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Structural, thermal, and physicochemical properties of ultrasound-assisted extraction of faba bean protein isolate (FPI)

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ABSTRACT

This study aimed to understand the impact of ultrasonication (24 kHz) at a fixed power (100 W) and duration (5–60 min) on the physicochemical, structural, and thermal properties of faba bean protein isolates. Ultrasound-relevant parameters such as acoustic density, intensity, and yield conversion were estimated. The average protein purity (~85% protein) and yield (up to 22 %) of the ultrasound-assisted protein isolate were higher than those of the control protein isolate. Compared to the original flour, the isolate had significantly lower levels of vicine and convicine. Electrophoresis revealed no substantial alterations in the primary structure of the native and sonicated faba bean isolates. SDS-PAGE and SE-HPLC showed that sonicated samples had profiles similar to those of the native protein isolate. FTIR spectra and X-ray diffraction (XRD) patterns were used to measure structural changes in all faba bean protein isolates (FBPI). XRD study revealed two distinct diffraction peaks at $2\theta = 10^{\circ}$ and $2\theta = 20^{\circ}$ for protein isolates, indicating that alteration in native conformational crystallite size was altered after the ultrasound application.

1. Introduction

Historically, animal proteins have been the primary protein source in human diet. However, animal-based diets are increasingly creating concerns about environmental sustainability. Alternative sources of protein can reduce the need for land and reduce greenhouse emissions. (Scarborough et al., 2023). Grain legumes are valuable for use as feed and food because of their high protein content. Faba bean has a high protein content compared to some legumes, outperforming peas (Pisum sativum L.) and other cool-season grain legumes. In 2016, 4.5 million tonnes of faba bean crop were grown globally, with China, Ethiopia, and Australia being the top producers (Khazaei et al., 2019). Among plant proteins, faba bean seeds are easy to grow, have better nitrogen-fixing ability, and have grown in popularity as a protein source (Dhull et al., 2022).

Despite their numerous benefits, faba beans produce anti-nutritional factors (vicine, phytic acid, saponins, condensed tannins and others) that can be detrimental by limiting nutrient digestibility and availability (Mayer Labba et al., 2021), thereby limiting their application in food and feed formulations (Badjona et al., 2023a). Vicine and convicine are the two major antinutrients found in faba beans. For people who are genetically deficient in glucose-6-phosphate dehydrogenase, the accumulation of aglycones could be hazardous and cause hemolytic anemia (Badjona et al., 2023b). Approximately 400 million individuals worldwide are afflicted by favism, with Asia, the Mediterranean, and Africa having the highest prevalence rates (Cappellini and Fiorelli, 2008). Nonetheless, faba bean protein is presently utilised in limited quantities in food systems because of its relatively lower solubility and functional properties when compared to conventional protein sources such as animal proteins (e.g., whey and egg proteins) (Badjona et al., 2023c). Protein isolates derived from cereals and pulses are generally classified based on their protein content, which is approximately 80 %. Proteins in pulses, such as faba beans, are mainly in their storage forms that can be categorized into 2S and 7S subunits based on their sedimentation coefficients (Lu et al., 2020). The hexameric holoproteins that make up 11S globulin proteins are different from the trimer of polymorphic subunits that encodes vicin (7S) that is made up of several gene families (Zheng et al., 2022).

Traditional methods for extraction of proteins from plant-based sources are limited because of low protein yield and limited functionalities due to the presence of cell walls and structural membranes, which limit the extraction of the maximum amount of proteins. In plant cell

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walls, proteins are usually structurally bound to biomolecules such as lipids, carbohydrates, and secondary metabolites, thereby limiting full extraction potential. Thus, to improve and maximize the extraction yield, pre-treatment or assisted extraction using ultrasonication can maximize structural collapse and improve protein yield with additional benefits of improved functionality. Ultrasound can boost the extraction yield and functionality of plant proteins by creating cellular wounds and disintegrating molecules attached to proteins (Ampofo and Ngadi, 2022). Ultrasound is a low-impact, environmentally friendly technology that shows promise and is frequently utilised in food processing and the extraction of biomolecules from plants, animals, and marine sources (Ojha et al., 2020). Improved yield, shorter processing times, less solvent use, and little environmental impact are just a few benefits of ultrasound-assisted extraction. Studies using ultrasound support for protein extraction have been performed on Capsicum seed, Rice bran, rapeseed meal and soybean (Jahan et al., 2022).

During ultrasonic treatment, two major reactions occur mainly protein solubilization from cell wall and transport of proteins from inside cells into the medium. Application of ultrasonic waves results in generating bubbles which collapse asymmetrically in the vicinity of the cells, enhancing solvent penetration thereby accelerating hydration and consequently resulting in the disintegration of cell walls. Following cell wall lysis, ultrasonication accelerates transport of proteins from inside the cell into the medium. Thus, ultrasound treatment as a micro mixing procedure can promote extraction of protein evidenced by the increase in extraction yield and protein purity of faba bean protein isolates.

Traditional alkaline isoelectric extraction of proteins is constrained by low protein recovery, low protein purity, and decreased protein quality and functionalities. However, these restrictions can be overcome by employing novel and sustainable physical treatments that reduce the amount of chemical reagents required. This improves protein quality and environmental sustainability, increases protein yield, and modifies the complex bonds of proteins (Hewage et al., 2022). The use of ultrasound to extract plant proteins, particularly from faba beans, has received limited attention in the literature. An in-depth investigation of the impact of ultrasonication on protein research is required to consider its eco-innovative nature and disruptive cavitation effect, as this information will help the food industry make tactical choices regarding the extraction and formulation of sustainable plant proteins. Protein extraction conditions should be selected and monitored based on their ability to extract proteins while preserving their structural integrity. Protein purity and yield can be used to measure the extraction efficiency. Mild and attainable extraction procedures that generate protein isolates with high protein purity and yield as well as adequate functionality require exploration. Thus, the goals of this study were to (1) evaluate faba bean protein extraction conditions to maximize protein purity and yield after ultrasound-assisted alkaline solubilization with isoelectric precipitation, and (2) characterize the influence of ultrasound conditions on protein structural, thermal, and physicochemical properties. Ultimately, the results of this study could influence the extraction of proteins from faba beans using eco-innovative procedures to maximize the yield and protein properties for various food applications.

2. Materials and methods

2.1. Raw material, chemicals, and reagents

Faba bean seeds were purchased from Whole Foods Earth (Kent, United Kingdom). Vicine standard, NaOH, Methanol (≥99.9 % pure), phosphate-buffered saline (PBS), and HCI were purchased from Sigma-Aldrich (United Kingdom). Ethylenediamine Tetraacetic Acid, Disodium salt, Sodium phosphate monobasic, sodium tetraborate, Tris (hydroxymethyl)-methylamine were purchased, Sodium dodecyl sulfate and Potassium chloride were purchased from Thermo Scientific. Faba beans seeds and chemicals were stored at 4 °C. All the chemicals and reagents used in this study were of analytical grade.

