Effect of extraction parameters on the isolation of fenugreek seed protein and characterization of fenugreek protein concentrate

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Received: 12 April 2022; Accepted: 24 June 2022; Published: 11 July 2022

ORIGINAL ARTICLE

Abstract

The present study was accomplished with the isolation of fenugreek seed protein. Effect of extraction parameters on the isolation of fenugreek seed protein from defatted fenugreek seed powder and characterization of the isolated protein was done. The pH (10, 11, 12, and 13), time (15 and 20 min), and rpm (7500 and 8000) were used to extract fenugreek protein from defatted fenugreek seed powder. The obtained protein was characterized for its functional, structural, morphological, and thermal properties. SEM, XRD, FTIR, and DSC revealed that the protein extracted at pH 12 had better functional, morphological, structural, and thermal properties. Color values indicated the dark color of fenugreek protein at alkaline pH. It was found that the protein extracted at pH 12, time 20 min, and rpm 8000 showed desirable functional, structural, morphological, and thermal properties as compared to others.

Keywords: DSC; fenugreek; functional properties; pH; protein extraction; SEM

Introduction

In recent years, due to population explosion, protein consumption has increased and also the demand for edible protein from plant sources has risen. Plant proteins are the most abundant and cheapest sources of protein. It is the best alternative for animal-based protein. The growing concern related to health benefits has led to greater demand for plant protein day by day (Vinayashree and Vasu, 2021). An animal-based food is regarded as an ecological burden. As a result, foodstuffs derived from more sustainable sources, like plant proteins, must be expanded. In order to overcome the challenge of functionalizing and making plant protein nutritionally suitable at a high quality, food from plant proteins needs to be developed (Tanger *et al*., 2020). It is very important to provide sufficient and high-quality protein when the world's population is expected to grow by 10 billion by 2050 (Kalaydzhiev *et al*., 2020). Furthermore, the FAO estimated that the global demand for food will increase by half by 2050. Therefore, the demand for inexpensive and more justified sources of plant protein is increasing these days. Increasing the production of animal protein would have an adverse impact on the environment (Hadidi *et al*., 2021). In recent years, plant proteins are being widely used due to ethical concerns and also religious preferences (Gao *et al*., 2020).

Fenugreek (*Trigonella foenum graecum*) has its place in *Leguminosae*. Fenugreek being a multiregional plant is cultivated in India, in the Mediterranean region, and in the North African countries. Fenugreek seeds are mainly

 ISSN 1120-1770 online, DOI 10.15586/qas.v14iSP1.1148

used for culinary and medicinal purposes. Fenugreek seeds have promising medicinal properties such as lowering blood glucose levels, anti-cancer, anorexia, hypocholesterolemic, and gastric stimulant, and are used for lactation aid and hepatoprotective effect. Fenugreek has the property to influence digestion (Wani and Kumar, 2018). Studies indicated that fenugreek seeds have an abundant protein content of about 25–38%. Owing to their physicchemical properties, plant proteins are highly demanded as food ingredients. An extensive sort of functional property is offered by plant proteins. The functional characteristics of protein arise from the three-dimensional structure, charge distribution, and molecular size (Feyzi *et al*., 2018). Fenugreek has higher protein content and essential amino acids when compared to soybean. The main protein in fenugreek seeds consists of albumin (43.8%), globulin (27.2%), glutelin (17.2%), and prolamine (7.4%), respectively. Albumin is majorly present in fenugreek protein. The quality of protein present in fenugreek is as good as soybean protein. The lysine content present in fenugreek protein is comparable to soybean protein (Feyzi *et al*., 2015). Fenugreek seed protein is a rich source of amino acids such as leucine, tyrosine, phenylalanine, glutamic acid, aspartic acid, etc. (Hidvegi *et al*., 1984). Biologically significant free amino acid (2S, 3R, 4S)-4-hydroxy isoleucine (HIL) present in fenugreek seed is the most studied. 4-hydroxyl isoleucine has a high potentiality for insulin stimulating action (Işıklı and Karababa, 2005). Fenugreek protein fractions are stable enough, and it is not affected by cooking (Srinivasan, 2006).

Fenugreek seed flour has a very poor economic value after oil extraction. Fenugreek seed flour has a rich protein concentration, making it an appealing and potential plant-based protein source. In recent days, much attention is being given to revalorization and waste utilization of the food processing byproducts and the attributes of their alternatives to the food industry applications. Fenugreek protein is a significant source of low cost material for edible protein production.

We can hypothesize that fenugreek seed protein concentrate can be used for food application if the protein has desirable functional properties. Only a few investigations on the extraction and characterization of fenugreek seed protein have been conducted recently. The purpose of this study was to evaluate the protein and determine the fenugreek protein concentrate's functional, structural, morphological, and thermal properties.

