scientific reports

Check for updates

OPEN

Nutritional value, antibacterial activity, ACE and DPP IV inhibitory of red pomegranate seeds protein and peptides

Zahra Akbarbaglu[1], Narges Mazloomi[2,3]✉, Laleh Karimzadeh[2,3], Khashayar Sarabandi[4]✉, Seid Mahdi Jafari[5,6] & Mohammad Ali Hesarinejad[7]

One of the challenges related to food and agriculture industries is the production of waste and by-products. In this study, red pomegranate seeds (PS) was selected as a by-product (from oil extraction) for the production of bioactive hydrolysate (with Alcalase, pancreatin, trypsin and pepsin). The composition of essential (~ 23.3%), hydrophobic (~ 32.9%), antioxidant (~ 13.9%) amino acids, and PER index (~ 2.1) especially in hydrolysates by alcalase (H-Al) indicated the nutritional value, antioxidant activity and high digestibility of hydrolysate. Secondary structures and amide regions (I, II and III) were identified in PS-protein. Enzymolysis led to the improvement of solubility, emulsification and foaming capacity of PS-protein, especially in acidic conditions. The water and oil holding capacity were also affected by the type of proteases. The most biological activities (DPPH, ABTS, OH, NO radicals scavenging, reducing power, total antioxidant and metal-ions chelating activities), also, Angiotensin I-converting enzyme (ACE) (50.1%) and Dipeptidyl peptidase-4 (DPP-IV) (61.2%) inhibition were achieved through hydrolysis using Alcalase and pancreatin. While, the highest antibacterial effect (*E. coli* and *S. aureus*) was obtained after hydrolysis with Alcalase. PS- hydrolysate can be considered as a natural nutritious, functional, antioxidant, preservative, blood pressure lowering and antidiabetic compounds in food formulations and dietary supplements.

Keywords ACE-inhibitory, Antibacterial activity, DPP-IV inhibitory, Bioactive hydrolysate, Pomegranate seeds

Abbreviations

PS	Pomegranate seeds
PER	Protein efficiency ratio
H-Al	Hydrolysates by alcalase
ACE	Angiotensin I-converting enzyme
DPP-IV	Dipeptidyl peptidase-4
BPs	Bioactive peptides
ABTS	(2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt)
DPPH	(1,1-Diphenyl-2-picrylhydrazyl)
Ferrozine	(3-(2-Pyridyl)-5-6-diphenyl-1,2,4-triazine-4′,4″ disulphonic-acid sodium salt)
SNP	Sodium nitroprusside
SA	Sulphanilamide
NEDD	Naphthyl-ethylenediamine-dihydrochloride

[1]Department of Food Science, College of Agriculture, University of Tabriz, Tabriz 5166616471, Iran. [2]Department of Nutritional Sciences, School of Health, Mazandaran University of Medical Sciences, Sari, Iran. [3]The Health of Plant and Livestock Products Research Center, Mazandaran University of Medical Sciences, Sari, Iran. [4]Department of Food Chemistry, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran. [5]Department of Food Materials & Process Design Engineering, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. [6]Halal Research Center of IRI, Iran Food and Drug Administration, Ministry of Health and Medical Education, Tehran, Iran. [7]Department of Food Sensory and Cognitive Science, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran. ✉email: samira.mazloomi@yahoo.com; kh.sarabandi@irfst.ac.ir; sarabandi_21@yahoo.com

TCA	Trichloroacetic acid
PSP	Pomegranate-seeds protein
PSPH	Pomegranate-seeds protein hydrolysates
BSA	Bovine serum albumin
DH	Degree of hydrolysis
WHC	Water holding capacities
OHC	Oil holding capacities
RP-HPLC	Reverse phase high performance liquid chromatography
TBA	Thiobarbituric acid
DW	Distilled water
FC	Foaming capacity
FS	Foam stability
PSPH-Al	Hydrolysis process using alcalase enzyme
PSPH-Pa	Hydrolysis process using pancreatin enzyme
PSPH-Tr	Hydrolysis process using trypsin enzyme
PSPH-Pe	Hydrolysis process using pepsin enzyme
EAA	Essential amino acids
HAA	Hydrophobic amino acids
AAA	Antioxidant amino acids
NO	Nitric oxide

Food proteins and their peptide hydrolysates have emerged as potent bioactive compounds and come under increased scrutiny. These compounds are typically inactive in their original protein form and are liberated through processes such as microbial fermentation by proteolytic microorganisms, enzymatic activities of plants and microorganisms, and digestion enzymes[1–3]. In this regard, various sources, including plant, marine, and animal proteins (such as Milk, egg, red meat, chicken, fish, shrimp, oyster, sea weed, crustaceans, seahorse, beans, wheat, barley, flaxseed, Peanuts, canola and algae), have been utilized for the production of bioactive peptides[4,5]. When proteins and peptides undergo hydrolysis, they can play a significant biological role by exhibiting antioxidant[6–8], antihypertensive[7,9,10], anti-inflammatory, anti-proliferative, cytomodulatory[11], antidiabetic[1,12], antibacterial[13] and anticancer properties[14,15]. The health advantages of bioactive peptides (BPs) are considerable enough that they are classified as pharmaceutical agents. Currently, there are over 80 BP medications available for the management of diabetes, cancer, osteoporosis, and multiple sclerosis, with many more in various stages of clinical and pre-clinical development. Additionally, more than 150 BPs are currently being evaluated in clinical trials, while around 600 are in pre-clinical research[16].

Generally, bioactive peptides derived from protein hydrolysates can serve as nutritional supplements or aid in the development of functional foods, often originating from underutilized resources including food industry by-products. These peptides can be utilized as nutritional supplements or functional enhancers in the creation of functional foods.

The by-products of the juice industry, such as peels, seeds, and pulps, account for approximately 50% of the raw fruit that is processed. If not properly managed, this potentially valuable resource can contribute to disposal challenges. For example, many studies have been conducted to evaluate the health-promoting properties of extracts from different parts of pomegranate (such as the peel and the seed). In those studies, properties such as antioxidant, antimicrobial, antifungal, antiviral, and anti-diabetic, anti-Alzheimer, and anti-glaucoma effects have been observed and reported[17,18]. However, each group of bioactive compounds has different mechanisms for demonstrating health-promoting properties and biological effects. On the other hand, the defatted flour protein from these seeds presents an economical source for the production of peptides through enzymatic hydrolysis, which have the potential to function as antioxidants, anticancer agents, antidiabetic compounds, or antihypertensive substances. For instance, pomegranate seeds, a significant by-product of the juice production industry, are noted for their protein content, which can reach 15.66–18.62% in seed flour[19,20].

Pomegranate is native to Iran, and a significant amount of seeds is generated as a byproduct of pomegranate juice production. This study investigates optimal conditions for enzymatic hydrolysis of pomegranate seed protein concentrate using various enzymes. Enzymatic hydrolysis is essential as it greatly affects the bioactive properties of the resulting peptides, with both the enzyme type and the amino acid profile influencing these properties. The hydrolyzed proteins are expected to exhibit beneficial health effects, including antioxidant activity, antihypertensive potential, and antidiabetic properties, alongside suitable functional and antimicrobial attributes. These benefits may enhance their use in functional foods and nutritional supplements. Thus, this study explores the effects of different enzymes on the bioactive properties of hydrolyzed pomegranate seed proteins while also assessing their functional, antimicrobial, and health-promoting potential. Additionally, an analysis of the amino acid profile was performed to evaluate nutritional properties and identify antioxidant factors.

Materials and methods

Materials

The chemicals including ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), DPPH (1,1-Diphenyl-2-picrylhydrazyl), Ferrozine (3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-4′,4″ disulphonic-acid sodium salt), Alcalase 2.4 L. (Novo Nordisk, Bagsvaerd, Denmark), Comasi brilliant blue (G250), Pepsin (porcine gastric mucosa), Sodium nitroprusside (SNP), Pancreatin from porcine pancreas (P1750, 4 × USP specifications), Trypsin (bovine pancreas), Sulphanilamide (SA), Pyrocatechol violet, and Naphthyl-ethylenediamine-dihydrochloride (NEDD) were provided from Sigma–Aldrich Co. (St. Louis, MO, USA).

Alpha-deoxyribose was supplied from Fluka (Stockholm, Sweden). Potassium persulphate, ferrous chloride, TCA (trichloroacetic acid), and other chemicals used were purchased from Merck (Darmstadt, Germany).

Protein extraction

The pomegranate seeds from the Saveh-region (Iran) were dried in the shade for 3 days. The moisture content of the seeds, determined by AOAC method 925.10 (2012), was approximately 7%. These dried seeds were then prepared, ground, and sieved with 30 mesh. Following the method described by Zheng et al.[3] a protein concentrate was obtained from the pomegranate seed flour. The process involved removing the oil from the flour using hexane, extracting the protein in a 0.1% salty solution with a pH of 9.5, precipitating the protein by adjusting the pH to 4.2 with 0.5 M HCl, neutralizing the mixture to pH 7 with 0.5 M NaOH, and finally The suspension was then centrifuged at 10,000 rpm, at room temperature for 15 min. The produced pellets were washed with 20 mL distilled water and then freeze-dried (Martin Christ, Germany).

Structural properties (FTIR) of protein

A mixture of PSP and KBr (potassium bromide) at a ratio of 1 to 100 was pressed to create a disk shape. Finally, chemical structure of the samples was evaluated at a frequency of 4000 to 400 cm^{-1} with a spectral resolution of 4 cm^{-1} and an aperture of 5.0 mm by a FTIR (Shimadzu 8400 S, Japan) spectrophotometer[21].

Preparation of protein hydrolysates

The process of dissolving the pomegranate-seeds protein (PSP) at a concentration of 5% w/v was conducted in 0.01 M PBS for 30 min at a temperature of 50 °C. Subsequently, the proteins were enzymatically hydrolyzed using Alcalase (protease from *Bacillus lichenformis*, 2.4 U/g., pH = 8, 50 °C), Trypsin (from bovine pancreas, enzyme activity: ≥10,000 units/mg protein, pH = 8, 37 °C), Commercial Pancreatin (pH = 8, 37 °C), and Pepsin (protease from porcine gastric mucosa, enzyme activity: ≥ 2,500 units/mg protein, pH = 2, 37°C) at an enzyme to substrate ratio of 2% for 120 min. The enzymatic activity was halted by transferring the reaction mixture to a water bath at 95 °C for 15 min. The resulting mixture was then centrifuged at 7,000 × g for 10 min, separating the supernatant containing the hydrolysates. The hydrolysates were freeze-dried (Martin Christ, Germany) at -20 °C under a pressure of 0.1 mbar and stored at -18 °C for preservation[22].

