealed the thermal stability of the protein isolates. As a result, the extraction process was optimized to achieve the highest extraction yield, which distinguishes it from other studies in the literature. The study also examined how each extraction condition affected the characteristics of protein isolates. In this study, unlike other studies in the literature, the extraction process was optimized to provide the highest extraction efficiency and it was revealed how each extraction condition affected the characteristics of protein isolates. The results showed that the functional properties of protein isolates obtained under different extraction conditions are competitive or even better than other plant-derived proteins in the literature. Based on these findings, fenugreek seed protein isolate, produced under optimum extraction conditions, may be an excellent alternative plant-based protein for several food applications. Its functional, structural, and thermal properties make it suitable for use in different formulations in many food processes.

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5. References

1.. N. Mohan, J. J. Mellem, *LWT-Food Sci. Technol.* **2020**, *129*, 109572. **DOI:**10.1016/j.lwt.2020.109572
2. S. Vinayashree, P. Vasu, *Food Chem*. **2021**, *340*, 128177. **DOI**:10.1016/j.foodchem.2020.128177
3. S. M. Mousavi, A. Jayedi, Y. Jalilpiran, M. Hajishafiee, A. Aminianfar, A. Esmaillzadeh, *Crit. Rev. Food Sci. Nutr*. **2022**, *62*, 1336–1349. **DOI:**10.1080/10408398.2020.1841730
4. S. R. Hertzler, J. C. Lieblein-Boff, M. Weiler, C. Allgeier, *Nutrients*. **2020**, *12*, 3704–3731. **DOI:**10.3390/nu12123704
5. S. Thakur, A. K. Pandey, K. Verma, A. Shrivastava, N. Singh, *Int. J. Food Sci. Technol.* **2023**, *59*, 488–497. **DOI:**10.1111/ijfs.16663
6. F. U. Akharume, R. E. Aluko, A. A. Adedeji, *Compr. Rev. Food Sci. Food Saf.* **2021**, *20*, 198–224. **DOI:**10.1111/1541-4337.12688
7. M. Pojić, A. Mišan, B. K. Tiwari, *Trends Food Sci. Technol.* **2018,** *75*, 93–104. **DOI:**10.1016/j.tifs.2018.03.010

