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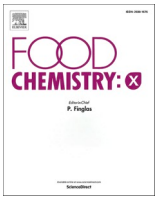

Characterisation and comparison of enzymatically prepared donkey milk whey protein hydrolysates

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ABSTRACT

This study evaluated the structural characteristics, processing properties, and antioxidant properties of hydrolysates prepared from donkey milk (DM) whey protein using different proteases (Alcalase, Neutrase, papain, and Flavourzyme). The results showed that enzymatic hydrolysis significantly increased hydrolysate solubility and reduced average particle size compared to those of DM whey protein. Neutrase and Flavourzyme hydrolysates exhibited higher degrees of hydrolysis (DH), along with elevated emulsification properties and surface hydrophobicity. The choice of protease influenced secondary and tertiary protein structures and amino acid composition. Enzymatic hydrolysis led to decreased molecular weight of DM whey proteins. Moreover, all hydrolysates exhibited higher fluorescence intensity at λ_{max} compared to DM whey protein, implying distinct properties due to the varied impacts of the four proteases on DM whey protein structure. The preparation of hydrolysates from DM whey proteins using proteases contributes to the development of integrated-value DM products.

1. Introduction

Donkey milk (DM) boasts nutritional advantages, low fat, and hypoallergenic properties, making it a commendable alternative to human milk with similarities in lactose and mineral content, fatty acids, and protein profiles. Additionally, DM whey protein, a valuable by-product in the dairy industry, is rich in nutrients, comprising proteins such as α-lactalbumin (α-La), β-lactoglobulin (β-Lg), immunoglobulins, and to a lesser extent, lysozyme and lactoferrin (Ambrosi, Polenta, Gonzalez, Ferrari, & Maresca, 2016). In particular, the lysozyme content of DM is significantly higher than that of human milk, bovine milk, and other milk sources. The presence of lysozyme imparts bactericidal properties to DM by disrupting bacterial cell walls. Enzymatic hydrolysis of DM whey proteins can generate bioactive peptides with various functional properties, such as antioxidant, antimicrobial, antihypertensive, anti-inflammatory, and immunomodulatory activities (Garhwal et al., 2022). These bioactive peptides have the potential to be used in functional foods, nutraceuticals, and pharmaceuticals. Therefore, the development and utilisation of DM whey proteins has great commercial value and potential. However, the utilisation of DM whey protein is currently limited to DM products, and its economic benefits remain underexplored.

In recent years, the preparation of bioactive peptides by enzymatic hydrolysis of proteins for application in food and other fields has attracted attention. Enzymatic hydrolysis has been widely recognised in the fields of food, medicine, and healthcare because of its high specificity, high product safety, and mild production conditions (Wang et al., 2023; Wu, Zhang, Jia, Kuang, & Yang, 2018). In addition, hydrolysates prepared using proteases improve several functional properties of proteins by cleaving peptide bonds, leading to a reduction in molecular weight, exposure of hydrophobic groups, and changes in hydrophobicity and polarity. Numerous studies have underscored the potential of bioactive peptides derived from whey proteins. For instance, research implementing *A. oryzae* LBA 01 (AO) protease to enzymatically hydrolyse whey proteins demonstrated an enhanced antioxidant capacity (de Castro & Sato, 2014). Another study also found that α-chymotrypsin

Abbreviations: CD, Circular dichroism; DH, Degrees of hydrolysis; DM, Donkey milk; DWPH, Donkey milk whey protein hydrolysates; EAI, Emulsifying activity index; ESI, Emulsion stability index; FC, Foaming capacity; FS, Foam stability; FT-IR, Fourier transform infrared spectrometry; PBS, Phosphate buffer solution; PDI, Polydispersity index; PPH, Peanut protein hydrolysates.

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hydrolysis of whey proteins produced hydrolysis products with the highest ACE inhibitory activity (Lourenço da Costa, da Rocha, Gontijo, & Netto, 2007). Alcalase, Neutrase, papain, Favourzyme and bromelain are often widely used in the preparation of whey protein hydrolysates. Several studies have shown that milk whey protein hydrolysates exert hypoglycaemic (Du et al., 2022), and antimicrobial activities (Abdel-Hamid, Goda, De Gobba, Jenssen, & Osman, 2016). DM whey proteins are abundant, rich in composition, hypoallergenic, and have a variety of biofunctional activities. Zhou et al. (2023) obtained DM whey protein peptides using neutrase and found that they regulated the gut microbiota and delayed aging in mice. However, reports on the effects of different proteases on the structural and processing characteristics of DM whey proteins are rare. Selecting the most suitable type of protease to maximise the functional properties of protein hydrolysis products is important in dairy processing.

Therefore, structural changes in DM whey protein hydrolysates were analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus, fluorescence spectrophotometry, Fourier transform infrared spectrometry (FT-IR), and circular dichroism (CD) spectrophotometry. Further comparisons of solubility, emulsification, and foaming were performed to investigate the changes in the processing properties. The antioxidant activity of DM whey protein hydrolysates was assessed by studying the scavenging effect on three free radicals: 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical (·OH), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS). This study serves as a valuable step toward exploring the application of DM whey protein bioactive hydrolysates in the food industry.

2. Materials and methods

2.1. Materials

DM whey protein was purchased from Kangze Biotechnology Co., Ltd. (Shaanxi, China). All reagents, including enzymes, were of analytical purity and purchased from Shenyang Lab Science and Trade Co., Ltd. (Liaoning, China). The enzyme exhibited an activity of 20,000 U/g.

2.2. Preparation of DM whey protein hydrolysates

The DM whey protein solution (6%, *w/v*) was heated in a water bath at 85 °C for 10 min to denature the DM whey protein. Subsequently, when the solution cooled, proteases were added to the solution for enzymatic hydrolysation. The DM whey proteins were digested with Alcalase (pH = 9.0, 55 °C), Neutrase (pH = 7.0, 50 °C), papain (pH = 7.0, 50 °C) and Flavourzyme (pH = 7.0, 50 °C) for 4 h at a constant temperature (E/S = 4:100). The hydrolysis conditions based on our previous studies.

During the enzymatic reaction, ultrasound was used for 20 min to assist the enzymatic process, and the pH of the solution was measured every 30 min. The desired pH was maintained by adding NaOH (1 M) or HCl (1 M). After hydrolysis, the protease was inactivated in a boiling water bath for 10 min, rapidly cooled to 25 °C, pH adjusted to 7, and centrifuged at 8000 ×*g* for 10 min at 4 °C. The donkey milk whey protein hydrolysates (DWPHs), namely Alcalase hydrolysates (Alcalase-DWPH), Neutrase hydrolysates (Neutase-DWPH), papain hydrolysates (papain-DWPH), and Flavourzyme hydrolysates (Flavourzyme-DWPH), were obtained by collecting and freeze-drying the supernatant.

2.3. Determination of protein content and solubility

Protein content: The protein content in the supernatant of the hydrolysates was determined using a BCA protein concentration assay kit (Suzhou Grace Biotechnology Co., Ltd., China) (Cortés-Ríos et al., 2020; Smith et al., 1985). A working solution of BCA was prepared by mixing reagent A (BCA, Na_2CO_3, $NaHCO_3$, dicinchoninic acid, and sodium tartrate in 0.1 M NaOH) with reagent B ($CuSO_4$, 4%) in a 50:1 ratio, as indicated by the manufacturer. The samples and the BCA working solution were incubated for 30 min, pipetted into a 96-well plate, and the OD value was measured at 562 nm. The protein content was calculated based on a standard curve.