Materials and Methods

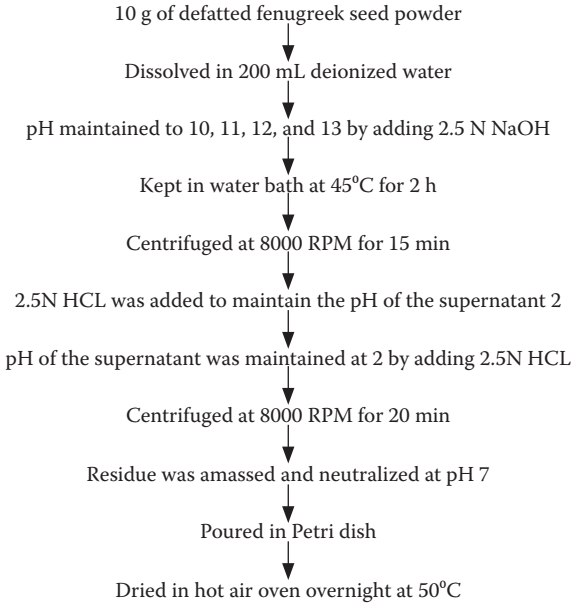
Raw materials and reagents

HSHM-57-Hisar Sonali, a specified variety, was selected from Haryana Agricultural University, Hisar, India. Analytical grade chemical reagents were bought from Sigma Aldrich, New Delhi, India. Adhered dust particles and dirt were removed from the fenugreek seeds manually. Seeds were also air dried and milled to flour. The milling process was carried out by the use of an electric mixer grinder. The milled flour was sieved after grinding and passed through an 80 mesh sieve size. The sieved flour was stored for further analysis.

Isolation of protein from defatted fenugreek powder

The extraction of proteins was carried out using the standardized method of Mir *et al.* (2019) with some modification. The defatted fenugreek seed powder was blended with water (deionized) (1:20, 10 g of defatted fenugreek in 20 mL of water, w/v). The pH of the solution was kept in the range of 10 to 13 by using a 2.5 N NaOH solution. The mix was then shaken while heating in a water bath for 2 h at a temperature of 45°C followed by centrifugation. The variables used for the extraction of proteins were pH, extraction time, and extraction rpm. The extraction pH was set at 10, 11, 12, and 13 (Fekria *et al*., 2012; Işıklı and Karababa, 2005; Lawal *et al*., 2007; Srinivasan, 2006), at two different extraction rpm (7500 × g and 8000 × g) and at two different extraction times (15 min and 20 min). The supernatant was accumulated and the sediment was disposed off. The pH of the supernatant was maintained at 2 by using 2.5 N HCL dropwise followed by isoelectric precipitation. Centrifugation was again performed on the precipitated protein at 8452 rpm for 20 min. The pH of the collected sediment was neutralized followed by drying at 50°C in a hot air oven for 24 h. The dried protein was stored in refrigerated conditions for further analysis.

Protein extraction flowchart



Functional, structural, morphological, and thermal analysis of protein powders

Emulsifying capacity (EC) and emulsifying stability (ES)

EC and ES were calculated by following the method of Lawal *et al*. (2007). Solution (5 mL) of 5% fenugreek protein was homogenized with 5 mL canola oil. The prepared emulsion was separated by centrifugation for 5 min at 4000 rpm. The length of the thickness of the two liquids and the entire length of the tubes were calculated. The EC of the fenugreek protein was evaluated as:

$$\textit{Emulsifying capacity}\ (\%) = \frac{\textit{length of the emulsified layer}}{\textit{length of the entire content of the tube}} \times 100 \quad (1)$$

ES was calculated by cooking the mix at 90°C for half-hour before centrifuging at 3500 rpm for 5 min. The ES was determined as:

$$\textit{Emulsifying stability}\ (\%) = \frac{\textit{length of the emulsified layer}}{\textit{length of the entire content in the tube}} \times 100 \quad (2)$$

Foaming capacity (FC) and foaming stability (FS)

FC and FS of protein concentrate extracted from fenugreek were estimated by following the method of Fekria *et al*. (2012) and Venktesh and Prakash (1993). Three grams of the protein concentrate was taken and mixed in 100 mL water. The solution was blended for 5 min at high speed in a mixer blender and then shifted into a cylinder. Foam volume was noticed. The FC was determined as:

$$FC\ (\%) = \frac{\textit{volume after whipping} - \textit{volume before whipping}}{\textit{volume before whipping}} \times 10 \quad (3)$$

Foaming stability was evaluated by calculating the deduction in the foam volume in the tube as a function of time, which was up to the interval of 30 min.

$$\textit{Foaming stability}\ (\%) = \frac{\textit{foam volume}}{\textit{initial foam volume}} \times 100 \quad (4)$$

Water binding capacity (WBC) and oil binding capacity (OBC)

WBC and OBC were determined by following the method of Shchekoldina and Aider (2014) with minor changes. For WBC, 1 g of the samples was combined with 10 mL of deionized water and agitated for 5 min. The mix was allowed to remain at room temperature for 30 min before being centrifuged at 4500 g for 30 min and the volume of the supernatant was determined. The supernatant was removed, and the tubes were inverted for 25 min at 45° to remove any remaining liquid. The ability to bind water was calculated as follows:

$$\textit{Water binding capacity}\ (\%) = \frac{a - b}{c} \quad (5)$$

Where,

A = Tube consisting of the protein concentrate and the water absorbed

B = Tube with protein concentrate

C = Protein concentrate

The OBC was also determined by the same method. One gram of the samples and 10 mL of canola oil was blended for OBC and stirred for 5 min. The solution was allowed to stay undisturbed for a time period of 30 min at ambient temperature and then centrifuged at 4500 × g for a period of 30 min and then the supernatant volume was calculated. The supernatant was separated, and the containers were tilted for 25 min at 45 °C to remove any remaining liquid. The ability to bind oil was calculated as follows:

$$\textit{Oil binding capacity}\ (\%) = \frac{a - b}{c} \quad (6)$$

Where,

A = Tube containing the protein concentrate and the oil absorbed

B = Tube with protein concentrate

C = Protein concentrate

Protein solubility (PS) and wettability

PS was evaluated by modifying the method of Mir *et al*. (2019). Protein isolate dispersions were prepared (2 g/100 mL); before using a magnetic stirrer, the slurries were mixed for 1 h. Centrifugation at 3000 g for 30 min was done. Micro-Kjeldahl technique was followed for the estimation of protein content in the centrifuged supernatant.

