Comparison of physicochemical characteristics, functional properties and biological activities of hemp seed proteins by different extraction methods

Hai-Hong Chen[1,*], Wei Lia, Qi-Xuan Zhou[1], Bing Xua[2], Yi Wang[3], Jun-Yu Liu[1], Jie Ren[1], Chong Zhang[3,4]， Xin-Hui Xing[1,2,3,4,*]

[1] *Institute of Biopharmaceutical and Health Engineering, Tsinghua Shenzhen International Graduate School, Shenzhen 518055, China*

[2] *Institute of Biomedical Health Technology and Engineering, Shenzhen Bay Laboratory, Shenzhen 440300, China*

[3] *Key Laboratory for Industrial Biocatalysis, Ministry of Education, Institute of Biochemical Engineering, Department of Chemical Engineering, Beijing 100084, China*

[4] *Center for Synthetic and Systems Biology, Tsinghua University, Beijing 100084, China*

*Corresponding authors: Xin-Hui Xing, Dr. Haihong Chen, E-mail: xhxing@tsinghua.edu.cn, chenhh@mail.tsinghua.edu.cn

Abstract

Objective: Protein extraction methods play a crucial role in the quality, functionality, and recovery efficiency of protein products, and directly affect their subsequent applications. Hemp seeds are rich in proteins and oils; however, the process of hemp seed protein extraction has rarely been studied. To bridge this knowledge gap, we aimed to explore the effects of the extraction conditions on the yield, physicochemical characteristics, functional properties, and biological activities of hemp seed proteins.

Methods: Alkaline extraction (AE), alkaline extraction combined with ultrasonication (AEU), salt extraction (SE), and salt extraction combined with ultrasonication (SEU) were used to extract the hemp seed proteins.

Results: The protein yields of SE and SEU were significantly higher than those of AE and AEU. All four types of protein extracts showed no obvious differences in protein purity, which was higher than 95%; however, significant differences were observed in protein physicochemical properties, functional properties, and biological

activities among the proteins obtained by the four extraction methods. The hemp seed proteins extracted by the SE (HPS) and SEU (HPSU) methods possessed better nutritional quality, digestion properties, higher fat absorption capacity, and a lower water-holding capacity. Moreover, we found that the proteolytic products of HPS and HPSU exhibited superior biological activities, including DPPH scavenging capacity and DPPIV inhibitory activity, but similar anti-inflammatory activities.

Conclusion: We concluded that SE was more suitable for the extraction of hemp seed protein because the SE method maintained the high nutritional value for the proteins. We believe that these findings contribute to a better understanding of the effects of extraction methods on protein quality.

Keywords

Alkaline Extraction; Salt Extraction; Physicochemical Properties; Functional Properties; Biological Activities

1 Introduction

In recent years, the prevalence of chronic diseases, including diabetes, cardiovascular diseases, and cancer, has increased owing to an aging population, urbanization, and lifestyle changes. However, maintaining a healthy lifestyle and healthy dietary habits can exert protective effects, thereby decreasing the morbidity of chronic diseases. A high-protein diet, particularly the consumption of plant-based proteins, is associated with a decreased risk of various chronic diseases [1]. The consumption of protein-based dietary supplements in developed countries has increased sharply in recent years [2, 3], and the global demand for protein is expected to double by 2050 [4]. However, domesticated animals and birds may not be an option to meet future needs as they have significant environmental impacts, including threats to biodiversity, greenhouse gas emissions, destruction of natural habitats, and destruction of terrestrial ecosystems [5]. Plant protein sources, including legumes, wheat, and sorghum, are widespread among vegetarian and vegan populations but do not meet the Recommended Dietary Allowance (RDA) level [6]. Therefore, there is a need to develop new natural resources containing high-quality proteins for the prevention of chronic diseases and healthcare.

The non-drug type Cannabis sativa, also called hemp or industrial hemp, is crucial to the industrial, medicinal, and food sectors [7-9]. Hemp seeds have been used as food and medicine in China for at least 3000 years [10-12]. Over the past 20 years, products (oil, meal, and protein powder) derived from hemp seeds have attracted interest owing to their nutritional and pharmaceutical values [11, 12]. The main focus is on the content of both macro- and micronutrients: polyunsaturated fatty acids of oil, proteins, total fiber, vitamins, and minerals in hemp seeds [9, 10, 13-15]. In particular, hemp seed proteins are characterized by good digestibility, contain all the essential amino acids in a balanced ratio, and have nutritional value comparable to that of other proteins, such as soy protein and egg white [9-11]. The presence of abundant proteins and other essential nutrients indicates that hemp seeds are an excellent alternative natural protein source for human health management.

Isoelectric precipitation and salt extraction are the most commonly used methods for the extraction of plant proteins. Isoelectric precipitation extraction involves the use of a powerful acidic or alkaline solution to enhance the charge on protein molecules, resulting in their solubilization. Salt extraction involves dispersing plant flours in a highly concentrated salt solution. This phenomenon causes proteins to aggregate through a salting-out phenomenon, resulting in the formation of a protein-rich precipitate phase that can be collected by centrifugation [16]. The characteristics of proteins extracted using different methods may vary significantly. For instance, Stone et al. [17] reported that the water-holding capacity of pea protein isolates depended on the extraction method adopted and that the water-holding capacity of proteins extracted by isoelectric precipitation was higher than that of salt extraction [17]. According to Karaca et al., the emulsifying capability of pulsed protein isolates obtained by isoelectric precipitation extraction is considerably greater than that of isolates obtained by salt extraction [18]. The methods used for extraction play a vital role in determining the functional characteristics of proteins. Alkaline and salt extraction combined with ultrasonication-assisted extraction is the most frequently employed method for the extraction of hemp seed proteins. However, studies regarding the impact of extraction conditions on physicochemical and nutritional properties, as well as bioactivity, are

rarely reported.

Based on these considerations, this study aimed to compare the physicochemical, functional, and biological properties of four protein extracts obtained via alkaline extraction, alkaline extraction combined with ultrasonication, salt extraction, and salt extraction combined with ultrasonication.

2 Materials and Methods

2.1 Materials

Industrial hemp seeds (Cannabis sativa L.) were provided by Bama Miao Autonomous County, Guangxi Zhuang Autonomous Region, China.

2.2 Chemicals

Ethanol was purchased from Sichuan Kolon Chemical Reagent Co., Ltd. (Chengdu, China). n-hexane, petroleum ether and n-butyl alcohol were purchased from Guangdong Guanghua Technology Co., Ltd. (Guangzhou, China). The kit to determine plant total phenolic content was purchased from Suzhou Comin Biotechnology Co., Ltd. (Suzhou, China). Hydrochloric acid and sodium hydroxide were obtained from China National Pharmaceutical Group Co., Ltd. (Beijing, China). Other conventional analytical grade chemicals and reagents such as ethylenediaminetetraacetic acid (EDTA), dithiothreitol, glycine, sodium tetraborate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), L-serine, decahydrate, and o-phthaldialdehyde were procured from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). The polyacrylamide gel electrophoresis (PAGE) fast preparation kit, 5× loading buffer supplemented with protease inhibitors, and pre-stained protein markers were obtained from Shanghai EpiZyme Biomedical Technology Co., Ltd. (Shanghai, China). Coomassie Blue G-250 staining buffer and Coomassie Blue destaining buffer were purchased from GenStar (Beijing, China).