8. M. Kumar, M. Tomar, J. Potkule, Reetu, S. Punia, J. Dhakane-Lad, S. Singh, S. Dhumal, P. C. Pradhan, B. Bhushan, T. Anitha, O. Alajil, A. Alhariri, R. Amarowicz, J. F. Kennedy, *Food Hydrocoll.* **2022**, *123*, 106986. **DOI:**10.1016/j.foodhyd.2021.106986
9. M. Hadnađev, T. Dapčević-Hadnađev, A. Lazaridou, T. Moschakis, A. M. Michaelidou, S. Popović, C. G. Biladeris, *Food Hydrocoll.* **2018**, *79*, 526–533. **DOI:**10.1016/j.foodhyd.2017.12.015
10. K. Kumar, S. Srivastav, V. S. Sharanagat, *Ultrason Sonochem.* **2021**, *70*, 105325. **DOI:**10.1016/j.ultsonch.2020.105325
11. L. J. Deleu, M. A. Lambrecht, J. Van De Vondel, J. A. Delcour, *Curr. Opin. Food Sci.* **2019**, *25*, 98–103. **DOI:**10.1016/j.cofs.2019.02.017
12. M. Du, J. Xie, B. Gong, X. Xu, W. Tang, X. Li, C. Li, M. Xie, *Food Hydrocoll.* **2018**, *76*, 131–140. **DOI:**10.1016/j.foodhyd.2017.01.003
13. D. Garg, S. Chakraborty, J. S. Gokhale, *LWT-Food Sci. Technol.* **2020**, *117*, 108630. **DOI:**10.1016/j.lwt.2019.108630
14. S. Sethi, D. N. Yadav, S. Snigdha, A. Gupta, *LWT-Food Sci. Technol.* **2021**, *137*, 110368. **DOI:**10.1016/j.lwt.2020.110368
15. R. Anusha, S. Deepti, P. Vinita, K. Rajendra, Y. Neelam, *Res. J. Biotech.* **2021**, *16*, 163–172.
16. N. Benzitoune, N. Kadri, M. Adouane, F. Berkani, A. Abbou, F. Dahmoune, H. Remini, S. Bensmail, *J. Food Process. Preserv.* **2022**, *46*, e16471. **DOI:**10.1111/jfpp.16471
17. N. A. El Nasri, A. H. El Tinay, *Food Chem.* **2007**, *103*, 582–589. **DOI:**10.1016/j.foodchem.2006.09.003
18. AOAC, Official methods of analysis of AOAC international. 17th edn. AOAC International, Md., USA, **2000**.
19. M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254. **DOI:**10.1016/0003-2697(76)90527-3
20. A. Kramer, W. H. Kwee, *J. Food Sci.* **1977**, *42*, 207–211. **DOI:**10.1111/j.1365-2621.1977.tb01253.x
21. Y. P. Timilsena, R. Adhikari, C. J. Barrow, B. Adhikari. *Food Chem.* **2016,** *212*, 648–656. **DOI:**10.1016/j.foodchem.2016.06.017
22. S. Feyzi, M. Varidi, F. Zare, M. J. Varidi, *J. Sci. Food Agric.* **2018**, *98*, 1880–1888. **DOI:**10.1002/jsfa.8669
23. V. Q. Neto, N. Narain, J. B. Silva, P. S. Bora, *Food/Nahrung.* **2001**, *45*, 258–262. **DOI:**10.1002/1521-3803(20010801)45:4<258::AID-FOOD258>3.0.CO;2-3
24. U. K. Laemmli, *Nature.* **1970**, *227*, 680–685. **DOI:**10.1038/227680a0
25. S. Feyzi, M. Varidi, F. Zare, M. J. Varidi, *J. Sci. Food Agric.* ***2015***, *95*, 3165–3176. **DOI:**10.1002/jsfa.7056
26. M. Jarpa-Parra, F. Bamdad, Y. Wang, Z. Tian, F. Temelli, J. Han, L. Chen, *LWT-Food Sci. Technol.* **2014,** *57*, 461–469. **DOI:**10.1016/j.lwt.2014.02.035
27. Z. Gao, P. Shen, Y. Lan, L. Cui, J. B. Ohm, B. Chen, J. Rao, *Food Res. Int.* **2020**, *131*, 109045. **DOI:**10.1016/j.foodres.2020.109045
28. V. Kiosseoglou, A. Paraskevopoulou, in: K. B. Tiwari, A. Gowen, B. McKenna (Ed.): Pulse Food: Processing, Quality and Nutraceutical Applications, Elsevier Inc., London, UK, **2011**, pp. 57–90. **DOI:**10.1016/B978-0-12-818184-3.00006-4
29. E. Tsaliki, U. Kechagia, G. Doxastakis, *Food Hydrocoll.* **2002**, *16*, 645–652. **DOI:**10.1016/S0268-005X(02)00030-9
30. M. Liu, J. A. Toth, M. Childs, L. B. Smart, A. Abbaspourrad. *J. Food Sci.* **2023**, *88*, 942–951. **DOI:**10.1111/1750-3841.16467
31. R. Kaur, G. Ghoshal, *Adv. Colloid Interface Sci.* **2022**, *306*, 102725. **DOI:**10.1016/j.cis.2022.102725
32. B. S. Yancheshmeh, L. M. Marvdashti, A. Emadi, A. Abdolshahi, A. Ebrahimi, N. Shariatifar, *Food Anal. Methods.* **2022**, *15*, 1187–1202. **DOI:**10.1007/s12161-021-02185-z
33. A. C. Karaca, N. H. Low, M. T. Nickerson, *Trends Food Sci. Technol.* **2015**, *42*, 5–12. **DOI:**10.1016/j.tifs.2014.11.002
34. Z. Yi-Shen, S. Shuai, R. FitzGerald, *Food Nutr. Res.* **2018**, *60*, 1290. **DOI:**10.29219/fnr.v62.1290
35. N. Singh, M. Kaur, K. S. Sandhu, *Drying Technol.* **2005**, *23*, 975–988. **DOI:**10.1081/DRT-200054253
36. A. A. Heywood, D. J. Myers, T. B. Bailey, L. A. Johnson, *J. Am. Chem. Soc.* **2002**, *79*, 1249–1253. **DOI:**10.1007/s11746-002-0635-y
37. M. Kaur, N. Singh, *Food Chem.* **2007**, *102*, 366–374. **DOI:**10.1016/j.foodchem.2006.05.029
38. R. Horax, N. Hettiarachchy, A. Kannan, P. Chen, *Food Chem.* **2011**, *124*, 545–550. **DOI:**10.1016/j.foodchem.2010.06.068
39. P. Kaushik, K. Dowling, S. McKnight, C. J. Barrow, B. Wang, B. Adhikari, *Food Chem.* **2016**, *197*, 212–220. **DOI:**10.1016/j.foodchem.2015.09.106
40. Y. Cattan, D. Patil, Y. Vaknin, G. Rytwo, C. Lakemond, O. Benjamin. *Innov. Food Sci. Emerg. Technol.* **2022**, *75*, 102903. **DOI:**10.1016/j.ifset.2021.102903
41. M. Felix, M. Cermeño, R.J. FitzGerald, *Food Hydrocoll.* **2019**, *97*, 105220. **DOI:**10.1016/j.foodhyd.2019.105220
42. Z. Nikolić, V. Dordević, A. Torbica, A. Mikić, *J. Food Compos. Anal.* **2012**, *28*, 75–80. **DOI:**10.1016/j.jfca.2012.08.005
43. M. Barać, S. Čabrilo, M. Pešić, S. Stanojević, M. Pavlićević, O. Maćej, N. Ristić, *Int. J. Mol. Sci.* **2011**, *12*, 8372–8387. **DOI:**10.3390/ijms12128372
44. I. Berrazaga, C. Bourlieu-Lacanal, K. Laleg, J. Jardin, V. Briard-Bion, D. Dupont, S. Walrand, V. Micard, *PLoS One.* **2020**, *15*, e0232425. **DOI:**10.1371/journal.pone.0232425
45. T. S. Withana-Gamage, J. P. Wanasundara, Z. Pietrasik, P. J. Shand. *J. Sci. Food Agric.* **2011**, *91*, 1022–1031. **DOI:**10.1002/jsfa.4277
46. Barth, C. Zscherp, *Q. Rev. Biophys.* **2002**, *35*, 369–430. **DOI:**10.1017/S0033583502003815
47. A. Barth, *Biochim. Biophys. Acta Bioenerg.* **2007**, *1767*, 1073–1101. **DOI:**10.1016/j.bbabio.2007.06.004
48. J. Kong, S. Yu, *Acta Biochim. Biophys. Sin.* **2007**, *39*, 549–559. **DOI:**10.1111/j.1745-7270.2007.00320.x
49. I. H. M. Van Stokkum, H. Linsdell, J. M. Hadden, P. I. Haris, D. Chapman, M. Bloemendal, *Biochem.* **1995**, *34*, 10508–10518. **DOI:**10.1021/bi00033a024
50. Y. Wang, Y. Wang, K. Li, Y. Bai, B. Li, W. Xu, *LWT-Food Sci. Technol.* **2020**, *129*, 109563. **DOI:**10.1016/j.lwt.2020.109563
51. K. Shevkani, N. Singh, A. Kaur, J. C. Rana, *Food Hydrocoll.* **2015**, *43*, 679–689. **DOI:**10.1016/j.foodhyd.2014.07.024