PS was evaluated as:

$$\textit{Protein solubility in}\ (\%) = \frac{SP}{PT} \times 100 \quad (7)$$

Where,

PT = total protein proportion in the sample calculated prior centrifugation, and SP is the protein proportion after centrifugation in the supernatant.

A static wetting test, as defined by Freudig *et al*. (1999), was used to measure the wettability of the protein powders. About 100 mL of water was filled into a beaker, and a glass funnel was set on top of the beaker in a ring stand.

A test tube was blocking the glass funnel's aperture. One gram of protein powder was filled in a funnel, and the test tube was quickly withdrawn to let the powder of protein to flow into the beaker. The time when all powder particles entered the water surface was recorded using a stopwatch.

Bulk density and tapped density

Followed by the method of El Nasri and Tinay (2007), the bulk density of the protein concentrate was evaluated. The sample was measured at 10 g and poured into a graduated cylinder. The cylinder was packed and tapped on the bench-top, sample volume reading was documented, and the bulk density was estimated in g/mL of the material. The tapped density was determined by beating the cylinder containing the sample on the tabletop 20 times. The result was expressed in g/mL.

Color measurement

Hunter Colour spectrophotometer (Model-Colour 15) was exploited for color measurements of fenugreek protein powder using $L^* a^* b^*$ values. Standardization of the instrument was done by the usage of black and white tiles before the sample measurement and the $L^* a^* b^*$ values were documented (Wani *et al*., 2021).

Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR analysis was obtained on a PerkinElmer Spectrum 400 (U.S). The FT-IR spectrum of the protein powder was recorded using an FT-IR spectrophotometer in the range of wavenumbers from 4000 to 400 cm^{-1}.

Scanning electron microscopy (SEM)

SEM was used for the determination of the surface morphology of the fenugreek protein concentrate. The concentrate was placed on the aluminium stubs by the use of adhesive tape with double-sided carbon. And then it was sputtered with a very fine layer of gold. 5 kV accelerating voltage was used for the testing of samples.

X-ray diffraction (XRD)

XRD of the fenugreek protein concentrate powder was evaluated by the use of Pan analytical-Xpert PRO; MRD, XRD with CoKa< 1 radiation. Diffractograms were observed in a scanning range between 5° and 70° at 1.20°/min for a period of 2 h and with a phase size of 0.05° (2 h).

Differential scanning calorimetry (DSC)

Thermal properties of the fenugreek protein concentrate powder were evaluated using DSC. Nitrogen gas was used as flushing or cooling gas at 20 mL/min. The fenugreek protein concentrate was taken around 3 to 4 mg and sealed using aluminium pans. Scanning of these pans was done at 20° to 200°C at 10°C/min. The peak temperature (Td) and the denaturation enthalpy (ΔH) were determined for all the samples.

Statistical analysis

For every experiment, three runs were carried out, and the data were statistically analyzed using StatSoft (Statistica 12.0). To assess significant ($p \leq 0.05$) differences, the data were subjected to an analysis of variance (ANOVA) and Duncan's multiple range test.

Results and Discussions

Effect of extraction parameter on the protein content of fenugreek protein concentrate

The effect of extraction pH, extraction rpm, and extraction time on the protein content of the FPC was studied. The extraction pH ranged from 10 to 13, and it was found that the protein content increased as the pH increased until pH 12 and then the protein content started decreasing. The extraction rpm also affected the protein content as it increased when the rpm increased from 7500 to 8000. Extraction time also played a significant role on the protein content of the FPC. As the extraction time increased from 15 min to 20 min, the protein content increased. The maximum protein content and yield at pH 10, 11, 12, and 13 were found at 8000 rpm and the extraction time of 20 min. The purity and yield of the extracted protein can be easily affected by processing conditions time, rpm, or g forces used for centrifugation (Russin *et al*., 2007). The protein content varied from 38.62 to 63.93% under experimental conditions. Maximum yield and protein content were found at pH 12, extraction rpm 8000, and the extraction time of 20 min. Zang *et al*. (2020) reported an increase in extraction yield from 33.58 to 61.25% with an increase in extraction pH from 8 to 13 for rapeseed protein, indicating that a higher concentration of alkaline media improves the extractability of proteins. The protein of fenugreek is more soluble at alkaline pH as reported by Meghwal and Goswami (2012). Alkaline conditions increase PS and extraction yield (Horax *et al*., 2011). Similarly, Gao *et al*. (2020) observed an increase in extraction yield with an increase in extraction pH. Ruiz *et al*. (2016) reported that at higher alkaline conditions the proteins are being negatively charged ascribed to the deprotonation of the amine groups and ionization of the carboxyl groups, in turn the electrostatic-repulsion among the negatively charged proteins is increased. Interaction between the protein and water increases and in turn the solubility increases. Similarly, according to Shen *et al*. (2008), the high alkali concentration aids in the detachment of hydrogen from carbonyl as well as sulfate groups by breaking down hydrogen bonds. It was observed that with a rise in pH from 10 to 12, the % of protein content and the weight of the protein concentrate increased but after pH 12 only the weight of the protein concentrate increased but the % of protein content

decreased. It led to the conclusion that a strong alkaline environment had an increased influence on the solubility of non-protein and extractability of non-protein compounds like polysaccharides as reported by Pickardt *et al*. (2009). Similarly, Ruiz *et al*. (2016) found that as the pH of extraction rises, the yield of protein also rises; however, the protein purity is reduced. Lesser protein content was found when samples were extracted at higher pH levels-(pH 13) because a greater amount of polysaccharide was added into the protein concentrate. Polysaccharides that are previously damaged by the milling practice are very prone to the strong alkaline conditions and as a result their solubility and extractability increase at higher pH values. When the extract solution's pH is reduced to the protein's isoelectric point, part of the solubilized polysaccharide precipitates with the protein (Han and Lim, 2004) due to which the weight and extraction yield increase but the purity of protein decreases.