2.3 Cells and culture medium

The Raw246.7 cell line was purchased from the China Center for Type Culture Collection (Wuhan, China). Dulbecco's modified Eagle’s medium (DMEM) was purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Foundation B™ fetal bovine serum (FBS) was purchased from Gemini BioProducts

Holding, Inc. (West Sacramento, CA, USA). Penicillin-streptomycin was purchased from Life Technologies Corporation (Grand Island, NY, USA). Lipopolysaccharide (LPS) and CCK-8 kits were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

2.4 Defatting of hemp seeds

In this study, to extract the hemp seed oil, solvent extraction was applied. Briefly, dehulled hemp seeds were ground using a grinding instrument (Supor, China). Next, 40 mL of different solvents (petroleum ether, n-hexane, n-butyl alcohol, ethanol, petroleum ether:n-butyl alcohol (5:2, v/v), n-hexane:n-butyl alcohol (5:2, v/v), petroleum ether:ethanol (5:2, v/v), and n-hexane: ethanol (5:2, v/v)) and 2 g of the pulverized hemp seed sample were mixed and stirred at room temperature for 12 h. The sample was centrifuged at 5000 rpm for 15 min to collect the precipitate, and the extraction procedure was repeated once. Finally, the precipitates were collected, heated at 60 °C by a water bath to volatilize residual solvents in the draught cupboard and kept at ambient temperature for subsequent examination. The recovery of the defatted powder was analyzed and calculated using the following formula:

$$\text{Recovery (\%)} = (\text{Weight of Defatted powder}) * 100 / (\text{Weight of hemp seed powder})$$

2.5 Extraction of hemp seed proteins

2.5.1 Alkaline extraction (AE) and alkaline extraction combined with ultrasonication (AEU)

A total of 2 g of defatted hemp seed powder was suspended in 40 mL of deionized water, and the pH was adjusted to 9.5 with NaOH (1.0 M). Subsequently, the mixture was magnetically stirred at 35 °C for 2 h or for 10 min, sonicated in an ultrasound bath (35 °C, 0.3 kVA) for 30 min, centrifuged at 5000 rpm for 20 min, and the supernatants were collected. The extraction was repeated once, and the supernatants were combined. The supernatant pH was decreased to 4.0 with HCl (1.0 M) to isolate the protein, and then centrifuged at 8000 rpm for 20 min. The sediments were rinsed with deionized water (pH 4.0) and centrifuged three times to eliminate any remaining salts and other non-protein substances. The precipitated proteins were dissolved in water (pH 7.0),

lyophilized, and stored at room temperature. The proteins obtained using AE and AEU were HPA and HPAU, respectively.

2.5.2 Salt extraction (SE) and salt extraction combined with ultrasonication (SEU)

A total of 2 g of defatted hemp seed powder was mixed with 20 mL of a 0.8 mol/L NaCl aqueous solution (pH 7.0). The mixture was magnetically stirred at 35 °C for 2 h or at 35 °C for 10 min, then sonicated the mixture in ultrasound bath (35 °C, 0.3 kVA) for 30 min, followed by centrifugation at 5000 rpm for 20 min, and the supernatants were collected. The extraction was repeated twice, and the supernatants were mixed. The isoelectric precipitation and lyophilization treatments for the isolate were the same as those described in section 2.5.1. Proteins obtained by SE were designated as HPS, whereas those obtained by SEU were designated as HPAS.

2.6 Physicochemical properties analysis

2.6.1 Determination of protein content

The total protein content of the four protein extracts was measured following the Kjeldahl nitrogen method using a Hanon K1160 Automatic Kjeldahl Protein/Nitrogen Analyzer (Hanon, China). The nitrogen conversion factor adopted in this study was 6.25.

2.6.2 Electrophoresis

Sodium dodecyl sulphate (SDS)-PAGE was used to analyze the protein/polypeptide composition of the four protein extracts. Briefly, the lyophilized protein extracts were suspended with phosphate-buffered saline (PBS) (10 mg/mL) and analyzed under reducing conditions (with β-mercaptoethanol). The electrophoresed gel was stained with Coomassie brilliant blue R-250 (0.1 %) for 30 min and then transferred to destained buffer for another one hour. Finally, the ChemiDoc MP Imaging System (Bio-Rad, USA) was used to capture the electrophoretic signals and analyze the molecular weights using the pre-stained marker.

2.6.3 Determination of free sulfhydryl contents

The free sulfhydryl (SH) contents of hemp seed protein samples were measured following Shen et al. [19]. Briefly, 15 mg of protein was mixed with 5 mL of Tris-glycine buffer (0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, pH 8.0) and 0.05 mL of Ellman's reagent (4 mg/mL DTNB in Tris-glycine buffer) and incubated at room

temperature in the dark for one hour. The absorbance of the mixture was measured at 412 nm using a UV-Vis spectrophotometer (Shanghai Yoke Instrument Co., Ltd., Shanghai, China). The calculation for levels of free sulfhydryl groups (μmol SH/g) was as follows:

$$SH\ (\mu mol/g) = (73.53 \times A_{412} \times D)\ /C$$

Where A412 is the absorbance at 412 nm, C is the protein extraction solution concentration (mg/mL), D is the dilution factor (73.53) from 106 /(1.36×104); 1.36×104 is the molar absorptivity constant ($M^{-1}cm^{-1}$).

2.6.4 Total phenolic content

Total phenolic content was measured using a commercial kit (Comin, China) according to the manufacturer's instructions. Briefly, 50 μL of a 1 mg/mL solution was mixed with 250 μL of reagent A, vortexed, and incubated at room temperature for 2 min. The solution was subsequently mixed with 250 μL of reagent B and 450 mL of deionized water, vortexed, then incubated at room temperature for 10 min and measured at 760 nm using an L6 UV–VIS spectrophotometer (Shanghai Yoke Instrument Co., Ltd, Shanghai, China). Deionized water (250 μL) was used instead of the reagent A as the control corresponding to the sample and tested, as previously described. Quantification was performed using the following formula:

$$\text{Total phenolic content}\ (mg/g) = \frac{Ab_{sample} - Ab_{control} - 0.0012}{5.615} \times V_{reaction} \div V_{sample} \div V_{total} \times W$$

Where Absample and Abcontrol are the absorbance values of the sample, control corresponding to sample at 340 nm, respectively; Vreaction was 1 mL; Vsample was 50 μL; Vtotal was 100 mL; W was 100 mg.

2.6.5 Amino acid composition analysis

2.6.5.1 Free amino acids quantification

The o-phthaldialdehyde (OPA) method was used to measure the free amino acid levels [20]. Briefly, the assay was carried out by adding 600 μL of L-serine standard solution or 1.0 mg/mL protein solution to 4.5 mL of OPA reagent, the mixture was vortexed and kept at room temperature for 2 min. Absorbance was measured at 340 nm using an L6 UV-Vis spectrophotometer (Shanghai Yoke Instrument Co., Ltd., Shanghai, China). Levels of free amino acids were calculated using the L-serine equivalent from

the calibration curve derived from serial dilutions (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM) of the L-serine standard solution.