2.2. Alkaline isoelectric precipitation of faba bean protein isolate

The faba bean isolate was prepared following the standard procedure employed by Sheikh et al. (2023) and Shen et al. (2017), with modifications. Whole faba beans were dehulled and milled into flour using a laboratory-type cyclone mill (sieve size of 0.5 mm). The control protein isolate (FBPI) was prepared as follows: 50 g flour was dispersed in 500 mL water (1:10 w/v) with 1 g of sodium chloride in the mixture. For alkaline solubilization, the suspension was stirred at 400 rpm using a magnetic stirrer for 60 min and adjusted to pH 11 using 1M NaOH. The mixture was centrifuged at 6000 rpm for 20 min at 25 °C. After centrifugation, the supernatant was collected and adjusted to pH 4.0, using 1 M HCI while stirring for 30 min. Precipitated protein pellets were obtained after centrifugation at 6000 rpm for 20 min at 25 °C. Isolates were re-dissolved (1:5 w/v) (to remove non-proteinaceous materials) while stirring for 10 min, after which the pH was adjusted to 4, followed by centrifugation at 6000 rpm for 20 min at 25 °C to obtain protein pellets (Fig. 1). The precipitated isolates were freeze-dried for 48 h, and the resulting protein was stored at −20 °C.

$$\text{Extraction yield } (\%) = \frac{m_i}{m_s} \times 100\ \% \quad \text{(Eq.1)}$$

$$\text{Protein yield } (\%) = \frac{m_i \times cp_i}{m_s \times cp_s} \times 100\ \% \quad \text{(Eq.2)}$$

The masses of the protein isolate and starting flour sample are denoted by m_i and m_s, respectively. The protein content of the isolate was denoted by cp_i, whereas the initial protein content of the flour sample was indicated by cp_s.

2.3. Ultrasonication-assisted alkaline extraction of faba bean protein isolate

Prior to the ultrasonic-assisted extraction (USAE), dispersions of 1:10 w/v were stirred at 25 °C for 20 min at 400 rpm. In the USAE treatment, pH was adjusted to 11, followed by ultrasonic treatment for 5–60 min with temperature maintained at 20–25 °C using an Ultrasonic (Teltow, Germany) with a S24d22D titanium horn. The mixture was centrifuged at 6000 rpm for 20 min at 25 °C after ultrasonic treatment. The supernatant was collected, and the pH was adjusted to 4.0 using 1 N HCI, and stirring was continued for 30 min. Precipitated protein pellets were obtained after centrifugation at 6000 rpm for 20 min at 25 °C. Isolates were re-dissolved (1:5 w/v) (to remove non-proteinaceous materials) while stirring for 10 min, after which the pH was adjusted to 4, followed by centrifugation at 6000 rpm for 20 min at 25 °C to obtain protein pellets. The precipitate was freeze-dried for 48 h, and the resulting protein was stored at −20 °C. The experimental parameters used for control and ultrasound-assisted protein extraction are listed in Table 1 (supplementary material).

2.3.1. Ultrasonic parameters

The actual acoustic power of the USAE extraction can be calculated using heat loss (Guimarães et al., 2020). The emitter surface area of the tip was 3.8 cm^2 and volume of the sonicated mixture was 550 mL. The calculated ultrasonic parameters are listed in Table 2 (supplementary material).

The acoustic power was calculated using Equation (3):

$$P = mC_p dT/_{dt}$$

The mass of the sonicated liquid and water specific heat capacity is denoted by m and C_p (4.18 J/g/° C). The temperature increase for the first 90 s during ultrasonication is denoted as dT/dt (° Cs^{-1}).

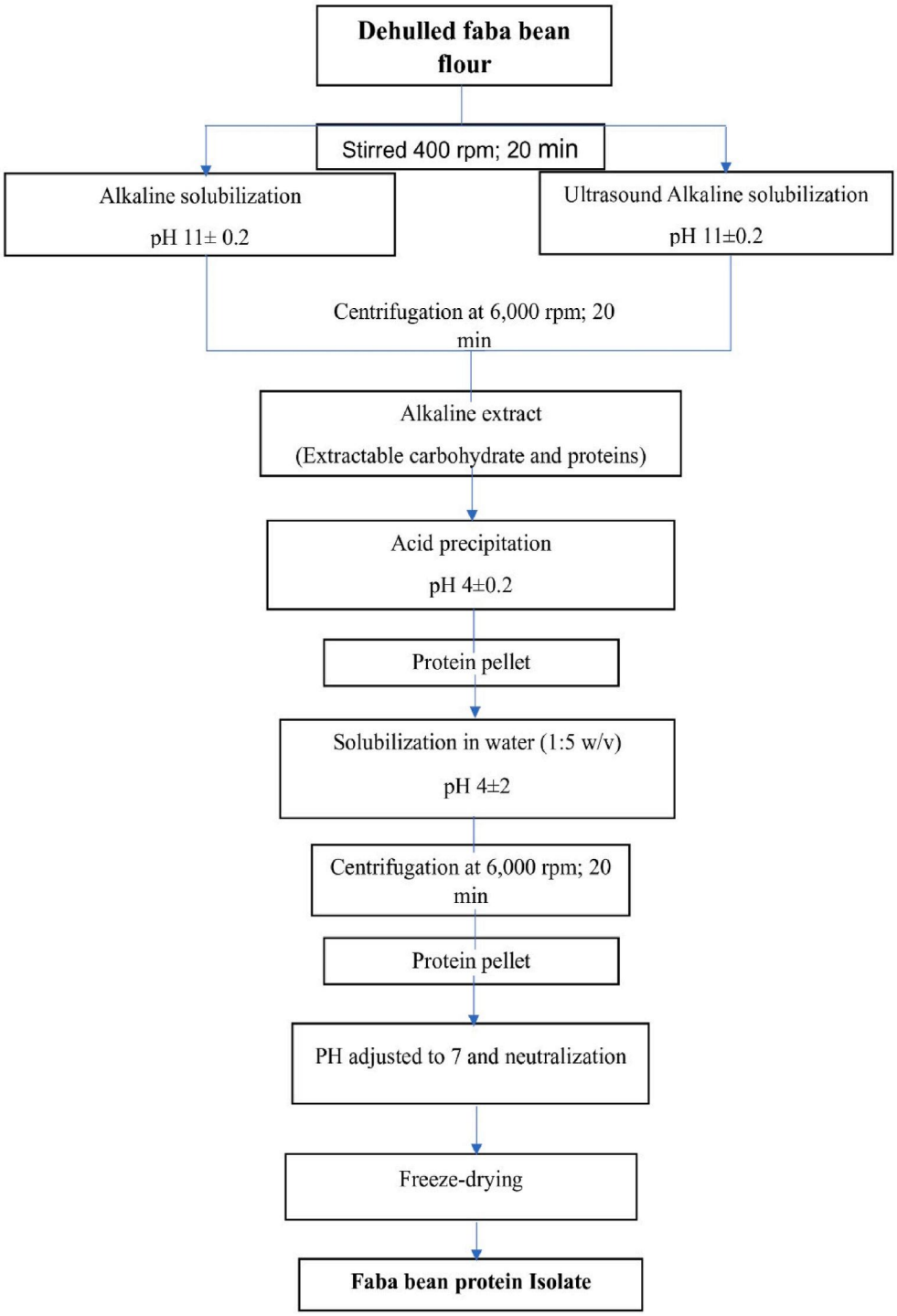

Fig. 1. Flowchart of protein isolation from faba beans with and without ultrasonication.

2.4. Physicochemical analysis

2.4.1. Gross composition analysis of flour

The proximate composition of the raw flour used in this study was analyzed in triplicate. Nitrogen content was determined using an Elementar Dumas system (Elemental, UK, Ltd) with a conversion factor of N × 6.25. Soxhlet extraction using petroleum ether was used to determine the fat content. Moisture analysis was performed by drying at 105 °C, while ash was obtained after incineration at 550 °C. Carbohydrate content was estimated using the difference method. The condensed tannins were measured using the method described by (Bento et al., 2021). A standard curve of (0–30 mg/ml) was used for quantification. The concentration of the condensed tannins was calculated and expressed in mg CE/g. A Mastersizer 3000 laser scattering particle size analyzer (Malvern, UK) equipped with a dry sampling system was used to measure the particle size distribution of the milled flour in triplicates (Jiang et al., 2016).