Protein solubility: The soluble content of the DWPHs was determined using the Folin–Lowry method (An et al., 2022). A solution of Folin–Ciocalteu Phenol Reagent A with Na_2CO_3, NaOH, $CuSO_4$, and potassium sodium tartrate was prepared. The sample solution (10 mg/mL) was mixed with an equal volume of 15% trichloroacetic acid (TCA), left to stand for 10 min, and then centrifuged (CR21N, Hitachi, Japan). The supernatant was diluted 10-fold with TCA. Subsequently, 1 mL of the diluted solution was mixed with 5 mL of Folin-Ciocalteu Phenol Reagent A solution, allowed to stand for 10 min, and then 0.5 mL of Folin-Phenol Reagent B (Beijing Solarbio Science & Technology Co, Ltd., Beijing, China) was added to the mixture and shaken immediately. The solution was incubated for 30 min at room temperature, and the absorbance was determined at 540 nm. DWPH content was calculated based on a previously established standard curve (y = 0.0621× + 0.0026). Protein solubility was determined as the ratio of protein content to DM whey protein content in the supernatant.

2.4. Degree of hydrolysis (DH) determination

DH of DM whey protein hydrolysates was determined using the O-phthaldialdehyde (OPA) method. Specifically, Serine (400 μL) was added to 3 mL of OPA reagent, and the reaction was carried out for 5 s. After vortex mixing (vortex-1, Shanghai Huxi Industrial Co., Ltd., China) for 5 s and incubating at room temperature for 2 min, the absorbance was measured at 340 nm, and a standard curve was plotted (y = 0.8871× + 0.092). A sample solution (5 mg/mL) was selected to determine the absorbance according to the above steps, and DH was calculated using the following formula (Nielsen, Petersen, & Dambmann, 2001):

$$DH\ (\%) = \frac{\frac{C \times N \times M}{m} - \beta}{\alpha h_{tot}} \times 100 \quad (1)$$

where h_{tot}, β, and α depend on the type of raw material and h_{tot} is the total number of peptide bonds in the protein. For whey protein, h_{tot} = 8.8, β = 0.40, and α = 1.000.

C represents serine concentration (mg/mL), *N* is the dilution factor, *V* is the volume of the sample solution (mL), and *m* is the sample mass (mg).

2.5. Amino acid composition analysis

According to the method described by Zhang et al. (2007), 20 mg samples (accurate to 0.1 mg) were added to a 20 mL vial and dissolved in 8 mL of 6 M HCl. Each vial was filled with nitrogen to prevent oxidation. After hydrolysis at 110 °C for 24 h, the sample was diluted to 50 mL. Subsequently, 2 mL sample was taken into an evaporating dish for evaporation, during which the concentration was rinsed several times with distilled water. The samples were then treated with 0.02 M sulfosalicylic acid and filtered through a 0.22 μm membrane filter. The amino acid composition of DWPHs was determined using an automatic amino acid analyser (L-8800, Hitachi, Ltd., Japan).

2.6. Analysis of structural characteristics

2.6.1. SDS-PAGE analysis

The electrophoresis mode of DWPHs sample was adjusted appropriately, according to the method described by Wang et al. (2014). Polyacrylamide gel included 15% separation gel and 5% concentrated gel. Hydrolysates samples (10 μL, 10 mg/mL) generated by different proteases were mixed with buffers (10 μL, SDS, DTT, Tris HCl,

bromophenol blue, and glycerol). The mixture was analysed using a dual vertical electrophoresis system (BG-verMIDI, Beijing Baygene Biotech Co., Ltd., Beijing, China). The initial voltage was 80 *V*, and the operation was continued at 120 V for 2 h. The gel images were analysed using an image lab (Gel Doc XR, Bio-Rad, USA).

2.6.2. Fourier transform infrared (FT-IR) spectroscopy

FT-IR (Nicolet, Thermo Electron, USA) was used to analyse the secondary structural characteristics of different DM whey protein hydrolysates. The samples were mixed with dried KBr (1:100, *w*/w) and pressed into thin slices. Thirty-two scans at 2 cm^{-1} resolution analyses were performed at the spectral range of 4000 cm^{-1} to 400 cm^{-1}.

2.6.3. CD spectroscopy

A 1 mg/mL hydrolysates solution with phosphate buffer solution (PBS) was analysed at 25 °C via CD spectrophotometry (JASCO J-810, Japan Spectroscopy Company), using a quartz cuvette with a 0.1 cm optical pathlength in the far-UV range (190–250 nm).

2.6.4. Fluorescence spectra measurement

Fluorescence spectra of the samples were detected using a fluorescence spectrophotometer (F-7100; Hitachi, Japan) with a 1 cm width quartz cuvette. Hydrolysate samples (5 mg/mL) were diluted with PBS (pH 7.0, 0.1 M) for measurement. The excitation and emission wavelengths of the fluorescence photometer were 280 nm and 300–460 nm, respectively. The working voltage was 750 *V*, and the slit width was set to 5 nm with a 300 nm/min scan rate.

2.6.5. Surface hydrophobicity

Surface hydrophobicity of the samples was determined using an ANS fluorescent probe. Samples (0.3 mg/mL) were dissolved in PBS (pH 7.0, 0.1 M). Subsequently, 10 μL of 8.0 mM ANS was mixed with 4 mL samples solution, and the fluorescence intensity of the shaken mixture was measured. The excitation wavelength was 350 nm, and the scanning wavelength range was 390–650 nm. Two slit widths were set (2.5 nm), and the working voltage was 750 *V*.

2.7. Analysis of processing characteristics

2.7.1. Foaming capacity and foam stability

The foaming capacity (FC) and foam stability (FS) of DWPHs were measured. DWPHs (0.4 g) were dissolved in 20 mL of PBS (pH 7.0, 0.01 M) in a 50 mL tube, and foam was prepared using a homogeniser (XHF-DY; Xinzhi Biotechnology Co. Ltd., China) at 10,000 ×g for 2 min. The foam volume was recorded at 0 (V_0) and 30 min (V_{30}) of standing. The FC and FS were calculated as follows (Tadesse et al., 2023):

$$FC(\%) = \frac{V_0 - V}{V} \times 100 \tag{2}$$

$$FS(\%) = \frac{V_{30} - V}{V} \times 100 \tag{3}$$

where V is the initial volume, V_0 is the total volume after homogenisation, and V_{30} is the total volume after standing for 30 min.

2.7.2. Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) were determined using the methods of Nalinanon, Benjakul, Kishimura, and Shahidi (2011) with some slight modifications. Soybean oil (7 mL) and DWPH solution (1 mg/mL, 21 mL) were mixed and homogenised (XHF-DY; NingBo Scientz Biotechnology Co., Ltd., China) at 10,000 ×g for 2 min. An aliquot of the emulsion (50 μL) was pipetted from the middle portion of the tube at 0 and 15 min after homogenisation and subsequently diluted 100-fold using 0.1% (*w/v*) SDS solution. The absorbance of diluted emulsions was measured at 500 nm after 5 s of shaking in a vortex mixer, and 0.1% SDS was used as the blank control. The EAI and ESI were calculated using the following formulas:

$$\mathrm{EAI}\left(m^2/\mathrm{g}\right) = \frac{2 \times 2.303 \times \mathrm{DF} \times \mathrm{A_0}}{\varphi \times \mathrm{c} \times \mathrm{L} \times 10000} \tag{4}$$

where A_0 is the absorbance at 0 min, *DF* is the dilution factor (100), *L* is the path length of the cuvette (cm), φ is the oil volume fraction (0.25), and *C* is the DWPH concentration (mg/mL).

$$\mathrm{ESI}(min) = \frac{\mathrm{A_0} \times \Delta \mathrm{t}}{\Delta \mathrm{A}} \tag{5}$$

where $\Delta A = A_0 - A_{15}$ and $\Delta t = 15$ min.