2.6.5.2 Quantification of hydrolyzed amino acids

Four types of protein extracts were placed into anaerobic hydrolysis tubes, filled with high-purity nitrogen for 20–30 seconds and hydrolysed with 6.0 M HCl at 110 °C for 22 hours, respectively. After hydrolysis, the amino acid levels were analyzed using an S334D automatic amino acid analyzer (Sykam, Germany) following standard procedures.

2.6.6 Scanning electron microscopy (SEM)

The molecular morphologies of the proteins were recorded using an MDTC-EQ-M18-01 (HITACHI, Japan) following standard procedures, with the following parameters: voltage of 3.0 kV, magnification at 500- or 1000-fold.

2.6.7 Measurement of internal fluorescence

The protein extracts were suspended in deionized water at a concentration of 1.0 mg/mL. Following this, 9 μL of the supernatant was loaded into the instrument high throughput protein stability analyzer (Unchained Labs, USA) to evaluate the protein extract's internal fluorescence.

2.6.8 Measurement of circular dichroism (CD) spectra

A J-1500 CD spectrometer (JASCO, Japan) was used to obtain the CD spectra. Briefly, protein samples were dissolved in deionized water at pH levels of 5.0, 7.0, and 10.0, to a concentration of 5 mg/mL. The samples were then centrifuged at 12000×g for 15 min and further diluted to the proper concentration for CD structural analysis within the range of 190–260 nm. CD Multivariate SSE software (optimized for 190–260 nm) was used to calculate the secondary structure fractions.

2.6.9 Fourier transform infrared (FTIR) spectroscopy analysis of proteins

The FTIR spectroscopic analysis of the proteins was performed using a Bruker Verter 70 spectrometer (Bruker, Germany). Prior to analysis, protein samples were dried to eliminate moisture. Samples were spread onto the sample plate, after which they were introduced into the optical scanning path and evaluated in the 400–4000 cm^{-1} range.

2.7 Functional properties analysis

2.7.1 Water holding capacity (WHC) evaluation

The WHC of the proteins was evaluated following the method described by Hadnadev et al. [21] with some modifications. Briefly, 0.1 g of protein and deionized water (10 g) were added to a weighed Eppendorf tube, vortexed for 2 min, kept at room temperature for 2 h, and centrifuged at 5000 rpm for 20 min. The supernatant was discarded, and the tube was inverted onto a filter paper for 30 min at room temperature. Finally, the total weight of the tube, including that of the precipitate, was recorded. WHC was calculated using the following formula:

$$WHC = (W2 - W1)/W1$$

Where W1 is the mass of the tube, including the dry sample, and W2 is the mass of the tube containing the obtained precipitate.

2.7.2 Fat absorption capacity (FAC) evaluation

The FAC of the proteins was evaluated using the method reported by Hadnadev et al. [21] with modifications. Briefly, 0.1 g of protein and oil (10 g) were added to a weighed centrifuge tube, vortexed for 2 min, left at room temperature for 2 h, and centrifuged at 5000 rpm for 20 min. The supernatant was discarded, and the tube was inverted onto a filter paper for 30 min at room temperature. Finally, the total weight of the tube, including that of the precipitate, was recorded. FAC was calculated using the following formula:

$$FAC = (W2 - W1)/W1$$

Where W1 is the mass of the tube, including the dry sample, and W2 is the mass of the tube containing the obtained precipitate.

2.7.3 Digestion properties analysis

The in vitro protein digestibility of the samples was determined according to the method described by Ohanenye et al. [22] with some modifications. Briefly, 10 mg lyophilized protein was mixed with 3 mL porcine pepsin (5000 U/mL, pH 3.0). The mixture was incubated with continuous shaking in a water bath at 37 °C for 2 h, then the mixture was adjusted to pH 7.0 and 3 mL of trypsin (100 U/mL) was added with continuous shaking at 37 °C for another 2 h. After digestion, the enzyme was

inactivated and treated at 100 °C water bath for 20 min. They were then centrifuged at 6000 rpm for 20 min, and the supernatants were collected for further analysis.

The degree of hydrolysis (DH) of the enzymolysis solutions was evaluated according to the procedure described by Wang et al. [23]. Briefly, 400 μL of diluted enzymolysis solution or serine standard solution was mixed with 3 mL of OPA reagent. The cells were then incubated in the dark for exactly 2 min, and the absorbance was measured at 340 nm using an L6 UV-Vis spectrophotometer (Shanghai Yoke Instrument Co., Ltd., Shanghai, China) with a 1 cm optical path length. 400 μL dH2O was used instead of the sample as the blank. The DH was calculated using the following formula:

$$Serine_{NH2} = \frac{Ab_{sample} - Ab_{blank}}{Ab_{s\tan d} - Ab_{blank}} \times \frac{0.9516}{c}$$

$$\mathrm{h} = \frac{Serine_{NH2} - \beta}{\alpha}$$

$$\mathrm{DH}(\%) = \frac{\mathrm{h}}{\mathrm{h}_{tot}} \times 100$$

SerineNH2 represents the milliequivalents of amino group per gram of protein; Absample, Abblank and Abstand are the absorbance values of the sample, blank and L-serine standard at 340 nm, respectively; c is the concentration of sample solution (g/L); α and β are 1.00 and 0.40, respectively; h is the number of peptide bonds hydrolysed; htot is the total number of peptide bonds of the protein. The htot depends on the amino acid composition of the protein. The htot value of hemp seed protein was 7.6.

In addition, the homogeneity and distribution of the enzymolysis solutions were evaluated using high-performance gel permeation chromatography (HPGPC), according to our previously described method [24].

2.8 Evaluation of bioactivities of extracted proteins enzymatic hydrolysates

2.8.1 Evaluation of antioxidant activity

DPPH free radical-scavenging ability was measured following the method reported by Wang et al. [23]. Briefly, 2 mL of 0.1 mM DPPH solution was mixed with 1 mL of diluted enzymolysis solution, shielded from light, incubated for 30 min, and the absorbance was measured at 517 nm. Water was used as blank control. The DPPH

radical-scavenging rate was calculated using the following formula:

$$\text{DPPH free radical scavenging rate\%} = \frac{Ab_{blank} - Ab_{sample}}{Ab_{blank}} \times 100$$

Absample and Abblank are the absorbance values of the sample and blank at 517 nm, respectively.

2.8.2 Evaluation of DPP IV inhibition activity

The DPP-IV inhibition assay was performed using a Protease Assay (G8350, Promega). Briefly, the reaction system was 100 μL, DPPIV-Glo™ Reagent volume to sample volume was 1:1, the final concentration of hDPP IV was 1 ng/mL, and concentrations of enzymatic products were 1000, 5000 and 10000 μg/mL, respectively. After incubation at room temperature for 30 min, the luminescence was measured using a microplate reader (EnVision 2105 HTS, USA). The inhibition ratios were calculated using the following formula:

$$\text{Inhibition ratio}\,(\%) = \frac{Lum_{blank} - Lum_{sample}}{Lum_{blank}} \times 100$$

Lumsample and Lumblank are the luminescence of the sample and blank respectively.