Degree of hydrolysis (DH)

We combined PSPH and TCA (0.44 M) in a 1:1 v/v ratio and then placed the mixture in a refrigerator at 4 °C for 10 min. Afterward, the mixture was centrifuged at 7,000 × g for 10 min. The concentration of soluble proteins was measured using the Bradford technique[23] and a standard curve was created using bovine serum albumin (BSA). The degree of hydrolysis (DH) was determined using the following equation [24]:

$$\mathrm{DH}\ (\%) = \frac{\mathrm{Protein\ (TCA\ +\ Supernatant)}}{\mathrm{Protein\ (hydrolysate\ suspension)}} \times 100 \tag{1}$$

Techno-functional properties

Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) as measures of emulsifying properties were determined using the method of Klompong et al.[25]. Briefly, aliquots of 15 mL hydrolysate solution (1%, w/v) and 5 mL grape seed oil were mixed and pH series of 3, 5, 6, 7 and 9 were adjusted. The mixture was homogenized using a polytron homogenizer (PT 10–35, Kinematica, Switzerland) at 20,000 ×g for 2 min to get an O/W emulsion. An aliquot of 50 µL of the obtained emulsion was pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 mL of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer (PJ Instruments, model T80, England). The absorbance measured instantly (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsifying activity index (EAI) and emulsion stability (ES) as follows:

$$EAI\ \left(m^2/\mathrm{g}\right) = \frac{(2 \times\ 2.303 \times\ A_0)}{0.25 \times\ \text{protein weight (g)}} \tag{2}$$

$$ESI\ (\%) = \frac{A_0 - A_{10}}{A_{10}} \times 100 \tag{3}$$

Foaming properties

The foaming capacity (FC) and foaming stability (FS) were measured using the method of Klompong et al.[25]. Briefly, an aliquot of 15 mL of sample solution (0.5%, w/v) was adjusted to pH of 3, 5, 6, 7 and 9, succeeded by homogenization at 19,000 ×g for 2 min to incorporate air bubbles. The whipped sample was instantly poured into a 25mL cylinder and the total volume was determined after 45 s. The foaming capacity was calculated on the basis of the following equation:

$$FC\ (\%) = \frac{\mathrm{Volume\ after\ whipping\ (mL)}}{\mathrm{Volume\ before\ whipping\ (mL)}} \times 100 \tag{4}$$

The whipped sample kept at 25 °C for 10 min and the volume of that was recorded. The foaming stability was calculated as follows:

$$FC\ (\%) = \frac{\text{Volume after standing}\ \ (\text{mL})}{\text{Volume before whipping}\ \ (\text{mL})} \times 100 \quad (5)$$

Water (WHC) and oil holding capacity (OHC)
Oil holding capacity (OHC) and water holding capacity (WHC) were determined according to Ge et al.[26]. Briefly, a sample of 0.5 g was combined with either soybean oil or distilled water in a ratio of 5:1 (5.0 g). This mixture was blended for 30 min to ensure uniform distribution of the sample. Following this, the emulsions were centrifugated at 4,000 × g for 20 min to separate the components and remove any insoluble particles. Different weights were used to calculate OHC and WHC.

Amino acid profile

In this analysis, 30 mg of the hydrolysates was subjected to hydrolysis with 6 N HCl at a temperature of 110 °C for 24 h. Sample was diluted to 250 mL and an aliquot was filtered through 0.22 µm MCE membrane syringe filter (Guangzhou Jet Bio-Filtration, China). The filtrate was derivatized using the AccQ-Fluor Reagent Kit (Waters, Milford, MA, USA). Finally, amino acids were quantified with an HPLC (Novapack C18, 4 µm, Waters, Milford, MA) equipped with an AccQ-Tag amino acids C18 column (Waters, Milford, MA, USA) and coupled to a UV detector (Waters, Milford, MA, USA). The absorbance was recorded at a wavelength of 248 nm. The mobile phase A was 100% AccQ-Tag Eluent, the mobile phase B was 100% acetonitrile HPLC-grade, and the mobile phase C was 100% water HPLC grade. The gradient employed was: 0–0.5 min 100% A; 0.5–18 min 99% A and 1% B; 18–19 min 95% A and 5% B; 19–28 min 91% A and 9% B; 28–35 min 83% A and 17% B; 35–38 min 60% B and 40% C; and 38–40 min 100% A; at a flow rate constant at 1 mL/min. The quantification was done by comparing the retention time and area of the Amino Acid Standard Hydrolysate (Thermo Scientific Pierce, Rockford, IL, USA) and Empower software (Waters, Milford, MA, USA)[27].

Antioxidant characterization

DPPH free radical scavenging activity
A mixture was prepared by combining 2 mL of PSPH (40 mg/mL) with 2 mL of 0.2 mM DPPH solution. The resulting blend was then kept in a dark environment for 30 min. Subsequently, the mixture was centrifuged at 5,000 × g for 10 min, and the absorbance of the supernatant was measured at 517 nm. This procedure was conducted following the protocol described[28].

$$\text{Inhibition}\ (\%) = 1 - \left(\frac{\text{sample Abs}}{\text{blank Abs}}\right) \times 100 \quad (6)$$

ABTS free radical scavenging activity
To create a mixture, ABTS and potassium persulfate were combined at concentrations of 7 mM and 2.45 mM, respectively. The blend was then stored in darkness for 12 h and later diluted with 0.2 M PBS (pH 7.4) until the final absorbance reached 0.70 at 734 nm. Afterward, 20 µL of the PSPH (10 mg/mL) or its peptide solutions (ranging from 1 to 20 mg/mL) were added to 2 mL of the ABTS solution. The mixture was vortexed for 10 s and left in the dark for 6 min. The absorbance was then measured at 734 nm[29].

Hydroxyl radical scavenging activity
The mixture of the reaction consisted of α-deoxyribose (10 mM; 0.5 mL), $FeSO_4$-EDTA (10 mM; 0.2 mL), PSPH (40 mg/mL; 0.2 mL), PBS (0.2 M, pH 7.4; 0.9 mL), and hydrogen peroxide (10 mM; 0.2 mL). The mixture was then incubated at 37 °C (1 h). Following the incubation, trichloroacetic acid (TCA) (3%; 1.0 mL) and thiobarbituric acid (TBA) (1.0%; 1.0 mL) were added. The resulting mixture was then boiled in water for 15 min and subsequently cooled in an ice bath. The absorbance was then measured at 532 nm[30].

Reducing power activity
The reaction mixture was prepared by combining PSPH (40 mg/mL; 0.5 mL), phosphate buffer (0.2 M, pH 6.6; 0.5 mL), and potassium ferricyanide (1%; 0.5 mL). The mixture was then incubated at 50 °C for 20 min. Following this, TCA (10%; 0.5 mL) was added, and the mixture was centrifuged at 3,000 ×g for 10 min. Subsequently, 1.0 mL of the supernatant was mixed with 1.0 mL of distilled water (DW) and ferric chloride (0.1%; 0.2 mL). The absorbance at 700 nm was then measured[31].

Fe^{2+} chelating activity
The solution was prepared by combining PSPH (40 mg/mL; 1 mL), double DW (1.8 mL), iron (II) chloride solution (2 mM; 50 µL), and Ferrozine solution (5 mM; 0.1 mL). After thorough mixing, the solution was allowed to stand at ambient temperature for 10 min, and then the absorbance was recorded at 562 nm, using the procedure described[27].

Cu^{2+} chelating activity
Initially, a solution containing $CuSO_4$ at a concentration of 0.2 mM was mixed with PSPH at a concentration of 40 mg/mL in a 1:1 ratio, and then allowed to sit at room temperature for 5 min. Subsequently, a 10% TCA solution (1 mL) was added to the mixture, which was then centrifuged at 2,000 × g for 10 min. The resulting supernatant (2 mL) was combined with pyridine (10%) and pyrocatechol violet (0.1%) in volumes of 1 mL and 20 µL, respectively. After thorough mixing using a vortex-mixer, the sample was incubated at ambient

temperature for 5 min. Finally, the sample absorbance was measured at 632 nm, following the method outlined by You, et al.[30].

Total antioxidant activity (TAA)
The total antioxidant capacity was determined following the method outlined by Akbarbaglu, et al.[32] with slight modifications. A blend of PSPH (10 mg/mL; 0.2 mL) and 2 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was incubated at 90 °C in a water bath for 90 min. Subsequently, the absorbance of the mixture was assessed at 695 nm. Increased absorbance levels correspond to greater total antioxidant capacity.

Nitric-oxide scavenging activity
NO-scavenging activity was determined following the method outlined by Tsai, et al.[33] with minor adjustments. A mixture of PSPH (40 mg/mL; 200 µL) and sodium nitroprusside solution (10 mM; 200 µL) was incubated for 150 min at ambient temperature in the presence of light. Subsequently, an equal amount of freshly prepared Griess reagent (composed of 1% sulphanilamide and 0.1% NEDD dissolved in 2.5% phosphoric acid) was added, and the absorbance was measured at 546 nm.

Angiotensin I-converting enzyme inhibitory activity
In the summary, every sample (2 mg/mL) was mixed with ACE (50 µL) and incubated at room temperature for 10 min. The resulting mix was then combined with Hip-His-Leu (8.3 mM, 250 µL) and incubated at 37 °C for 30 min. To halt the reaction, 1 N HCl (250 µL) was added. The released hippuric acid was extracted using ethyl acetate (500 µL). After evaporating the ethyl acetate, the acid was dissolved in DW (1 mL), and the absorbance was assessed at 228 nm using a UV–visible spectrophotometer (Cecil CE 2021, Lab Equip Instruments Ltd). The activity of each sample was tested in triplicate. For comparison, a control assay without protein hydrolysate was carried out. A 100% inhibitory activity would denote complete enzyme suppression without any rise in absorbance value[34,35].

Dipeptidyl peptidase IV (DPP-IV) inhibitory activity
Samples were first dissolved in 100 mM Tris-HCl buffer at pH 8.0 to reach a final concentration of 1.25 mg/mL. Then, the sample (25 µL) was mixed with substrate Gly-Pro p-nitroanilide hydrochloride (6 mM; 25 µL) in a 96-well microplate and incubated at 37 °C for 10 min. The colorimetric reaction began with the addition of human DPP-IV (4.5 unit/mL; 50 µL) and was allowed to proceed at 37 °C for 60 min. The reaction was halted by introducing sodium acetate buffer (1 M, pH 4.0; 100 µL). The absorbance of the released p-nitroanilide was detected at 405 nm using a Multiskan™ FC Microplate Photometer (Waltham, MA, USA). The absorbance of the sample was adjusted by subtracting the blank, replacing DPP-IV with Tris-HCl buffer (100 mM, pH 8.0). The positive control, without an inhibitor, utilized buffer in place of the PSPH sample. The negative control, indicating no DPP-IV activity, employed buffer instead of the DPP-IV solution[36]. The percentage of DPP-IV inhibition was calculated using the equation:

$$\text{DPP} - \text{IV inhibition } (\%) = 1 - \frac{\text{Abs Sample} - \text{Abs Sample blank}}{\text{Abs positive control} - \text{Abs negative control}} \times 100 \quad (7)$$

Antibacterial activity of hydrolysate
The effectiveness of the hydrolysate in inhibiting bacterial growth was assessed using the agar well diffusion method. Initially, a solution of hydrolysate was prepared in DW at a concentration of 50 mg/mL. Bacterial suspensions were created by diluting an overnight culture in DW to achieve a turbidity equivalent to 0.5 McFarland standards. Each type of bacteria was spread onto the surface of a nutrient agar plate by applying 200 µL of the bacterial suspension. A sterile corn borer was used to create a well in the agar, into which 200 µL of the peptide solution (at a final concentration of 10 mg per well) was added. Positive control wells contained oxytetracycline solution (50 µg per well), while negative control wells contained DW. The plates were then incubated at 28 °C for 24 h, and the diameter of the zones of inhibition around each well was measured[37].

