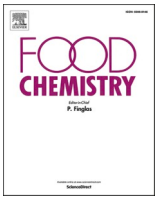
Food Chemistry 493 (2025) 145852

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



The digestibility of rapeseed protein isolate prepared by salt and alkali extraction: The importance of protein composition

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ARTICLE INFO

Keywords:
Rapeseed protein isolate
4D label-free proteomic
Salt extraction
Alkali extraction
Protein quality

ABSTRACT

In this study, four rapeseed protein extracts were prepared from intact/dehulled rapeseed using salt and alkaline extraction. Their nutritional quality was assessed and linked to protein composition through 4D label-free proteomics. Salt-extracted protein isolates had lower glucosinolate (0.47 μmol/g) and phytic acid (0.59 %) content than the alkaline-extracted protein isolates, whereas the alkaline-extracted isolates demonstrated higher digestibility (93 %). The inconsistency between the trend of non-protein anti-nutritional factors and protein digestibility suggests that protein composition may play a more significant role than anti-nutritional factors. Proteomic analysis showed that both extraction methods yielded similar globulin (cruciferin) abundance, but the salt-extracted isolate contained more protein anti-nutritional factors, including albumin (napin) and other protease inhibitors. These findings highlight the importance of protein composition in determining digestibility, showing the need for a protein composition-based approach in evaluating (plant) protein digestibility.

1. Introduction

The food industry faces the challenge of meeting the growing global demand for protein while mitigating environmental impacts. Heavy reliance on animal proteins exacerbates environmental pressures and threatens biodiversity, driving interest in alternatives such as plant, microbial, marine, and insect proteins to ensure global food security. Among these alternatives, plant proteins have gained significant attention as substitutes for animal-based proteins (Fasolin et al., 2019). One promising crop for protein extraction is rapeseed, which is the second most abundant oilseed crop after soybean (*FAOSTAT 2022*). After oil extraction, the resulting by-product, known as a meal or cake, contains about 30–40 % protein on a dry matter basis (Rommi et al., 2014), and is mainly used as animal feed or fertilizer (Rommi et al., 2014). Previously, researchers highlighted the promising techno-functional properties of the rapeseed proteins, including foaming (Yang, Berton-Carabin, Nikiforidis, van der Linden, & Sagis, 2022), emulsifying (Ntone, Bitter, & Nikiforidis, 2020), and gelation (Wang, Zhang, Chen, He, & Ju, 2020). In addition, rapeseed proteins are rich in lysine and sulfur-containing essential amino acids (Shahidi, 2012). Despite these advantages, challenges remain regarding industrial-scale production and protein digestibility (Mupondwa, Li, & Wanasundara, 2018), which this study aims to address.

Rapeseed proteins consist mainly of two groups: globulins and albumins (Wanasundara, 2011). Cruciferin, the major globulin (11/12S), is salt-soluble and belongs to the cupin superfamily (Du et al., 2025; Shen, Yang, Nikiforidis, Mocking-Bode, & Sagis, 2023). Its structure comprises six protomers formed by two trimers, each protomer containing two polypeptide chains. These chains are disulfide-linked, while trimers are stabilized by non-covalent interactions (Jiang, Nie, Sun, & Xiong, 2021). In contrast, napin (1.7/2S albumin) is a water-soluble prolamin superfamily member (Du et al., 2025; Shen et al., 2023). Mature napin subunits consist of a 4-kDa small chain and a 9-kDa large chain, connected by two inter-chain disulfide bonds. Notably, the large chain contains two intra-chain disulfide bonds (Jiang et al., 2021). Currently, salt-based extraction is the most widely industrialized method for obtaining edible rapeseed protein ingredients (Zheng et al., 2021), but this method primarily extracts the salt-soluble globulins from

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https://doi.org/10.1016/j.foodchem.2025.145852
Received 23 April 2025; Received in revised form 12 July 2025; Accepted 4 August 2025
Available online 7 August 2025

the rapeseed meal. After the solid-liquid separation, the resulting globulin extract is membrane-filtrated to remove non-proteinaceous solutes (*e.g.*, salts, sugars, and phenols), while concentrating the protein solution. Rapeseed protein products that are industrialized based on this method include Supertein™, Puratein®, Isolexx™, Vitalexx™, and CanolaPro™, which are produced by the companies of Burcon, BioExx, and Royal DSM (Chmielewska et al., 2021; Mupondwa et al., 2018).

However, a drawback of this salt-based extraction method is that high salt concentrations accelerate metal equipment corrosion, which largely limits its large-scale implementation. To overcome this limitation, researchers are investigating the alkaline extraction process as an alternative. The traditional alkaline extraction-acid precipitation method (AEP) uses alkaline conditions to enhance the solubility of the albumin and globulin proteins. An acid precipitation step then precipitates the globulins near its isoelectric point, but this approach induces protein aggregation, thus reducing protein functionality (Lie-Piang, Yang, Schutyser, Nikiforidis, & Boom, 2023). Another limitation of AEP is the use of high alkaline pH, often above 9.0 (Vahedifar & Wu, 2022). Although the extreme alkaline conditions slightly increase the protein solubility, for instance, by 15 % when increasing the pH from 9.0 to 13.0 (Zhang et al., 2020), they will induce protein-phenol interactions, which cause off-flavors, color changes, and protein aggregation (thus reducing functionality) (Naczk, Amarowicz, Sullivan, & Shahidi, 1998). These drawbacks can be mitigated by mild protein extraction (Lie-Piang et al., 2023), where less alkaline conditions are used and the acid precipitation is replaced by membrane filtration (Ntone et al., 2020; Yang et al., 2021). A mild rapeseed extraction method, which is comparable to the salt-extracted method, is included in this study.

Rapeseed protein digestibility is a key concern due to the presence of anti-nutritional factors, such as glucosinolate, phytic acid, and phenols (Karabulut et al., 2025). These compounds not only affect human health and nutrient absorption but also influence the flavor and color of the final products (Vahedifar & Wu, 2022). For example, Zheng et al. (2021) effectively reduced the content of glucosinolates, phytic acid, and phenols in rapeseed protein using the ethanol-phytase method, thereby improving the protein extract's whiteness and bioavailability. Jiang et al. (2021) successfully lowered phenol content and improved digestibility by combining ethanol washing and pH-shift treatments. In addition, the role of protein composition on digestion cannot be neglected (Du et al., 2025). In oil seeds, albumin exhibits high resistance to gastrointestinal digestion, as seen in rapeseed and sunflower seed proteins (Beaubier, Albe-Slabi, Beau, Galet, & Kapel, 2025). Several proteins, such as protease inhibitors, may further reduce digestibility. These inhibitors interfere with mammalian digestive enzymes (*e.g.* trypsin), thereby reducing their activities and affecting protein digestion in the gastrointestinal tract (Kadam, Kumar, & Kasara, 2021; Miranda et al., 2019). Also, the high heat resistance of the inhibitors is concerning, as processing will not deactivate them, as shown by Vioque et al. (2001), where protease inhibitors retained 50 % of their activity after heating at 70 °C for 45 min. Therefore, understanding the abundance and composition of both proteinaceous and non-proteinaceous anti-nutritional factors in rapeseed protein isolates is critical. This knowledge can help improve the digestibility of rapeseed protein products and guide the production process of rapeseed protein and targeted breeding.

In this study, the rapeseed protein isolates from rapeseed meal were extracted by salt and alkaline extraction methods. Here, dehulled and non-dehulled rapeseed meal is used, which allows us to study the role of rapeseed hulls in introducing anti-nutritional factors, as the hull is rich in those. Afterwards, we compared the *in-vitro* protein digestibility (IVPD) of the obtained rapeseed protein isolates. Additional analyses included color, anti-nutritional factors, amino acid profiles, and process performance indicators, such as protein content, protein recovery rate, and protein composition. Finally, proteomics was used to characterize the relative abundance and composition of specific rapeseed proteins.