Characterization of fenugreek protein concentrate

On the basis of the highest protein content in FPC, samples obtained at different pH were named FPC10, FPC11, FPC12, and FPC13 according to their extraction pH of 10, 11, 12, and 13, respectively. Furthermore, these FPC were characterized for various properties.

Emulsifying capacity and emulsifying stability of fenugreek protein concentrate

EC and ES were used to determine the emulsion activity of the FPC powder. Emulsifying activity may be defined as the capability of proteins to adsorb at oil–water interface based on the hydrophobicity and ionic charge of the protein, and emulsifying stability may be defined as the capability of proteins to form and stay at oil–water interface upon storage of emulsion depending on the requisite balance between the flexibility and molecular size, surface hydrophobicity, and charge (Zang *et al*., 2020). The EC and ES of FPC ranged from 40.06 to 56.66% and 46.6 to 58.02%, respectively, as shown in Table 2. The EC was found to be increasing as the extraction pH increased from pH 10 to pH 12 and then at pH 13 the EC decreased. The reason could be the protein content (%) of the FPC. Maximum EC was observed for the FPC obtained at pH 12 (56.66%) as the protein content of the FPC was higher at pH 12. Similarly, increases in emulsifying properties were observed with an increase in extraction pH for chia protein isolate (Lopez *et al*., 2018). The EC of FPC was found to be higher than sunflower protein (49.56%) obtained by Malik and Saini (2018). The discrepancies in FPC extracted at various pH might be linked to the fact that fenugreek proteins isolated at various alkaline pH had varying powers to induce repulsive contacts among oil droplets. Colloidal interactions between oil droplets are influenced by surface activity, electrical charges, and surface hydrophobicity. High solubility, in particular, is known to be required for fast migration to the oil–water interface. As a result, larger soluble protein content may improve oil droplet coverage, promoting colloid stability (Lopez *et al*., 2018). The maximum ES was found for FPC 12 (58%) which was similar to the findings of Malik and Saini (2018) for sunflower protein isolate (55.52%). Emulsifying stability of FPC was lowest for the protein extracted at pH 10 (46%) and highest for the protein extracted at pH 12 (58.57%). Emulsion stability is

Table 1. Influence of extraction parameter on the protein content of fenugreek protein concentrate.

Experiment no.	pH	Rpm	Extraction Time (min)	Yield (%)	Protein content (%)
1	10	7500	15	2.9 ± 0.15^{p}	38.62 ± 0.21^{p}
2	10	7500	20	3.4 ± 0.11^{o}	40.82 ± 0.18^{o}
3	10	8000	15	3.6 ± 0.13^{n}	43.25 ± 0.14^{n}
4	10	8000	20	4.2 ± 0.10^{m}	44.82 ± 0.17^{m}
5	11	7500	15	5.0 ± 0.16^{l}	51.78 ± 0.20^{k}
6	11	7500	20	5.9 ± 0.12^{k}	52.96 ± 0.17^{i}
7	11	8000	15	6.9 ± 0.15^{j}	55.26 ± 0.14^{g}
8	11	8000	20	8.1 ± 0.11^{i}	57.62 ± 0.16^{e}
9	12	7500	15	9.8 ± 0.17^{h}	58.87 ± 0.13^{d}
10	12	7500	20	12.0 ± 0.10^{g}	59.92 ± 0.18^{c}
11	12	8000	15	14.1 ± 0.14^{f}	60.66 ± 0.15^{b}
12	12	8000	20	16.0 ± 0.11^{e}	63.93 ± 0.13^{a}
13	13	7500	15	16.8 ± 0.16^{d}	48.87 ± 0.16^{l}
14	13	7500	20	17.5 ± 0.13^{c}	52.26 ± 0.19^{j}
15	13	8000	15	18.2 ± 0.11^{b}	55.06 ± 0.17^{h}
16	13	8000	20	19.3 ± 0.15^{a}	55.98 ± 0.15^{f}

The mean ± SD readings in the columns preceded by various letters differ considerably ($P \leq 0.05$). The results are the averages of three determinations.

Table 2. Emulsifying capacity, emulsifying stability, foaming capacity, and foaming stability of fenugreek protein concentrate.

Sample	EC (%)	ES (%)	FC (%)	FS (%)
FPC10	40.06 ± 0.52^{d}	46.78 ± 0.50^{c}	40.21 ± 1.52^{d}	82.40 ± 0.47^{d}
FPC11	50.03 ± 0.55^{b}	48.02 ± 0.45^{a}	60.66 ± 1.06^{b}	87.50 ± 0.50^{b}
FPC12	56.66 ± 0.51^{a}	58.57 ± 0.44^{b}	66.29 ± 1.00^{a}	90.33 ± 0.57^{a}
FPC13	49.86 ± 0.52^{c}	46.61 ± 0.51^{d}	50.89 ± 1.26^{c}	84.93 ± 0.30^{c}

The values reported as mean ± SD in the column with different characters show significant (P ≤ 0.05) differences. The results are the averages of three determinations.

increased by increasing the concentration of protein. This is because a rise in pH results in a rise in the columbic revulsion between adjacent drops associated with an increase in hydration of energy-containing protein molecules. Followed by the combination of emulsion droplets and deduction in interface energy, the protein accounted for higher ES as explained by Chavan *et al*. (2001).