2.8.3 Evaluation of anti-inflammatory activity

2.8.3.1 Cell viability

Cell viability was evaluated using a CCK-8 kit (Solarbio, China). Raw264.7 cells were inoculated at a density of 1.0 × 105 cells/cm2 and counted using a hemocytometer (Bodboge, China) in 96-well plates and cultured with DMEM (10 % FBS and 1 % antibiotics) for 24 h. The medium was then replaced with DMEM that contains various concentrations of enzymatic products (0, 50, 100, 200, 500, 2000, 5000, 10000 mg/mL) for 24 h. Afterwards, the cells were rinsed once with PBS, and 100 μL fresh DMEM and 10 μL CCK-8 solution were added. The plates were incubated further at 37 ℃ for another 2 h and then the absorbance at 450 nm was measured.

2.8.3.2 Detection of inflammatory cytokines

Raw264.7 cells were inoculated at a density of 5 × 104 cells/cm2 in 48-well plates and cultured with DMEM (with the addition of 10 % FBS and 1 % antibiotics) for 24 h. The medium was then changed into DMEM contains various concentrations and

pre-treated for 1 h followed by the addition of LPS (final concentration of 1 μg/mL), co-incubated for 12 h to collect the supernatant for measuring NO and cytokines (TNF-α, TNF-1β) content. NO and cytokine levels were determined using an ELISA Kit (Elabscience, China).

2.9 Statistical analysis

SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Significant differences ($p \leq 0.05$) were evaluated by One-factor analysis of variance (ANOVA), Tukey test. Data were represented as the mean ± standard error.

3 Results and discussion

3.1 Results of defatting of hemp seeds

To ensure the efficient retrieval of plant seed proteins, it is essential to perform degreasing or defatting, as the presence of oil invariably results in the creation of lipid-protein complexes and diminishes the extraction efficiency. Solvent extraction is a common method for degreasing oils. In this study, we selected eight different solvent/solvent mixtures to remove oil from hemp seeds and examined the protein extraction conditions. The results showed that extraction with n-butyl alcohol and ethanol resulted in a highly efficient oil removal ratio compared to the other six solvents (Fig. 1a); n-butyl alcohol had the highest removal ratio; however, the defatting powder gave an unpalatable smell, so ethanol was applied as a solvent during the degreasing of hemp seed.

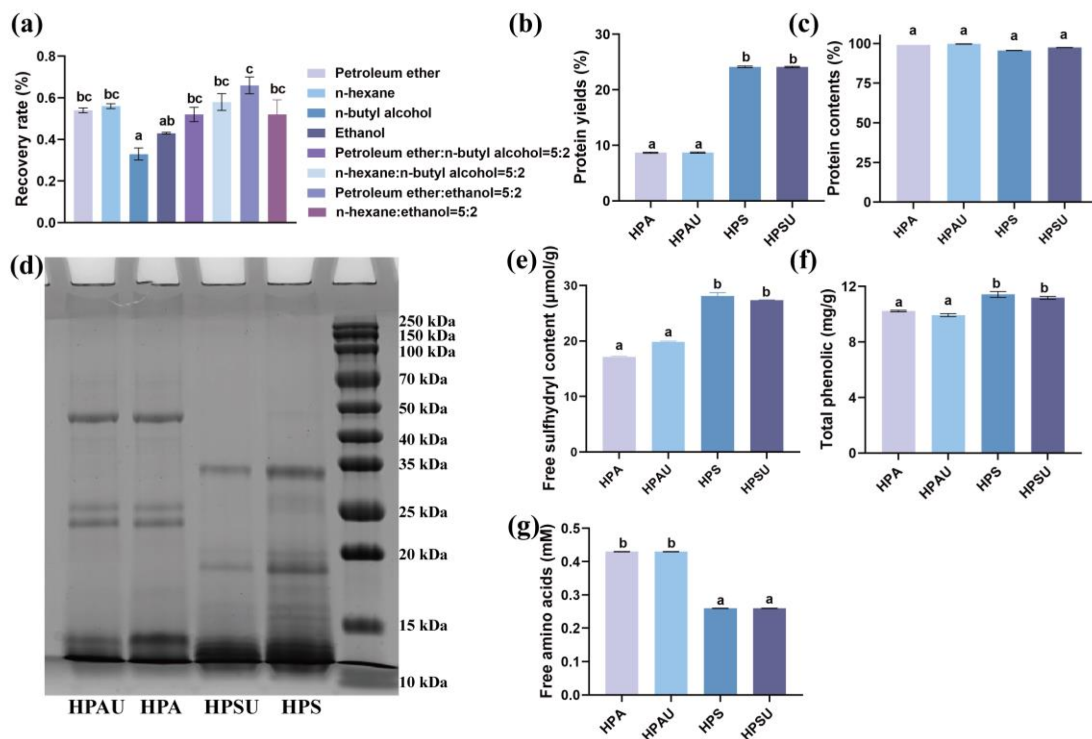

Fig. 1 Hemp seeds powder recovery for different oil extraction methods (a); Protein yields for different extraction methods (b); Protein purity measurement (c); Electropherograms of the four types of protein extracts (d); Contents of free sulfhydryl content (μmol/g), total phenolic content (mg/g), free amino content (mM) (e-g).

3.2 Effects of extraction methods on protein physicochemical properties

3.2.1 Effects of different extraction methods on protein extraction yield and protein purity

Four different protein extraction methods, AE, AEU, SE, and SEU were used. The protein yield and content were measured and are shown in Fig. 1b and c respectively. The results showed that the protein yields extracted by both SE and SEU were significantly higher than those extracted by AE and AEU; however, no significant difference was observed between AE and AEU. The same was observed between SE and SEU, indicating that the extraction buffer had a noticeable effect on protein extraction yield. This may be attributed to the difference in buffer pH and high salt concentration, as hemp seeds mainly consist of albumin (25–37 %) and the legumin edestin (67–75 %) [10, 12, 25]. Edestin is soluble in neutral saline and salt solutions [26]. The protein content of the four types of extracts were also measured, and the

results showed that all four types of protein extracts possessed a high protein content (greater than 95 %). The protein contents of HPS and HPSU were slightly higher than those of HPA and HPAU but showed no significant difference among them (Fig. 1c).

3.2.2 Effects of different extraction methods on protein composition

The composition of the four proteins was determined using SDS-PAGE. The results showed that HPA and HPAU mainly consisted of 15, 25, and 50 kDa protein constituents, whereas the molecular weights of HPS and HPSU were 10–15, 20, and 35 kDa, respectively (Fig. 1d), indicating that the protein extraction conditions affected the protein composition or quality. In particular, edestin is composed of six identical subunits, and each subunit consists of an acidic (AS) and a basic (BS) subunit linked together by a disulfide bond; AS is characterized by a molecular weight of 33–34 kDa, and the BS consists of two subunits of approximately 20 and 18 kDa, respectively [27, 28]. In addition, the edestin fraction was identified as salt-soluble [27], indicating that the electrophoresis bands corresponding to molecular weights of approximately 20 and 33 kDa in HPS and HPSU were presumably the components of edestin protein fractions, one AS subunit, and two BS subunits, respectively, which is similar to previous findings. Both HPA and HPAU contained a protein constituent of approximately 45 kDa, corresponding to the subunit of soy β-conglycinin, which was in agreement with observation over hemp seed protein isolates extracted under alkaline conditions reported previously [28, 29]. All four protein extracts contain minor bands corresponding to Mw <15 kDa, which is likely to be the albumins as previously reported [29, 30].