2. Materials and methods

2.1. Materials and reagents

Rapeseed (variety: zhongyouza 39) was provided by Oil Crops Research Institute, Chinese Academy of Agricultural Sciences (WuHan, China). Myrosinase was bought from Sigma-Aldrich Company (St. Louis, MO, USA). Glucosinolate standard was purchased from BioSun Company (ShangHai, China). Cellulose dialysis bags and BCA protein assay kit were obtained from Beyotime Institute of Biotechnology (ShangHai, China). Other chemicals and reagents were of analytical grade or higher level.

2.2. Extraction of rapeseed protein isolates

The extraction process of rapeseed protein isolate is shown in **Fig. S1**. The rapeseed was dehulled by a rapeseed dehulling machine (Independently developed by Oil Crops Research Institute, WuHan, China). Dehulled or intact defatted rapeseed meal was obtained by a low-temperature pressing system (Independent assembly by Oil Crops Research Institute, WuHan, China), followed by defatting by subcritical extraction using solvent 4# (mixed alkane solvent mainly composed of butane (>90 %)) at 45 °C. The fat content of defatted rapeseed meals is less than 0.5 %. After crushing using a grinder (800 A, REDSUN, JinHua, China) and refining by a 60-mesh sieve, the dehulled or intact defatted rapeseed meal (batch size: 100 g) was dissolved in an aqueous solution at room temperature at a ratio of 100 g/L (w: v) and stirred for 2 h at a set pH. During the stirring process, the pH of the solution would decrease as the protein dissolves. Thus, the pH was adjusted every 20 min to maintain the initially set pH using 1 M NaOH solution. Next, the dispersion was centrifuged at 8000*g* for 30 min at 4 °C, and then the supernatant was poured into 3.5 kDa cellulose dialysis bags for 48 h. Ultrapure water was used throughout the dialysis process, with water changes every 2 h in the first 12 h and an interval of 8 h thereafter, with a total of 10 times water changes. The retained solution (pH 5.9 ± 0.1) was stirred and freeze-dried to obtain a protein sample.

The differences between the different methods were the aqueous solution and the extraction pH. The aqueous solution for the salt extraction was a 200 mM $CaCl_2$ solution with a pH of 5.8. The aqueous solution for alkaline extraction was ultrapure water with a pH of 8.5. The salt concentrations and pH values in salt and aqueous solutions were determined based on the optimized results of preliminary experiments, with comprehensive consideration of protein recovery, color, and non-protein antinutritional factor content. The freeze-dried protein samples were extracted from intact rapeseed using salt and alkaline extraction, which were named salt extraction intact (SEI) and alkaline extraction intact (AEI), and from dehulled rapeseed, which were designated as salt extraction dehulled (SED) and alkaline extraction dehulled (AED), respectively. The defatted meal powder produced by the intact and dehulled was labeled as intact meal (IM) and dehulled meal (DM), respectively. Salt extraction was adapted from a patent (*WO2018007492A1*) and authorized by the Royal DSM company.

2.3. Color determination

The L^*, a^*, and b^* values of the sample (powder) were measured by a colorimeter (CR-400, Minolta, Tokyo, Japan), and calibrated against a white plate. The color information was quantified by values of L^* (brightness), a^* (greenness (−) → redness (+)), and b^* (blueness (−) → yellowness (+)). The whiteness value (WI) calculation formula (1) was as follows (Ge et al., 2023):

$$WI = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2} \quad (1)$$

2.4. Chemical composition measurements

The protein content was determined by the Kjeldahl method using DigiPREP TKN Systems (Hanon K9860, Jinan, China). The nitrogen conversion factor was 6.25.

The extraction rate was calculated by the following formula (2):

$$Dry\ matter\ recovery\ (\%) = \frac{m_p}{m_d} \times 100 \quad (2)$$

The protein recovery rate was calculated by the following formula (3):

$$Protein\ recovery(\%) = \frac{c_p \times m_p}{c_d \times m_d} \quad (3)$$

where m_p, m_d, c_p, and c_d represent the mass of protein extract, mass of defatted rapeseed meal, protein content of protein extract, and protein content of defatted rapeseed meal, respectively.

2.5. Non-protein anti-nutritional factors measurements

The total content of glucosinolate was determined according to the *NY/T 1582-2007* method based on high-performance liquid chromatography (LC-6 CE, Shimadzu, Tokyo, Japan). A rapid method described by *Variant a* of Haug and Lantzsch (Haug & Lantzsch, 1983) was used to determine phytic acid content according to the reaction between phytic acid and acidic ferric iron solution. The absorbance of the test sample solution was determined at 519 nm against ultrapure water. Calculation was done using standard phytic acid solutions at varying concentrations. According to the method described by Li, Nie, and Li (2008), the phenolic content was determined based on the Folin phenol colorimetric method.

2.6. Amino acid composition measurements

About 200 mg freeze-dried protein sample was put into 10 mL 6 M HCl and sealed after nitrogen flushing for 30 s, and hydrolyzed at 110 °C for 48 h. The hydrolyzed solution was filtered with a 0.45 μm filter membrane (Luer Syringe Filter) and diluted to 50 mL. Then, 2 mL diluted solution was transferred into a rotary evaporator for deacidification at 45 °C until a little solid or stain was left at the bottom of the bottle. Finally, 2 mL sodium citrate buffer solution (pH 2.2) was added to fully dissolve it, which was filtered through a 0.45 μm filter. The amino acid composition was determined by an amino acid analyzer (Biochrom30+, Cambridge, UK).

2.7. In-vitro protein digestion measurements

The determination of the *in-vitro* protein digestion (IVPD) was slightly modified based on the method reported by the previous report (Brodkorb et al., 2019). Individual rapeseed protein isolate samples were weighed and dissolved in ultrapure water at 10 mg/mL. The rapeseed protein isolate solution was mixed with an equal amount of simulated oral digestive fluid (15.1 g/L KCl, 3.7 g/L H_2PO_4, 13.6 g/L $NaHCO_3$, 1.1 g/L NaCl, 0.15 g/L $MgCl_2(H_2O)_6$, 0.5 g/L $(NH_4)_2CO_3$, 0.3 g/L $CaCl_2$, 0.6 g/L mucin, pH 7.0) and the pH was adjusted to 7.0 using 0.5 M HCl. Samples were then placed in a constant temperature shaker (THZ-C, China) and vibrated at 150 rpm for 5 min at 37 °C. The mixture was added with gastric simulation fluid (6.9 g/L KCl, 0.9 g/L H_2PO_4, 25 g/L $NaHCO_3$, 47.2 g/L NaCl, 0.12 g/L $MgCl_2(H_2O)_6$, 0.5 g/L $(NH_4)_2CO_3$, 0.15 g/L $CaCl_2$, 0.97 mg/mL pepsin, pH 3.0), and then the pH was adjusted to 3.0 using 0.5 M HCl. The mixed sample was then placed in a constant temperature shaker and continuously vibrated at 150 rpm for 2 h at 37 °C. Part of the mixture was removed and centrifuged at 4000*g* for 30 min to calculate the IVPD in the stomach. After gastric digestion, the mixed solution pH was adjusted to 7.0 using 0.5 M NaOH. Appropriate amounts of small intestine simulation fluid (6.8 g/L KCl, 0.8 g/L H_2PO_4, 85 g/L $NaHCO_3$, 38.4 g/L NaCl, 0.33 g/L $MgCl_2(H_2O)_6$, 0.5 g/L $(NH_4)_2CO_3$, 0.6 g/L $CaCl_2$, 14.39 mg/mL trypsin, pH 7.0) were added and then transferred to a constant temperature shaker and shook at 150 rpm for 2 h at 37 °C. The volume ratio of rapeseed protein isolate solution, oral simulation solution, gastric simulation solution, and small intestine simulation solution added during the simulated digestion process was 1:1:2:2. After cooling to room temperature, the final mixture was centrifuged at 4000*g* for 30 min. Soluble proteins in the supernatant were considered digestible components. The nitrogen content in the supernatant was measured using the Kjeldahl method. The IVPD was calculated using the equation (Pan et al., 2020) (4):

$$IVPD(\%) = \frac{(N_T - N_e) - N_t}{N} \times 100 \quad (4)$$

where N_t and N_T are the nitrogen contents before and after simulated *in vitro* digestion, respectively, N_e is the blank contrast, and N is the total protein nitrogen content.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGA experiment was tested using a reagent kit. Briefly, 2 g/L rapeseed protein solution was mixed with a sample lysis buffer containing β-mercaptoethanol in a 4:1 volume ratio and boiled in water for 5 min. Then, 10 μL mixture was added to the gel wells (including 12 % separating gel and 5 % stacking gel) and run at 60 V for 30 min, followed by an adjustment to 110 V for another 60 min. Staining was performed with 0.02 % Coomassie brilliant blue R-250.