Statistical analysis
The data was statistically analyzed using SPSS software (version 19.0, SPSS Inc., Chicago, IL) and one-way analysis of variance (ANOVA). The analysis was repeated three times. Furthermore, the average values were compared using Duncan's test with a significance level of 5%.

Results and discussion
Structural properties (FTIR) of PS-protein
Figure 1 shows the functional groups, conformational and chemical structure of PS-protein. The main groups were as below: (1) the spectrum related to O-H stretch at 3739 cm^{-1}; (2) Amide A region (N-H stretch) at the frequency 3417 cm^{-1}; (3) Amide B region (C-H and O-H stretch) at the frequencies 2927 cm^{-1} and 2857 cm^{-1}; (4) The second part of spectroscopy included the structural regions of protein. In this section, the spectra related to amide I (especially C = O stretch) and amide II (N-H deformation and C-N stretch) at frequencies 1744 cm^{-1}, 1652 cm^{-1} and 1542 cm^{-1} were observed, respectively. Also, some other significant regions (such as α-helix, β-sheet, beta tern and random coil structures) can be seen in addition to the amide I region in the region related to amide III. The main spectrum of this region was mainly detected at 1238 cm^{-1} frequency. At the end of the spectrum, the presence of some polysaccharides and C = O stretch (1161 cm^{-1}) as well as N-H bending

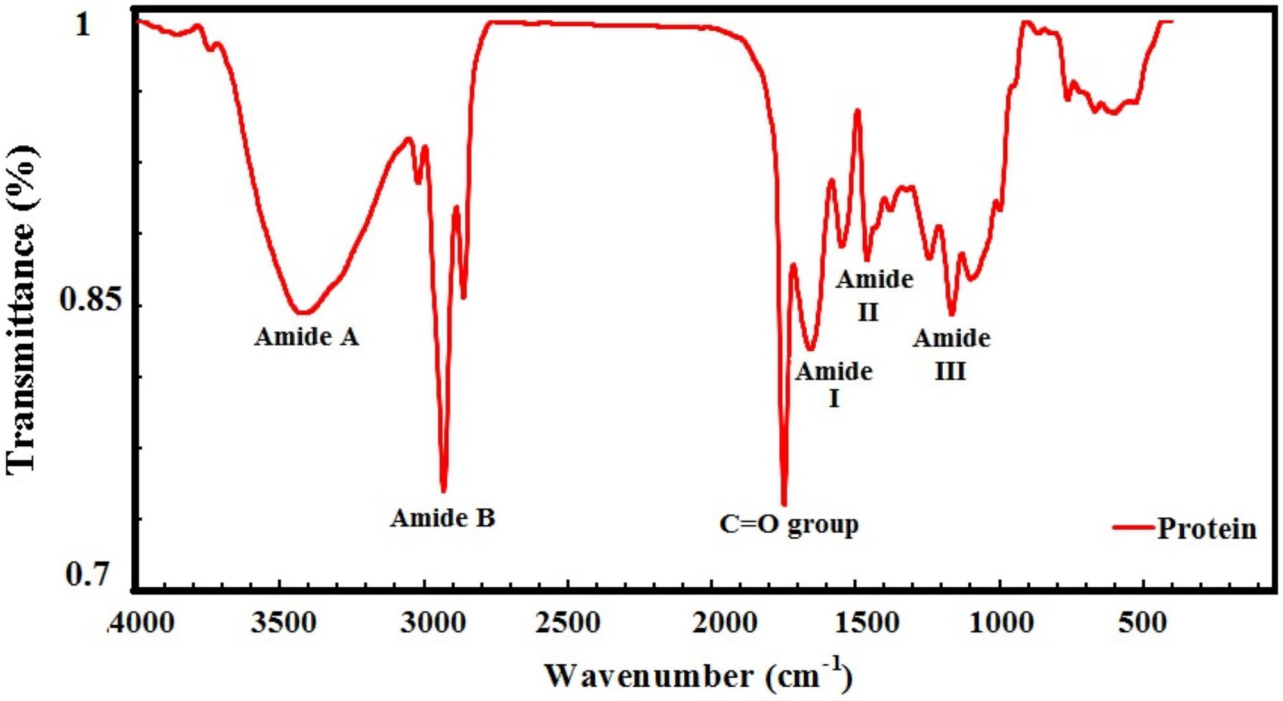

Fig. 1. Chemical structure (FTIR) of pomegranate seed protein.

(663 cm^{-1}) were observed, respectively[38,39]. Also, similar structures were found in research conducted on mung bean[34], wheat glutenin[2], *Spirulina* algae[39], *Chlorella* hydrolysates[40] and wild pistachio[41] proteins were reported.

Degree of hydrolysis (DH)

Protein hydrolysis is commonly evaluated based on the DH, which indicates the functional characteristics of the protein hydrolysate[3]. In this study, pomegranate seed protein underwent treatment with various enzymes to determine the DH. The results demonstrate that the DH values increased when different enzymes were used ($p < 0.05$). The amounts of DH in PSPHs were as follows: H-Pa (~31%) > H-Al (~28.6%), and H-Tr (~27.9%) > H-Pe (~22.7%). The difference in the amount of DH can be attributed to the difference in the proteolytic activity of enzymes and their performance in breaking peptide bonds[2]. In another study, mushroom protein hydrolysis was performed with Alcalase, Pancreatin, Flavourzyme and alcalase combination with each of the other enzymes (for 30–240 min). In the first minutes of hydrolysis, the maximum breaking rate of peptide bonds and the increase of DH were observed. Among the single-enzyme hydrolysates, the highest degree of hydrolysis was related to the samples treated with alcalase (~18%), followed by the samples obtained from pancreatin (~13%) and Flavourzyme (~4%)[42].

Techno-functional properties

In this study, the effect of enzymatic hydrolysis on the solubility of PSP (as a key functional index) was investigated (Fig. 2). The lowest value of this index was obtained at the point close to isoelectric (pH 5) and the highest at pH 9. Hydrolysis led to a significant increase in the value of this index and a decrease in the sensitivity of proteins to precipitation in acidic conditions. The amount of these changes was also affected by the type of enzyme and DH. The cause of these findings can be attributed to the breaking of disulfide bonds, the opening of insoluble protein masses and the greater access of hydrophilic groups to water[22,43].The results of the study, as depicted in Fig. 3, demonstrate the effect of enzymatic hydrolysis on the emulsifying activity index and emulsion stability index of PSPH. Notably, the hydrolysates of pepsin exhibited a significant increase ($p < 0.05$) in both emulsifying activity and emulsion stability. This indicates that the emulsifying activity of PSPH is enhanced after enzymolysis and is influenced by the degree of hydrolysis ($p < 0.05$). Furthermore, changes in pH were found to significantly affect ($p < 0.05$) the emulsifying capacity and its stability. The lowest and highest emulsifying capacities and emulsion stability index were observed at a pH of 5 and 9, respectively. Generally, the emulsifying activity is dependent on the degree of hydrophobicity and molecular weight of the hydrolysates. Samples with hydrophobic amino acids and longer chains tend to exhibit higher emulsifying activities. This is attributed to the presence of hydrophobic and hydrophilic sequences in the hydrolysates, which are crucial for interfacial properties[44]. The hydrolysates exhibited better emulsifying activity at pH 9, which can be attributed to the presence of hydrophobicity residues. These residues have the ability to quickly spread and be absorbed onto the surface of newly formed oil droplets during the homogenization process[3]. It is widely acknowledged that emulsion stability is strongly influenced by the size of the droplets, which can be affected by the protein emulsifier used[45].

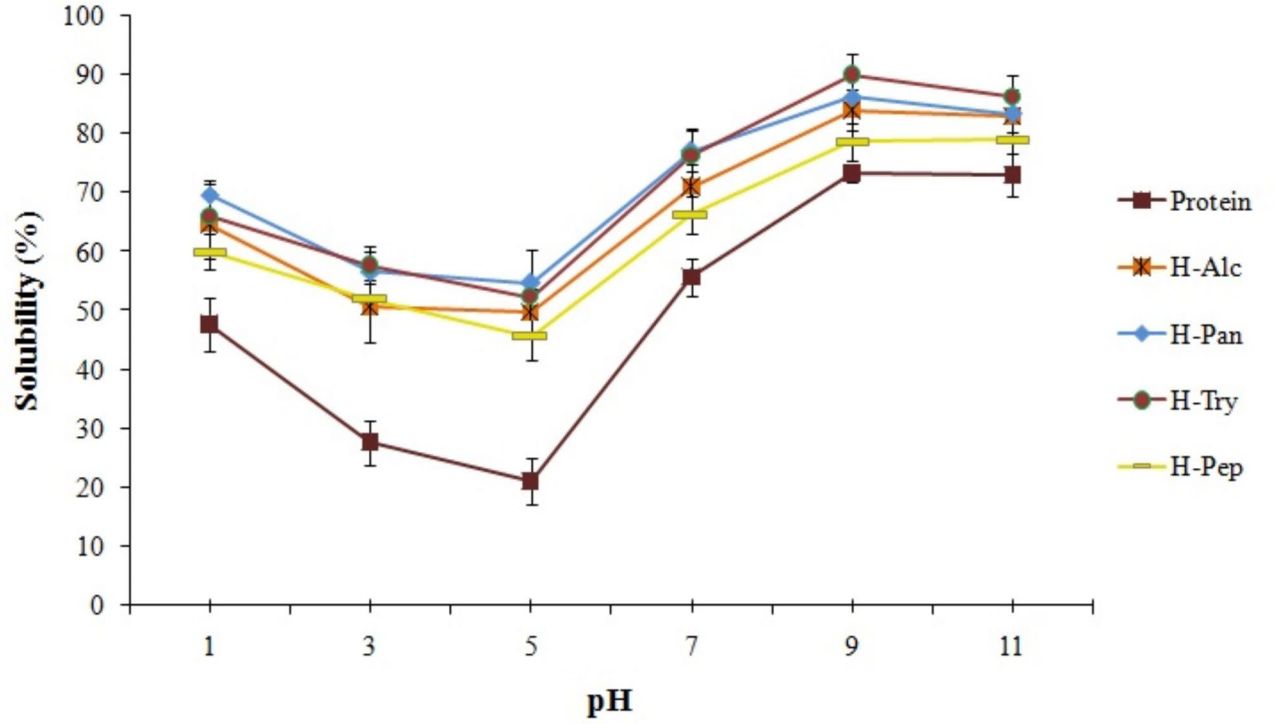

Fig. 2. Effects of enzyme type on the solubility of pomegranate seed protein at different pH values.