3.2.3 Free sulfhydryl contents

Free sulfhydryl content is one of the decisive factors affecting the functional properties (gelling, emulsifying, foaming, etc.) of proteins and is also a key indicator for estimating conformational changes in proteins [30]. Moreover, the differences in functional properties may be attributed to the distinct protein composition and aggregation properties of glycinin and the edestin fractions [29]. As shown in Fig. 1e, the free sulfhydryl contents of HPS and HPSU were higher than those of HPA and HPAU (Fig. 1e). A previous study showed that disulfide bonds and free sulfhydryl

groups can be transformed into each other [31]. It has been indicated that the SH groups are rather active, particularly under alkaline conditions [30]. The aggregation or conformational change triggered by covalent disulfide bonds resulted in the surface SH being buried in the interior of the protein structure, which might be the reason for the lower free sulfhydryl content of the hemp seed protein extracted by alkaline extraction in this study [29-31].

3.2.4 Total phenol quantification

The total phenolic content of the four types of protein extracts were evaluated, and the results show that the total phenolic contents in HPS and HPSU were notably higher than those in HPA and HPAU, which could be attributed to the consumption of the Maillard reaction under alkaline conditions (Fig. 1f). The speed of the Maillard reaction, formed by the glycosylation of food proteins with carbohydrates as conjugates, also increases with pH [32].

3.2.5 Amino acid composition analysis

3.2.5.1 Free amino acids content

Free amino acids in the four protein extracts were characterized using the OPA method. As shown in Fig. 1g, the free amino acid contents of HPAU and HPA were notably higher than those of HPS and HPSU. There was no difference between HPA and HPAU, which indicated that alkaline condition promotes protein hydrolysis, hence playing an effect on protein structural, while ultrasound treatment has almost no effect on protein hydrolysis (Fig. 1g).

3.2.5.2 Hydrolyzed amino acids quantification

The amino acid composition directly influences the nutraceutical and functional properties of proteins. The amino acid composition of the four protein extracts were measured, and the results are shown in Table 1. These results suggest that hemp seed proteins are rich in functional amino acids, including arginine, glutamic acid, methionine, and cysteine, in line with previously reported data [10, 28, 29]. In particular, the cysteine, methionine, histidine, and lysine contents of HPA and HPAU were higher than those of HPS and HPSU, whereas amino acids, including aspartic acid, alanine, valine, leucine, phenylalanine, and arginine, showed the opposite trend (Table 1). In addition, we found that HPS and HPSU contained higher levels of essential

amino acids, than HPA and HPAU, except for methionine and lysine. This finding may be attributed to the ionic strength of the NaCl solution, which attracts and preferentially retains several types of protein fractions in the solution. In addition, we found that ultrasonic treatment resulted in the reduction of essential amino acids (EAA), suggesting that salt extraction may be an optimal choice to preserve the nutritional properties of hemp seed proteins.

Table 1. Hydrolyzed amino acids profiles of the protein extracts

Amino acids	HPA (%)	HPAU (%)	HPS (%)	HPSU (%)
Aspartic acid	7.77	7.57	10.87	10.3
Threonine	2.9	2.78	3.34	3.21
Serine	4.99	4.78	5.09	4.88
Glutamic acid	19.82	18.03	18.76	17.85
Proline	3.28	3.19	3.47	3.43
Glycine	3.42	3.36	4.01	3.83
Alanine	2.94	3.05	4.18	3.97
Cystine	2.35	2.51	0.98	1.03
Valine	3.79	3.60	4.92	4.66
Methionine	2.77	2.22	1.89	1.41
Isoleucine	3.02	2.99	3.94	3.74
Leucine	5.41	5.46	6.47	6.15
Tyrosine	3.00	3.08	3.60	3.45
Phenylalanine	3.69	3.52	4.74	4.51
Histidine	3.10	3.10	2.61	2.50
Lysine	4.24	3.95	2.69	2.63
Arginine	11.38	11.08	13.88	13.05
EAA	25.82	24.52	27.99	26.31
PAA	62.97	60.24	65.83	62.73
NPAA	24.9	24.03	29.61	27.87
SCAA	5.12	4.73	2.87	2.44
AAA	27.59	25.6	29.63	28.15
BAA	18.72	18.13	19.18	18.18

EAA: essential amino acid; PAA: polar amino acid; NPAA: non-polar amino acid; SCAA: sulfur-containing amino acid; AAA: acidic amino acid; BAA: basic amino acid.

3.2.6 Effects of different extraction methods on protein morphology

The morphology of the four types of protein samples was observed via SEM, and the results are displayed in Fig. 2, showing that there was a significant difference in morphology among the four types of proteins. HPA and HPS displayed a consistent

spherical form, forming spherical protein complexes, but compared with HPA, HPS formed smooth lump-like aggregates, while HPA formed rough lump-like aggregates. Compared to HAP, HPAU was well dispersed, and the proteins formed into with proteins forming particles that resembled flower-like structures, albeit with varying sizes and folds, and were well dispersed into small and smooth ball-like aggregates, indicating that ultrasonic treatment would disrupt the integrity of the proteins.

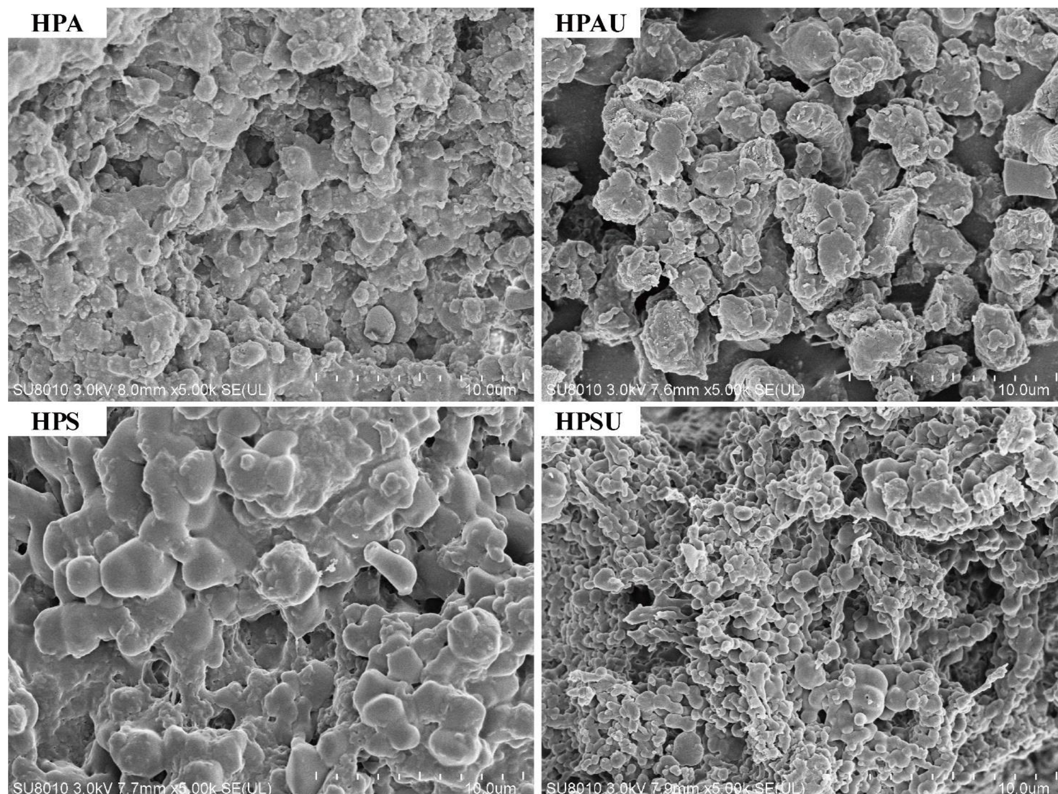

Fig. 2 SEM micrographs of HPA, HPAU, HPS and HPSU.