2.9. Quantitative proteomic analysis

The 4D-label-free-based quantitative proteomic analysis of the rapeseed protein isolate sample was carried out by Jingjie PTM Biolabs Inc. (Hangzhou, China). Rapeseed protein isolate powders were mixed with approximately four times the volume of lysis buffer (8 M urea, 1 % protease inhibitor), and then the dispersion was treated by ultrasound. The mixture was centrifuged at 4 °C and 12,000*g* for 10 min, and the protein content in the supernatant was determined using the BCA reagent kit. The final concentration of 20 % trichloroacetic acid was slowly added, vortex mixed, and precipitated at 4 °C for 2 h. The sample was centrifuged at 4500 *g* for 5 min; the supernatant was discarded, and the precipitate was washed three times with pre-cooled acetone. After drying the precipitate, tetraethylammonium bromide with a final concentration of 200 mM was added, and the precipitate was dispersed by ultrasound. Trypsin was added at a 1:50 (protease: protein, w: w) ratio and hydrolyzed overnight. The system was added with dithiothreitol to a final concentration of 5 mM, which was then reduced at 56 °C for 30 min, followed by the addition of iodoacetamide to a final concentration of 11 mM, and incubation at room temperature away from light for 15 min.

The peptide segment was dissolved in liquid chromatography mobile phase A (peptide concentration: 0.125 g/L) and separated using ReproSil-PurC18 (Ammerbuch, Germany) by the NanoElute ultra-high-performance liquid chromatography system. Mobile phase A was an aqueous solution containing 0.1 % formic acid and 2 % acetonitrile. Mobile phase B was an acetonitrile aqueous solution containing 0.1 % formic acid. Liquid phase gradient setting (loading volume: 2 μL): 0–8 min, 9–24 %; B, 8.0–12.0 min, 24–35 %; B, 12.0–16.0 min, 35–80 %; B, 16.0–20.0 min, 80 % B, with a flow rate maintained at 450 nL/min. The peptide segments were separated by an ultra-high performance liquid chromatography system and injected into a capillary ion source for ionization, followed by data collection by timsTOF Pro 2 (Bruker) mass spectrometry. The ion source voltage was set to 2.0 kV, and the peptide parent ions and their secondary fragments were detected and analyzed using time of flight (TOF). The data collection mode used the data-

independent parallel cumulative serial fragmentation (dia PASEF) mode, with the primary mass spectrometry scanning range set to 100–1700 *m/z*. After collecting a primary mass spectrometry, 10 times of PASEF mode collection were performed. The secondary mass spectrometry scanning was conducted in the range of 100–1700 m/z, with windows of every 25 m/z. The output data were filtered at 1 % false discovery rate.

Database matches for each of the four samples identified by peptide search of the DIA-NN (v 1.8). Blast_Brassica_napus_3708_PR_20221125. FASTA databases were downloaded. In the present study, ontology-based pathway analysis was conducted using the Gene Ontology (GO), protein domains. Subcellular localization prediction, functional enrichment, and cluster analysis were all performed using GO terms. The different descriptions, mass spectrometry signal intensity, and relative quantification values (R) of each protein are shown in Table S1 in the supplementary information (SI). R was the result of the intensity value changing through centralization.

2.10. Statistical analysis

All experiments were performed in triplicate. Differences between mean values were evaluated using the *t*-test by the SPSS software or GraphPad Prism 9.0 software and were considered significant at $p < 0.05$.

3. Results and discussions

3.1. Chemical composition

The protein content of the dehulled meals (DM) (42.50 ± 0.3 %) was slightly higher than that of intact meals (IM) (44.86 ± 0.3 %), which can be attributed to the fact that the protein content in rapeseed hulls is lower than that in kernels after defatting. This is consistent with the result reported by Ghodsvali, Khodaparast, Vosoughi, and Diosady (2005).

Table 1 and Table S2 summarize the content of dry matter content of the main components, dry matter recovery, and protein recovery rate of the proteins extracted by salt extraction and alkaline extraction before and after dialysis. The letter I or D in the abbreviation stands for intact or dehulled, respectively, which is related to the starting material of the extraction. We observed an increase in protein content from 35.6 to 44.8 to over 80 %, suggesting a significant reduction in non-proteinaceous components. As reported by Rommi et al. (2014), monosaccharides and sucrose account for 86.3 % of the water-soluble carbohydrates in rapeseed meal, while the ratio is 89.3 % in dehulled rapeseed meal. Therefore, most of the components removed by dialysis were small-molecular-weight sugars, as well as salts, small-molecular-weight proteins/peptides, and anti-nutritional factors. The removal of small-molecular-weight proteins/peptides or nitrogen-containing non-proteinaceous components most likely explains the slight reduction in protein recovery after dialysis, which ranged from 2.2 % to 5.4 % for all protein extracts.

The protein content of all dialyzed rapeseed protein isolates exceeded 90 %, with values ranging from 90.5 % to 92.0 %, suggesting comparable protein content regardless of the extraction method. However, protein recovery differed significantly, with salt extraction yielding about 20 % higher protein recovery than alkaline extraction. This difference may be due to the more effective solubilization of salt-soluble globulins, whereas the pH of 8.5 used in the alkaline method (without additional salt) was insufficient for optimal solubilization. A higher pH could improve protein recovery in the alkaline method, but this approach carries the risk of increased polyphenol oxidation, potentially leading to insoluble rapeseed-polyphenol complexes and darkening the final protein color (Vahedifar & Wu, 2022). As shown in Table S3, dehulling had a minor effect on the color of the final rapeseed protein extracts, while there was only a slight difference in whiteness index (WI) between salt- and alkaline-extracted protein. This indicates that extracting protein at pH 8.5 can achieve an acceptable protein color. In addition, the salt-extracted protein had more green and blue hues, while the alkaline-extracted protein had more red and yellow hues.

As shown in Table 1, the proportion of essential amino acids in all rapeseed protein isolates exceeded 40 %, consistent with previous reports (Kapel et al., 2025). Dehulling had little effect on their amino acid composition, regardless of whether salt or alkaline extraction was used. For different extraction methods, most amino acid compositions exhibited little difference. Notably, the salt-extracted protein isolates had higher contents of histidine, lysine, and proline, but lower contents of threonine and aspartic acid, with all relative differences below 25 % (Table S4).

Currently, salt extraction in combination with ultrafiltration/filtration is the most common extraction method for rapeseed protein, typically using NaCl solution for solubilization. Jia, Rodriguez-Alonso, Bianeis, Keppler, and van der Goot (2021) solubilized dehulled rapeseed meal in a 200 mM NaCl solution and obtained rapeseed protein isolate with 94 % protein content through two-step dialysis and ultrafiltration (using a semipermeable membrane with a molecular weight cutoff ≤3.5 kDa). However, producing such a high-purity isolate requires extensive processing, resulting in extensive protein loss, thus a low protein recovery of 36 %. Ghodsvali et al. (2005) obtained highly pure rapeseed protein extracts by dissolving protein from three rapeseed varieties at pH 12, followed by isoelectric point precipitation after ultrafiltration, achieving protein contents of 81.3–86.2 % with recoveries of 12.3–15.4 %. In comparison, the alkaline extraction-based protein extracts in this work not only achieved higher protein content but also had a protein recovery of about 2.5 times higher, which could be the result of replacing the acid precipitation step with dialysis. A reason could be the retention of globulin and albumin proteins using membrane filtration or dialysis, while acid precipitation removes the albumins, as shown for several plant protein sources (Giménez-Ribes, Yang, He, Habibi, & Sagis, 2023; Sagis & Yang, 2022).