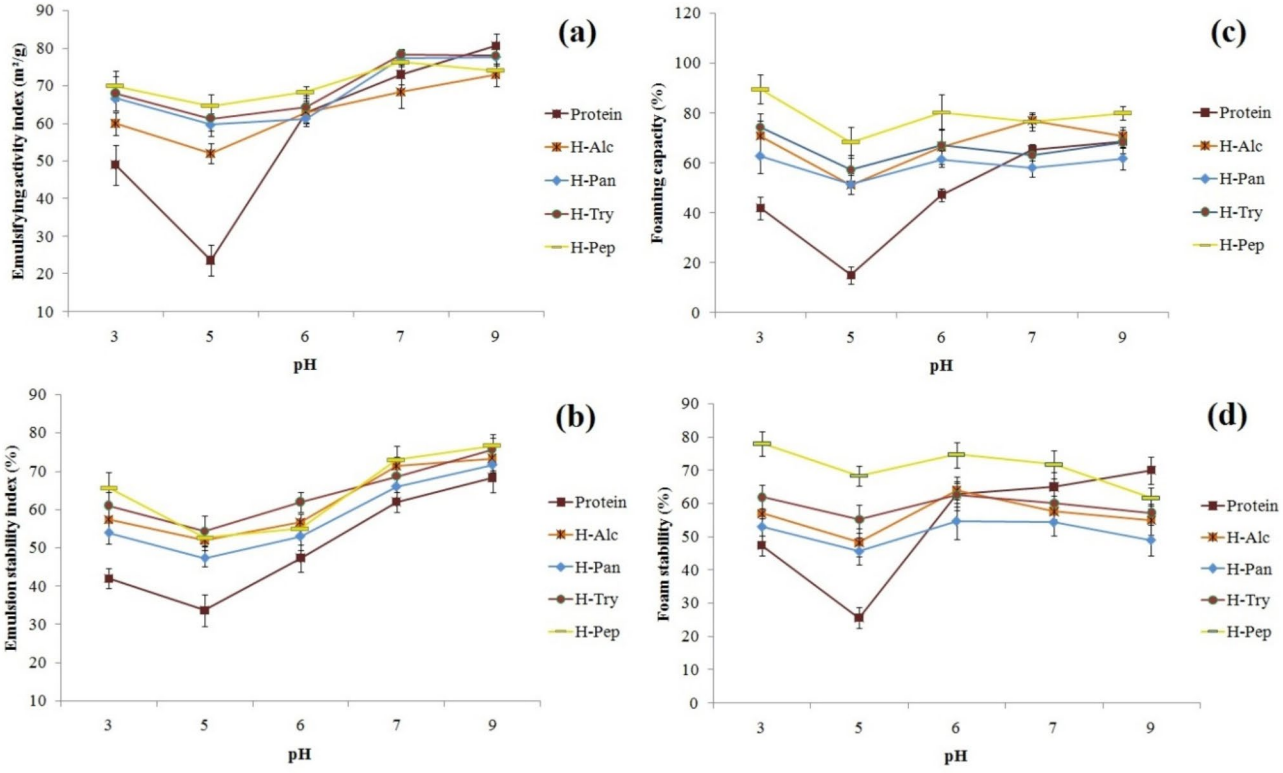

Fig. 3. Techno-functional properties of pomegranate seed protein and its hydrolysates at different pH values. (a) Emulsifying activity; (b) emulsion stability; (c) foaming capacity and (d) foaming stability.

The results regarding the effect of enzymatic hydrolysis foaming capacity and foaming stability of PSPH are presented in Fig. 3. It was observed that both the foaming capacity (Fig. 3c) and foaming stability (Fig. 3d) of all the hydrolysates were significantly higher ($p < 0.05$) compared to PSPH alone. Additionally, changes in pH were found to have a significant effect ($p < 0.05$) on both foaming capacity and foaming stability. The lowest foaming capacity and foaming stability index were observed at a pH of 5, while the highest values were observed at a pH of 9. These findings suggest that enzymatic hydrolysis generates more flexible protein components, which facilitate rapid film formation at the air/water interfaces. This, in turn, leads to improved film viscoelasticity at the interfaces[46]. The foaming properties of the hydrolysates were influenced by the pH level and the specific enzyme utilized for hydrolysis. The highest foaming capacity (FC) was observed for H-Pep at pH 3 and for H-Alc at pH 7. Although H-Pep had the highest FC at pH = 3, its FS decreased at the same hydrolysis pH. The absence of stable foam in the solution could be attributed to the release of smaller hydrolysates at longer hydrolysis pH and with high DH. These smaller hydrolysates may incorporate more air into the solution compared to larger hydrolysates. However, it should be noted that stable foam was not observed in this case[46]. Generally, proteins and hydrolysates with suitable FC do not possess the ability to form stable foams. It is evident that FC and foam stability (FS) are influenced by different molecular properties of hydrolysates, which often exhibit contrasting characteristics. For instance, FC is affected by factors such as flexibility, hydrophobicity, and adsorption rate, while FS depends on rheological properties of films, including protein concentration, thickness, desirable intermolecular interactions, and hydration[47]. Similar observations have been reported in studies on hydrolyzed Jackfruit leaf protein, bean protein, and hydrolyzed peanut protein, where limited hydrolysis significantly improved foaming properties[46,48,49].

The effects of enzymatic hydrolysis had a significant impact ($p < 0.05$) on the WHC and OHC of Pomegranate Seeds Protein Hydrolysate (Fig. 4). The solubility of protein and hydrolyzed proteins plays a role in determining the OHC and WHC, as higher solubility indicates a smaller molecular size, resulting in reduced absorption of oil and water[50]. The findings indicate that the crude protein exhibited the highest OHC and WHC ($p < 0.05$), with values of 5.4 and 5.9 g/g, respectively. Enzymatic hydrolysis generally increases the presence of polar groups such as COOH and NH_2, which may decrease the WHC in the protein hydrolysate[50]. The changes in the absorbed amounts of water and oil can be attributed to the hydrophilic polar side chains. The WHC and OHC of proteins and hydrolysate are crucial for maintaining the texture and organoleptic properties of products. Proteins and hydrolysate with high WHC are beneficial in reducing moisture loss in packaged bakery products and preserving freshness[26].

Amino acid profile and nutritional quality

The analysis of amino acids has gained significant importance due to the nutritional value of essential amino acids and their impact on the functional properties and health effects of hydrolyzed proteins[51]. Referring to Table 1, the hydrolysis process using different enzymes (PSPH-Al, PSPH-Pa, PSPH-Tr, and PSPH-Pe) resulted in changes in the levels of specific amino acids. For instance, the amounts of serine and glycine decreased after hydrolysis by

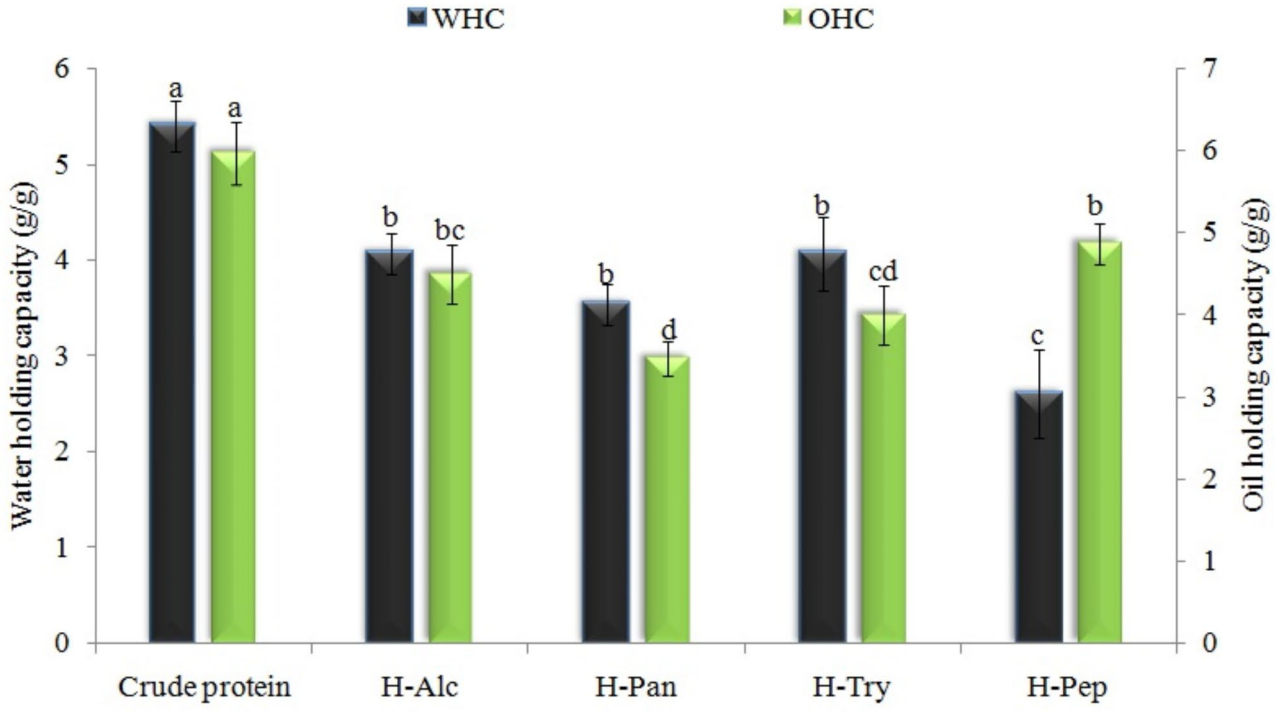

Fig. 4. Effects of enzymes type on the water (WHC) and oil (OHC) holding capacity of pomegranate seed protein.

Amino acid	Crude protein	PSPH-Al	PSPH-Pa	PSPH-Tr	PSPH-Pe
Aspartic (Asp)	50.7	53.4	52.1	52.8	51.6
Glutamic (Glu)	146.3	152.3	150.7	151.2	151.8
Asparagine (Asn)	4.2	6.1	6.7	5.9	4.8
Histidine (His)*	16.7	19.3	20.4	18.3	17.2
Serine (Ser)	35.5	33.1	37.6	34.4	37.5
Arginine (Arg)	84.5	87.2	89.1	87.3	89.6
Glycine (Gly)	46.4	43.7	45.9	47.3	44.9
Threonine (Thr)*	23.7	25.4	26.8	26.5	24.6
Alanine (Ala)	30.4	33.5	33.2	35.9	34.7
Tyrosine (Tyr)	21.2	25.1	25.4	23.5	24.2
Methionine (Met)*	8.6	10.5	11.2	9.8	9.4
Valine (Val)*	28.9	32.2	33.7	32.6	30.8
Phenylalanine (Phe)*	26.5	30.9	28.8	29.5	27.3
Isoleucine (Ile)*	24.9	27.1	26.5	25.8	26.4
Leucine (Leu)*	48.6	51.5	50.3	52.3	49.5
Lysine (Lys)*	26.6	28.7	27.5	29.2	30.1
Tryptophan (Trp)*	9.4	9.7	8.6	9.8	9.5
EAA	144.1	156.4	157.1	156.8	150.2
NCAA	197.0	205.7	202.8	204.0	203.4
PCAA	127.8	135.2	137.0	134.8	136.9
HAA	198.5	220.5	217.7	219.2	211.8
AAA	82.5	93.3	93.1	90.6	90.4
PER	1.52	2.08	1.55	1.66	1.53
TAA	633.1	669.7	674.5	672.1	663.9

Table 1. Amino acid composition of pomegranate seeds protein and hydrolysates (mg amino acid/g dry sample). PSPH-Al: Pomegranate seeds protein hydrolyzed with Alcalase; Pa: Pancreatin; Tr: Trypsin; Pe: Pepsin. *Essential amino acids (EAA); negatively charged amino acids (NCAA) = asx (asparagine and aspartic acid) and glx (glutamine and glutamic acid); positively charged amino acids (PCAA) = Arg, His, and Lys; Hydrophobic amino acids (HAA) = Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro and Met; Antioxidant amino acids (AAA) = Trp, Met, His, Tyr and Lys; Total amino acids (TAA).