2.4.2. Analysis of vicine and convicine content

Protein isolate and flour were extracted with 1 mL of 50 % methanol, vortexed for 1 min, sonicated for 30 min (temperature maintained between 25 and 40 °C), and centrifuged at 14,000×*g* for 15 min. The

Table 1
Acoustic variables applied in this study. [b](Gao et al., 2022).

	Water 100 W (550 mL)	Flour suspension 100 W (550 mL)	Pea isolate solution [b] 200 W (25 mL)
dT/dt ($^oCs^{-1}$)	3 ± 0.18	0.01 ± 0.00	0.12 ± 0.007
Acoustic power (W)	76.63 ± 0.21	21.29 ± 0.74	12.59 ± 0.81
Acoustic intensity (Wcm^{-2})	20.17 ± 0.24	5.60 ± 0.19	11.14 ± 0.72
Yield of power conversion (%)	76.63 ± 0.24	21.29 ± 0.74	6.29 ± 0.41
Acoustic density(W/mL)	0.14 ± 0.16	0.04 ± 0.00	0.50 ± 0.03

extracts were recovered in a vial for HPLC-UV analysis. The remaining pellets were re-extracted three more times, and the supernatants were pooled for analysis. An HPLC-UV method was developed to quantify vicine and convicine content. Separation was performed using a Poroshell 120 column (4.6 × 150 mm) with C18 phase. Elution was performed in an isocratic run for 10 min with 0.1 % formic acid in MilliQ water at a flow rate of 0.8 ml/min. UV absorption was observed at 273 and 280 nm. Quantification of vicine and convicine was achieved at 273 nm and analyzed using the Agilent 1200 online software.

2.4.3. Protein solubility of fava protein isolates

The absolute solubilities of native and sonicated FBPI were investigated as described by Adebiyi & Aluko (2011). Protein concentration in the supernatant was determined using the Qubit Assay. The protocol given by the manufacturer was followed for analysis. Briefly, 20 μL of the isolates was added to 180 μL of reagent mix (1 μL of dye was diluted in 199 μL of buffer). The reaction mixture was analyzed using a Qubit Fluorometer after 15 min of incubation. Each sample was measured in triplicates (Contreras-Martos et al., 2018).

2.5. Structural properties

2.5.1. Fourier-transform infrared spectroscopy analysis

The FTIR study was carried out using an Attenuated total reflectance (ATR)-FTIR spectrophotometer (CA, USA). Prior to the experiment, the absorbance spectrum of the air was recorded and automatically removed from the sample spectra. Spectroscopic studies were carried out with freeze-dried faba bean protein isolates in the range 4000–400 cm^{-1} at a resolution of 4 cm^{-1} with 64 scans.

2.5.2. Protein profiling by gel electrophoresis

Electrophoresis was carried out on SDS-PAGE in a reducing solution of β-mercaptoethanol (Laemmli, 1970) for all protein isolates. An Apelex's Vertical Mini-Vertigel 2 electrophoresis device and a PS 608 power supply were also employed. Protein isolates were dissolved, vortexed in Eppendorf tubes with a reducing solution containing 10 % 2-mercaptoethanol. The samples were heated for 4 min at 90 °C, cooled, and centrifuged at 13000×*g* for 2 min. Aliquot was injected into the pocket of the Bio-Rad 4 % acrylamide stacking gel and 12 % acrylamide was used to separate the Precast Gels. DC separation at a current of 25 mA was performed for 1 h at a voltage of 120 V. SDS-PAGE Molecular Weight Broad Range™ Unstained Protein Standard was used as standard. The gels following Instant Blue staining (Expedeom) while deionized water was used to destain the gels. Destained gel was scanned using the Gel analyze software.

2.5.3. Molecular weight distribution (SE-HPLC)

Sequential extractions of protein fractions were done by the method described by DuPont et al. (2008). Briefly Albumin and globulin rich fraction was extracted from 200 mg of protein isolate with 6 mL of 100 mM KCI solution on ice for 5 min. The solution was centrifuge for 20 min and labelled as KCI extract. The remaining pellet was then re-extracted with 6 mL 0.25 % SDS in 50 mM borate buffer. The centrifuged mixture (40000×*g* for 30 min) was referred to as 0.25 % SDS extract. The remaining polymeric proteins were obtained after stirring for 2 h at 60 °C with 2 % SDS in borate buffer. The centrifuged and recovered supernatant was referred to as 2 % SDS extract. The extracts were injected to a Superdex 200 10/300 GL column. The injection volume was 50 μL. Elution was done on an HPLC-UV using 0.1 M sodium phosphate buffer containing 0.1 % SDS at 0.3 mL/min. The detector was set at 280 and 210 nm.

2.5.4. X-ray diffraction

XRD studies were carried out with a X'pert PRO X-ray diffractometer. A 0.04 mm anti-scatter slit and 1 mm divergence and receiving slits were employed. Diffractogram was measured from 5 to 70 ° (2θ) at 2θ min^{-1} with 0.05 ° step size. Native and USAE faba bean isolates diffractograms were used to observe changes in crystallite size.

2.5.5. Ultraviolet–visible (UV–vis) spectroscopy

Protein dispersions (1 mg/mL) of native and UAE faba bean isolates were dispersed in PBS (10 mM, pH 7.4) and gently stirred for 3 h, allowed to stand overnight. The resultant mixture was centrifugated for 10 min at 5000 rpm for 10 min. The supernatant was passed through a 0.22 μm filter and read using a UV–Vis spectrophotometer 200–900 nm at 25 °C.

2.6. Thermal properties

2.6.1. Thermogravimetric analysis (TGA)

TGA was done with a Thermogravimetric analyser model Q50 (TA Instruments, New Castle, Germany). Sample weight of about 2 mg were heated from 30 to 900 °C under atmospheric nitrogen (200 $mLmin^{-1}$). The degradation temperature was assigned to the second step of weight loss while the first was assigned to water loss due to evaporation.

2.6.2. Differential scanning calorimetry (DSC)

The thermal profile of the protein isolates was determined using differential scanning calorimetry. About 10–20 mg of protein isolates were closed hermetically in aluminum pans. Samples were subjected to heat ramp from 25 to 180 °C at heating rate of 10 °Cmin^{-1} under inert atmospheric condition (40 mL/min of dry N_2). Calibration was carried out with indium. The reference was a void aluminum pan.

2.7. Statistical analysis

Apart from TGA analysis, which was done using approved procedure and thus analyzed just once and data shown without standard deviation, all analyses were performed in triplicate unless otherwise specified. Results were analyzed with Origin software and a one-way analysis was used to investigate differences between samples with criteria $p < 0.05$.

3. Results and discussion

3.1. Chemical composition of faba bean flour

Faba bean flour gross chemical profile used in the present study is displayed in Table 3 (supplementary material). The moisture content of raw faba bean flour was 10.35 %. Similar moisture content (9 %) was obtained by (Mattila et al., 2018), but slightly higher moisture content

was obtained by Millar et al. (2019). The difference in moisture content could be related to cultivation conditions, seed variety and storage conditions after harvest. The ash content was 3.03 %. Similar results have been recorded to be 4.0 % by Millar et al. (2019). With regards to fat content, a value of 1.15 % was recorded. Results from other studies have shown similar results (2.1 %) by Mattila et al. (2018). Protein content in raw faba bean flour was 29.09 % similar to that of Nosworthy et al. (2018) however lower compared to Mattila et al. (2018) (35 %) but higher than those reported by Millar et al. (2019). A carbohydrate content of 56.38 % was reported in this study. Condensed tannin content and total phenolic content were found to be 0.14 mg CE/g and 57.80 mg GA/100g which is lower than other varieties reported in the literature. Total phenolic content was lower than those reported by De Angelis et al. (2021) (see Fig. 1).