3.2. Non-protein anti-nutritional factors

The presence of anti-nutritional factors in rapeseed protein has been a long-term concern of researchers, as they significantly impact its edibility (Chmielewska et al., 2021). In 2014, the European Commission set standards for rapeseed protein as a novel food ingredient, setting the

Table 1
Protein content, dry matter recovery, protein recovery, essential amino acids ratio, glucosinolate content, phytic acid content, and total phenol content of rapeseed protein isolates (SEI: salt-extracted intact; SED: salt-extracted dehulled; AEI: alkaline extraction intact; AED: alkaline extract dehulled) after dialysis.

Sample	Protein content / %	Dry matter recovery / %	Protein recovery / %	Essential amino acids ratio / %	Glucosinolates /μmol·g^{-1}	Phytic acid content /%	Total phenol content /%
SEI	91.31 ± 1.14ab	25.75 ± 0.25b	54.24 ± 0.24b	42.96 ± 0.07ab	0.47 ± 0.11d	0.75 ± 0.11d	0.54 ± 0.01b
SED	92.01 ± 0.45a	27.73 ± 0.20a	57.25 ± 0.25a	42.54 ± 0.03b	1.23 ± 0.01b	0.59 ± 0.01b	0.30 ± 0.02a
AEI	90.50 ± 0.01b	17.27 ± 0.27d	36.20 ± 0.30d	43.77 ± 0.41a	1.15 ± 0.02c	1.00 ± 0.02d	0.54 ± 0.01d
AED	91.96 ± 0.14ab	19.85 ± 0.16c	39.87 ± 0.13c	42.53 ± 0.24b	1.67 ± 0.25a	0.78 ± 0.25a	0.32 ± 0.02c

Note: The data were expressed as mean ± SD. The values with different superscript letters between different samples with the same treatment are significantly different. "-" represents data unavailable or not measured.

limits for the content of glucosinolates and phytic acid as <1 μmol/g and < 1.5 % (*COMMISSION IMPLEMENTING DECISION of 1 July 2014*). As shown in Table 1, the glucosinolate content of SEI and the phytic acid content of all protein samples mentioned above meet this standard.

Table 1 and Table S2 display that dehulling removed about 8.6 % of the phytic acid from rapeseed meal, with little effect on total protein content. The glucosinolate content in DM was significantly higher than that in IM (15.2 μmol/g *vs.* 9.2 μmol/g), indicating that rapeseed kernels contain more glucosinolates, which is consistent with the results of Mińkowski (Mińkowski, 2002). After solubilization of rapeseed meals with salt solution or alkaline solution, the glucosinolates and phytic acid contents in the four samples significantly decreased to 2.1–4.0 umol/g and 0.7–2.3 %, respectively, as the various components were removed in the pellet upon centrifugation or during the dialysis step. Compared to alkaline extraction, salt extraction was more effective in removing glucosinolates (by 5.0–11.1 %) and phytic acid (by 18.6–29.8 %), possibly due to 1) The tendency of glucosinolates and proteins to undergo hydrophobic interaction in an alkaline environment, enriching glucosinolates in the aqueous extract (Akbari & Wu, 2015); 2) The precipitation of phytic acid as calcium phytate in weakly acidic conditions, allowing its removal by centrifugation (Akbari & Wu, 2015; Grynspan & Cheryan, 1983; Zhang et al., 2020). The content of non-protein anti-nutritional factors in rapeseed protein extract before dialysis is shown in Table S2.

For all samples, more than 87 % of glucosinolates and 74 % of phytic acid were removed (compared to the meal) during solubilization and dialysis, following a consistent trend before and after dialysis. Regarding phenol content, all extracts showed high phenol content before dialysis, and dialysis removed over 40 % of phenols, suggesting that salt and alkaline extractions extracted phenols similarly. The higher total phenolic content in SEI and AEI compared to SED and AED after dialysis may be due to a greater proportion of polyphenols in the rapeseed hull. Overall, salt extraction was more effective in removing glucosinolates and phytic acid than alkaline extraction, while dehulling and dialysis effectively removed polyphenols.

3.3. *In-vitro* protein digestion

The protein digestibility of the rapeseed protein isolates was analyzed by simulating the digestion process and expressed as the *in-vitro* protein digestion (IVPD). Fig. 1A shows that the IVPD values of SEI, SED, AEI, and AED after passing through the oral and gastric regions are 74.2 %, 78.3 %, 75.3 %, and 76.4 %, respectively. This demonstrates that the extraction method had a minimal effect on gastric digestion rates. Fig. 1B represents the IVPD of four rapeseed protein isolates after oral, gastric, and small intestinal simulated digestion. The SEI exhibited the lowest IVPD at 80.3 %, which increased to 83.5 % for SED when dehulled seeds were used, slightly lower than the digestibility of CanolaPro™ (87 %) (*GRAS 2016*). The AEI and AED showed the highest IVPD, at 88.1 % and 92.5 %, respectively. The high IVPD of the extracted rapeseed protein isolates can be attributed not only to their low non-protein anti-nutritional factor content but also to the relatively lower degree of aggregation in dialysis compared to isoelectric precipitation, which provides more accessible sites for digestive enzymes (Zhang et al., 2024).

Salt- and alkali-extracted rapeseed protein isolates showed similar proportions in simulated gastric digestion, likely due to a balance between two opposing factors. Napin, with its compact structure, is more resistant to pepsin hydrolysis than cruciferin (Napieraj, Lutton, Perez, Boué, & Brûlet, 2024). As demonstrated later, salt-extracted proteins contain a higher abundance of napin, which would tend to reduce their IVPD. In contrast, phytate lowers digestibility by binding to basic amino acid residues (*e.g.*, histidine) in the active site of pepsin, hindering substrate access to the catalytic pocket (Han et al., 2025). Here, salt-extracted proteins had lower phytate levels, a factor that would otherwise enhance IVPD (Table 1). These counteracting effects likely resulted in the comparable gastric digestion proportions observed. The salt-extracted SEI and SED showed an IVPD increase of 6.1 % and 5.2 %, respectively, after the small intestinal phase, while the alkaline-extracted AEI and AED increased by 12.8 % and 16.1 %, respectively. This suggests that the key IVPD differences between alkaline- and salt-extracted proteins occur during the intestinal digestion stage.

Previous studies on rapeseed protein digestibility identified amino acid composition and non-protein anti-nutritional factors as crucial digestion-determining factors (Chmielewska et al., 2021). Table S4 shows that the four rapeseed protein isolates had similar amino acid compositions. Thus, the IVPD differences may be less affected by amino acid variations.

Non-protein anti-nutritional factors, especially phytic acid, phenolics, glucosinolates, and their breakdown products, may affect protein digestibility through two mechanisms (Chmielewska et al., 2021; Vahedifar & Wu, 2022). 1) Binding to digestive enzymes, thereby reducing enzymatic activity; 2) Interacting with proteins, hindering proteolytic hydrolysis. Additionally, fiber is a potential factor affecting protein digestibility, with two primary mechanisms (Bournazel et al., 2018; Joehnke, Sørensen, Bjergegaard, Markedal, & Sørensen, 2018): 1) Insoluble fibers (*e.g.*, cellulose, lignin) form dense networks that encapsulate rapeseed protein particles, preventing their contact with digestive enzymes; 2) Soluble fibers can increase the viscosity of protein solutions, thereby affecting intestinal peristalsis. However, in the context of this study, two considerations apply. Firstly, Rommi et al. (2014) reported that soluble fiber contents in intact and dehulled rapeseed meal are comparable, accounting for less than 10 % of total fiber, while most insoluble fibers are removed during centrifugation. Secondly, the effects of soluble fibers on digestibility are more likely to