PSPH-Al, while glycine and tryptophan decreased after hydrolysis by PSPH-Pa, and serine and glycine decreased after hydrolysis by PSPH-Tr and PSPH-Pe, respectively. However, it is noteworthy that protein hydrolysis led to an overall increase in other amino acids, including essential amino acids, resulting in an increase in the total amino acid content of pomegranate seeds. The content of essential amino acids (EAA) in all hydrolysates ranged from 144.1 to 157.1 mg/g of dry sample. Among them, leucine was found to be the highest EAA in PSPH-Tr (52.3 g/g dry sample) and PSPH-Al (51.5 g/g dry sample), while tryptophan had the lowest EAA content, recorded in PSPH-Pa (8.6 g/g dry sample). The predominant amino acids found in all samples are glutamic acid and arginine. The protein derived from pomegranate seeds and its hydrolysate exhibit low levels of asparagine. The hydrolysates produced by the Alcalase enzyme showed significantly higher amounts of hydrophobic amino acids (HAA) and antioxidant amino acids (AAA) compared to other methods ($p < 0.05$). The values obtained were 220.5 mg amino acid/g dry sample for HAA and 93.3 mg amino acid/g dry sample for AAA. These findings align with previous research that identified tryptophan, histidine, phenylalanine, alanine, tyrosine, methionine, glycine, leucine, and valine as key amino acids associated with peptides possessing antioxidant properties[34,52]. Additionally, the hydrophobic amino acid content in the hydrolysates positively correlates with their antioxidant and antimicrobial activities[43]. The specific sequence of amino acids in the hydrolysates obtained by different enzymes varies due to the physical interactions between the substrate and the enzyme during hydrolysis in an aqueous medium[2].

Antioxidant characterization

DPPH/ABTS radical scavenging

The effects of enzymatic hydrolysis of PS proteins had a significant impact ($p < 0.05$) on the scavenging activities of ABTS and DPPH radicals (Fig. 5). The results indicated a notable increase ($p < 0.05$) in antioxidant activity when using hydrolysates produced by the Alcalase enzyme for DPPH radical scavenging and hydrolysates produced by the Pancreatin enzyme for ABTS radical scavenging. These hydrolysates with high antioxidant activity were found to contain exposed hydrophobic amino residues, which are capable of interacting with and trapping DPPH radicals[3]. Consistent with previous studies the ABTS and DPPH radical scavenging activities of protein hydrolysates were influenced by the type of enzyme and hydrolysis conditions used[31]. All hydrolysates exhibited greater ability to scavenge ABTS radicals compared to DPPH radicals, likely due to the hydrophilic nature of the ABTS radical and its interaction with the hydrolysates. However, a higher degree of hydrolysis was

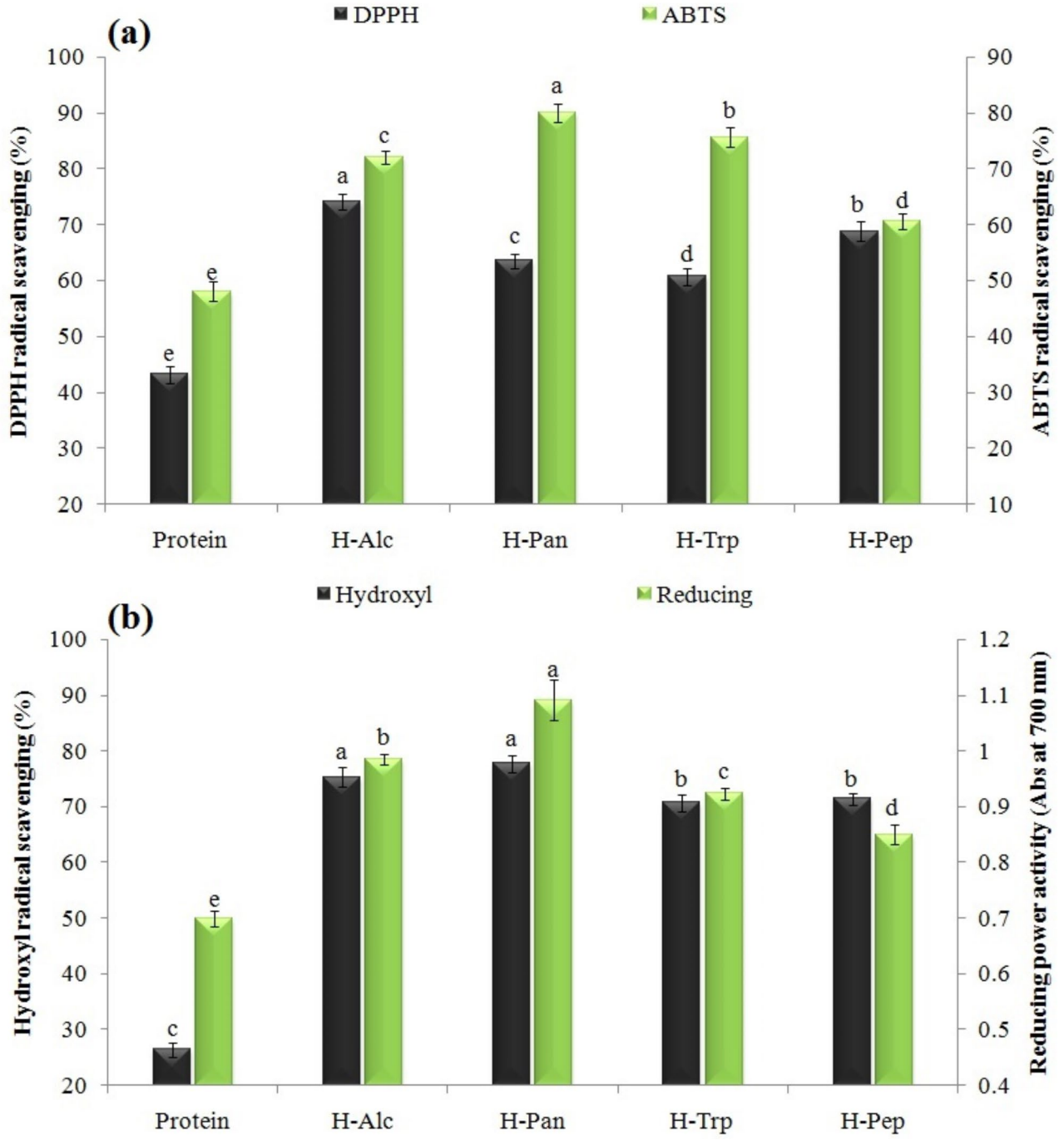

Fig. 5. Effects of different enzymes on (a) DPPH, and ABTS; (b) Hydroxyl radical scavenging, and Reducing power activity of pomegranate seed protein.

required for the DPPH method, possibly due to the lower solubility of hydrolysates in the ethanol-containing DPPH solution[46]. Similar findings have been reported by other researchers[22,27,31,42,53] who studied the radical scavenging activities of shrimp shell, mung bean, rice bran, mushroom and poppy-pollen peptides, respectively.

OH radical scavenging and reducing power

The effects of enzymatic hydrolysis of PS proteins had a significant impact ($p < 0.05$) on the hydroxyl radical scavenging activity and reducing power activity of PSPH. The results indicate that there is no significant difference in hydroxyl radical scavenging activity between the hydrolysates produced by the Alcalase enzyme and the hydrolysates produced by the Pancreatin enzyme. However, Fig. 5b shows a notable increase ($p < 0.05$) in antioxidant activity, specifically in hydroxyl radical scavenging activity and reducing power activity, in the hydrolysates produced by the Pancreatin enzyme. Overall, these findings confirm that the antioxidant activities

exhibited by the hydrolysates vary depending on the enzyme and the DH. Hydrolysates generated by different enzymes can result in hydrolysate with distinct amino acid sequences, which contribute to their ability to scavenge free radicals such as DPPH, OH, and ABTS[13]. The results of this study demonstrate that hydrolysate produced by Pancreatin and Alcalase enzymes act as hydrogen donors, leading to the scavenging of ABTS, OH, and DPPH free radicals, respectively. Similar observations have been made with other hydrolysates, such as fish protein by-products produced by the alcalase enzyme[54] or Protamex[13].

Nitric-oxide radical scavenging and total antioxidant activity (TAA)

Nitric oxide (NO) is a free radical produced through the oxidation of L-arginine to L-citrulline by NO synthase. Excessive production of NO and pro-inflammatory cytokines can lead to various diseases, including chronic inflammation, atherosclerosis, cancer, rheumatoid arthritis, and asthma[32]. Inhibitors targeting this radical have proven effective in reducing tissue and cell damage. The results depicted in Fig. 4 demonstrate significant differences ($p < 0.05$) the effects of enzymatic hydrolysis of PS proteins on NO inhibition in pomegranate seeds protein (Fig. 6a). The unprocessed primary protein exhibited low NO inhibition, with no clear distinction observed between the primary protein and the hydrolysates generated by the Pepsin Enzyme. Among the different enzymes tested, the hydrolysates produced by alcalase showed the highest inhibitory effect on NO, while those generated by pepsin displayed the lowest. The degree of hydrolysis had a notable impact on this measure. Research has consistently shown that hydrophobic amino acids like valine, leucine, phenylalanine, isoleucine, and alanine play a significant role in NO inhibition by facilitating peptide access to hydrophobic regions and interacting with the cell membrane[55]. Similar findings have been observed in other hydrolysates, such as peptides derived from black bean protein through enzymolysis with the Flavourzyme enzyme[56] or *Arthrospira platensis* produced by alcalase enzyme[32].

The measurement of total antioxidant capacity is based on the ability of the sample to reduce molybdate and form a green molybdate/phosphate complex under acidic conditions[57]. The results indicate that the hydrolysis process using enzymes led to an increase in the total antioxidant power of pomegranate seeds protein (Fig. 6a). In this study, no significant differences were observed between trypsin and pancreatin hydrolysates, while trypsin, pancreatin, and pepsin hydrolysates exhibited the highest and lowest total antioxidant power, respectively. Similar findings have been reported for other hydrolysates, such as sweet potato protein hydrolysates[58] and *Arthrospira platensis* protein hydrolysates[32]. These studies suggest that the presence of hydrophobic amino acids like valine, phenylalanine, isoleucine, and leucine in hydrolyzed proteins and hydrolysate plays a crucial role in their total antioxidant activity[1].

Fe^{2+} and Cu^{2+} chelating activity

Fe and Cu are trace elements that serve as cofactors for many enzymes. In their free forms, they act as pro-oxidants by generating hydroxyl radicals. However, molecules that bind to these elements can reduce their pro-oxidant properties and enhance their biological function[59]. The chelating activities of Fe^{2+} and Cu^{2+} in protein hydrolysates were significantly influenced by different enzymes used in their production ($p < 0.05$) (Fig. 6b). Notably, the chelating activity of Fe^{2+} increased significantly in the hydrolysates produced by alcalase enzyme, while the chelating activity of Cu^{2+} increased significantly in the hydrolysates produced by trypsin enzyme. Enzymolysis also resulted in increased chelating activities of primary pomegranate protein. The variations in Fe^{2+} and Cu^{2+} chelating activities can be attributed to the action of enzymes, which affect the amino acid sequencing, degree of hydrolysis, and the number of active sites involved in chelation of these metal ions. Enzymatic hydrolysis leads to the release of acidic and basic amino acids, thereby increasing the presence of carboxylic and amino groups, which facilitate the removal of metal ions from the environment[31]. These findings align with the studies conducted by[39], who investigated the Fe^{2+} and Cu^{2+} chelating activity of *Spirulina platensis* using different enzymes.