3.2. Protein contents, extraction and protein yields of the isolates

The impact of ultrasonication on protein extraction yield, protein purity and protein content of untreated and treated isolates are shown in Fig. 2. In comparison to the protein extraction yield of control faba bean isolate, assisted-ultrasonic treatment under the given conditions (Table 1) generally increased protein yield. Control showed protein yield of 18.27 ± 0.23 % with a gradual increased in extraction yield from 5 min to 45 min (Fig. 2A), however prolonged ultrasound extraction duration (60 min) showed a slightly lower extraction yield compared to 45 min treatment. Enhanced acoustic cavitation due to prolonged duration could cause soluble proteins to denature with concomitant re-aggregation and thus lower protein extraction efficiency as observed in extraction duration of 60 min. There was difficulty observed with controlling temperature for extended extraction duration (60 min) but no significant increase in extraction yield. Since the cavitational effects produced by ultrasound can speed up chemical reactions by improving mass transfer at the microscale, the traditional alkaline-isoelectric extraction strategy used magnetic stirring, which concentrated on the macro mixing scale.

When comparing the protein content of the control and ultrasound-assisted protein isolates, there was a significant difference between native and sonicated protein isolates with the exception of 5 min ultrasound treatment. Protein content of control and treated isolates ranged from 83.56 to 88.77 %. The highest protein was observed in ultrasonic treatment for 45min. Protein content of about 80–90 % has been achieved using alkaline extraction of faba bean isolates (Krause et al., 2023). The creation of cavitation bubbles during ultrasonication enables localised breakdown of the cell wall and greater alkaline solvent penetration into the cellular components, leading to the release of intracellular products (Carpentieri et al., 2021). Additionally, sonication generates heat and, when paired with mechanical impacts, encourages the formation of pores in the flour suspeension. This raises the yields of the extraction process and the protein content (Kingwascharapong et al., 2021; Mushtaq et al., 2020). Comparable patterns were also observed for protein yield and purity. as shown in Fig. 2B&C.

3.3. Physicochemical properties

3.3.1. Vicine and convicine

Faba bean seed cotyledon contain a family of species-specific compounds known as vicine and convicine. These two glycosides cause of favism, a disorder marked by a deficit in glucose-6-phosphate dehydrogenase (Badjona et al., 2023b). Faba bean flour and protein isolates were investigated for levels of pyrimidine glycosides (v-c) as shown in Table 2. Individuals who consume faba bean products with excessive levels of vicine and convicine contents risk developing serious and possibly fatal hemolytic anaemia (Luzzatto and Arese, 2018; Martineau-Côté et al., 2022). In the raw flour used the V–C content was

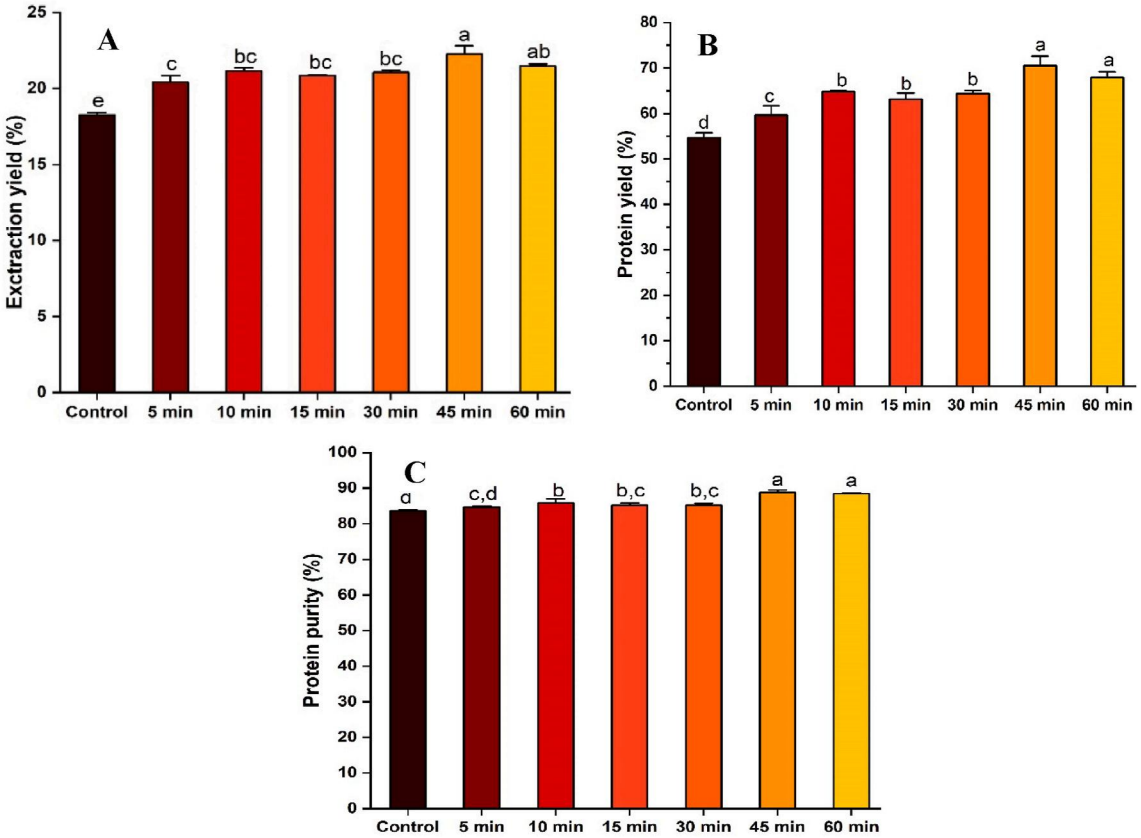

Fig. 2. Effect of ultrasonic time on (A) extraction yield (B) protein content (C) Protein purity of different ultrasonic durations.

Table 2
Content of pyrimidine glycosides (mg/g) in native and ultrasonic-assisted faba bean protein isolate.

Sample	Vicine	Convicine	Total
Raw flour	4.92 ± 0.03 [a]	3.91 ± 0.02	8.83 ± 0.02 [a]
Native protein	3.8 ± 0.04 [b]	n.d.	3.8 ± 0.04 [b]
5min	3.67 ± 0.01 [c]	n.d.	3.67 ± 0.01 [c]
10min	3.58 ± 0.05 [b]	n.d.	3.88 ± 0.05 [b]
15min	3.58 ± 0.02 [c]	n.d.	3.58 ± 0.02 [c]
30min	3.64 ± 0.07 [c]	n.d.	3.64 ± 0.07 [c]
45min	3.64 ± 0.06 [c]	n.d.	3.64 ± 0.06 [c]
60min	3.56 ± 0.04 [c]	n.d.	3.56 ± 0.04 [c]

Data shown as average ± SD (n = 3). Tukey's test ($p < 00.05$); Values denoted by different letters differ significantly (n.d. = not detected).

4.92 and 3.91 mg/g respectively (total 8.83 mg/g). Different cultivars of faba beans have been demonstrated to content varied concentrations of V–C, thus classified as low and high V–C cultivars. The literature (Ivarsson and Neil, 2018; Khamassi et al., 2013; Mayer Labba et al., 2021) reported different concentrations of vicine and convicine for faba beans from 2 to 8 mg/g (vicine - convicine). Differences in vicine content could also be attributed to differences in cultivar used in the study as well as extraction protocol used. In our case 3 times extraction was optimised to ensure maximum extraction of v-c from flour while 2 times extraction was used for isolates. No recognised threshold for the amount of faba beans that must be consumed to cause favism in populations with G6PD deficiency has been documented in the literature. This may be because the severity of the genetically determined deficit varies.