52. C. H. Tang, Z. Ten, X. S. Wang, X. Q. Yang, *J. Agric. Food Chem.* **2006**, *54*, 8945–8950. **DOI:**10.1021/jf0619176

53. Y. Deng, L. Huang, C. Zhang, P. Xie, J. Cheng, X. Wang, S. Li. *Food Chem.* **2019**, *283*, 539–548. **DOI:**10.1016/j.foodchem.2019.01.083

Povzetek

Namen študije je optimizacija postopka ekstrakcije in karakterizacija beljakovin, ki jih najdemo v semenih triplata. Zmogljivosti zadrževanja vode in olja, vsebnost koaguliranih proteinov, penjenje in emulgiranje izoliranih proteinov so bile raziskane pri vseh pogojih ekstrakcije. Določene so bile tudi topnost, molekulske mase, strukturne in toplotne lastnosti. Pri ekstrakcijskih postopkih, izvedenih pri različnih pH (pH 6,0–12,0) in razmerjih trdna snov:topilo (20–60 g/L), je bilo ugotovljeno, da je bil največji izkoristek ekstrakcije (94,3 ± 0,3 %) dosežen pri pH 11,47 in razmerju med trdno snovjo in topilom 34,50 g/L. Določeni so bili trije različni pasovi (46, 59 in 80 kDa) v območju 22–175 kDa za proteinski izolat semena triplata, pridobljenega pri optimalnih pogojih ekstrakcije. Sekundarne strukture proteinov so bile določene z uporabo Fourierove transformacijske infrardeče spektroskopije (FT-IR) in ugotovljeno je bilo, da so bile dobro zastopane β-planarne strukture. Poleg tega sta bili izračunani temperatura in entalpija denaturacije kot ~119 °C, oziroma 28 mJ/g.