2.7.3. Particle size and polydispersity index (PDI)

The average particle size of the DWPH samples were determined using a Zetasizer (Nano-ZS; Malvern Ltd., UK) instrument. The concentration of the samples was diluted to 1 mg/mL with 0.1 M PBS (pH 7.0). Average particle size was measured at room temperature.

2.8. Antioxidant activity

2.8.1. DPPH scavenging activity

Following the method outlined by Wali et al. (2019), a 0.1 mM DPPH solution was prepared using ethanol. Each sample (3 mL, 2.5 mg/mL) was mixed with a DPPH (2 mL) solution and ethanol (2 mL). The mixture was incubated for 30 min after vortexing (vortex-1, Shanghai Huxi Industrial Co., Ltd., China), and the absorbance was measured at 517 nm. The DPPH scavenging activity was calculated as follows:

$$DPPH\ scavenging\ effect\ (\%) = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100 \tag{6}$$

where, A_0 is the absorbance of the DPPH solution without the sample, A_1 is the absorbance of the sample mixed with the DPPH solution, and A_2 is the absorbance of ethanol without DPPH.

2.8.2. ·OH scavenging activity

Approximately 2 mL of $FeSO_4$ (6 mM) and 2 mL a salicylic acid–ethanol solution (6 mM) was added to 2 mL of DWPH solutions (2.5 mg/mL). Finally, 2 mL of H_2O_2 (6 mM) was added to initiate the reaction, and the mixture was stored at 37 °C for 30 min. Absorbance of the mixture was measured at 510 nm. The capability of ·OH scavenging was measured using the following equation:

$$\cdot OH\ scavenging\ effect\ (\%) = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100 \tag{7}$$

where A_0 is the absorbance of the control group (H_2O + salicylic), A_1 is the absorbance of the sample group (sample + salicylic), and A_2 is the absorbance of the control group (sample + H_2O).

2.8.3. ABTS radical scavenging activity

ABTS radical scavenging activity was determined as previously described (Wang et al., 2021). A 10 mL solution of 7 mM ABTS and 176 μL of 2.45 mM potassium persulfate were allowed to react at 4 °C for 14 h in the absence of light. The mixture was then prepared as a stock solution with ethanol to give an absorbance of 0.7 ± 0.02 (734 nm). Subsequently, 0.8 mL of DWPH samples were mixed with 3.2 mL stock solution and incubated at room temperature for 6 min in the dark. The absorbance was measured at 734 nm, and the ABTS radical scavenging activity was calculated as follows:

$$ABTS\ scavenging\ effect\ (\%) = \left[1 - \frac{A_1}{A_0}\right] \times 100 \tag{8}$$

where A_0 is the absorbance of ethanol and the ABTS radical and A_1 is the absorbance of the sample and the ABTS radical.

2.9. Statistical analysis

Each sample was measured in triplicate, and data were presented as means ± standard deviation. Statistical analyses were performed using SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA). Differences among means were detected using Duncan's multiple range test ($P < 0.05$).

3. Results and discussion

3.1. Protein content and solubility

The standard curve for protein content calculations, y = 0.188×-0.0046 (R^2 = 0.9987), was obtained using the BCA Protein Content Assay Kit. Based on the standard curve, the concentration of DM whey protein was 0.8942 mg/mL. The results of the hydrolysates obtained using the four enzymatic methods are shown in Fig. 1A. The protein concentrations of the four DWPHs were 0.6331 (Alcalase-DWPH), 0.7571 (Neutrase-DWPH), 0.8048 (papain-DWPH), and 0.8016 (Flavourzyme-DWPH) mg/mL ($P < 0.05$). Notably, Neutrase-DWPH, papain-DWPH, and Flavourzyme-DWPH exhibited the highest concentrations, possibly attributed to the extensive peptide bond cleavage by papain and flavour enzymes, resulting in a greater diversity of small proteins/peptides and increased protein concentrations (Nalinanon et al., 2011).

Solubility is mainly associated with the distribution of hydrophobic and hydrophilic amino acids in proteins and is also influenced by the thermodynamics of protein–water interactions (Kristinsson & Rasco, 2000). As illustrated in Fig. 1A, the solubility of Neutrase-DWPH, papain-DWPH, and Flavourzyme-DWPH were close to 80%, all of which were higher than those of Alcalase-DWPH (71.11%) and DM whey protein (47.74%) ($P < 0.05$). A previous study applied porcine trypsin, papain, and Neutrase to the enzymatic hydrolysis of bovine whey proteins to compare solubility and found that the type of enzyme and pH affected solubility, with the hydrolysate of trypsin being completely soluble at a neutral pH (Monti & Jost, 1978). It has been suggested that when proteins are enzymatically hydrolysed, the formation of smaller peptides or proteins, as well as the presence of newly exposed amino and carboxyl groups during bond breaking, promote more interactions with water and increase hydrophilicity, which in turn improves the solubility of proteins (Kristinsson & Rasco, 2000). Our results showed that enzymatic hydrolysis significantly increased the solubility of DM whey proteins ($P < 0.05$). Furthermore, the effectiveness of the four proteases varied when acting on DM, with Alcalase being significantly less effective than the other three proteases on DM whey proteins ($P < 0.05$).

3.2. DH

DH, representing the extent of enzymatic hydrolysis of proteins or polypeptides, is depicted in Fig. 1B. DH values varied among different DWPHs depending on the enzyme used. The DH values of the DWPHs showed significant differences ($P < 0.05$), with the highest being that of Flavourzyme (39.46%), followed by that of Neutrase (30.36%), and the lowest being that of papain-DWPH (12.73%). Wu et al. (2018) investigated the effect of ultrasonic pretreatment on the physicochemical properties of bovine whey protein enzymatic hydrolysis and found that DH of the ultrasonic whey protein hydrolysate was <15%. In a separate study on bovine whey protein hydrolysis, after treatment with Alcalase and papain, the DH was found to be 15.9%. This discrepancy may arise from differences in enzymatic hydrolysis, site of action, and the number of hydrolysed peptide bonds.

Furthermore, according to a previous study, DH is positively correlated with the ability of the hydrolysis process to disrupt peptide bonds, with a higher DH value representing a higher number of short-chain peptides in the hydrolysate (Charoenphun, Cheirsilp, Sirinupong, & Youravong, 2012).

In a prior study on the enzymatic hydrolysis of myofibrillar proteins, papain demonstrated a higher DH than Alcalase and Flavourzyme, attributed to its broader range of action and its tenderising function (Najafian & Babji, 2015). In our current study focusing on DM whey protein enzymatic hydrolysis, the altered target may have influenced the site of action, leading to a greater extent of hydrolysis by Flavourzyme, resulting in potentially higher numbers of short-chain peptides in Flavourzyme-DWPH. Despite this, the enzyme's specificity in site of action prevents achieving a DH of 100% (Ningsih, Raharjo, Haryadi, & Wikandari, 2023). This result was similar to previous reports in which whey proteins of goat milk were hydrolysed by trypsin, and their DH values did not reach 100% (Ningsih et al., 2023). Thus, Flavorzymes and Neutrases are more hydrolytic in DM.