3.2.7 Effects on protein internal fluorescence

Proteins containing aromatic amino acid residues, including tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe), emit fluorescence upon excitation at 280 nm. Trp, Tyr, and Phe exhibit distinct fluorescence spectra due to their unique side chain chromophores, and significant fluorescence emission peaks can be observed at 348, 303, and 282 nm, respectively [33]. Nevertheless, the fluorescence intensity of Phe is extremely low and rarely observed as it is difficult to excite. Therefore, the intrinsic fluorescence of the protein primarily arises from Trp and Tyr residues. However, in cases where proteins contain both Trp and Tyr, the fluorescence of Tyr residues is

suppressed owing to the transfer of energy from Tyr residues to Trp residues [34]. The results displayed in Fig. 3a show that a strong fluorescence signal of Trp can be observed for the HPS and HPSU samples at wavelengths of approximately 350 nm, whereas the fluorescence peaks of Tyr and Trp can be seen in both the HPA and HPAU samples at nearly 300 and 350 nm. The height of the peak indicated the relative content of these amino acids in the sample; therefore, HPS and HPSU were estimated to have a higher content of Trp residues.

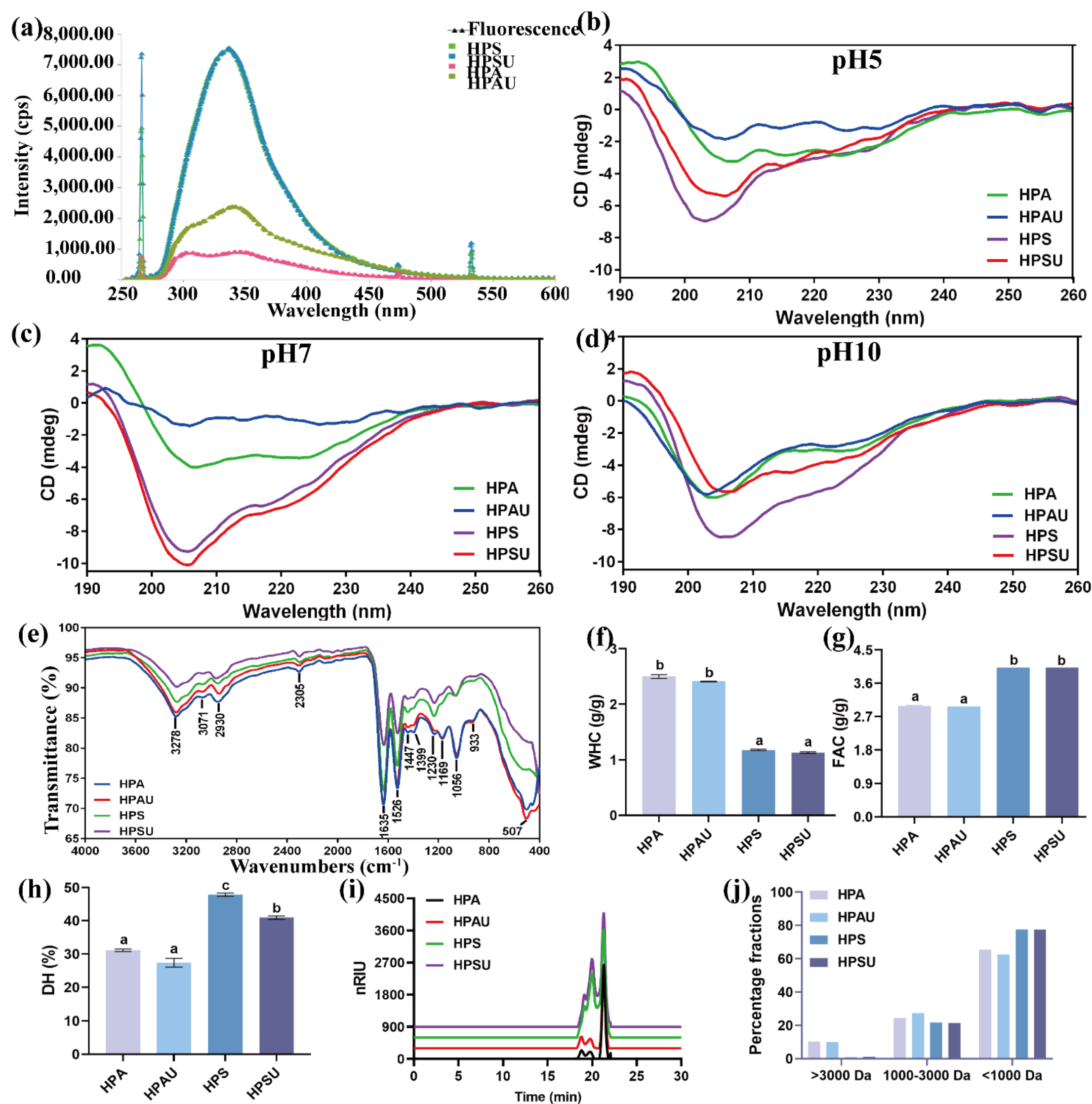

Fig. 3 (a) Internal fluorescence of hemp proteins prepared by different methods under reducing conditions, molecular masses of standards are indicated on the right; (b–d) Circular dichroism spectra of the proteins; (e) The FTIR spectra of the purified protein; (f–h) Measurement of WHC, FAC, and DH; HPGPC chromatogram (i) and percentage of the fractions (j).

3.2.8 Secondary structural composition of the proteins

Proteins are macromolecules with optical activity and exhibit varying absorption of circularly polarized light. Proteins and peptides can be analyzed using CD spectroscopy, which primarily relies on exciting electronic transitions in amide groups within a wavelength range of 180–250 nm. Secondary structures such as α-helices, β-pleated sheets, turns, and disordered sections with specific Φ, Ψ, dihedral angles and H-bond patterns have an impact on the CD spectrum of the peptide backbone. CD spectroscopy is extensively used in the investigation of protein secondary structures, and various algorithms have been established to estimate the secondary structure composition from CD spectra [35]. Circular dichroism was used to evaluate the impact of the extraction methods and pH on the secondary structural conformations of the hemp seed protein, and the results are displayed in Fig. 3b–d and Table 2. The findings indicated that the proteins derived from hemp seeds were primarily characterized by the α-helix conformation, particularly at pH levels of 5.0 and 10.0 (Table 2). This observation is further supported by the significant ellipticity observed between 200 and 220 nm (Fig. 1b-d). There was a significant decrease in α-helix conformation domination, and with an increasing domination of β-turn and unordered composition at pH 7.0, indicating weaker inter-hydrogen and intra-hydrogen bonds at pH 7.0. With the increase of pH, the α-helix conformation domination increased, which may be attributed to the increase in negative charge as the pH becomes more alkaline. This creates a more hydrophilic environment, promoting structural rearrangements that move aromatic residues away from the protein surface into hydrophobic environments. In addition, we found that HPAU and HPSU were more sensitive to pH than that of HPA and HPS, as with the changing of pH, the former showed a more significant decrease in the domination of α-helix conformation, which may be attributed to ultrasound extraction having a negative effect on the integrity of proteins.