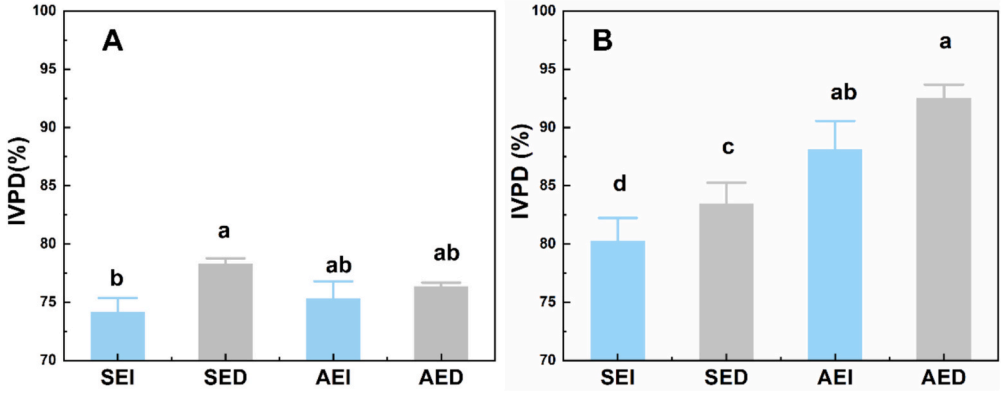

Fig. 1. The *in-vitro* protein digestion (IVPD) of rapeseed protein isolates after simulating the gastric (A) and small intestine (B) (SEI: salt-extracted intact; SED: salt-extracted dehulled; AEI: alkaline extraction intact; AED: alkaline extract dehulled) after dialysis. The data were expressed as mean ± SD. The values with different superscript letters are significantly different.

exhibit differences in *in-vivo* experiments. Thus, fiber content is unlikely to be the primary factor contributing to differences in IVPD.

Fig. 1 shows that rapeseed protein extracted from dehulled meal (DM) exhibited significantly higher IVPD than protein extracted from intact meal (IM), regardless of the extraction method. This suggests that higher phytic acid levels in rapeseed protein isolates extracted from IM, as well as phenol-protein complexes, may play a role in inhibiting the digestive enzyme activity (Z. Zhang et al., 2020). Surprisingly, salt-extracted protein had significantly lower contents of glucosinolates and phytic acid than alkaline-extracted protein, yet their IVPD values were lower. This raises questions about the potential influence of extracted proteins and protein composition, which will be discussed in the following sections.

3.4. SDS-page

As can be seen in Fig. 2, the SDS-PAGE profile of all four rapeseed protein isolates showed five bands at 10, 18–25, and 26–37 kDa below 37 kDa. The results were in agreement with the findings of Akbari and Wu (2015) that the globulin cruciferin dissociates into α-polypeptides (26.7–37.1 kDa) and β-polypeptides (18.3–22.9 kDa) chains in the presence of 2-mercaptoethanol. In addition, the albumin napin dissociated into two bands of 5 and 10 kDa. Fig. 2 also showed that the intensity of the four protein samples in 18–25 and 26–37 kDa was rather similar, and the band at 10 kDa of protein was significantly thinner than that of the salt-extracted protein. This suggests a lower napin concentration for the alkaline-extracted protein isolates. The next step is the in-depth analysis of the protein present in the protein isolates using proteomics.

3.5. Proteomics

3.5.1. Differences and similarities in rapeseed protein isolates

In proteomics experiments, 15,089 peptides and 3104 proteins were identified for the four protein isolates. Among them, 3099 proteins were defined as comparable proteins (the number of proteins quantified through specific peptides), which accounted for 99.84 % of identified proteins. Principal component analysis (PCA) was performed on the protein data matrix to find variable relationships (Fig. 3A). PC_1 explained the highest variance (52.3 %), whereas PC_2 accounted for only 17.6 %, suggesting that dehulling had a smaller impact on protein composition compared to the extraction method. Fig. 3B shows that the comparison between SEI and AEI gave the highest number of differential proteins, while the AEI and AED comparison group showed a greater number of differential proteins than the SEI and SED comparison group.

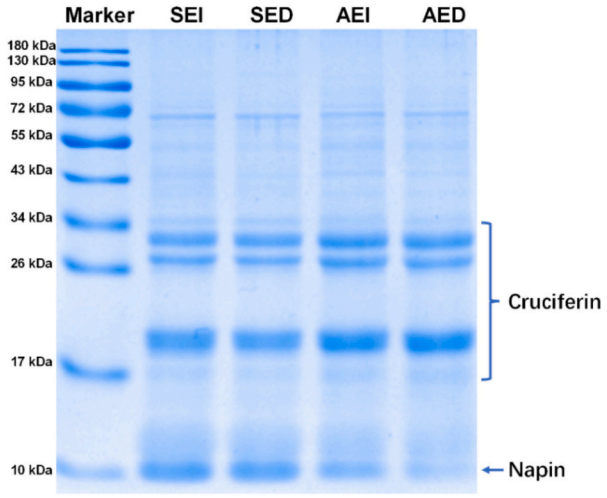

Fig. 2. SDS-PAGE profiles of rapeseed protein isolates (SEI: salt-extracted intact; SED: salt-extracted dehulled; AEI: alkaline extraction intact; AED: alkaline extract dehulled) under rescuing conditions.

A Venn diagram was created using the top 50 proteins from each sample based on GO terms of molecular function from UniProt (Fig. 3C) to reveal overlapping proteins in each extraction. It should be noted that some proteins had multiple annotations and might appear several times in the same analysis. The overlapping regions among the four samples contained 36 proteins, 12 of which were labeled as nutrient reservoir activity, corresponding to storage proteins that typically accounted for over 90 % of rapeseed protein (Vahedifar & Wu, 2022).

For the difference between salt and alkaline extraction, AEI and AED shared proteins mainly comprising of enzymes with catalytic activity, while SEI and SED shared two identified endopeptidases, two carbohydrate-binding glycoproteins, and one sodium channel inhibitor. The sodium channel inhibitor contained a Knottin scorpion toxin-like domain, which might confer antibacterial properties during storage (Chalhoub et al., 2014).

Notably, no differential proteins were identified in the regions shared by SEI and AEI or SED and AED, confirming again that dehulling had a minimal impact on the biggest group of extract proteins. Additionally, three thioglucosidases were identified within the top 50 proteins, which may catalyze the glucosinolate hydrolysis into isothiocyanate and other toxic substances, potentially reducing the protein quality (Z. Zhang et al., 2020).

3.5.2. Storage protein analysis

In rapeseed, 12S globulin (cruciferin) and 2S albumin (napin) are predominant storage proteins, with reported cruciferin-to-napin ratios ranging from 0.6 to 2.0 (Chmielewska et al., 2021). Due to the absolute dominance of storage proteins in rapeseed protein composition, variations in storage proteins can significantly impact protein recovery rates. Through a comprehensive comparison of multiple databases, 12 cruciferins (11 of which were comparable) and 9 napins were annotated (see Table S1 in the SI). The subcellular localization of these proteins was mainly in the endoplasic reticulum, vacuolar membrane, and chloroplast, and minor distribution in the cycloplasm and extracellular matrices (see Table S1 in the SI). Fig. 3A–D shows the distribution of napin and cruciferin intensity in the four samples, revealing no significant effect of dehulling on albumin and globulin distribution. However, the albumin proportion in salt-extracted protein isolates was about 10 % higher than that in alkaline-extracted ones. The observed differences may stem from interactions between cruciferin and napin. Mudau, Moutkane, Chassenieux, Lund, and Nicolai (2024) reported that under conditions of pH > 7 and high napin content, electrostatic interactions drive the formation of aggregates that are removable by centrifugation, resulting in a reduced proportion of napin in alkali-extracted rapeseed protein isolates.

Fig. 4E–H presents variations in storage protein abundance. Fig. 4E and H show 14 and 15 proteins with log2FC < 0, respectively, all belonging to the top 50 proteins (see Fig. 4G), supporting the observation that dehulling enhances protein dissolution, which is consistent with Table 1.