Foaming capacity and foaming stability of fenugreek protein concentrate

The FC of FPC ranged from 40.21 to 66.29%. The lowest FC (40.21%) was obtained for the FPC extracted at pH 10. The reason could be that, at this moment, the molecules tend to be more compact as compared to FPC extracted at other pH points. The FC of FPC increased considerably, especially at pH 11 and 12, reaching 60.66 and 66.66%, respectively. Similarly, Cui *et al*. (2020) observed increasing FC with an increase in extraction pH from 8.5 to 10 due to higher protein content. The capability to adsorb quickly at the air–water boundary at the time of bubble formation and the capacity to endure quick conformational change and rearrangement at the boundary is the basic necessity for proteins to be a worthy foaming agent (Fidantsi and Doxastakis, 2001). The status of FC contrary to pH for FPC was somewhat comparable to the nitrogen solubility profile attributed to the rise in the proteins net-charge weakening the hydrophobic interaction and increased protein flexibility and solubility letting the protein to rapidly expand to the air–water interface by encapsulation of air, resulting in increased formation of foam as documented by Lawal *et al.* (2007) for African fenugreek. The FPC possesses a stronger FC than other plant proteins such as lupine 58%, as reported by Alsohaimy *et al.* (2007). The FS value was maximum for the protein extracted at pH 12 which was 90.33% standing for 30 min at ambient temperature. The value obtained is greater than the value reported by Wang *et al.* (2010) for chickpea and pea protein (30 to 40%). An increase in extraction pH enhanced the FS of the protein ascribed to more surface activity and solubility of the soluble protein. The minimum FS was observed for FPC 10 (82.40%). That may be owing to the low protein content in FPC 10 and the presence of additional non-protein components that prevent foam formation in FPC.

Water binding capacity and oil binding capacity of fenugreek protein concentrate

The WBC and OBC are two essential parameters for texture and flavor in foods consisting of proteins. The WBC is the property to absorb water by means of hydrogen bonds among water molecules and polar groups of proteins and the OBC may be defined as the property to absorb oil depending on the bonding of the hydrocarbon chain of oil and the non-polar part of proteins (Zang *et al*., 2020). The protein concentrate of fenugreek had a WBC ranging from 3.07 to 3.58 mL H_2O/g of protein, as indicated in Table 3. Results are similar to the results observed by Abdel-Aal *et al*. (1986) which was 3.52 mL H_2O/g of protein for Egyptian fenugreek. The FPC showed good water holding capacity. This might demonstrate that the protein concentrate has a good capability to inflate, unfold, and disassociate disclosing more binding sites. WBC is one of the most significant properties of proteins in sticky foods like dough, soups, baked goods, custards, and so on, and it is attributed to the protein's ability to absorb water without dissolving, resulting in thick and viscous (Adeyeye *et al*., 1994; Seena and Sridhar, 2005). The OBC of protein concentrate of fenugreek was observed to be between 1.09 and 1.89 mL oil/g of protein extracted at varied extraction pH, which is close to that of cowpea protein isolate (1.66 mL oil/gram) revealed by Mwasaru *et al*. (1999). Kinsella (1979) proposed a physical trapping of oil as the mechanism of OAC. OAC is an essential determinative factor of flavor. The fat emulsion activity and stability are salient attributes for additives for fat emulsion stabilization. The acquired result in this analysis showed that the FPC had very good OAC.

Solubility and wettability of fenugreek protein concentrate

One of the most prerequisite functional properties of protein is solubility because it greatly affects other properties like emulsification, gelation, and foaming properties (Gao *et al*., 2020). The factors on which the solubility of protein depends are the composition, surface features of basic amino acids, molecular weight as well as the environmental elements like ionic strength, temperature, and pH (Culbertson, 2005). Protein solubility of FPC ranged from 48.49 to 76.53%, as indicated

Table 3. Water binding capacity, oil binding capacity, solubility, and wettability of fenugreek protein concentrate.

Sample	Water binding capacity (g/g of protein)	Oil binding capacity (g/g of protein)	Solubility (%)	Wettability (minutes)
FPC10	3.35 ± 0.08^{b}	1.89 ± 0.12^{a}	48.49 ± 0.31^{d}	19.17 ± 0.51^{a}
FPC11	3.20 ± 0.06^{c}	1.09 ± 0.08^{d}	57.23 ± 0.35^{b}	16.40 ± 0.54^{b}
FPC12	3.58 ± 0.09^{a}	1.55 ± 0.11^{c}	76.53 ± 0.36^{a}	12.27 ± 0.57 ^{d}
FPC13	3.07 ± 0.06^{d}	1.68 ± 0.09^{b}	65.75 ± 0.37^{c}	14.22 ± 0.59^{c}

The values reported as mean ± SD in the column with different characters show significant (P ≤ 0.05) differences. The results are the averages of three determinations.

Table 4. Bulk density, tapped density, and color of fenugreek protein concentrate.