Table 2 CD-derived protein secondary structure composition of hemp seed protein at different pH (n=3)

pH	Samples	α-helix (%)	β-sheet (%)	β-turn (%)	Unordered (%)
pH5.0	HPA	98.60±1.98	0.00±0.00	1.40±1.98	0.00±0.00

	HPAU	98.70±1.84	0.00±0.00	1.30±1.84	0.00±0.00
	HPS	92.97±1.76	0.00±0.00	7.03±1.76	0.00±0.00
	HPSU	96.20±4.25	0.00±0.00	3.80±4.25	0.00±0.00
pH7.0	HPA	73.27±2.21	0.00±0.00	17.53±4.45	9.20±1.30
	HPAU	69.90±4.60	0.00±0.00	3.80±1.50	27.40±7.20
	HPS	81.00±5.20	0.00±0.00	19.00±5.20	0.00±0.00
	HPSU	66.40±1.60	0.00±0.00	22.83±1.55	10.75±0.05
pH10.0	HPA	92.90±4.30	0.00±0.00	0.95±0.58	6.15±3.45
	HPAU	96.19±0.11	0.00±0.00	0.00±0.00	3.81±0.11
	HPS	93.00±3.60	0.00±0.00	4.43±2.84	2.57±0.75
	HPSU	85.90±4.18	0.00±0.00	4.63±1.47	9.47±2.12

Moreover, FTIR experiments were conducted to assess the effects of the extraction methods on the protein structural characteristics, and the results are shown in Fig. 3e. The findings indicated that in all FTIR spectra, there were two prominent absorption bands observed at 3278 and 2930 cm-1. These two bands were linked to the amide A and B regions, representing N-H stretching vibrations and C-N stretching vibrations, respectively. As shown in Fig. 3e, HPA and HPAU exhibited stronger absorption than HPS and HPSU, which can be attributed to the alkaline environment that enhanced the Maillard reaction and facilitated the formation of Amadori compounds through the arrangement of a Schiff base [36]. The number of N-H groups in the protein increases after undergoing a structural rearrangement, similar to the Maillard reaction [37]. The saccharides caused absorption peaks in the range of 1100–1000 cm-1 due to the stretching vibration of C-OH and C-C [37], and the results displayed in Fig. 3e show that HPA and HPAU had stronger absorption peaks at 1056 cm-1 than that of HPS and HPSU, which indicated that saccharide modifications appeared much more marked in the HPA and HPAU proteins. The FTIR spectra contained three regions that were highly responsive to changes in the protein structure. The amide I region, which is generated by the stretching vibration of the C=O bond in the peptide backbone, spans 1700–1600 cm-1. The amide II region is the result of the stretching vibration of C-N and the bending vibration of N-H, spanning from 1600 to 1500 cm-1. Additionally, the amide III region, also caused by the stretching vibration of C-N and the bending vibration of N-H, ranges between 1360 and 1200 cm-1 [38]. In our study, the FTIR spectra revealed

absorption peaks at 1635, 1526, and 1230 cm-1 for the amide I, II, and III regions (Fig. 3e). In particular, the amide I region showed the strongest correlation with each secondary structural element of the protein. From Fig. 3e we can see that HPA and HPAU have stronger absorption peaks at 1635 and 1526 cm-1, but weaker absorption peaks at 1230 cm-1.

3.3 Effects on protein functional properties

3.3.1 Effects on protein WHC

WHC pertains to the quantity of water soaked in proteins, which is crucial in influencing the functional characteristics, palatability, and shelf life of protein-based food products [39]. WHC is affected by several parameters, including protein molecular weight, subunit composition, and conformation [39]. Protein molecules can undergo conformational alterations that may reveal previously enclosed amino acid side chains, allowing them to interact with water [40]. The balance between the water-attracting and water-repelling properties of amino acids in proteins, along with factors like the pH and ionic strength of the environment, and the ability of polar amino groups in proteins to dissolve in water, which are crucial for protein-water interactions, are all vital [27]. The effects of the extraction methods on the WHC were evaluated in this study, and the results displayed in Fig. 3f show that the WHC of HPA and HPAU were markedly higher than those of HPS and HPSU. In other words, the HPS and HPSU extracts had a lower ability to hold water against centrifugal forces than HPA and HPA. Several studies have also reported that protein extracted by an NaCl solution has a lower WHC than that extracted by an alkaline solution [40, 41]. This may be attributed to the extensive conformational changes caused by alkali extraction, including partial protein denaturation and exposure to additional hydrophilic binding sites for hemp proteins [21, 42].

3.3.2 FAC

FAC, which indicates how well the hydrophobic groups of proteins can interact with lipids, is an important functional property as it is closely associated with protein taste and flavor retention [27]. Proteins with a high FAC can be used as ingredients in food formulations [39]. Hemp seeds are typically abundant in over 30 % of the oil,

accounting for over 80 % of the polyunsaturated fatty acids [10]. In this study, the FAC of HPS and HPSU were notably higher than those of HPA and HPAU (Fig. 3g). Similar trends have been observed in protein isolates from guava [43], flaxseed [41], and wild Lupinus campestris seeds [40], indicating that an NaCl solution may improve the exposure of several surface-enclosed hydrophobic groups/non-polar side chains from protein extracts. This in turn facilitates the effective absorption of oil by binding to the hydrocarbon chains of the fats [40].

3.3.3 In vitro digestion properties

The breakdown of proteins into peptides by enzymes in the gastrointestinal tract may be necessary for the fragments to be taken up by the bloodstream, and may have either a beneficial or detrimental effect on health. To assess the digestive properties of the protein extracts, we used an in vitro static model to simulate gastrointestinal digestion and measured the degree of hydrolysis (DH). As shown in Fig. 3h, the DH values of HPS and HPSU were notably higher than those of HPA and HPAU. In addition, the results of the distribution and homogeneity evaluation showed that the degree of hydrolysis for HPS and HPSU was higher than that for HPA and HPAU (Fig. 3i).The percentages of the <1000 Da fractions for HPA, HPAU, HPS, and HPSU were 65.37 %, 62.56 %, 77.53 %, and 77.42 %, respectively (Fig. 3j), indicating better digestion properties of proteins extracted using a NaCl solution than those of proteins extracted under alkaline conditions. This may be attributed to the higher molecular weights of the protein subunits in HPA/HPAU than in HPS/HPSU (Fig. 1d).