Fig. 4F indicates minimal differences in cruciferin abundance within the top 50 proteins (A0A078GOQ2, A0A078GA98, A0A078HBQ3, A0A078FMU9, A0A078FAN5, and A0A078FRG0) identified in SEI and AEI. Except for AOAO78FRG0, all other globulins in the top 50 had absolute values of log2FC of <0.1. In addition, identified napin abundance within the top 50 proteins (A0A078GUQ3, A0A078IBF3, A0A078GW3, Q42469, A0A078H4D, and A0A0A78IPA5) showed significant differences, with log2FC values ranging from 0.54 to 0.97. The difference in storage proteins between SED and AED also showed a similar trend (see Fig. 4G). This phenomenon confirmed that salt- and alkaline-extracted protein isolates had similar types and abundance of cruciferins, and their difference in storage proteins was mainly attributed to napins.

Joehnke et al. (2018) demonstrated that napin exhibits strong trypsin inhibitory activity, which is closely associated with its structural

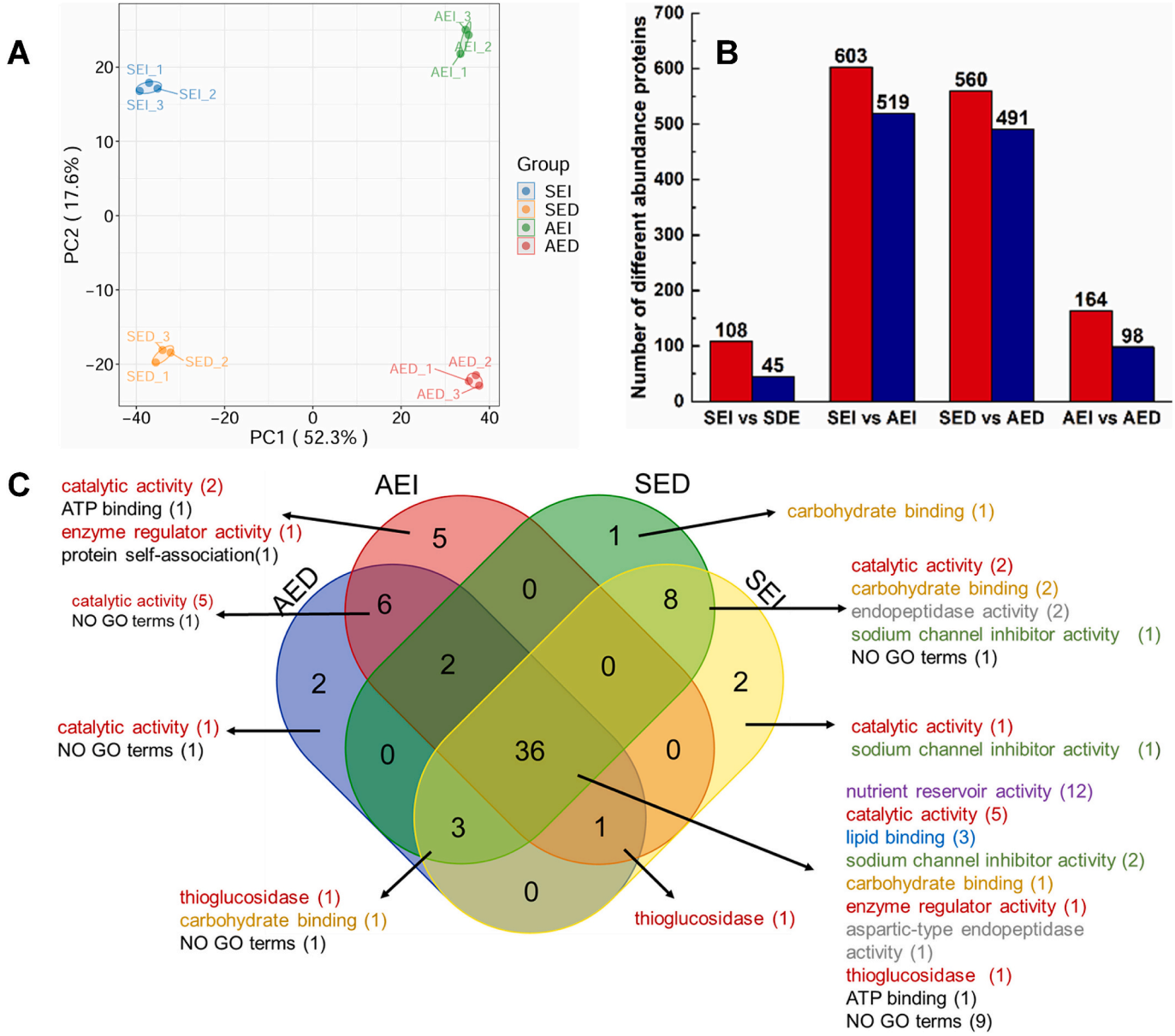

Fig. 3. (A) PCA score plots demonstrate the separation of the protein level data (log10 transformed and Pareto scaled) and the extraction method (PC_1) and dehulling (PC_2) dependent relationships (each point in the chart describes a biological replicate). (B) Number of different proteins identified by mass spectrometry. Red represents the protein number with significantly higher relative abundance in the former, while blue represents the opposite. (C) Venn diagram showing the top 50 most abundant protein database matches of rapeseed protein extracts (SEI: salt-extracted intact; SED: salt-extracted dehulled; AEI: alkaline extraction intact; AED: alkaline extract dehulled) after dialysis. GO terms in each category are indicated, along with the number of proteins identified in parentheses. Functional classifications in multiple overlaps are indicated by colored text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

features. Napin forms a heterodimeric structure composed of two polypeptide chains linked by disulfide bonds, adopting a compact globular fold with five α-helices and a C-terminal loop (Svendsen, Nicolova, Goshev, & Genov, 1989). These disulfide bonds stabilize its tertiary structure, ensuring the rigidity of the reactive site loop and facilitating its interaction with proteases. For instance, the 2S albumin from *Ricinus communis* (a homolog of napin) exerts trypsin inhibition through a conserved disulfide-rich core that maintains its inhibitory conformation (Souza et al., 2016). Furthermore, the rapeseed napin gene shares homology with the trypsin inhibitor gene from barley, suggesting that napin may retain such inhibitory activity (Ericson et al., 1986). Notably, the alkaline nature of napin promotes electrostatic interactions with trypsin, a serine protease with an acidic active site (Mudau et al., 2024). Studies on napin homologs have highlighted that positively charged residues (*e.g.*, Arg, Lys) in the reactive site loop are critical for binding to the negatively charged substrate-binding pocket of trypsin (Svendsen et al., 1989). This trypsin inhibitory activity of napin implies that the higher proportion of napin in salt-extracted protein isolates could account for their lower digestibility compared to alkaline-extracted counterparts.

3.5.3. *Protease inhibitor analysis*

The next step is the evaluation of the impact of extraction methods and dehulling on the proportion of lower-abundance protein (excluding storage protein) and protein edibility. Therefore, we inferred, summarized, and annotated the structural domains and molecular functions in the four samples from multiple databases, and compared the distribution of the top 15 GO terms.