Sample	Bulk density (g/mL)	Tapped density (g/mL)	*L**	*a**	*b**
FPC10	0.97 ± 0.02^{a}	0.81 ± 0.03^{a}	61.12 ± 0.02^{a}	3.69 ± 0.03^{d}	18.87 ± 0.02^{a}
FPC11	0.98 ± 0.02^{a}	0.83 ± 0.02^{a}	53.50 ± 0.04^{b}	4.41 ± 0.01^{a}	13.79 ± 0.03^{b}
FPC12	0.96 ± 0.01^{a}	0.85 ± 0.01^{a}	52.04 ± 0.01^{c}	4.14 ± 0.01^{b}	11.96 ± 0.01^{c}
FPC13	0.99 ± 0.03^{a}	0.84 ± 0.02^{a}	49.12 ± 0.02^{d}	4.04 ± 0.03^{c}	9.48 ± 0.02^{d}

The values reported as mean ± SD in the column with different characters show significant (P ≤ 0.05) differences. The results are the averages of three determinations.

in Table 3. Protein solubility increased with an increase in extraction pH. Similarly, Zang *et al*. (2020) observed an increase in PS with an increase in extraction pH. Protein molecules have a net negative or positive charge when pH levels are greater or below the isoelectric point, which considerably increases the protein–water interaction, resulting in improved solubility. Seena and Sridhar (2005) revealed that protein possesses net negative and positive charges at highly alkaline pH which is advantageous in the molecules repulsion, consequently increasing the PS. This can be ascribed to electrostatic repulsive force between the proteins carrying a positive charge, which helps them to stay apart resulting in increased protein-solvent interactions (Mao and Hua, 2012).

The potential of water to adhere to or expand on the surface of a solid in the company of other nonmiscible fluids is wettability. It is affected by many elements like surface polarity, topography, texture area, particle size as well as the structure of protein molecule (Hägerdal and löfqvist, 1978). The presence of amphipathic chemicals, porosity, density, surface area, surface charge, and the contact angle among the penetrating water and powder surface are all factors to consider. The highest wettability (19.17 min) was reported in FPC 10 extracted at pH 10, whereas the lowest value was for FPC12 extracted at pH 12. The reason for lesser time might be the company of more water loving groups in the protein of fenugreek protein to imbibe water (Mir *et al*., 2019). After 20 min, all the wetted samples had sunk to the bottom.

Bulk density and tapped density of fenugreek protein concentrate

Bulk density may be defined as the heaviness of the flour sample. Bulk density of FPC ranged from 0.97 to 0.99 g/mL which is more than reported by El Nasri and Tinay (2007) for FPC protein concentrate and the tapped density ranged from 0.81 to 0.85 g/mL. However, there was not much difference in the bulk density and tapped density value, as shown in Table 4. The extraction pH did not affect the bulk density and tapped density.

Color of fenugreek protein concentrate

*L**, *a**, and *b** values of FPC were evaluated and shown in Table 4. The *L** value represents lightness, the higher the *L* value the lighter is the sample. Positive *a** value represents the red color and positive *b** value represents yellowness. The values showed the dark and yellowish colors of protein concentrate. The FPC extracted at pH 10 had *L** value (61.12), *a** value (3.69), and *b** value (18.87). The *L** and *b** values decreased as the extraction pH increased from pH 10 to pH 13. This was because extraction at higher pH results in the darker color of protein. The minimum *L** value was 49.12, *a** value was 4.04, and *b** value was 9.48, observed at pH 13. Similarly Zang *et al*. (2020) reported a decrease in *L** value with increasing pH indicating the color change of rapeseed protein from bright yellow to black. The dark color of FPC may be ascribed to polyphenols and pigments with an increasing alkaline medium which might bind to the fenugreek protein, resulting in an oxidized colored product (Lopez *et al*., 2018). Kaur and Singh (2007) reported *L** value (58.63 to 61.33), *a** value (1.88 to2.21),

and b^* value (22.46 to 24.55) for chickpea protein concentrate similar to FPC.

Fourier-transform infrared spectroscopy analysis of fenugreek protein concentrate

FTIR is a very valuable tool for the determination of proteins' secondary structure based on infrared bands (Cai and Singh, 1999). The infrared spectral data are generally explained with reference to vibrations of the repeat component. The repeated units of polypeptide bring about nine characteristic infrared absorption bands, which are amide A, amide B, and amide I to VII. The two most prominent vibrational bands out of these are amide I and amide II bands of the protein backbone (Adochitei and Drochioiu, 2011). The FT-IR spectra of fenugreek proteins extracted at pH 10, pH 11, pH 12, and 13 were analyzed in the range of 600–4000 cm^{-1} and the spectra are displayed in Figure 1. The intermolecular interactions between the bonds were assessed. It can be depicted that as the extraction pH increases the concentration of protein also positively affects the bonding. The reason for increased bonding at higher pH is possibly due to the steady unfolding of the tertiary structure of a protein that was extracted at upper pH, i.e., alkali (Chen, 2013). As the extraction pH increased, a shift in bond length was observed. The shift specifies the chemical process of protein unfolding which may weaken the hydrogen bond of the β-sheet structure (Coelho *et al*., 2018). Variation in the wavenumber of protein could result from variation in the composition of amino acids, functional groups, and interactions between them (Kudre *et al*., 2013). FTIR of FPC extracted at different pH had strong bonding as shown by the presence of Amide-A (3267.64 cm^1) and Amide-B (2925.40 to 2928.32 cm^1) ascribed due to NH stretching vibrations. The frequency is affected by hydrogen bonding (Barth and Zscherp, 2002). Amide I band was observed at (1641.02–647.95 cm^1) which is attributed to C = O, C-N stretching, and this band is considered to be the most informative band about the secondary structure of the protein. Amide II band was observed at (1537.99–156.24cm^1) which corresponds to C-N stretching and N-H bending. The two major bands Amide I and Amide II are considered conformationally sensitive bands of the protein infrared spectrum (Krimm and Bekar, 1986). Amide III band was observed associated with various peptide conformations. The band below 1400 represents Amide III (1396.90cm^1). The peaks observed at (604.00–698.67cm^1) represent Amide IV which attributes to the O-C-N bending.