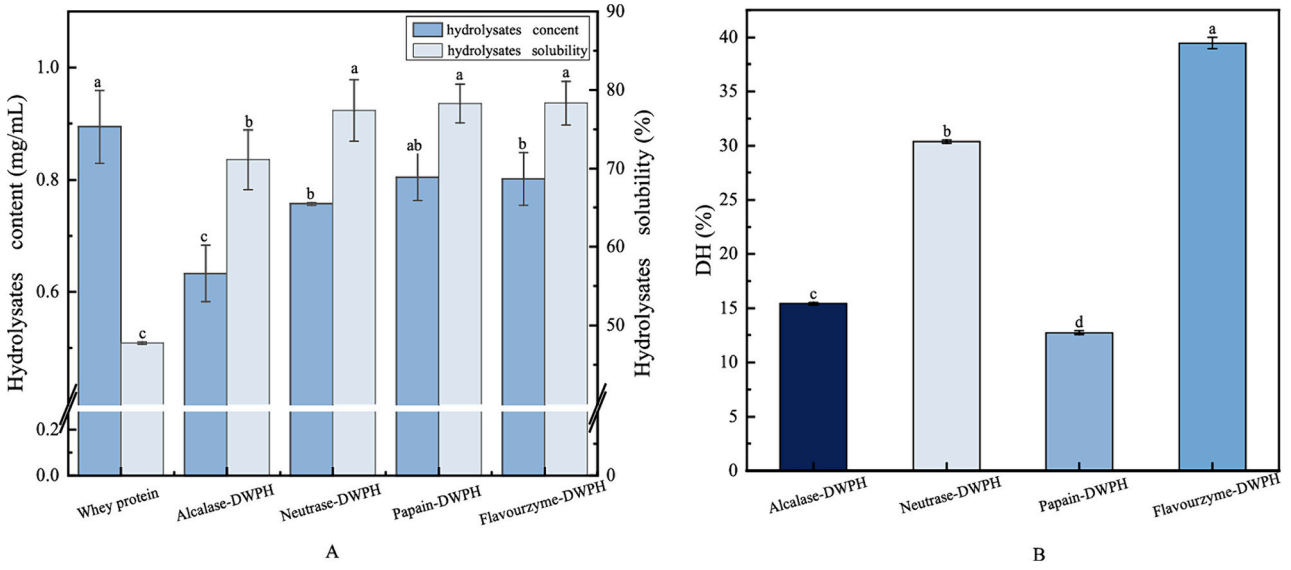

Fig. 1. The content and solubility of DWPHs and whey protein hydrolysed by different protease (A). The DH of DWPHs hydrolysed by different protease (B). For the data of Fig. 1. (A, B), values were means ± SD (three replicates). Different letters were used to show significant differences ($P < 0.05$).

3.3. Amino acid composition analysis

The amino acid compositions of DM whey protein and its hydrolysates are shown in Table 1. The total amino acid content of DWPHs was significantly higher than that of DM whey proteins ($P < 0.05$). This indicates that enzymatic hydrolysis promotes the release of low-molecular-weight peptides and free amino acids. The free amino acid content in Neutrase-DWPH and Flavourzyme-DWPH was much higher than that in the other two hydrolysates, which positively correlated with the DH results. A previous study examined the hydrolysis of tree peony seed proteins using different proteases and similarly found a positive correlation between DH and the amino acid content of the hydrolysates (Wang et al., 2021). DM whey protein and DWPHs had the same amino acid composition, with Glu, Leu and Asp being the most abundant amino acids among the samples. In particularly, Thr, Glu, Ala, Pro, and Lys were the most abundant amino acids in the Neutrase, Papain, and Flavourzyme hydrolysates, but not in Alcalase-DWPH ($P < 0.05$), indicating that the high content of these amino acids might be attributed to differences in the cleavage sites. A study comparing the essential amino acid (Val, Leu, Ile, Thr, Met, Phe, Tyr, and Lys) composition in donkey, bovine, and human milk proteins showed that DM has a higher proportion of the eight essential amino acids in the protein than the other two (Guo et al., 2007). Therefore, the amino acid composition of DM is relatively more suitable for human consumption. In particularly, enzyme hydrolysis reduces the molecular weight of DM whey protein and increases the amino acid content, which is easier for intestinal digestion and absorption.

Table 1
Amino acid contents of whey protein and its hydrolysates prepared with Alcalase, Neutrase, Papain, and Flavourzyme.

AA	Whey protein (mg/g)	A-DWPH (mg/g)	N-DWPH (mg/g)	P-DWPH (mg/g)	F-DWPH (mg/g)
Asp	21.74 ± 0.93^{c}	32.32 ± 1.48^{b}	35.17 ± 0.90^{a}	33.51 ± 0.43^{ab}	35.93 ± 2.08^{a}
Thr	8.94 ± 0.36^{c}	18.75 ± 0.96^{b}	20.34 ± 0.60^{ab}	20.11 ± 1.43^{ab}	20.84 ± 1.30^{a}
Ser	9.49 ± 0.34^{c}	13.53 ± 0.64^{b}	14.39 ± 0.30^{ab}	14.85 ± 0.58^{a}	14.83 ± 0.74^{a}
Glu	25.39 ± 1.25^{c}	51.50 ± 2.43^{b}	57.19 ± 1.55^{a}	55.03 ± 0.21^{ab}	58.76 ± 2.98^{a}
Gly	14.66 ± 0.34^{a}	4.99 ± 0.19^{b}	5.08 ± 0.11^{b}	5.39 ± 0.21^{b}	5.32 ± 0.20^{b}
Ala	6.74 ± 0.23^{c}	12.01 ± 0.45^{b}	13.18 ± 0.37^{a}	13.14 ± 0.31^{a}	13.65 ± 0.52^{a}
Cys	8.39 ± 0.27^{a}	7.67 ± 0.14^{b}	7.79 ± 0.16^{b}	7.72 ± 0.12^{b}	7.52 ± 0.12^{b}
Val	9.77 ± 0.35^{c}	16.70 ± 0.98^{b}	17.93 ± 0.48^{ab}	17.76 ± 0.37^{ab}	18.53 ± 0.83^{a}
Met	2.57 ± 0.24^{b}	7.27 ± 0.22^{a}	8.04 ± 0.16^{a}	7.93 ± 0.44^{a}	7.88 ± 0.69^{a}
Ile	8.21 ± 0.35^{c}	17.07 ± 0.97^{b}	19.08 ± 0.54^{a}	18.98 ± 0.89^{a}	19.63 ± 0.85^{a}
Leu	13.78 ± 0.67^{c}	35.83 ± 2.24^{b}	38.66 ± 1.09^{ab}	37.86 ± 0.79^{ab}	39.72 ± 1.92^{a}
Tyr	11.32 ± 0.28^{b}	12.62 ± 0.30^{a}	12.56 ± 0.23^{a}	13.15 ± 0.36^{a}	12.92 ± 0.55^{a}
Phe	4.30 ± 0.24^{b}	7.05 ± 0.53^{a}	7.52 ± 0.23^{a}	7.78 ± 0.60^{a}	7.76 ± 0.53^{a}
Lys	12.13 ± 0.53^{c}	24.65 ± 1.31^{b}	26.49 ± 0.69^{a}	25.69 ± 0.38^{ab}	27.17 ± 1.19^{a}
His	3.22 ± 0.17^{c}	4.13 ± 0.22^{b}	4.42 ± 0.08^{ab}	4.62 ± 0.27^{a}	4.54 ± 0.24^{ab}
Arg	10.27 ± 0.38^{a}	8.25 ± 0.56^{b}	8.90 ± 0.24^{b}	8.96 ± 0.40^{b}	8.55 ± 0.55^{b}
Pro	7.76 ± 0.21^{c}	15.26 ± 1.03^{b}	17.01 ± 0.67^{a}	17.47 ± 0.75^{a}	17.41 ± 0.73^{a}
TAA	178.68	289.62	312.93	309.93	320.95

Asp: Aspartic acid; Thr: Threonine; Ser: Serine; Glu: Glutamic acid; Gly: Glycine; Ala: Alanine; Cys: Cysteine; Val: Valine; Met: Methionine; Ile: Isoleucine; Leu: Leucine; Tyr: Tyrosine; Phe: Phenylalanine; Lys: Lysine; His: Histidine; Arg: Arginine; Pro: Proline.
A-DWPH: Alcalase-DWPH; N-DWPH: Neutrase-DWPH; P-DWPH: Papain-DWPH; F-DWPH: Flavourzyme-DWPH. Results were expressed as the mean ± standard deviation ($n = 3$). Different letters in the same row correspond to a significant difference at $P < 0.05$.