In this present study, protein isolates showed the lowest vicine content values were observed in ultrasound-assisted protein isolates compared to control protein isolate. R^2 of vicine calibration curve was 0.9917 (Fig. 3. A-C). Vicine calibration was used for quantification of convicine due to lack convicine standard (Shi et al., 2022). With regards to convicine content there was no convicine detected. Vicine content was found to range from 3.56 to 3.8 mg/g with the highest in control and lowest observed in 60 min extraction treatment. The results showed significant difference between individual and total v-c (mg/g sample) control and ultrasound treated samples except 10 min treatment. Protein isolates were examined by Shi et al. (2022), who observed that the isolate from Malik and Saini, 2018 had values of 2.40 and 1.45 mg/g and 3 - 1 mg/g, for vicine and convicine content respectively. When compared to dry fraction processing wet extraction procedures leach away vicine-convicine through medium solubilization and acid treatment (Sharan et al., 2021). Given that total glycoside levels was found in lower levels compared to flour; isoelectric precipitation effectively reduced these antinutrients which is promising when incorporated in different food products for individuals with G6PD deficiency.

3.3.2. Protein solubility

Unsurprisingly, the native FBPI showed comparatively lower absolute solubility compared to ultrasound extracted faba bean protein isolate (Fig. 4A). One of the main problems for native FBPI's application in food industries is their poor solubility, which was observed in this study. All ultrasound-assisted protein isolates (10–60 min) increased the solubility of FBPI. Highest absolute protein concentration in the soluble extract was observed after 30–60 min. The increased solubility was not brought on by the hydrolysis of the FBPI's peptide link as observed in SDS-PAGE as well as SEC-HPLC, but rather by the exposure of the hydrophilic regions due to structural alterations and increase amount of soluble protein aggregates.

3.4. Structural properties

3.4.1. UV–vis spectra

The UV–Vis spectra of native and sonicated FBPIs are shown in Fig. 4. C. A distinctive peak at 290 nm was observed for all protein isolates. Aromatic amino acids such as tryptophan and tyrosine residues are responsible for absorption at 280 nm (Grimsley and Pace, 2003; C. Ren et al., 2018). This result indicates that increasing the ultrasonic extraction duration increased the absorption peak intensity of the isolates at 290 nm compared to native faba bean isolates. The absorbance intensity of the FBPI increased after the USAE treatment, depicting the exposure of buried hydrophobic groups. Similar observation has been found by X.

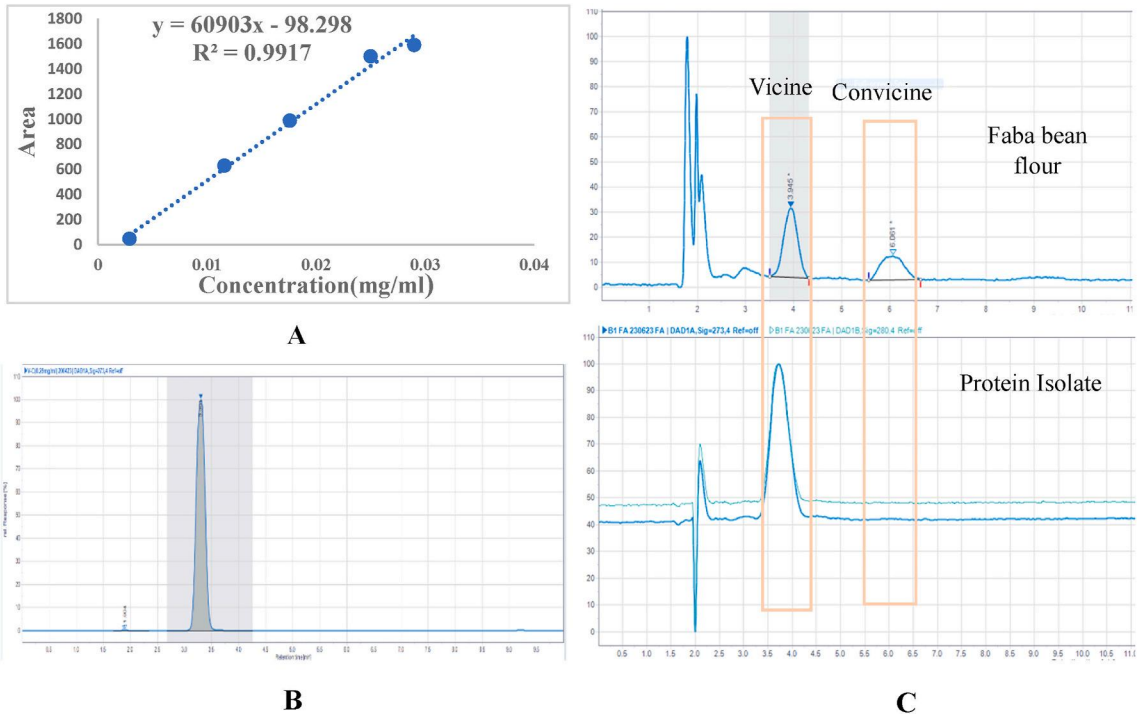

Fig. 3. (A) Calibration curve for vicine standard; (B) HPLC chromatogram of vicine standard; (C) faba bean flour and protein isolate. Detection was carried out at 273 nm. The retention time (RT) windows for vicine and convicine are shaded in orange.

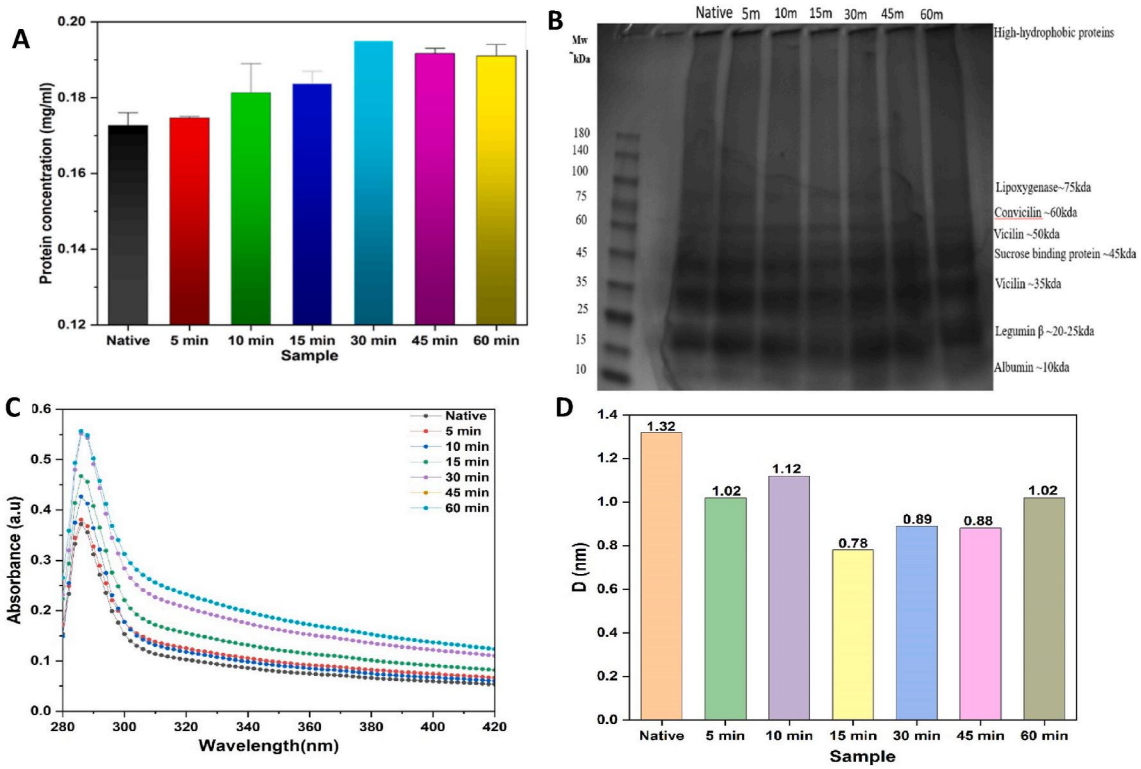

Fig. 4. (A) Protein solubility; (B) SDS-PAGE under reducing; (C) UV–Vis spectra; (D) crystallite size obtained from XRD diffractogram native and ultrasound-assisted faba bean protein isolate.