3.4 Biological activities evaluation of enzymatic products of the extracted protein

3.4.1 DPPH free radical scavenging capacity

The antioxidant activities of the four protein enzymatic products were evaluated by measuring their DPPH free radical scavenging ability. The results displayed in Fig. 4a illustrate that the DPPH free radical scavenging abilities of HPS and HPSU were notably higher than those of HPA and HPAU (Fig. 4a). Previously reported findings have indicated that digestion leads to the release or synthesis of novel peptides or amino acids possessing antioxidant properties. Furthermore, these compounds exhibit an enhanced capacity to absorb oxygen free radicals, as evidenced by their increasing

categories and contents during reactions [44, 45]. Peptides with low molecular weights can readily interact with and eliminate free radicals due to their small size [44]. Previous research on the simulated digestion of purple kidney bean (Phaseolus vulgaris L.) protein and Z. bungeanum seed proteins in vitro demonstrated that the antioxidant activity of digestive products improved with an increase in the degree of hydrolysis [44, 46], which was in accordance with the trend observed in this study.

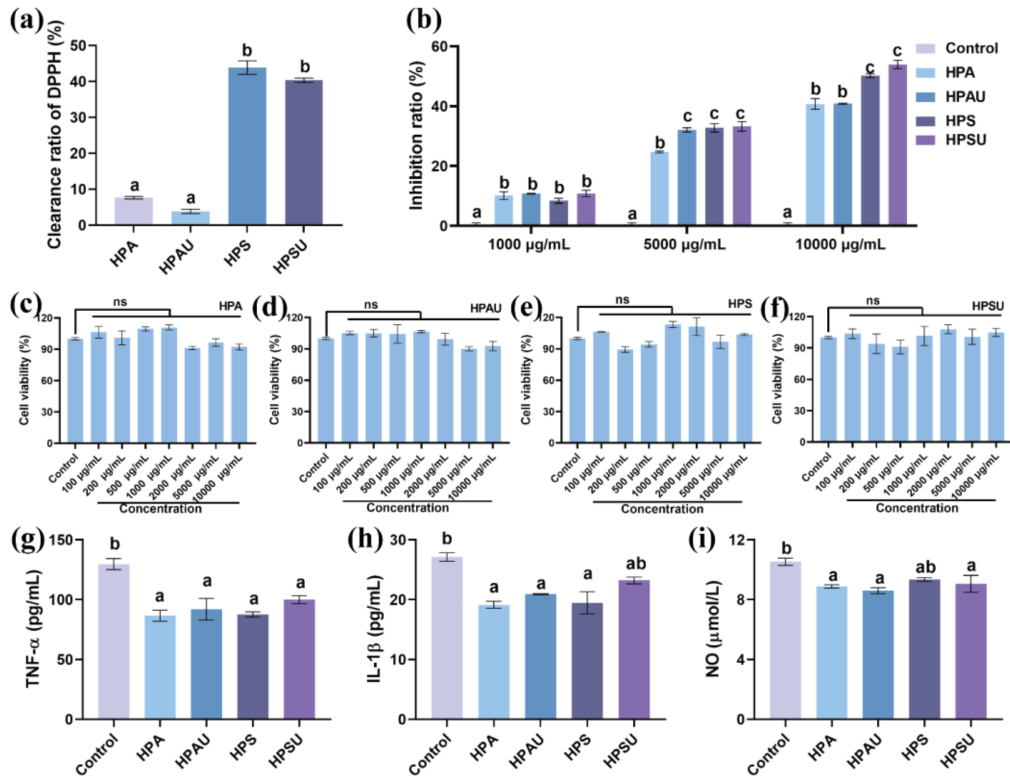

Fig. 4 Biologic activities of hemp seed protein enzymatic products. (a) DPPH scavenging ability, (b) DPP IV inhibition, (c–f) Enzymatic products on Raw264.7 cell viability evaluation, (g–i) concentrations of TNF-α, IL-1β and NO, respectively.

3.4.2 DPP IV inhibition evaluation

Glucagon-like peptide-1 (GLP-1), a hormone known as incretin, is released by L cells in the intestine. It plays a vital role in maintaining glucose balance by stimulating the secretion of insulin, enhancing the mass of beta-cells, suppressing the release of glucagon, delaying stomach emptying, decreasing intestinal movement, and reducing food consumption [47, 48]. However, DPP-IV secreted by intestinal L cells causes rapid degradation (with a half-life of 1–2 min) of GLP-1 [49]. Therefore, DPP-IV inhibitors, which are known to hinder DPP-IV activity and prolong the lifespan of active hormones, are regarded as effective agents for regulating the glucose balance. The results displayed in Fig. 4b illustrate that after proteolysis, all four protein extracts could inhibit DPP-IV activity to a degree, and with increasing concentrations of enzymatic products, the inhibition of DPP-IV activity increased in a dose-dependent manner. In addition, the inhibitory effects of HPS and HPSU were notably higher than those of HPA and HPAU at levels of 10, 000 μg/mL. This may be attributed to the higher values of DH of HPS and HPSU.

3.4.3 Anti-inflammatory activity

Raw264.7 cells are widely used because of their high sensitivity to external stimuli and secretion of inflammatory cytokines. In our study, Raw264.7 cells were used to evaluate the anti-inflammatory activity of the four kinds of protein extract enzymatic products. First, we treated Raw264.7 cells with various concentrations of enzymatic products for 24 h, and cell viability was measured using a CCK-8 kit (). The results showed that all enzymatic products showed no toxicity below 10, 000 μg/mL (Fig. 4c–f), hence, 5000 μg/mL of enzymatic products was used in the subsequent experiments. After aggregation growth for 24 h, the cells were treated with 5000 μg/mL of enzymatic products and 1 μg/mL lipopolysaccharide for one hour. It has been reported that when the Raw264.7 cells were subjected to inflammatory stimulants including LPS, the levels of NO and cytokines (TNF-α and IL-1β) can participate in the immune response with abnormal secretion [50]. In our study, we also found that after treatment with LPS, the concentrations of TNF-α, IL-1β, and NO were significantly increased; however, they were decreased by the treatment of enzymatic products (Fig.

4g–i), indicating the potential anti-inflammatory effects of HPA, HPAU, HPS, and HPSU enzymatic products. No significant difference was observed in anti-inflammatory activity among the four types of enzymatic products.

4 Conclusion

In this study, we used four different methods (alkaline extraction, alkaline extraction combined with ultrasonication, salt extraction, and salt extraction combined with ultrasonication) to extract the hemp seed proteins. The results illustrated that an extraction buffer plays a vital role in releasing protein physicochemical and nutritional properties, as well as its bioactive properties, and that salt extraction protein products including HPS and HPSU possessed significantly higher total phenolic content, FAC, DPPH free radical scavenging, and DPP IV inhibiting ability, as well as more effective digestibility than alkali extraction protein products (HPA and HPAU). Ultrasonic treatment may result in a decrease in hemp seed nutritional quality, as it leads to the distribution of protein integrity, a more significant Maillard reaction, and a reduction of EAA, suggesting that salt extraction may be the optimal choice to preserve the nutritional properties, functional characteristics, and biological activities of hemp seed proteins. These findings contribute to developing an economically viable and sustainable extraction process of proteins from hemp seed.

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Declaration of competing interest

All the contributing author(s) report(s) no conflict of interests in this work.

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