3.4. SDS-PAGE electrophoretic profiles of DWPHs

The SDS-PAGE profiles of the DM whey proteins and DWPHs were characterised based on their protein constituents, as shown in Fig. 2A and Fig. 2B, respectively. The molecular weight profiles of DWPHs were different from those of DM whey protein. As depicted in Fig. 2, the molecular weight of DM whey protein was concentrated in the range of 50–100 kDa, which were mainly composed of serum albumin, immunoglobulins, and lactoferrin, and α-lactalbumin and β-lactoglobulin were distributed between the range of 15–30 kDa (Ozturkoglu-Budak, 2018). The electrophoretogram of Neutrase-DWPH (lane 2) and Flavourzyme-DWPH (lane 4) in Fig. 2B shows four major and clear bands at molecular weights of approximately 25, 23, 10, and 6.5 kDa, and a weak low-intensity band at 3 kDa. The intensities of the four protein bands in Alcalase-DWPH and papain-DWPH (lanes 1 and 3) weakened or partially disappeared. Moreover, papain and Alcalase hydrolysates exhibited almost absent bands, while more weak bands accumulated near the 4.2 kD band, especially in lane 3. These variations in the protein bands among the hydrolysates may be related to bond specificities and different conditions of protease action. The presence of high-intensity bands in Neutrase-DWPH and Flavourzyme-DWPH suggests that these two hydrolysates were more extensive than those produced by the other two proteases, confirming the DH results. A study of the enzymatic hydrolysis of camel whey protein with papain revealed two new low-molecular-weight bands (17–20 kDa) in the hydrolysate in the SDS-PAGE spectra, suggesting that enzymatic hydrolysis was effective in reducing the molecular weight of whey protein. Furthermore, Lourenço da Costa et al. (2007) used Alcalase, a-chymotrypsin, or Proteomix for the enzymatic degradation of whey proteins, observing the formation of low-molecular-weight peptides (two bands with an average molecular weight of about 5.6 and 7.8 kDa) in the hydrolysates. In conclusion, enzymatic hydrolysis can reduce the molecular weight of DM whey proteins, yielding small-molecule peptides below 5 kDa.

3.5. FT-IR analysis

FT-IR is widely applied in a variety of fields, providing not only the characteristic vibrational absorption bands of molecular groups but also the detection of changes in molecular groups and their surroundings (Hou et al., 2017). The secondary structures of the DWPHs were determined via FT-IR spectroscopy to study the relationship between structure and function. As shown in Fig. 3A, there was strong absorption at wavenumbers between 3440 and 3070 cm^{-1} which are generally associated with the vibrations of the hydroxyl group and the N—H bond vibration. Generally, the N—H expansion vibration ranges from 3400 to 3440 cm^{-1}, recognised as amide A band. When N—H is bonded to the carbonyl group in the peptide chain, the wavenumber experiences a blue shift to approximately 3300 cm^{-1}. Protein repeats units exhibit several characteristic infrared absorption bands, including those of amides A, B, and I-VII. The amide I band (1700–1600 cm^{-1}) is closely related to the secondary structure of proteins, with broad peaks in the O—H stretching vibration indicating the presence of intramolecular or intermolecular hydrogen bonds (Wang et al., 2023). Moreover, absorbance bands at 1642 cm^{-1} are associated with C═C stretching vibration (Li et al., 2020). Within the amide I band, characteristic spectral bands are mainly related to C═O stretching vibrations and N—H bending vibrations. The amide II band, occurring between 1600 and 1500 cm^{-1}, is primarily generated by N—H stretching vibrations. The amide III band, situated within the 1320–1200 cm^{-1} range, is mainly associated with C—H stretching vibrations.

In addition, the distinct bands formed by the amide I band

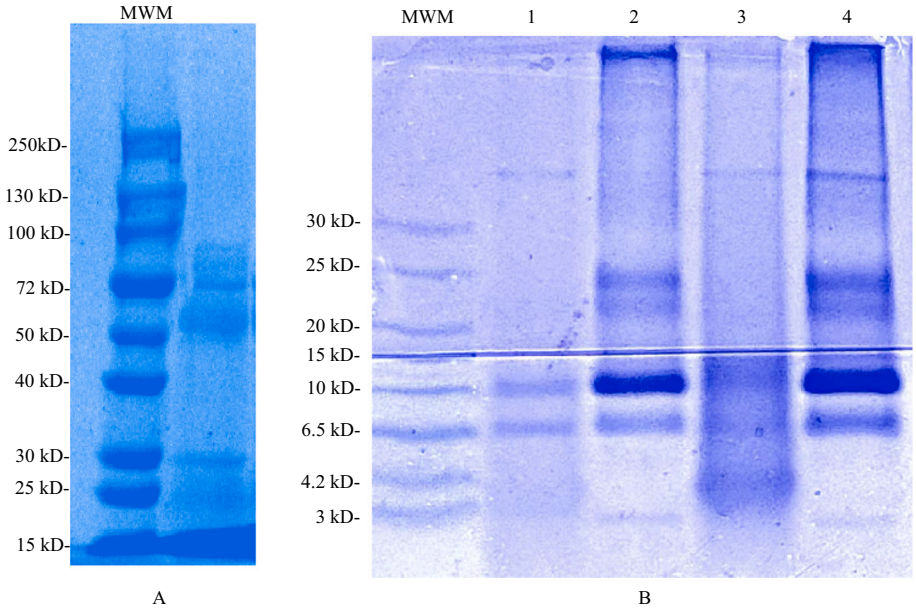

Fig. 2. The SDS-PAGE profiles of whey protein (A). Lanes: (1) molecular weight marker (MWM), with sizes in kDa indicated on the left, (2) donkey whey protein. The SDS-PAGE profiles of DWPHs extracted by different protease (B). Lanes: (1) molecular weight marker (MWM), with sizes in kDa indicated on the left, (2) From 1 to 4 were Acalase-DWPH, Neutrase-DWPH, Papain-DWPH, Flavourzyme-DWPH, respectively.

correspond to the various secondary protein structures, including α-helix (1645–1662 cm^{-1}), β-sheet (1615–1638 cm^{-1}), β-turn (1662–1682 cm^{-1}), and random coil (1638–1645 cm^{-1}), as reported by Hou et al. (2017). Usually, the α-helix is considered an ordered structure, and its content is sensitive to conformational changes. The β-sheet and β-turn structures belong to relatively stretched arrangements that enable polypeptide chains to bend and fold upon themselves. The random coil structure is associated with a disordered arrangement (Wang et al., 2021). In the present study, the type of enzyme exhibited minimal impact on changes in the structure of DWPHs, with only slight differences in secondary content ($P < 0.05$). The secondary structures of the DWPHs were deconvoluted from the amide I region, and Gaussian curve fitting was used to determine the approximate distribution of the secondary structures, as shown in Table 2.