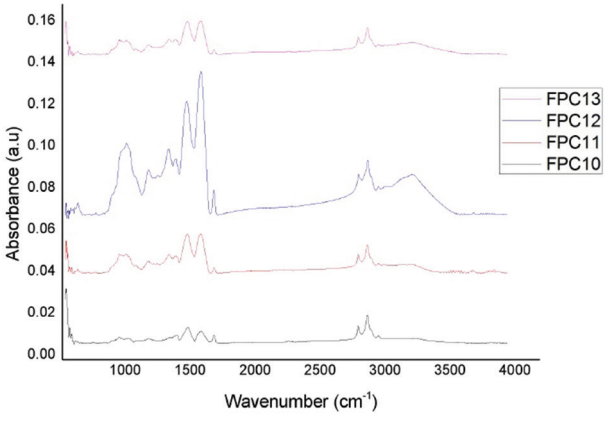


Figure 1. Fourier-transform infrared spectroscopy spectrum fenugreek protein concentrate obtained at pH 10, pH 11, pH 12, and pH 13.

Surface morphology of fenugreek protein concentrate

The structural morphology of the FPCs extracted at different pH was explored by the scanning electron microscope (SEM), as depicted in Figure 2. The surface of the FPC appeared rough and irregular. The rough and irregular surface of the FPC might be due to alkaline extraction and the isoelectric precipitation technique. This might have changed the protein microstructure. It was observed that as the extraction pH increased the compactness decreased. The reason could be the higher alkaline condition which might have decreased the compactness in the protein structure ascribed to the unfolding and uncoiling of protein structure. A similar surface morphological structure was observed in quinoa protein by Mir *et al*. (2019).

X-ray diffraction of fenugreek protein concentrate

Two diffraction peaks in the range of (12.49°–12.58°) small intensity and other in the range of (18.48°–20.49°) high intensity were observed for all the four powders extracted at different pH values, namely, 10, 11, 12, and 13, as shown in Figure 3. The FPC represented only two diffraction peaks which depict less amount of crystalline structure present in the powder. The broader peak in FPC represents a smaller crystallite size or partial crystalline structure of the material. The partial crystalline structure of FPC may be due to oven drying of the protein powders for a longer duration of time. Similarly, Joshi *et al*. (2011) reported two diffraction peaks (10° and 24°) for lentil protein isolate.

Differential scanning calorimetry of fenugreek protein concentrate

The differential scanning calorimeter was performed to investigate the thermal properties of the FPC extracted at different pH values. Differential Scanning Calorimetry is considered as the most frequently used technique to determine the thermal stability of proteins. The thermal stability of proteins functionally indicates their resistance to aggregation in response to heating (Horax *et al*., 2004). Tm is the onset temperature, Td is the peak denaturation temperature, Tc is the end set temperature, and ΔH is the heat of transition and enthalpy. All these factors were determined. Exothermic reactions were observed through the thermographs. Td is the temperature where the transition occurs, and it is a measure of thermal stability. ΔH