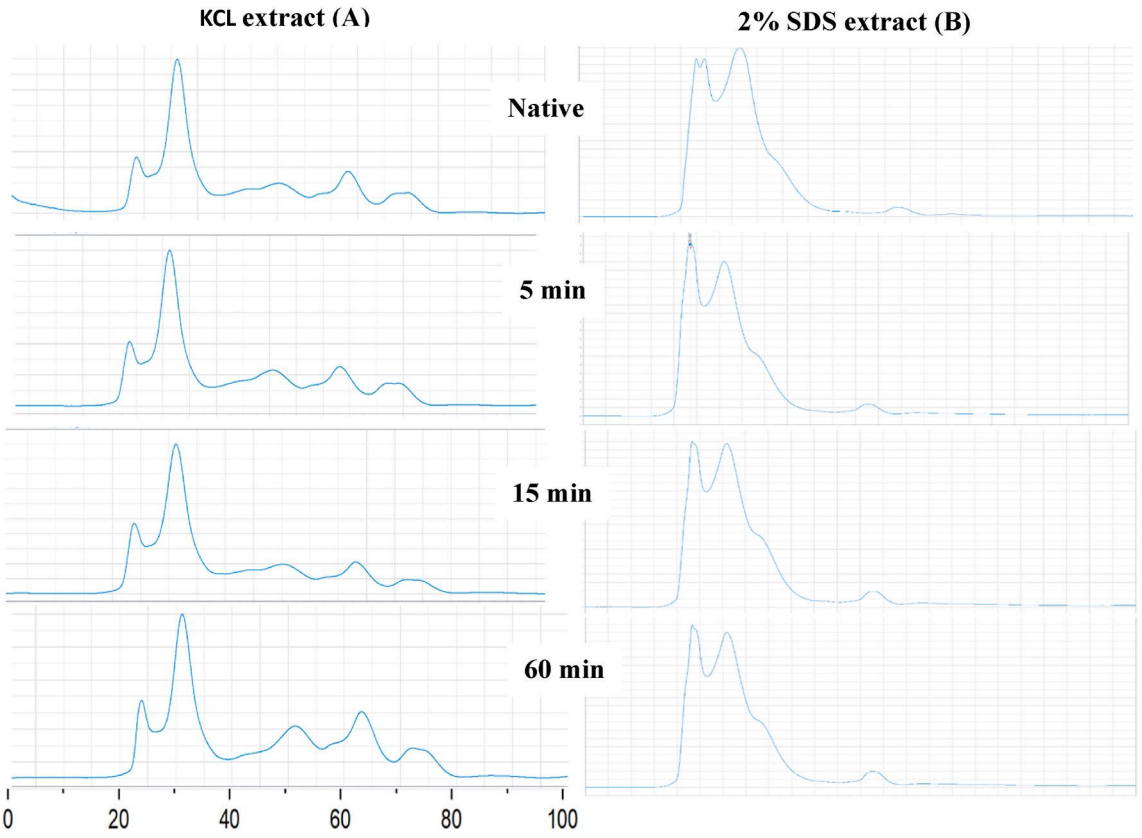

Fig. 5. SEC chromatogram of the control and ultrasound-assisted protein isolates (**A**) KCI extract (albumins and globulins) (**B**) 2 % SDS extract (other proteins).

Yang et al. (2017) for rice protein. After 45 min ultrasonic treatment no observable differences in the uv–vis spectra was observable in comparison with 60 min treatment time.

3.4.2. Effect of extraction conditions on protein profile (SDS-PAGE)

Primary structures of faba bean isolates obtained from ultrasound-extracted isolates was compared with that of native sample by the patterns of reducing condition on PAGE (Fig. 4. B). Approximately 70–78% of the proteins in pulses are globulins, followed by 10–20% of albumins (Bessada et al., 2019; Singhal et al., 2016). Overall, SDS-PAGE pattern of native FBPI mainly consisted of vicilin, legumin, albumin, sucrose binding protein and lipoxygenase. Native and treated FBPI samples showed three strong bands at 45, 35 and 20–25 kDa. Similar subunit bands were also observed across all FBPI from ultrasound treatments, indicating that the bands were not split during the sonication process. Vicilin was identified in its dissociated form ~35 kDa(α/β) and 47–50 kDa (α/β/γ), whereas legumin was obbserved in its β 20–25 kDa subunits. In the upper portion of their respective lanes, the control FBPI and ultrasonicated samples both showed smearing, indicating the existence of protein aggregation. The high molecular weight protein band (>180 kDa) in the first band, which may be related to the high degree of polymerization involving not only disulfide bridges but also other covalent bonds, revealed the high hydrophobic nature of the faba bean proteins (Famuwagun et al., 2020), thus such proteins could not enter the gel. The findings support the observation by (Xiong et al., 2018) who indicated that ultrasonication did not alter the primary structures of pea protein isolates. Similar findings was observed in various plant proteins, including soy and Moringa oleifer protein isolates (X. Ren et al., 2020; Tang et al., 2021). It could be hypothesieds that the shear stress turbulences during sonication in this studies investigation could not break peptide bonds and modify the primary structures of FBPIs because ultrasonic treated FBPI had the similar subunit composition being independent time.

3.4.3. Molecular weight distribution (SE-HPLC)

Size exclusion (SE-HPLC) is a useful technique to analyze proteins with varying molecular weight. The chromatogram (Fig. 5) demonstrates the existence of proteins with various molecular weight distributions that correspond to the globulins and albumins in native and ultrasound-assisted protein isolates for KCI extract. Globulins are divided into two groups: legumin and vicilin, with a third component named convicilin being debatable (Barac et al., 2010; Kornet et al., 2022). Despite small changes in peak area and intensity between samples, the elution chromatogram of ultrasound-treated samples were roughly separated into six fractions, which was similar to native FBPI. Similar observation was observed in the 2 % SDS extract (Fig. 5B). Compared to the untreated sample, no new peak was observed in the FBPI acquired via ultrasound-assisted extraction. This supports the SDS-PAGE findings that the primary structure of the proteins was unaltered. Peaks from SEC can be regarded as globulin fractions as iso-electric precipitation does not extract albumins (S. Yang et al., 2020).