The initial analysis of DM whey protein indicated the presence of α-helix (14.50%), β-sheet (29.69%), β-turn (27.75%), and random coils (28.06%). Following enzymatic hydrolysis, Neutrase and Flavourzyme increased the proportion of β-sheet while reducing the β-turn content, with no significant change in the content of random coils ($P > 0.05$). Conversely, Alcalase-DWPH and papain-DWPH exhibited a significant reduction in β-sheet content, transforming into α-helix and random coils ($P < 0.05$). This transformation is likely attributed to the unfolding, dissociation, and rearrangement of DWPHs during hydrolysis (Wang, Yang, Fan, Zhang, & Chen, 2019). Furthermore, the exposure of hydrophobic and hydrophilic regions of proteins to new environments, along with cross-linking between formed hydrolysates, leads to changes in secondary structure, even the loss or reduction of specific structures due to bond breakage (Meziani et al., 2011). Supporting this, a study by Wang et al. (2023) indicated that the combined proportions of α-helical and β-sheet structures reflect the compactness of protein structure. When hydrolysing peanut proteins with different enzymes, they observed a decrease in the proportions of β-sheet and β-turn structures, along with an increase in the proportions of random coils in the resulting hydrolysates. This conclusion is similar to that of the secondary structural changes induced by the hydrolysis of DM whey protein using papain. Other proteases show different changes in secondary structures owing to their different action sites (Wang et al., 2023). In our study, Alcalase and papain were found to exert a more pronounced effect on the ordered structure of DM whey proteins, resulting in partial unfolding of the conformation and imparting flexibility and looseness to the hydrolysate structure.

3.6. CD analysis

CD spectroscopy was used to analyse the secondary structures of the DWPHs, and the results are shown in Fig. 3B. The CD spectra encompass two regions: near-UV (245–320 nm) and far-UV (185–245 nm). The peptide bond absorption peaks were mainly in the far-UV region, where the conformation of the main chain was observed. The CD spectra of native proteins contain a positive peak at 190 nm and a negative peak at 205–235 nm, which affects the conformation of the main chain (Hou et al., 2017). Generally, the α-helix of proteins appears near 192 nm in the positive band and has two shoulder peaks at 222 nm and 208 nm in the negative band. β-sheets appear with a negative peak at 216 nm, while β-turns exhibit a positive peak near 206 nm. Fig. 3B. illustrates a negative band at approximately 210 nm, representing the α-helix conformation. A reduction in the negative peak at 210 nm and a blue shift of the peak wavelength occurred for the four enzymatic hydrolysates, indicating a loss of α-helix content, especially for Acalase-DWPH which has the greatest structural impact (Jiang, Chen, & Xiong, 2009). The peak intensities of all the DWPHs varied; however, the intensities of Neutrase-DWPH and Flavouryzyme-DWPH were higher than those of the remaining two DWPHs, showing that better results were being achieved. This suggests that enzymatic hydrolysis alters the secondary structure of DM whey proteins and has different effects depending on the type of enzyme and site of action.

3.7. Fluorescence spectra

Aromatic amino acids (especially Trp residues) inside a protein molecule can produce fluorescence, and proteins are usually surrounded by multiple nonpolar amino acid residues (Vivian & Callis, 2001). As shown in Fig. 3C, the overall trend of DWPHs enzymatically cleaved by Neutrase and Flavourzyme was consistent with that of DM whey protein. However, the maximum fluorescence intensities of Neutrase-DWPH and Flavourzyme-DWPH showed a higher increase than that of DM whey protein, but less than those of Alcalase-DWPH and papain-DWPH. This phenomenon might have been caused by the enzymatic hydrolysis of

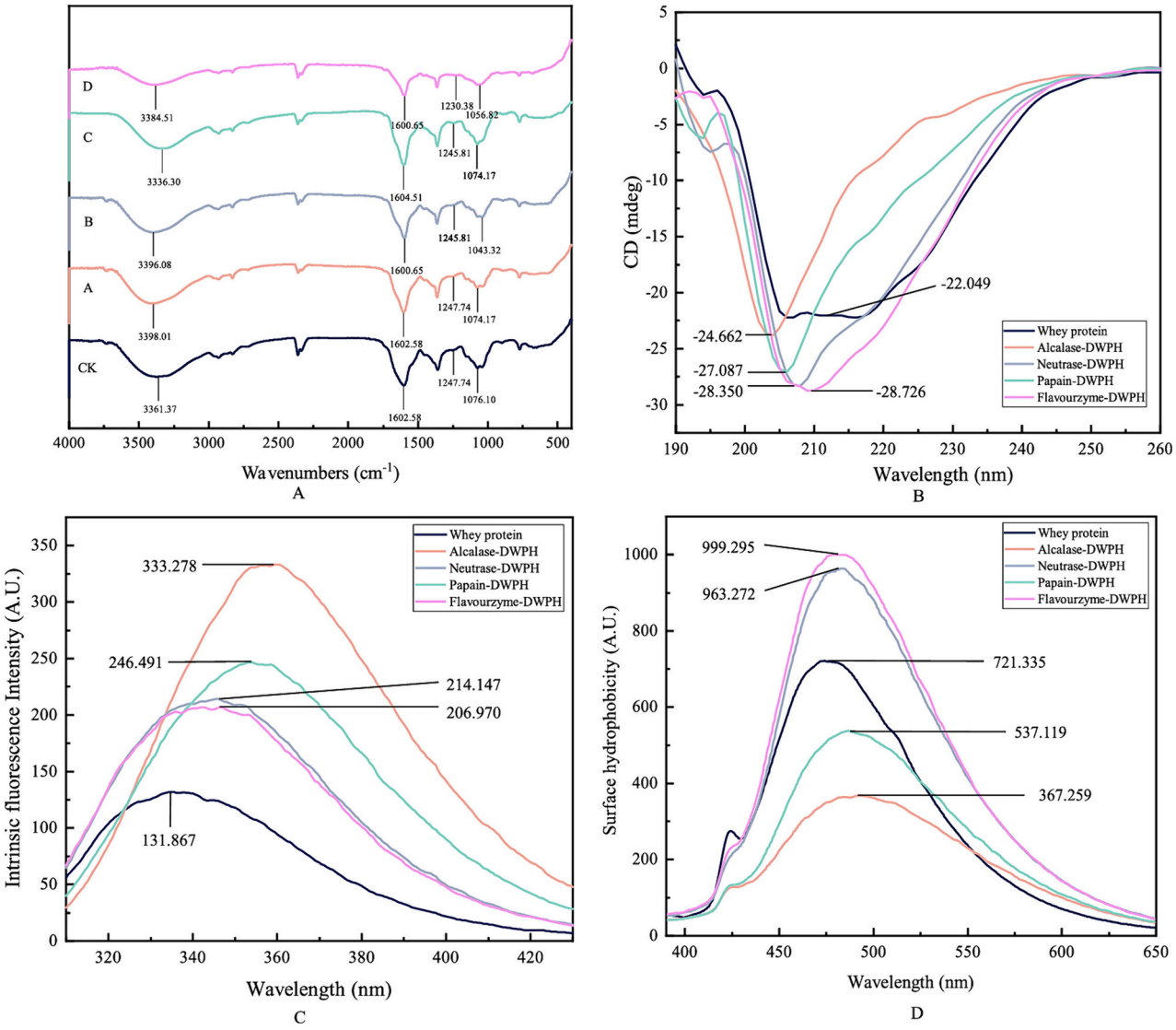

Fig. 3. The FT-IR spectrum of whey protein and DWPHs hydrolysed by different protease. Transimisson was obtained in the wavenumber range of 4000 to 400 cm-1. The KBr spectrum was taken as background. Groups A-D was Alcalase-DWPH, Neutrase-DWPH, Papain-DWPH, Flavourzyme-DWPH, namely. And CK group was donkey whey protein (A). The CD spectra of whey protein and DWPHs hydrolysed by different protease (B). Changes in intrinsic fluorescence spectra of whey protein and DWPHs hydrolysed by different protease (C). The surface hydrophobicity of whey protein DWPHs hydrolysed by different protease (D).

Table 2
The content of the secondary structure of whey protein and DWPHs.