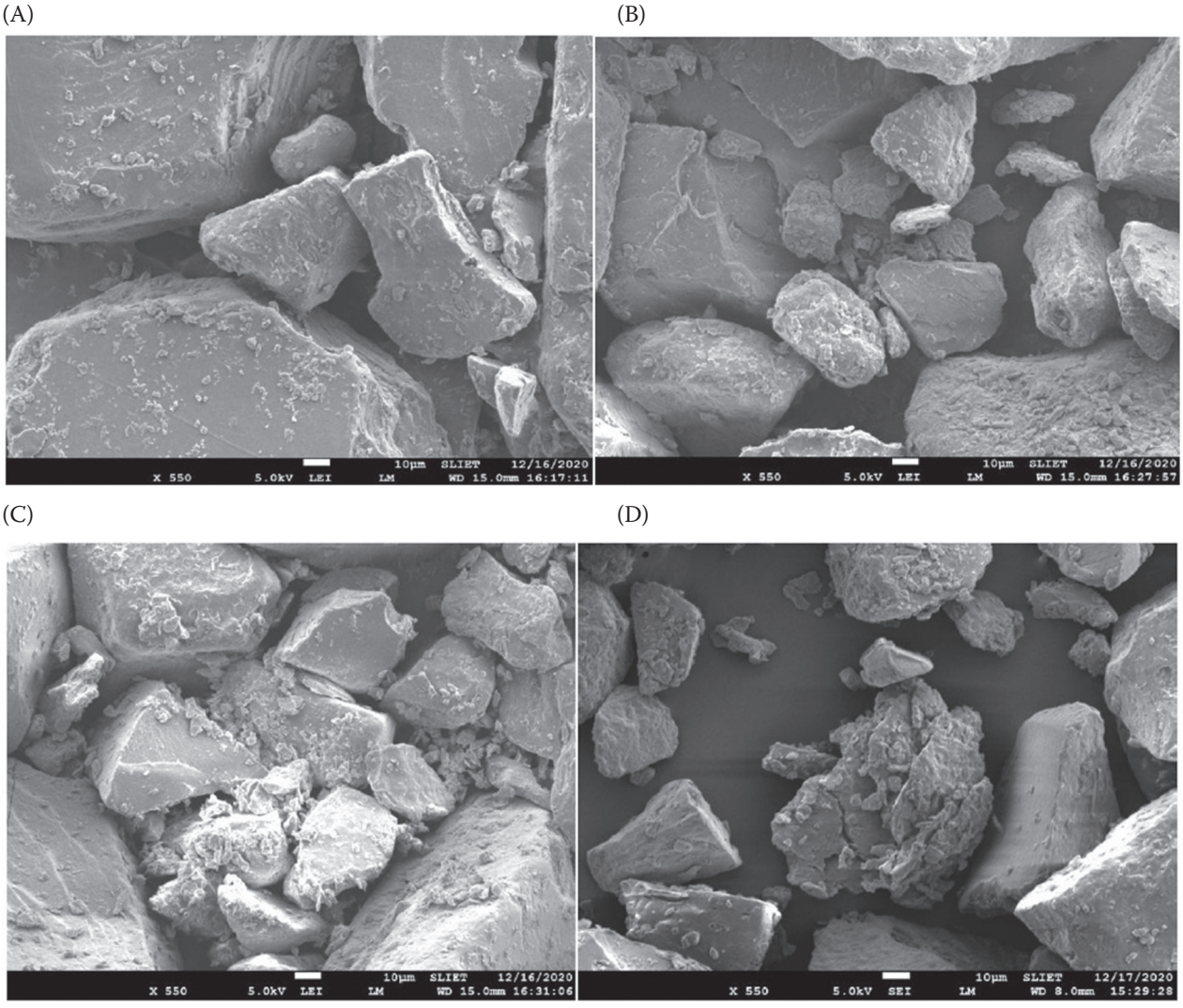


Figure 2. SEM image (×550 magnification) of fenugreek protein concentrate obtained at pH 10, pH 11, pH 12, and pH 13.

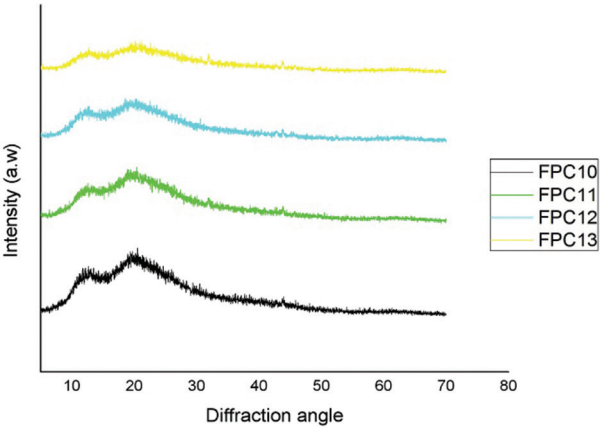


Figure 3. X-ray diffraction of fenugreek protein concentrate obtained at pH 10, pH 11, pH 12, and pH 13.

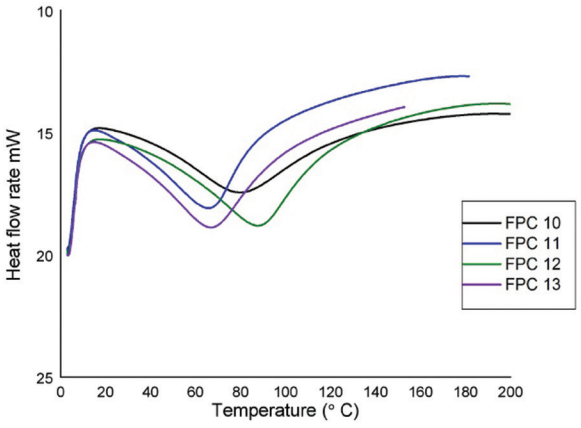


Figure 4. Differential scanning calorimetry of FPC extracted at pH 10, pH 11, pH 12, and pH 13.

indicates hydrophobic and hydrophilic interaction as well as the compactness of proteins (Ma and Harwalkar, 1991). The proportion of proteins that do not denature during the process is monitored by ΔH (Biliaderis, 1983).

Tm (onset temperature) for FPC extracted at different pH ranged from 38.89°C to 49.61°C. Td (thermal denaturation temperature/peak temperature) was observed in the range of 80.21°C–88.02°C, as shown in Figure 4. ΔH

(heat transition/enthalpy) ranged between 183.3832 J/g and 310.7540 J/g. Similarly, Mir *et al*. (2019) reported Td to be in the range of 75.90°C–84.56°C for chickpea protein isolate. The heat stability of protein is controlled by polar and non-polar balance and protein with higher heat stability (higher Td value) has higher non-polar proportion (Biliaderis, 1983).

Conclusions

The current study unveiled the impact of extraction pH-10, 11, 12, and 13 that significantly affected the functional properties of the FPC. The protein obtained from fenugreek displayed better functional properties, and it can be further used for edible applications. Dark-colored proteins were obtained due to alkaline pH, resulting in lower L^* values. Differences in extraction-pH led to variation in the morphological, thermal, structural, and functional properties manifested by the samples. Fenugreek protein extracted at pH 12 had the highest protein content and displayed better functional properties. SEM revealed unfolding and uncoiling of protein structure at higher alkaline-pH. FTIR and DSC depicted strong chemical bonding and better thermal resilience property for the protein extracted at pH 12, respectively. The study, therefore, dispenses information on possible manipulation of extraction pH variation to achieve desired food functionalities.

Funding: None

Conflicts of Interest: None

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