Correlation coefficients between various properties of pomegranate-seeds protein hydrolysates

Table 2 presents the correlation coefficients among key properties of pomegranate-seed protein hydrolysates, including the degree of hydrolysis (DH%), antioxidant activities, total amino acid content, metal chelation, and solubility across varying pH levels. These coefficients reflect the strength and direction of the linear relationships between these properties. A notable observation is the strong correlation between DH% and both antioxidant activities and solubility across all pH levels, suggesting that increased hydrolysis enhances both the antioxidant properties and solubility of the protein hydrolysates. Solubility, particularly at acidic (pH 1 and pH 3) and alkaline (pH 9 and pH 11) pH levels, shows robust positive correlations with DH% and antioxidant activities, underscoring the critical role of solubility in determining the functional properties of the hydrolysates. Specifically, solubility is strongly influenced by the degree of hydrolysis, antioxidant activity (particularly ABTS), total amino acid content, and metal chelation (especially iron). Across all pH levels, solubility exhibits very strong positive correlations with DH% (0.87-1.00), ABTS (0.94–0.99), total amino acids (0.91-1.00), and iron chelation (0.87–0.94). Interestingly, solubility at acidic pH levels (pH 1 and pH 3) displays stronger correlations with DH%, ABTS, and total amino acids compared to alkaline pH levels (pH 9 and pH 11). In terms of antioxidant activities, ABTS shows strong correlations with solubility, total amino acids, and reducing power, indicating that ABTS activity is closely linked to solubility and amino acid content. Conversely, DPPH exhibits stronger correlations with hydroxyl activity (0.90) and iron chelation (0.92) but weaker correlations with ABTS (0.63) and total amino acids (0.62), implying that different mechanisms govern DPPH activity. Hydroxyl activity, in turn, demonstrates strong correlations with reducing power, iron chelation, and solubility, highlighting its close relationship with these properties.

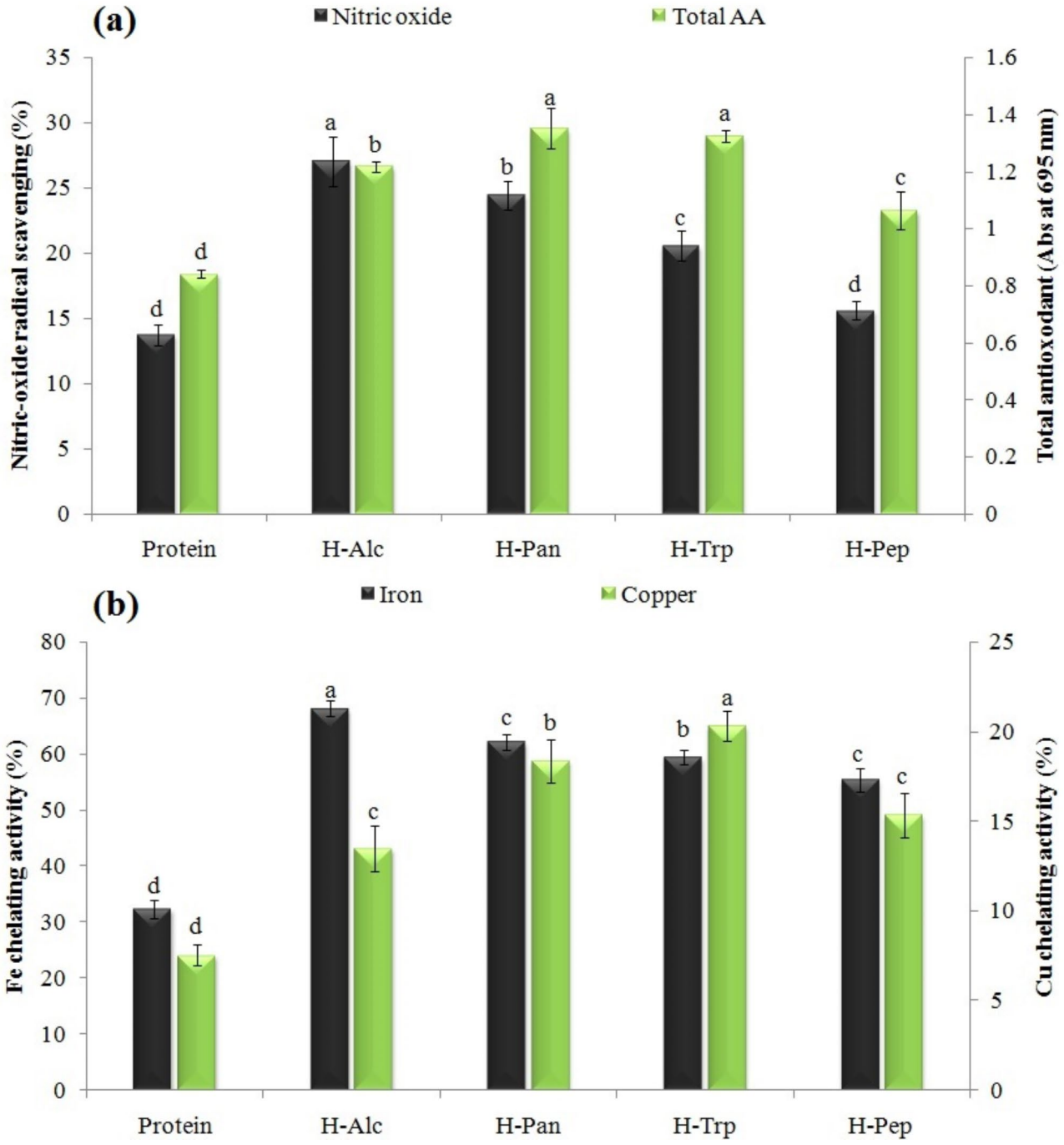

Fig. 6. Effects of different enzymes on (a) Nitric-oxide scavenging, Total antioxidant activity; (b) Fe^{2+}, and Cu^{2+} chelating activities of pomegranate seed protein.

ACE- inhibitory

The figure presented the effects of enzymatic hydrolysis of PS proteins on ACE-inhibitory activity (Fig. 7a). The highest ACE inhibitory activity (49.43%) was observed in the hydrolysates produced by the Alcalase enzyme ($p < 0.05$). The variations in ACE inhibitory effect among the treatments can be attributed to variances in the molecular weight and amino acid sequence of the hydrolysate. The presence of hydrophobic amino acids in the peptide sequences appears to be associated with ACE inhibitory activity. This is because hydrophobic hydrolysates have better access to the active site of ACE, making it more reachable. Significant amino acids in hydrolysate exhibiting ACE inhibitory activity include Pro, Glu, His, Ser, and Tyr. Research suggests that hydrophobic amino acid residues such as Ala, Tyr, Val, Phe, Leu, or Trp can act as competitive ACE inhibitors by selectively binding to the catalytic sites of ACE. It is considered ideal to have aromatic amino acids like phenylalanine near the end of a protein chain, specifically at any of the three positions closest to the C-terminal. Furthermore, hydrolysate with strong antihypertensive properties often contain positively charged amino acids like arginine and lysine at

	DH%	DPPH	ABTS	Hydroxyl	Reducing	Nitric-oxide	Total AA	Fe	Cu	Sol. pH 1	Sol. pH 3	Sol. pH 5	Sol. pH 7	Sol. pH 9	Sol. pH 11
DH%	1.00														
DPPH	0.84	1.00													
ABTS	0.94	0.63	1.00												
Hydroxyl	0.98	0.90	0.86	1.00											
Reducing	0.92	0.69	0.95	0.87	1.00										
Nitric-oxide	0.79	0.68	0.84	0.72	0.88	1.00									
Total AA	0.94	0.62	1.00	0.87	0.92	0.79	1.00								
Fe	0.97	0.92	0.88	0.96	0.88	0.86	0.87	1.00							
Cu	0.86	0.53	0.86	0.82	0.73	0.47	0.91	0.72	1.00						
Sol. pH 1	0.98	0.74	0.98	0.94	0.95	0.80	0.98	0.92	0.89	1.00					
Sol. pH 3	0.97	0.77	0.90	0.96	0.82	0.62	0.92	0.89	0.95	0.95	1.00				
Sol. pH 5	1.00	0.81	0.94	0.98	0.90	0.74	0.95	0.94	0.90	0.98	0.99	1.00			
Sol. pH 7	0.95	0.63	0.99	0.89	0.91	0.75	1.00	0.87	0.93	0.98	0.94	0.96	1.00		
Sol. pH 9	0.87	0.52	0.95	0.78	0.79	0.72	0.97	0.79	0.91	0.91	0.87	0.88	0.96	1.00	
Sol. pH 11	0.92	0.65	0.94	0.86	0.81	0.74	0.96	0.87	0.91	0.93	0.92	0.93	0.97	0.99	1.00

Table 2. Correlation coefficients between various properties of pomegranate-seeds protein hydrolysates. * Correlation is significant at the 0.05 level.

their C-terminal position[1,60]. In general, hydrolysate with ACE inhibitory activity can function in two potential ways. Firstly, these hydrolysate can attach themselves to the active site of the ACE enzyme. Alternatively, they can attach to inhibitory sites present on the ACE enzyme. These attachments induce structural changes in the protein, ultimately preventing the substrate (angiotensin) from binding to the enzyme's active site[61]. The findings of this study are consistent with the research conducted by Maqsoudlou et al. (2019), who examined the ACE inhibitory activity in alcalase-based pollen hydrolysates[62]. Similarly, Mazloomi et al. (2021) measured the ACE inhibitory activity in orange seed protein hydrolysates using alcalase and pepsin enzymes[1].

Dipeptidyl peptidase IV (DPP-IV) inhibitory

The evaluation of the antidiabetic properties of pomegranate-seed proteins, hydrolyzed using different enzymes, was conducted based on their DPP IV inhibitory activity. The results are presented in Fig. 7a. The study found that the highest DPP IV inhibitory activity (51.56%) was observed in the hydrolysates produced by alcalase and pancreatin enzymes ($p < 0.05$). There was no significant difference in DPP-IV inhibitory activity between the hydrolysates produced by alcalase and pancreatin enzymes. The hydrolysates with potential antidiabetic properties were found to contain notable amounts of Ser, Asp, and Glu. Additionally, the presence of aromatic residues (such as Phe, Trp, and Tyr) in hydrolysate was identified as crucial for their antidiabetic potential[63] and Jonker et al.[64] discovered that the breakdown of casein and whey proteins leads to the production of peptides with antidiabetic properties[64]. These peptides were observed to effectively reduce blood glucose levels in individuals with type-2 diabetes. Yang et al.[65] conducted a study demonstrating that incorporating peptides derived from the breakdown of soybean protein into the diet of rats with type I diabetes enhances the effects of insulin and improves the liver's sensitivity to insulin in diabetic rats[65].