Samples	β-sheet (%)	β-turn (%)	α-helix (%)	Random coils (%)
Whey protein	29.69 ± 2.18^{b}	27.75 ± 2.62^{b}	14.50 ± 1.39^{bc}	28.06 ± 1.76^{b}
Alcalase-DWPH	0.52 ± 0.59^{d}	49.14 ± 7.86^{a}	17.90 ± 7.59^{ab}	32.44 ± 4.32^{b}
Neutrase-DWPH	49.24 ± 9.16^{a}	16.94 ± 5.33^{b}	8.33 ± 2.36^{c}	25.46 ± 1.50^{b}
Papain-DWPH	15.85 ± 3.22^{c}	18.97 ± 8.01^{b}	22.88 ± 2.17^{a}	42.30 ± 6.95^{a}
Flavourzyme-DWPH	31.79 ± 6.32^{b}	16.13 ± 3.23^{b}	24.79 ± 0.75^{a}	27.30 ± 2.69^{b}

Results were expressed as the mean ± standard deviation (n = 3). Different letters in the same column correspond to a significant difference at $P < 0.05$.

proteins, which exposed the Trp residues to water and increased the fluorescence intensity. However, different fluorescence intensities resulted because of the varied effects of the enzymatic action. This is similar to the results obtained for antioxidant peptides produced from whey protein (Ma et al., 2023). The λ_{max} of Alcalase, Neutrase, papain, and Flavourzyme hydrolysates were 358, 346, 353, and 344 nm, respectively. Notably, the fluorescence emission spectra of all DWPHs exhibited a redshift of 9–23 nm compared to that of DM whey protein (335 nm). Consistent with a prior study (Wang et al., 2021), our results indicate a redshift in the λ_{max} of the hydrolysates. Moreover, higher fluorescence intensities at λ_{max} were also observed for these hydrolysates compared to that of DM whey protein, highlighting the positive impact of the four proteases on the hydrolysis of DM whey protein. During enzymatic hydrolysis of proteins, the unfolding spatial structure of the molecule and the exposure of aromatic amino acid residues buried in the interior increase the polarity of the environment surrounding the aromatic amino acids, leading to a redshift in their absorption peak (λmax) (Wang et al., 2021). It is noteworthy that changes in enzyme site of action, the type of hydrolysates produced, and the exposed amino acids can affect the fluorescence peak and λ_{max} values (Ai et al., 2019).

3.8. Surface hydrophobicity

Surface hydrophobicity is closely related to protein conformation, stability, and functional properties and can be used to evaluate changes in protein structure. The fluorescence emission spectra of ANS after binding to DM whey protein and DWPHs are shown in Fig. 3D. Surface hydrophobicity is related to the emulsification characteristics of proteins (Ding et al., 2021). The highest fluorescence emission spectra among the hydrolysates were observed with Flavourzyme, displaying the maximum fluorescence intensity (999.36), followed by Neutrase (968.77), while the lowest fluorescence peak was noted for the Alcalase hydrolysate (368.92). These peaks serve as indicators of hydrophobic strength, offering insights into the spatial structure and functional characteristics of proteins. These results indicated that papain-DWPH and Alcalase-DWPH reduced the surface hydrophobicity of DM whey protein, whereas Flavourzyme-DWPH and Neutrase-DWPH improved surface hydrophobicity. This aligns with the findings regarding emulsification properties and DH in our study. The decrease in surface hydrophobicity probably resulted from protease hydrolysis, which destroyed hydrophobic regions and exposed hydrophilic groups, thereby improving the dispersion of the hydrolysate products in water (Wang et al., 2021). In parallel studies, the impact of six proteases on the gel properties of preserved egg white gel revealed that hydrolysis products of Flavourzyme exhibited a higher likelihood of forming anionic sites with pronounced hydrophobicity compared to other proteases (Ai et al., 2019). In conclusion, Flavourzyme is more suitable for the enzymatic hydrolysis of DM whey proteins.

3.9. FC and FS

Protein foaming occurs at the liquid/vapour interface. When proteins dissolve in the aqueous phase, they envelop bubbles by forming a continuous intermolecular polymer. The stability of foam formed by proteins is closely related to protein intermolecular cohesion and elasticity (Tang, Hettiarachchy, Horax, & Eswaranandam, 2003). The FCs and FSs of DWPHs are shown in Fig. 4A. Flavourzyme-DWPH showed superior FC than those of other samples ($P < 0.05$). The FCs of Neutrase-DWPH and papain-DWPH were similar but much higher than that of Alcalase-DWPH ($P < 0.05$). Generally, FC is affected by the diffusion and denaturation rates of proteins at the interface. However, DM whey protein demonstrated better FS, substantially higher than that of its hydrolysate. This shift may be attributed to the alteration in the

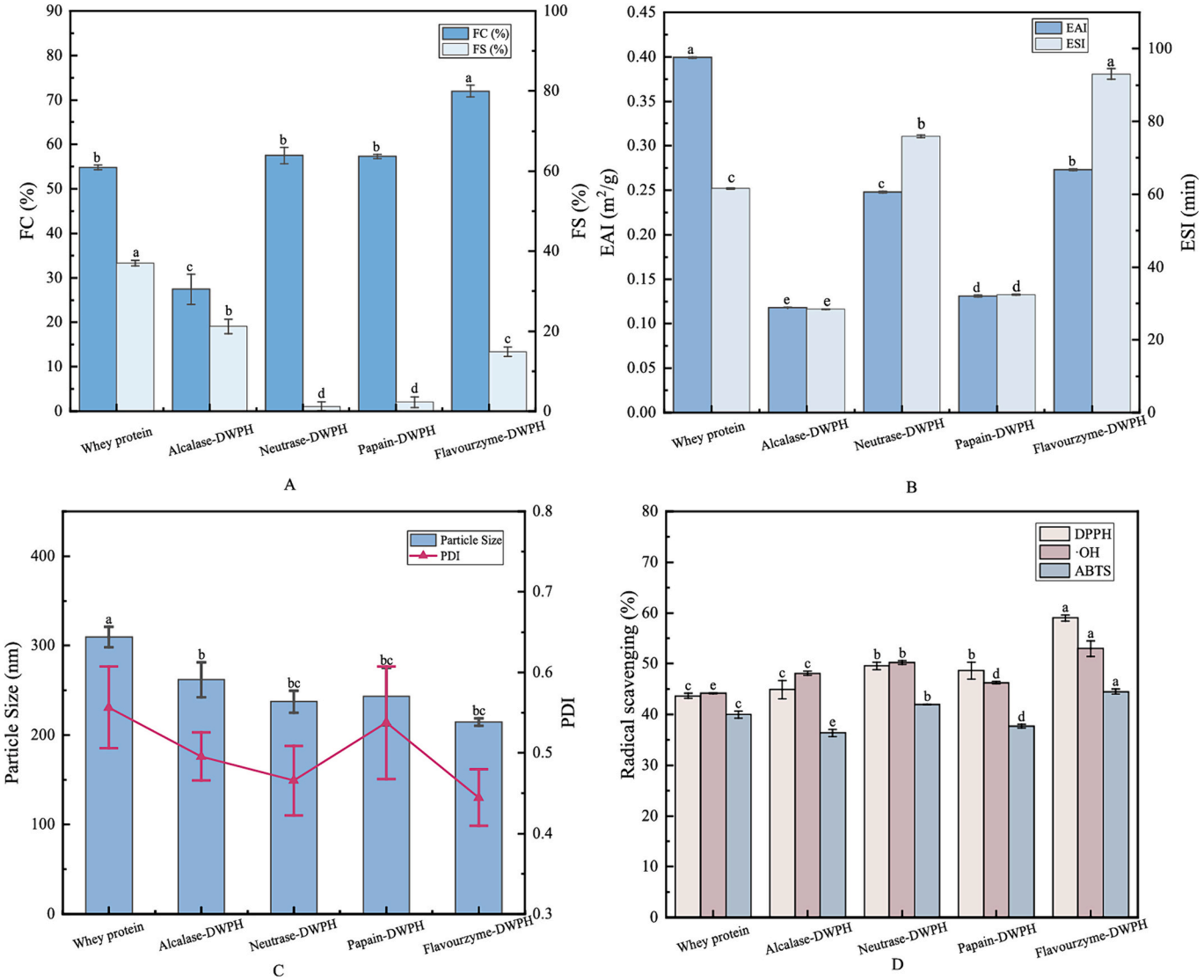

Fig. 4. FC and FS of whey protein DWPHs hydrolysed by different protease (A). EAI and ESI of whey protein DWPHs hydrolysed by different protease (B). The particle size and PDI of whey protein DWPHs hydrolysed by different protease (C). The DPPH, ·OH and ABTS radical scavenging activity of whey protein and its hydrolysates at concentration 2.5 mg/mL (D). For the data of Fig. 4 (A, B, C, D), values were means ± SD (three replicates).