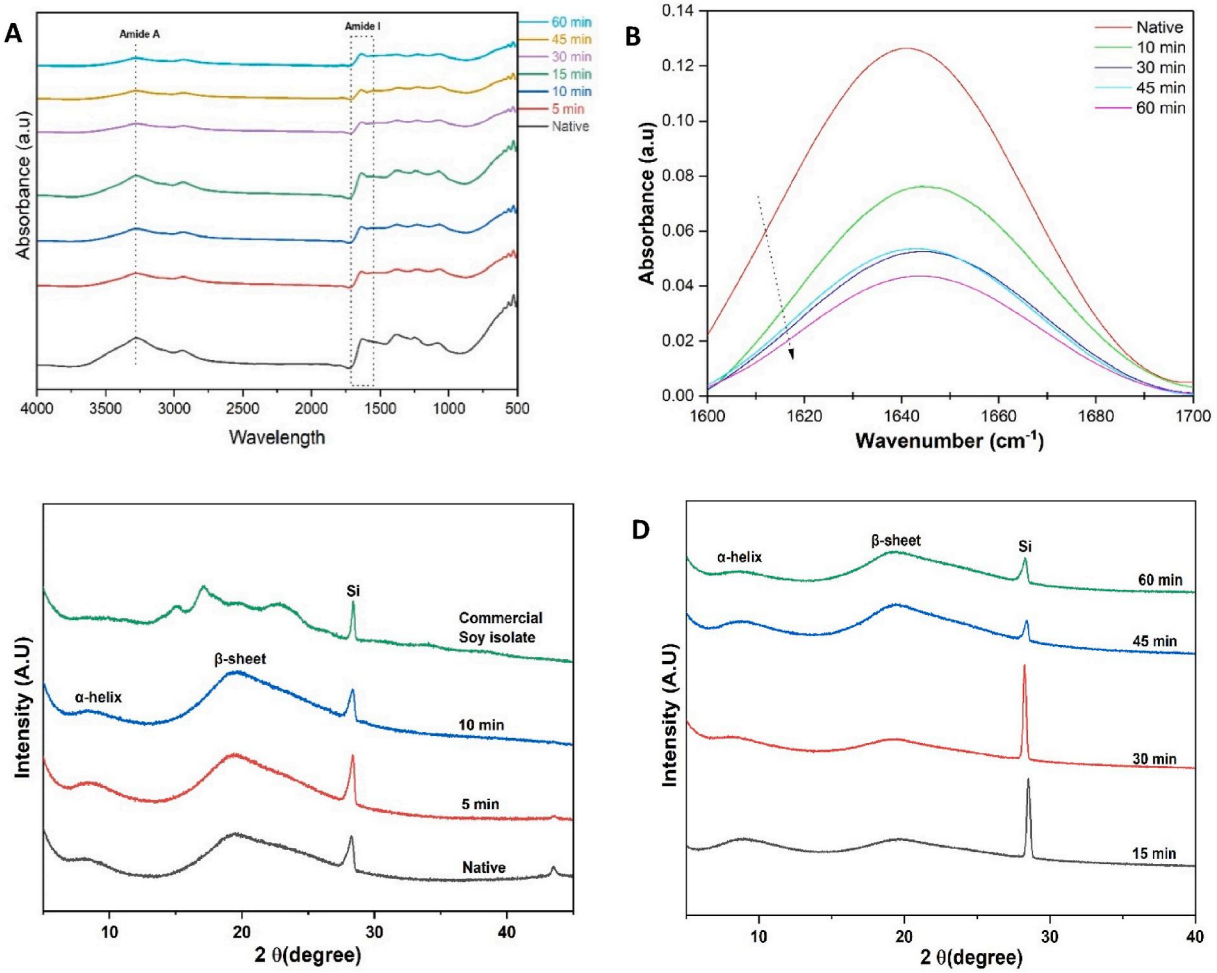

Fig. 6. (A–B) Fourier transform infrared (ATR-FTIR) spectroscopy; (C–D) XRD patterns of native and ultrasound-assisted faba bean protein isolate. Si represent silicon peaks used for validation, A_1 (α-helix) and A_2 (β-sheet).

3.4.4. Infrared spectroscopy (FTIR)

The impact of ultrasound on the structure of native and ultrasound-assisted protein isolates was investigated using FTIR, a relevant method for characterizing the secondary structure of proteins. The spectral positions of amide I are principally attributed to C–O stretching of protein molecules, mostly sensitive to changes in protein secondary conformation (Tian et al., 2020). Peaks corresponding to the amide I region in various protein isolates confirm the presence of α-helix, β-sheet, β-turn, and random coil structures. Protein samples treated with ultrasound showed variations in the secondary structure when compared to the native sample, as illustrated in Fig. 6. A. The peak of amide I shifted from 162 5 cm^{-1} after ultrasound treatment. Longer sonication times (10–60 min) resulted in lower peak intensities in the amide one region of the protein isolate compared to the control protein isolate (Fig. 6B), indicating that the vibrational and stretching states of the protein structure were affected by the ultrasound treatment, which altered the secondary structure. The notable peak of amide A lies between 3000 and 3500 cm^{-1}, and it results from N–H bending and O–H stretching of polypeptide's main chain (Yan et al., 2021). The position of amide A shifted for all samples after ultrasonication. In a similar finding, Nazari et al. (2018), found that ultrasonic treatment caused the spectra peak of millet protein isolate to shift from 3286 to 3418 cm^{-1}.

3.4.5. X-ray diffraction

The impact of ultrasonication on the crystalline structure of FBPI was studied by XRD. XRD patterns of the seven samples are presented in Fig. 6C-D and the quantitative data are presented in Table 6. Sharp peak shapes have been associated with crystalline fractions, according to the XRD spectrum, while broad and diffused background is linked with amorphous fractions (Farrokhi et al., 2018) The native and sonicated protein isolates samples' XRD patterns showed a weak peak at 8°. Additionally, both untreated and ultrasound treated FBPI samples' XRD patterns revealed a prominent peak at about 20 °. These two distinct peaks in the XRD profile of all protein isolates at roughly $2\theta = 8.90$ ° and $2\theta = 19$ ° indicates the presence of α-helical and β-sheets structures (Ghobadi et al., 2021). In contrast, commercial soy protein isolate showed numerous peaks from $2\theta = 8.90$ ° and $2\theta = 25$ °. The presence of diffused peaks in soy isolates could be attributed to harsh processing conditions used during production of protein isolates. The intensity of the two peaks and their relative ratio of the protein structures of native and treat FBPI between the values of the different ultrasound treatment confirmed the unequal distribution of the α–helix and β–sheet structures. There was an observed changes in intensity and peak position indicates modification of crystalline properties of treated FBPI samples (Table 6) (Li et al., 2021). Additionally, there is a clear correlation between peak intensity and particle size. The crystal's size affects the diffraction angle and peak intensity which elucidate potential conformational changes in protein structures and interactions between them (Sahni et al., 2020). Ultrasound treatment reduced the crystallite size of ultrasonicated FBPIs indicating a reduction in particle size (Fig. 4D) (Fadimu et al., 2022). A similar XRD pattern was obtained by Li et al. (2021); & Mir et al., 2021 for different proteins. Hence, XRD pattern was useful in elucidating structural changes after ultrasound-assisted protein extraction from faba beans flour.

3.5. Thermal studies

3.5.1. DSC thermograms of faba bean protein isolates

The functioning of proteins and, consequently, their usability in food systems are significantly influenced by their heat stability during processing. It is crucial to the food sector since the majority of processing chains include heating processes that have the potential to alter the end