Antibacterial activity

Figure 7b illustrates the antimicrobial activity of PSPH (protein hydrolysates from industrial byproducts of *Stripped weakfish*) using different enzymes. All of the tested enzymes exhibited inhibitory effects against the microorganisms *E. coli* and *B. cereus*. The highest inhibitory actions against *E. coli* (15 mm ± 0.5) and *S. aureus* (11.7 mm ± 0.4) were observed with the enzyme H-Alc. Generally, the hydrolysis process carried out by various enzymes showed significant inhibitory properties compared to the crude protein ($p < 0.05$). Similar findings were reported in a study by Lima et al.[13], where peptides derived from enzymolysis of protein hydrolysates from Stripped weakfish using pepsin and pancreatin showed antimicrobial activity, with the highest activity observed for alcalase hydrolysate against *E. coli* O_{157}:H_7 (5.50 ± 0.17 mm). In contrast, the enzymes pancreatin and trypsin hydrolysate did not exhibit any noticeable difference in inhibitory effect against B. *cereus*[13].

Conclusion

This research aimed to assess the antioxidant activities (DPPH and ABTS free radical prevention, hydroxyl radical inhibition, reducing power, total antioxidant activity), Fe^{2+} and Cu^{2+} chelating activities, nitric oxide inhibition, ACE and dipeptidyl peptidase IV inhibitory activities. Additionally, antibacterial activity of hydrolysate and functional properties (emulsification, foaming, water and oil holding capacity), along with amino acid profile analysis of all hydrolysates, were conducted. The most potent biological activity (antioxidant, antibacterial, ACE and dipeptidyl peptidase IV inhibitory activities) ($p < 0.05$) was achieved through Alcalase and pancreatin enzyme hydrolysis, while the lowest and highest functional properties were observed at pH 5 and 9, respectively. The study highlighted that the degree of hydrolysis, enzyme type, amino acid composition, and pH affected the biological and functional properties of hydrolysates. Pomegranate seed protein hydrolysates are rich in hydrophobic amino acids, contributing to their high antioxidant and antimicrobial activities. The highest

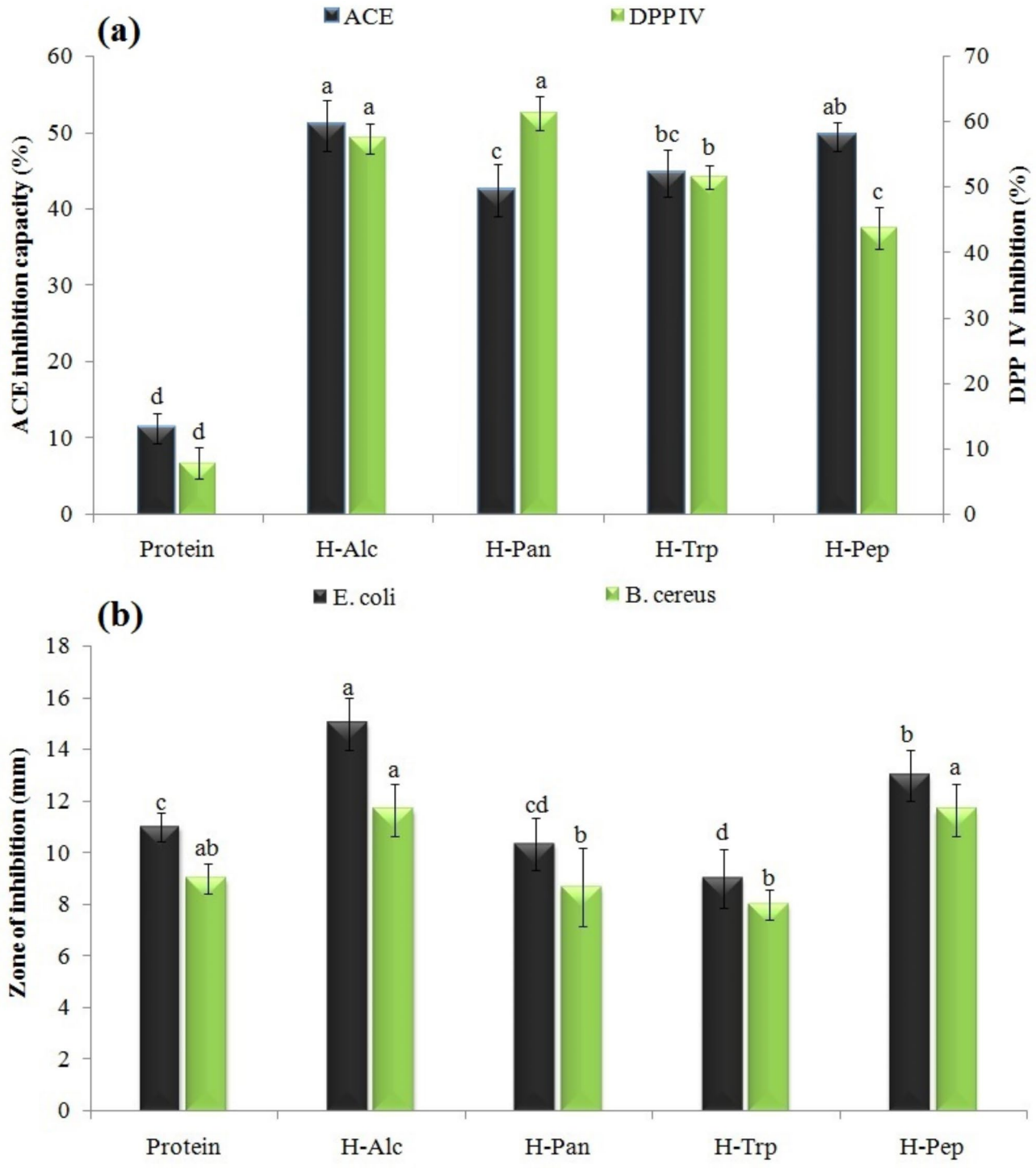

Fig. 7. Effects of different enzymes on (a) ACE and DPP-IV inhibitory; (b) antibacterial activities of pomegranate seed protein.

inhibitory effect of hydrolysates against *E. coli* and *S. aureus* bacteria (H-Alc) was observed in hydrolysates that were rich in amino acids such as aspartic acid, glutamic acid, arginine, glycine, and leucine. Generally, red-pomegranate (*Punica granatum L.*) seed protein hydrolysate could be used as a health-promoting ingredient to help in the reduction of blood pressure and the regulation of diabetes, as well as valuable insights as functional ingredients in various food products. As a result these bioactive compounds can have a high potential to improve the health of consumers as a natural product of food origin, but future clinical studies in vivo would be necessary to confirm the health beneficial effect of red-pomegranate seed protein hydrolysate. These compounds also have a bitter taste and attract moisture. Therefore, to mask the bitter taste, reduce the moisture attraction of bioactive proteins, improve bioavailability, and protect the hydrolyzed protein component with the highest health-giving

activity, it is imperative to utilize new technologies such as nano and microencapsulation to ensure the stability of these bioactive compounds in food. This study also offers valuable insights into the functional properties of pomegranate hydrolysates, suggesting their suitability as functional ingredients in various food products, particularly those requiring strong foaming and emulsifying capacity, stability, and extended shelf life, such as bread, cakes, cookies, sausages, and sauces. However, isolation, sequencing, and identification of the active and effective moieties in peptides and hydrolysates can provide more information. Hence, the characteristics of peptide fractions and their sequences can be considered for future studies.

Data availability

All data generated or analyzed during this study are included in this published article.

Received: 3 August 2024; Accepted: 19 March 2025
Published online: 28 March 2025

References

1. Mazloomi, S. N. et al. Pepsin hydrolysis of orange By-Products for the production of bioactive peptides with Gastrointestinal resistant properties. *Foods* **10**, 679 (2021).
2. Bozkurt, F., Bekiroglu, H., Dogan, K., Karasu, S. & Sagdic, O. Technological and bioactive properties of wheat glutenin hydrolysates prepared with various commercial proteases. *LWT* **149**, 111787 (2021).
3. Zheng, Z., Li, J., Li, J., Sun, H. & Liu, Y. Physicochemical and antioxidative characteristics of black bean protein hydrolysates obtained from different enzymes. *Food Hydrocoll.* **97**, 105222 (2019).
4. Bhat, Z. F., Kumar, S. & Bhat, H. F. Bioactive peptides of animal origin: a review. *J. Food Sci. Technol.* **52**, 5377–5392 (2015).
5. Patil, S. P., Goswami, A., Kalia, K. & Kate, A. S. Plant-derived bioactive peptides: A treatment to cure diabetes. *Int. J. Pept. Res. Ther.* **26**, 955–968 (2020).
6. Selamassakul, O., Laohakunjit, N., Kerdchoechuen, O., Yang, L. & Maier, C. S. Isolation and characterisation of antioxidative peptides from bromelain-hydrolysed brown rice protein by proteomic technique. *Process. Biochem.* **70**, 179–187 (2018).
7. Jo, C., Khan, F. F., Khan, M. I. & Iqbal, J. Marine bioactive peptides: types, structures, and physiological functions. *Food Rev. Int.* **33**, 44–61 (2017).
8. De Domenico, S., De Rinaldis, G., Paulmery, M., Piraino, S. & Leone, A. Barrel jellyfish (Rhizostoma pulmo) as source of antioxidant peptides. *Mar. Drugs.* **17**, 134 (2019).
9. Mazloomi, S. N. et al. Impact of simulated Gastrointestinal digestion on the biological activity of an alcalase hydrolysate of orange seed (Siavaraze, citrus sinensis) by-products. *Foods* **9**, 1217 (2020).
10. Soleymanzadeh, N., Mirdamadi, S., Mirzaei, M. & Kianirad, M. Novel β-casein derived antioxidant and ACE-inhibitory active peptide from camel milk fermented by leuconostoc lactis PTCC1899: identification and molecular Docking. *Int. Dairy. J.* **97**, 201–208 (2019).
11. Tkaczewska, J. Peptides and protein hydrolysates as food preservatives and bioactive components of edible films and coatings-A review. *Trends Food Sci. Technol.* **106**, 298–311 (2020).
12. Chakrabarti, S., Jahandideh, F. & Wu, J. Food-derived bioactive peptides on inflammation and oxidative stress. *Biomed Res. Int.* **2014** (2014).
13. Lima, K. O. et al. Bioactivity and bioaccessibility of protein hydrolyzates from industrial byproducts of stripped weakfish (Cynoscion guatucupa). *Lwt* **111**, 408–413 (2019).
14. Aslam, M. Z. et al. Role of bioactive peptides in reducing the severity of hypertension with the Inhibition of ACE. *Int. J. Pept. Res. Ther.* **25**, 1639–1649 (2019).
15. Yan, J., Zhao, J., Yang, R. & Zhao, W. Bioactive peptides with antidiabetic properties: A review. *Int. J. Food Sci. Technol.* **54**, 1909–1919 (2019).
16. Mazloomi, N. et al. Loading bioactive peptides within different nanocarriers to enhance their functionality and bioavailability; In vitro and in vivo studies. *Adv. Colloid Interface Sci.* **334**, 1–16 (2024).
17. Ali, M. Y., Jannat, S. & Chang, M. S. Discovery of potent Angiotensin-Converting enzyme inhibitors in pomegranate as a treatment for hypertension. *J. Agric. Food Chem.* **71**, 11476–11490 (2023).
18. Karagecili, H., İzol, E., Kirecci, E. & Gulcin, İ. Determination of antioxidant, anti-alzheimer, antidiabetic, antiglaucoma and antimicrobial effects of Zivzik pomegranate (punica granatum)—a chemical profiling by LC-MS/MS. *Life* **13**, 735 (2023).
19. Ayoubi, A., Balvardi, M., Akhavan, H. R. & Hajimohammadi-Farimani, R. Fortified cake with pomegranate seed powder as a functional product. *J. Food Sci. Technol.* **59**, 308–316 (2022).
20. Gül, H. & Şen, H. Effects of pomegranate seed flour on dough rheology and bread quality. *CyTA-Journal Food*. **15**, 622–628 (2017).
21. Kristoffersen, K. A. et al. FTIR-based hierarchical modeling for prediction of average molecular weights of protein hydrolysates. *Talanta* **205**, 120084 (2019).
22. Sarabandi, K., Akbarbaglu, Z., Peighambardoust, S. H., ayaseh, A. & Jafari, S. M. Physicochemical, antibacterial and bio-functional properties of Persian poppy-pollen (*Papaver bracteatum*) protein and peptides. *J. Food Meas. Charact.* 1–12 (2023).
23. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 (1976).
24. Akbarbaglu, Z. et al. Biological conversion of red Garlic (Allium sativum L) protein to bioactive peptides: ACE-DPP-IV inhibitory, and Techno-functional features: bioactive peptides derived from red Garlic. *Appl. Food Res.* 100551 (2024).
25. Klompong, V., Benjakul, S., Kantachote, D. & Shahidi, F. Antioxidative activity and functional properties of protein hydrolysate of yellow Stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. *Food Chem.* **102**, 1317–1327 (2007).
26. Ge, J. et al. Physicochemical and pH-dependent functional properties of proteins isolated from eight traditional Chinese beans. *Food Hydrocoll.* **112**, 106288 (2021).
27. Liu, F. F. et al. Physicochemical, functional and antioxidant properties of mung bean protein enzymatic hydrolysates. *Food Chem.* 133397 (2022).
28. Song, W. et al. Antioxidant and antibacterial activity and in vitro digestion stability of cottonseed protein hydrolysates. *LWT* **118**, 108724 (2020).
29. Moghadam, M. et al. Physicochemical and bio-functional properties of walnut proteins as affected by trypsin-mediated hydrolysis. *Food Biosci.* **36**, 100611 (2020).
30. You, L., Zhao, M., Regenstein, J. M. & Ren, J. Changes in the antioxidant activity of loach (Misgurnus anguillicaudatus) protein hydrolysates during a simulated Gastrointestinal digestion. *Food Chem.* **120**, 810–816 (2010).
31. Ambigaipalan, P. & Shahidi, F. Bioactive peptides from shrimp shell processing discards: antioxidant and biological activities. *J. Funct. Foods.* **34**, 7–17 (2017).