conformation of DM whey protein from a compact to a loose structure, impacting its surface hydrophobicity and foaming ability (Zhang et al., 2019). During enzymatic hydrolysis, higher solubility can promote peptide–peptide interactions, which lead to the rapid migration of hydrolysates to the air–water interface and the formation of a wide intermolecular network, ultimately enhancing FC values (Eckert et al., 2019). The higher solubility of Flavourzyme-DWPH, papain-DWPH, and Neutrase-DWPH presented a higher FC, a result similar to that of a previous pepsin hydrolysis product study (Eckert et al., 2019). The FS was significantly lower for all DWPHs than for DM whey proteins ($P < 0.05$), especially for Neutrase-DWPH and papain-DWPH, where the FS was almost negligible. This may be due to the lack of secondary and tertiary structures forming cohesive layers around the air droplets. It is also possible that the hydrolysates produced from Neutrase and papain are less capable of forming a cohesive layer for stabilising the foam (Tang et al., 2003).

3.10. Emulsifying properties

EAI and ESI are generally used to investigate the emulsifying properties of proteins in food. The EAI and ESI of the DWPHs and DM whey proteins are shown in Fig. 4B. The EAIs of DWPHs were significantly lower than those of the DM whey proteins ($P < 0.05$). This proves that protease hydrolysis can reduce the emulsifying performance of oil–-water systems to a certain extent. This may be because DM whey proteins are more tightly packed than enzyme hydrolysates and are better able to interadsorb oil (Li, Wang, Chen, Sun, & Li, 2019). Similar results have been previously reported (Wang et al., 2021). Neutrase-DWPH and Flavourzyme-DWPH (75.94 ± 0.34 min and 93.05 ± 1.45 min, respectively) exhibited better emulsion stability than that of DM whey protein (61.62 ± 0.20 min) ($P < 0.05$). In contrast, Alcalase-DWPH and papain-DWPH showed similar trends in EAI and ESI ($P > 0.05$), both registering lower values than DM whey protein ($P < 0.05$). In addition, DH was found to influence EAI and ESI (Klompong, Benjakul, Kantachote, & Shahidi, 2007). The DHs of Neutrase- and Flavourzyme-DWPHs were higher than those of the other DWPHs, and the hydrophobic groups within the hydrolysates were more exposed, thereby improving their emulsification properties.

3.11. Particle size and PDI

The average particle sizes (Fig. 4C) of Alcalase, Neutrase, papain, and Flavourzyme hydrolysates were 261.97 ± 19.67 nm, 214.57 ± 4.26 nm, 243.10 ± 32.12 nm, and 237.37 ± 12.25 nm, respectively. The particle size of Neutrase-DWPH and Flavourzyme-DWPH, although not significantly different from each other ($P > 0.05$), was smaller compared to DM whey protein (309.57 ± 11.32 nm). This observation can be attributed to the proteases' ability to convert whey protein into a relatively higher number of short peptides or amino acids, particularly evident with Neutrase and Flavourzyme, which exhibited lower average particle sizes consistent with their higher DH.

PDI served as an indicator for assessing the dispersive performance of macromolecular polymers. Lower PDI values for DWPHs indicate better dispersibility in water. The hydrolysates and amino acids generated after protein hydrolysis tend to aggregate into fragments or particles through chemical bonding, thereby exhibiting varied dispersions. Fig. 4C shows lower PDI values for the DWPHs than for the DM whey proteins. This indicated that the dispersion of the protease hydrolysates was superior to those of DM whey protein (0.56 ± 0.05), especially those of Neutrase-DWPH (0.47 ± 0.04) and Flavourzyme-DWPH (0.44 ± 0.03), consistent with previous reports (Ding et al., 2021).

3.12. Antioxidant activities of DM whey protein and DWPHs

Generally, free radicals are scavenged by binding to hydrogen donors, causing a change in solution colour. Thus, the antioxidant potential of natural compounds is assessed in terms of their scavenging activity against free radicals (Shimada, Fujikawa, Yahara, & Nakamura, 2002). Fig. 4D shows the DPPH, ·OH, and ABTS radical scavenging activity of DM whey protein and its hydrolysates (2.5 mg/mL). The results showed that the DPPH and ·OH radical scavenging rates of all DWPHs were higher than that of DM whey protein ($P < 0.05$), indicating that enzymatic hydrolysis enhances the antioxidant capacity by efficiently terminating the free radical chain reaction through electron donation (Lorenzo et al., 2018). A previous study investigated the antioxidant properties of protein hydrolysates extracted from eggshell membranes by lactic acid bacteria and found similar results (Jain & Anal, 2017). The differences in antioxidant activity between DWPHs may be attributed to the number of hydrolysates exposed to side chains produced by varying DH (Najafian & Babji, 2015). The antioxidant results of Neutase-DWPH and Flavourzyme-DWPH determined using the three assay methods were better, especially for Flavourzyme-DWPH. The highest DPPH, ·OH, and ABTS radical scavenging activities were found in Flavourzyme-DWPH (59.00 ± 0.58%, 52.97 ± 1.55%, and 44.50 ± 0.48%, respectively; $P < 0.05$). Moreover, DWPHs were more effective in scavenging DPPH and ·OH radicals than ABTS.

The antioxidant properties can be determined by the scavenging rate of free radicals, but also by the reducing ability, chelating capacity of metal ions, etc. The present study is not sufficiently comprehensive in terms of only reacting the antioxidant capacity of DM whey protein hydrolysates for the free radical clearing ability. Therefore, further investigation of the comprehensive antioxidant capacity of DWPHs is needed. In addition, Flavourzyme and Neutrase were found to have similar effects on the structure and some processing properties of DM whey proteins, which might be related to the site of action of the proteases, and further studies are needed to investigate the mechanism of their reaction with donkey whey proteins.

4. Conclusions

This study investigated the structural characteristics, processing characteristics, and antioxidant capacities of DM whey protein hydrolysates prepared using different proteases. According to these results, the enzymatic hydrolysis of DM whey protein improves functional properties, promotes the exposure of hydrophilic groups, reduces particle size and molecular weight, enriches amino acid content, and increases the looseness of the structure. Additionally, antioxidant peptides in protein hydrolysates can be used as functional ingredients in food applications. It was found that Flavorzyme and Neutrase acted similarly on DM whey proteins, displaying higher levels of DH, emulsification characteristics, and smaller particle sizes. However, the deeper changes and underlying mechanisms require further exploration. These findings could provide strong support for donkey whey hydrolysates as an antioxidant food additive and suggests a potential application of donkey milk whey hydrolysates as nutritional supplements to improve protein absorption and application.

CRediT authorship contribution statement

Chong Ning: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Funding acquisition. **Yifei Ren:** Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Fengqing Hu:** Supervision, Resources, Project administration, Data curation. **Mingxia Wang:** Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Weixuan Li:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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