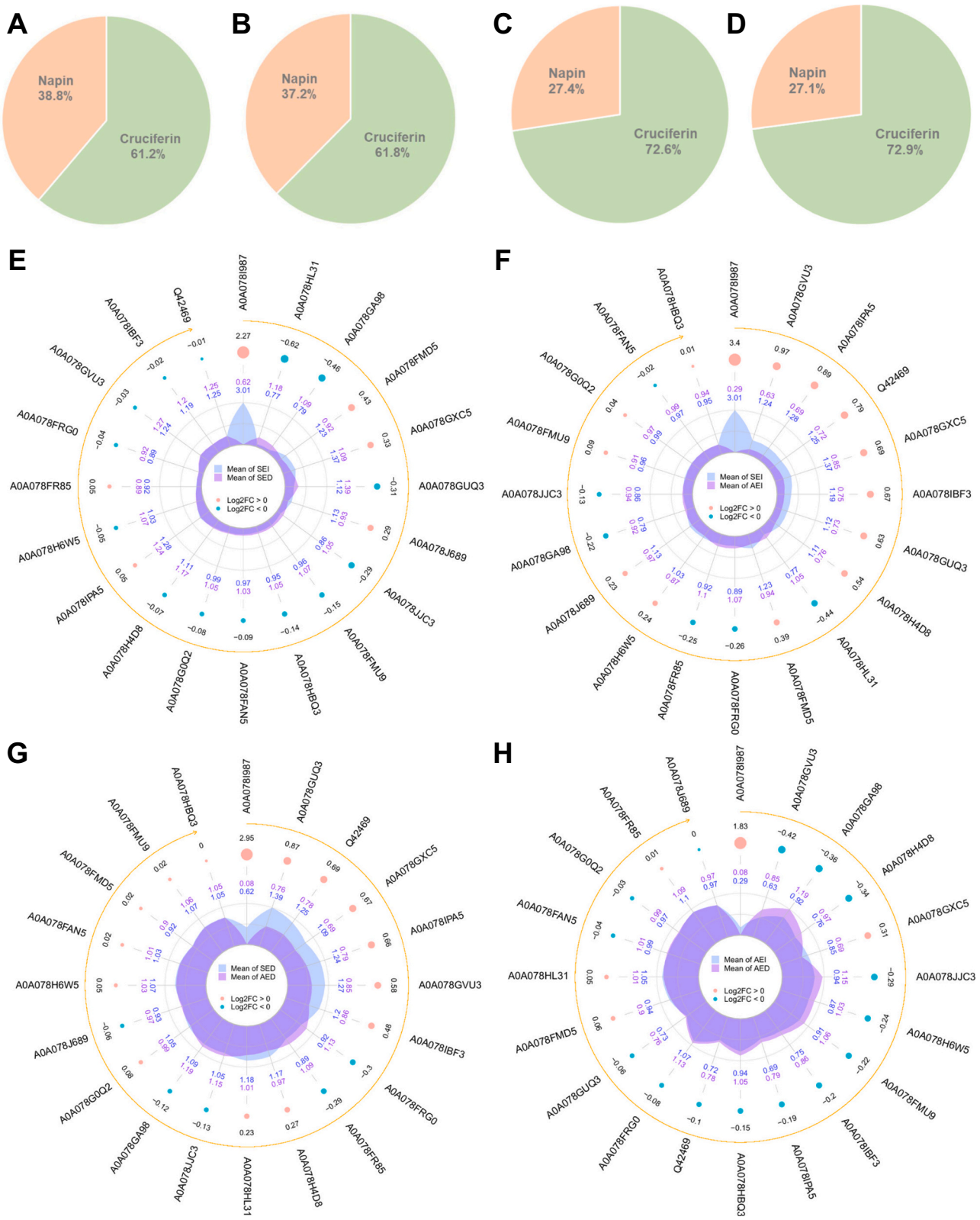

Fig. 4. (A ~ D) Pie charts for the proportion of mass spectrometry signal intensity of napin and cruciferin in SEI, SED, AEI, AED. (E ~ H) Radar maps reflect the differential levels of napin and criciferin in SEI *vs.* SED, SEI *vs.* AEI, SED *vs.* AED, and AEI *vs.* AED. From the outside to the inside, the first circle represents the protein accession. The orange arrow in the second circle indicates the absolute values of the differential protein difference multiples after Log2 conversion, sorted from highest to lowest. The third circle represents the differential protein difference multiples after Log2 conversion, with larger dots indicating greater difference multiples. Pink represents the high abundance of the former, and baby blue represents the high abundance of the latter. The fourth circle represents the R values of the two groups. Log2FC was calculated as $\log_2$ (The ratio value is obtained by dividing the R values of two samples). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As shown in Fig. 5, for the four samples, the cupin domain had the highest annotation frequency. Notably, trypsin and protease inhibitor ranked fourth in both SEI and SED, accounting for 9.33 % and 7.77 % of the top 15 GO terms, respectively, but they were not annotated, thus absent, in AEI and AED. Besides, the intensity of protease inhibitor/seed storage/LTP family accounted for 5.07 % and 5.32 % for SEI and SED, respectively, which was higher than that of AEI (3.77 %) and AED (4.13 %). The higher protease inhibitor content in salt-extracted protein isolates could contribute to their lower IVPD values than those of alkaline-extracted protein isolates.

Protease inhibitor is a key anti-nutritional factor in plant protein extracts (Kong, Li, & Liu, 2022). By comparing and predicting the domain structure, molecular function, biological processes, and 3D structure of proteins from multiple databases, 18 protease inhibitors (17 comparable proteins) were identified (excluding storage proteins) (Fig. 6**)**. Here, A0A078GU58 was exclusively present in SEI and SED (Table S1 in the SI). Several high-abundance inhibitors, such as A0A078GZH0. A0A078FVB1, A0A078I535, and A0A078J0B3 were more abundantly present in salt-extracted proteins than alkaline-extracted ones (Fig. 4G). Among them, A0A078GZH0 and A0A078I535 belonged to the kunitz type protease inhibitors; A0A078FVB1 was a bi-functional inhibitor; and A0A078J0B3 was a sodium channel inhibitor (https://www.uniprot.org/). Moreover, serine protease inhibitors (A0A078J386 and A0A078G608) and cysteine proteinase inhibitors (A0A078IMU3 and A0A078F5V0) were more easily obtained by alkaline extraction. Table S5 in the SI exhibited the subcellular localization of 18 identified protease inhibitors. Here, an interesting pattern was that all annotations indicating salt extraction were more favorable (A0A078GZH0, A0A078FVB1, A0A078I535, A0A078J0B3, A0A078JIJ5, A0A078JP16, A0A078FY61, A0A078G1J7, A0A078FY61) came from two labels: chloroplast and extracellular. This demonstrates that salt extraction was more likely to induce protein dissolution at these two positions. The specific mechanism of this phenomenon requires further exploration.

Overall, regardless of salt or alkaline extraction, more types of protease inhibitors exhibited high abundance in protein isolates obtained from DM. However, it should be noted that the abundance of A0A078I535 in SEI was much higher than that in SED. The subcellular localization of this protein was related to chloroplasts, and it could be speculated that this protein is a salt-soluble protein, mainly present in rapeseed hull cells. In summary, salt-extracted proteins had a higher abundance of protease inhibitors than alkaline-extracted proteins, which could explain their lower IVPD. However, the effects and activities vary greatly among different protease inhibitors, and cannot be evaluated solely by abundance. Therefore, further experiments are needed to prove the impact of various protease inhibitors in rapeseed protein on its edibility.

4. Conclusion

In this study, both salt- and alkali-extraction methods successfully extracted rapeseed protein isolates with high purity and a reasonable protein recovery. Dehulling treatment proved to be an effective approach for reducing total phenolic content. Based on the results of *in-vitro* simulated protein digestion, salt-extracted protein isolates show lower digestibility than alkaline-extracted protein isolates. This finding was unexpected, as salt-extracted proteins contained significantly lower levels of anti-nutritional factors, such as glucosinolates, phytic acids, and phenols, which are known to impair protein digestibility. Further proteomics analysis revealed that salt-extracted protein isolates contained higher levels of napin proteins and protease inhibitors, which could have contributed to the lower digestibility. This brings us to the key insight of this study: protease-inhibiting proteins should be considered alongside anti-nutritional factors when assessing plant protein digestion. In addition, heating may partially eliminate the effect of protease inhibitors, so the digestion behavior of rapeseed protein after thermal processing is the focus of later research. Finally, to make rapeseed protein available for human or pet consumption/good digestion, further control of anti-nutritional factors and protein inhibitors for additional heat treatment or other processes may be required during the production process.