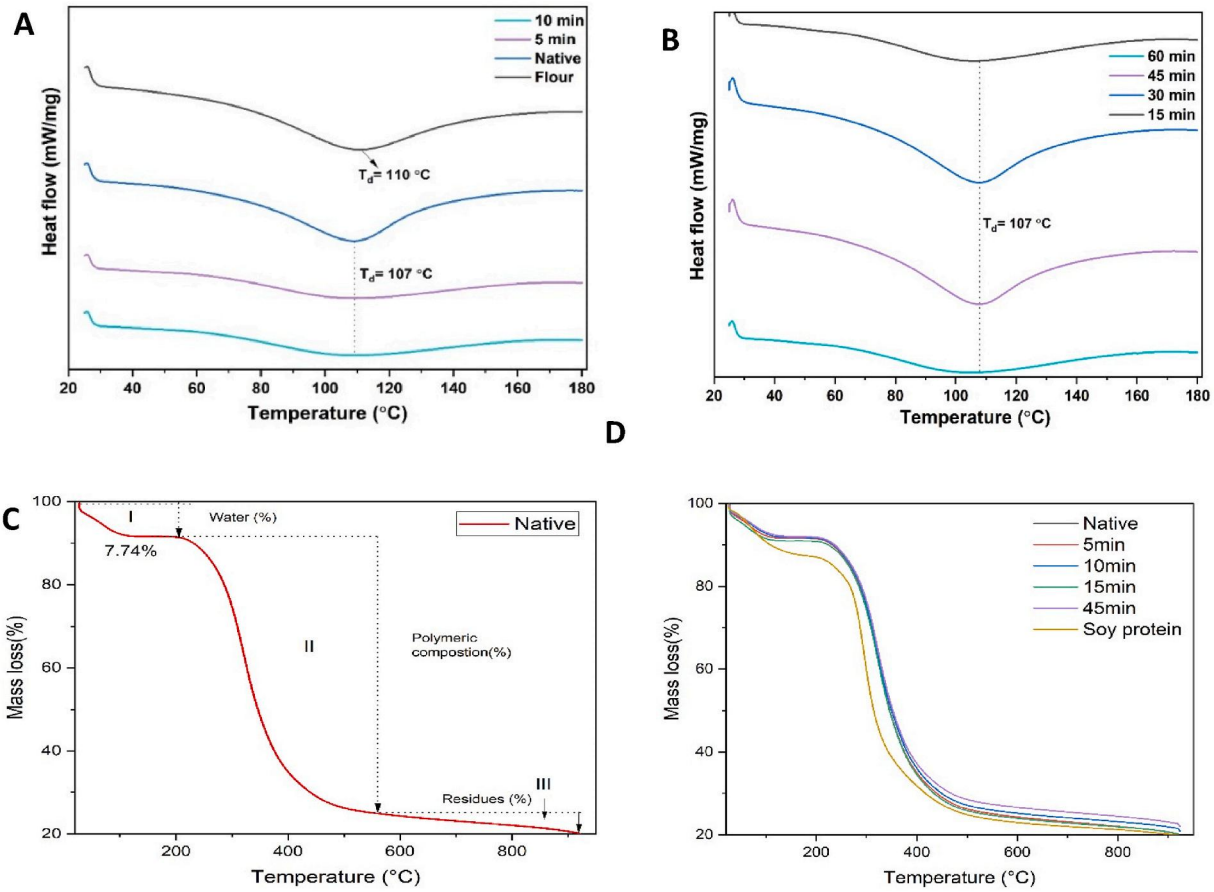

Fig. 7. (A–B) Differential scanning calorimeter profile; (C–D) Thermogram patterns of native and ultrasound-assisted faba bean protein isolate.

product's functional and nutritional qualities. Furthermore, TGA allows for the in-depth investigation of events including mass loss associated with proteins, lipids, carbohydrates, water, and volatile compounds.

Protein denaturation is seen as an endothermic peak on the thermogram in the DSC (Fig. 7. A-B). The processing conditions for a food's protein depend on its thermal stability; as a result, an increase in thermal stability can broaden the protein's processing time (Feng et al., 2021). No major difference in denaturation temperature between native protein isolate and ultrasound-treated protein isolate (107 °C) was observed while the T_d value of faba bean flour was to 110 °C which was slightly lower compared to extracted protein isolates. These values are comparable to previously reported values of faba bean isolates (Arntfield et al., 1985) who reported values between 90 and 140 °C for faba bean vicilin and legumin indicating that the processing conditions did not have a harsh effect on protein quality. The denaturation peak at 107 °C can be attributed to legumin protein fraction. However lower T_d values have been reported by (Shevkani et al., 2015; Sosulski et al., 1985).The variations in T_d can be attributed to the various faba bean varieties utilised, which differ in their amino acid makeup, or to the protein state as a result of past processing, which results in a variable protein structure (Kudre et al., 2013). There is a high denaturation temperature for storage proteins from seeds of other plants. As example, the globulin denaturation temperatures in chia and oat seeds are 105 and 112 °C, respectively (C.-Y. Ma and Harwalkar, 1988; Sandoval-Oliveros and Paredes-López, 2013).

3.5.2. Thermogravimetric analysis of ultrasound-assisted faba bean proteins

The thermal stability of native and ultrasound-assisted protein isolates with varying protein content was analyzed using thermogravimetric analysis. The mass loss profile of the FBPIs with temperature are displayed in Fig. 7C-D. Additionally, Table.5 (supplementary material) shows relevant thermal degradation features, such as the onset temperatures at which deterioration begins for each isolate and the maximum temperatures at each phase, as well as the percentage of mass losses attained at each of these stages. Mass loss at temperatures 27–190 °C) is due to moisture evaporation by protein isolates and the breakdown of low molecular weight volatiles. The loss of molecular weight protein fractions (protein and CNCs degradation) corresponds to the second degradation stage (190–500 °C). Finally, the third stage (>590 °C) represent residual components (Osorio-Ruiz et al., 2019). Native and USAE protein isolates began disintegrating at approximately 190–500 °C, resulting in a significant weight loss, mostly due to breakdown of the protein that constitutes the main component. With increasing temperature peptide bonds are ruptured, culminating in the complete collapse of the FBPPI protein backbone (Lqari et al., 2002). All ultrasound treated FBPIs were less thermally resistant than native protein, indicating a lower heat required for thermal denaturation of USAE protein isolates, whereas all FBPIs were more thermally stable than commercial soy protein isolates. The results revealed that the thermogravimetric characteristics of the USAE FBPIs were altered by varied ultrasonic treatment times. Overall, the results showed that ultrasound treated FBPIs were less thermally resistant than native FBPIs, emphasizing the role of sonication in protein structural modification.

4. Conclusion

In an effort to address the growing market for alternative proteins, attempts to isolate proteins from faba beans which are profitable and environmentally sustainable have been investigated. Due to functional constraints and presence of antinutrients, the inclusion of faba bean as a dietary food source has been limited. In this current research, ultrasonic-assisted isolation on the protein purity, physicochemical and thermo-structural properties of faba bean protein isolate (FBPI) was comprehensively studied. In general, the FBPI purity and protein content were increased by ultrasound-aided extraction compared to FBPI extracted based on conventional approaches. The resultant higher yields were attributed to the mass transfer and high shear effects produced by the sonic cavitation. Levels of pyrimidine glycosides(v-c) were dramatically reduced compared to raw faba beans flour indicated that isoelectric precipitation or other aqueous extraction may be necessary if a high protein purity is required as shown in this study. In addition. Protein extraction may be useful to generate sustainable products for individuals prone to favism. According to SDS-PAGE and SEC-HPLC studies, there was no substantial modification in the primary structure of FBPI. Changes in secondary structure were altered after sonication as observed by FTIR and Uv–vis spectra. Native and treated FBPIs were characterized by X ray diffraction peaks with varying intensities (~8.5° and 19.5°) associated with α-helix and β-sheet. It can be concluded that the modifications through ultrasound treatment can generate FBPIs with improved protein yield, different physical and structural properties which might affect their functionalities for different food applications. Therefore, ultrasonication is an effective non-thermal technology that could be useful in the food industry to increase protein yield as well as modify the structural and functional properties of plant-based proteins. The development of ultrasonic technology for industrial use necessitates parameter optimisation and in-depth investigation into the impact of acoustic therapy on food production in large quantities. Larger-scale studies should take into account the risks associated with ultrasound use for individuals as well as its safety features.

CRediT authorship contribution statement

Abraham Badjona: Writing – review & editing, Data curation, Formal analysis, Methodology, Writing – original draft. **Robert Bradshaw:** Investigation, Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Caroline Millman:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Martin Howarth:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Bipro Dubey:** Conceptualization, Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfoodeng.2024.112082.

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