32. Akbarbaglu, Z. et al. Chemical structure and bio-functional properties of arthrospira platensis peptides produced by ultrasonication-enzymolysis: Their emulsification capabilities. *Process. Biochem.* (2023).
33. Tsai, P. J., Tsai, T. H., Yu, C. H. & Ho, S. C. Comparison of NO-scavenging and NO-suppressing activities of different herbal teas with those of green tea. *Food Chem.* **103**, 181–187 (2007).
34. Xie, J., Du, M., Shen, M., Wu, T. & Lin, L. Physico-chemical properties, antioxidant activities and angiotensin-I converting enzyme inhibitory of protein hydrolysates from mung bean (Vigna radiate). *Food Chem.* **270**, 243–250 (2019).
35. Jia, J. et al. The use of ultrasound for enzymatic Preparation of ACE-inhibitory peptides from wheat germ protein. *Food Chem.* **119**, 336–342 (2010).
36. Hall, F., Johnson, P. E. & Liceaga, A. Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein. *Food Chem.* **262**, 39–47 (2018).
37. Balouiri, M., Sadiki, M. & Ibnsouda, S. K. Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal.* **6**, 71–79 (2016).
38. Chen, Y. et al. Physicochemical and functional properties of proteins extracted from three microalgal species. *Food Hydrocoll.* **96**, 510–517 (2019).
39. Akbarbaglu, Z., Ayaseh, A., Ghanbarzadeh, B. & Sarabandi, K. Techno-functional, biological and structural properties of spirulina platensis peptides from different proteases. *Algal Res.* 102755 (2022).
40. Gharehbeglou, P., Homayouni-Rad, A., Jafari, S. M., Sarabandi, K. & Akbarbaglu, Z. Stabilization of chlorella bioactive hydrolysates within biopolymeric carriers: Techno-functional, structural, and biological properties. *J. Polym. Environ.* 1–17 (2023).
41. Sarabandi, K. et al. Nutritional, functional, biological and antibacterial properties of wild pistachio (*P. khinjuk*) nuts peptides. *J. Food Meas. Charact.* 1–13 (2023).
42. Kimatu, B. M. et al. Antioxidant potential of edible mushroom (Agaricus bisporus) protein hydrolysates and their ultrafiltration fractions. *Food Chem.* **230**, 58–67 (2017).
43. Islam, M. S. et al. Degree of hydrolysis, functional and antioxidant properties of protein hydrolysates from grass turtle (Chinemys reevesii) as influenced by enzymatic hydrolysis conditions. *Food Sci. Nutr.* **9**, 4031–4047 (2021).
44. Tan, Y., Chang, S. K. C. & Meng, S. Comparing the kinetics of the hydrolysis of by-product from channel catfish (Ictalurus punctatus) fillet processing by eight proteases. *LWT* **111**, 809–820 (2019).
45. He, W., Yang, R. & Zhao, W. Effect of acid deamidation-alcalase hydrolysis induced modification on functional and bitter-masking properties of wheat gluten hydrolysates. *Food Chem.* **277**, 655–663 (2019).
46. Calderón-Chiu, C., Calderón-Santoyo, M., Herman-Lara, E. & Ragazzo-Sánchez, J. A. Jackfruit (Artocarpus heterophyllus Lam) leaf as a new source to obtain protein hydrolysates: physicochemical characterization, techno-functional properties and antioxidant capacity. *Food Hydrocoll.* **112**, 106319 (2021).
47. Damodaran, S. *Food Proteins and their Applications* (CRC, 2017).
48. Wani, I. A., Sogi, D. S. & Gill, B. S. Physico-chemical and functional properties of native and hydrolysed protein isolates from Indian black gram (Phaseolus mungo L.) cultivars. *LWT-Food Sci. Technol.* **60**, 848–854 (2015).
49. Jamdar, S. N. et al. Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chem.* **121**, 178–184 (2010).
50. Noman, A. et al. Influence of enzymatic hydrolysis conditions on the degree of hydrolysis and functional properties of protein hydrolysate obtained from Chinese sturgeon (Acipenser sinensis) by using Papain enzyme. *Process. Biochem.* **67**, 19–28 (2018).
51. dos Santos, S. D., Martins, V. G., Salas-Mellado, M. & Prentice, C. Evaluation of functional properties in protein hydrolysates from Bluewing searobin (Prionotus punctatus) obtained with different microbial enzymes. *Food Bioprocess. Technol.* **4**, 1399–1406 (2011).
52. Sanjukta, S., Rai, A. K., Muhammed, A., Jeyaram, K. & Talukdar, N. C. Enhancement of antioxidant properties of two soybean varieties of Sikkim Himalayan region by proteolytic Bacillus subtilis fermentation. *J. Funct. Foods.* **14**, 650–658 (2015).
53. Phongthai, S., D'Amico, S., Schoenlechner, R., Homthawornchoo, W. & Rawdkuen, S. Fractionation and antioxidant properties of rice Bran protein hydrolysates stimulated by in vitro Gastrointestinal digestion. *Food Chem.* **240**, 156–164 (2018).
54. Zamora-Sillero, J., Ramos, P., Monserrat, J. M. & Prentice, C. Evaluation of the antioxidant activity in vitro and in hippocampal HT-22 cells system of protein hydrolysates of common carp (Cyprinus carpio) by-product. *J. Aquat. Food Prod. Technol.* **27**, 21–34 (2018).
55. Ryu, B., Qian, Z. J. & Kim, S. K. Purification of a peptide from Seahorse, that inhibits TPA-induced MMP, iNOS and COX-2 expression through MAPK and NF-κB activation, and induces human osteoblastic and chondrocytic differentiation. *Chem. Biol. Interact.* **184**, 413–422 (2010).
56. López-Barrios, L., Antunes-Ricardo, M. & Gutiérrez-Uribe, J. A. Changes in antioxidant and antiinflammatory activity of black bean (Phaseolus vulgaris L.) protein isolates due to germination and enzymatic digestion. *Food Chem.* **203**, 417–424 (2016).
57. Yang, B., Yang, H., Li, J., Li, Z. & Jiang, Y. Amino acid composition, molecular weight distribution and antioxidant activity of protein hydrolysates of soy sauce lees. *Food Chem.* **124**, 551–555 (2011).
58. Zhang, M., Mu, T. H. & Sun, M. J. Purification and identification of antioxidant peptides from sweet potato protein hydrolysates by alcalase. *J. Funct. Foods*. **7**, 191–200 (2014).
59. Esfandi, R., Willmore, W. G. & Tsopmo, A. Peptidomic analysis of hydrolyzed oat Bran proteins, and their in vitro antioxidant and metal chelating properties. *Food Chem.* **279**, 49–57 (2019).
60. Mora, L., Aristoy, M. C. & Toldrá, F. Bioactive peptides in foods. (2016).
61. Moayedi, A. et al. ACE-inhibitory and antioxidant activities of peptide fragments obtained from tomato processing by-products fermented using Bacillus subtilis: effect of amino acid composition and peptides molecular mass distribution. *Appl. Biochem. Biotechnol.* **181**, 48–64 (2017).
62. Maqsoudlou, A. et al. Peptide identification in alcalase hydrolysated pollen and comparison of its bioactivity with Royal jelly. *Food Res. Int.* **116**, 905–915 (2019).
63. Siow, H. L., Lim, T. S. & Gan, C. Y. Development of a workflow for screening and identification of α-amylase inhibitory peptides from food source using an integrated Bioinformatics-phage display approach: case study–Cumin seed. *Food Chem.* **214**, 67–76 (2017).
64. Jonker, J. T. et al. Effects of low doses of casein hydrolysate on post-challenge glucose and insulin levels. *Eur. J. Intern. Med.* **22**, 245–248 (2011).
65. Yang, H. J., Kwon, D. Y., Kim, M. J., Kang, S. & Park, S. Meju, unsalted soybeans fermented with Bacillus subtilis and Aspergilus Oryzae, potentiates insulinotropic actions and improves hepatic insulin sensitivity in diabetic rats. *Nutr. Metab. (Lond).* **9**, 1–12 (2012).

Acknowledgements

This work was supported by a research project (Project number: 18130; Approval ID: IR.MAZUMS.REC.1402.18130) at the Health of Plant and Livestock Products Research Center, Mazandaran University of Medical Sciences, Iran.

Author contributions

Zahra Akbarbaglu: Methodology, Investigation, Writing-original draft. Narges Mazloomi: Project administration, Data curation, Investigation. Laleh Karimzadeh: Methodology. Khashayar Sarabandi: Supervision, Data curation, Review & editing. Seid Mahdi Jafari: Review & editing. Mohammad Ali Hesarinejad: Review & editing. All authors have read and agreed to the published version of the manuscript. All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to N.M. or K.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.