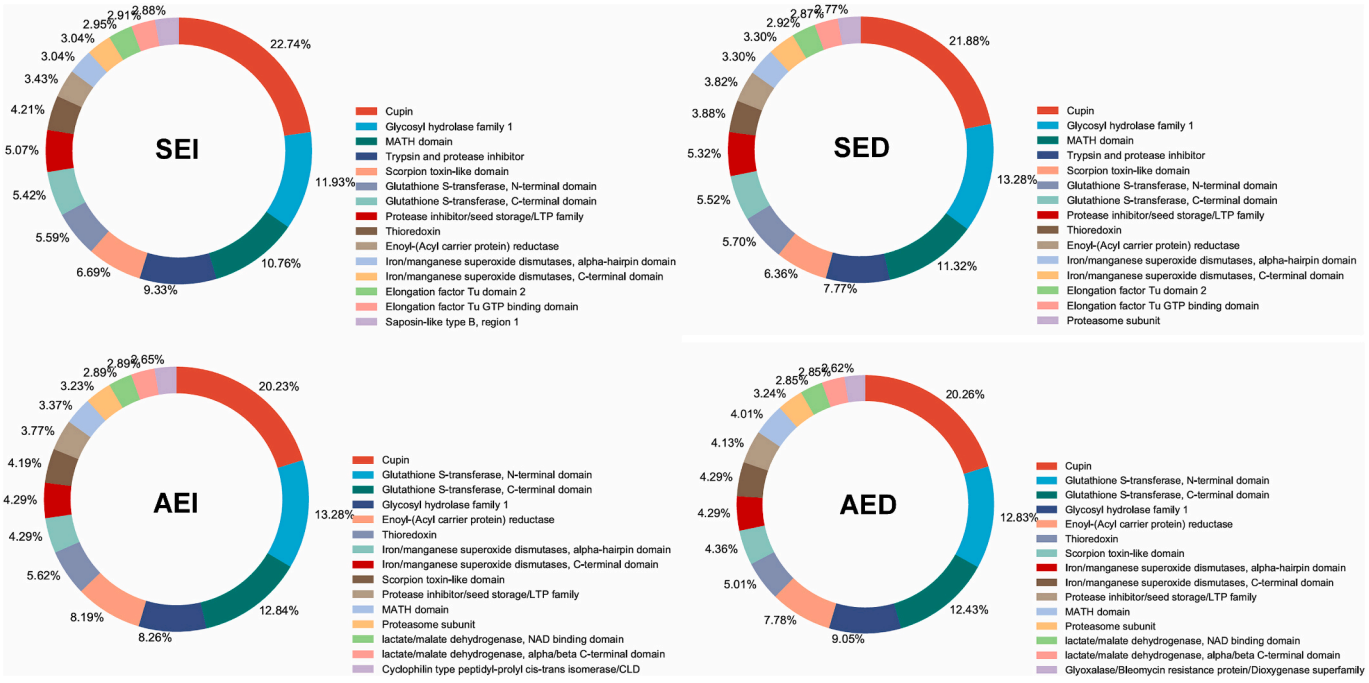

Fig. 5. Domain description distribution of the top 15 GO terms (excluding storage protein) for rapeseed protein isolate (SEI: salt-extracted intact; SED: salt-extracted dehulled; AEI: alkaline extraction intact; AED: alkaline extract dehulled) after dialysis. The colors in the figure are sorted according to the proportion of identified structural domains.

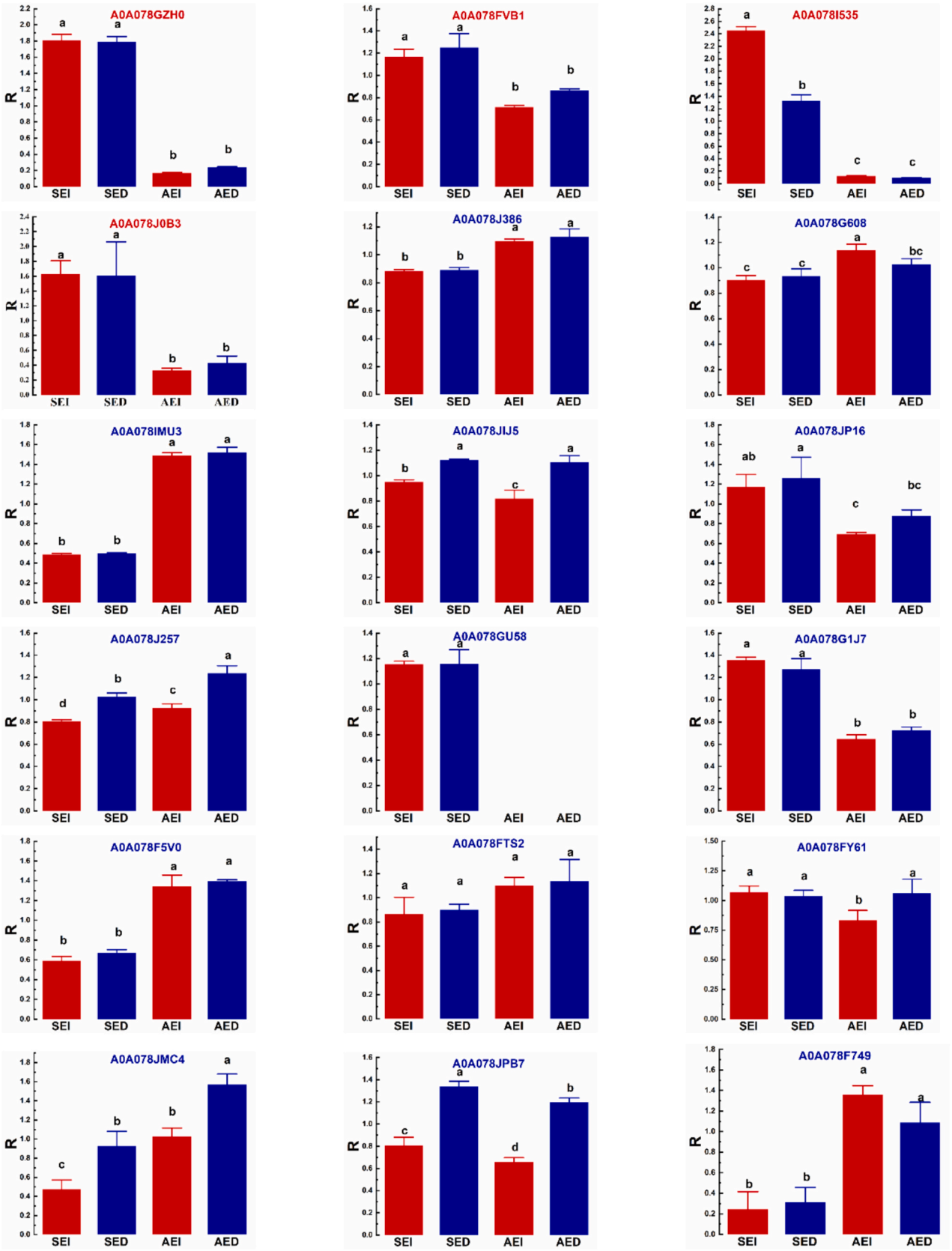

Fig. 6. Column charts of 18 protease inhibitors (excluding storage protein) identified in SEI, SED, AEI, and AED. From the left to the right, there is a descending order in the abundance of the protease inhibitors identified from SEI. The protein accession labeled in dark red was identified as the top 50 in Fig. 3C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CRediT authorship contribution statement

Kangyu Li: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Dengfeng Peng:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Jiaqi Shao:** Writing – review & editing, Formal analysis. **Fenghong Huang:** Writing – review & editing, Formal analysis. **Weiping Jin:** Methodology, Formal analysis. **Xia Wan:** Methodology, Formal analysis. **Jack Yang:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Qianchun Deng:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Funding

This research was funded by the National Key Research and Development Program of China (2021YFD2100400), the Opening Project of Key Laboratory of Oilseeds Processing, Ministry of Agriculture and Rural Affairs, China (202402), and the Xinjiang Uygur Autonomous Region (2022A02004). This work thanks to the financial support from the "Young Talents of Chinese Academy of Agricultural Sciences" and "Wuhan Yellow Crane Talents".

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.145852.

Data availability

Data will be made available on request.

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