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Research Article

Comparative Analysis of Protease-Assisted Extraction and Three-Phase Partitioning for Protein Recovery from Thai Black Soybean Okara

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ABSTRACT

Okara, a by-product of soy-based food production, has gained increasing interest, particularly that derived from black soybean, due to its higher protein and nutrient contents than yellow soybean. However, its utilization remains challenging due to its high perishability. Limited studies have addressed okara bioactive composition, bioactivity, and the environmental impacts of different extraction methods. This study aimed to compare environmentally friendly extraction methods and evaluate their efficiency and functional properties for recovering bioactive proteins from Sukhothai 3 black soybean okara, using protease-assisted extraction (Protease-P6SD & Protease-A2SD) and three-phase partitioning (TPP). Extraction yield, amino acid composition, and antioxidant activity were evaluated. TPP showed a significantly higher extraction yield (26.61 ± 2.24%) than protease-assisted extraction (16.49 ± 0.95% and 15.36 ± 1.63% for Protease-P6SD and Protease-A2SD, respectively) (p<0.05). In contrast, the amino acid composition analysis showed that extracts obtained by protease-assisted extraction contained a broader range of essential amino acids than those obtained by the TPP method. This may be attributed to the higher specificity of proteolytic enzymes toward target proteins compared with chemical extraction methods. Antioxidant assays (DPPH and ABTS) revealed significantly lower IC_{50} values, approximately two-fold lower, in protease-derived extracts compared to TPP (p<0.05), indicating stronger antioxidant activity. These findings demonstrate that a higher extraction yield does not necessarily correspond to superior protein quality. While TPP provides a higher extraction yield, protease-assisted extraction enhances the amino acid profile and bioactivity. Therefore, enzymatic extraction represents a promising green approach for enhancing the utilization of black soybean okara. This study highlights the potential of Sukhothai 3 black soybean okara as a valuable biofunctional ingredient for functional food applications.

Key words: Black soybean, okara, soy protein, Sukhothai 3, three-phase partitioning (TPP)

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INTRODUCTION

Okara, the insoluble residue generated during soy-based beverages and tofu production (Cech *et al.*, 2022), is produced in large quantities but remains underutilized despite its nutritional value. More than half of the original soybean material remains as okara after processing, whereas about 45% is recovered as the final food product. In practice, okara is commonly directed to low-value applications such as animal feed and biofertilizers while a considerable amount is discarded due to its high moisture content and rapid perishability, leading to handling challenges and environmental concerns (Kamble & Rani, 2020). Okara is recognized as an inexpensive source of nutrients, particularly proteins, dietary fiber, lipids, and bioactive constituents (Kamble & Rani, 2020). Among these, proteins are of particular interest due to the presence of essential amino acids such as leucine, threonine, and lysine. Soy proteins exhibit high digestibility (up to 80% *in vitro*), comparable to that of cow's milk (Eze, 2019), highlighting their potential for use in functional food applications (Cech *et al.*, 2022).

Despite this potential, protein recovery from okara remains challenging due to the entrapment of proteins within a complex matrix rich in insoluble dietary fiber and other cellular components. Various extraction techniques, including chemical, physical, and enzymatic methods, have been investigated (Colletti *et al.*, 2020).

Among these, protease-assisted extraction is considered a promising green approach because it operates under mild conditions and enhances protein release efficiency. In particular, protease-assisted extraction is advantageous due to its ability to hydrolyze peptide bonds and disrupt protein–matrix interactions, thereby improving extraction efficiency (Zhao *et al.*, 2022). However, enzymatic extraction still presents limitations, including complex process control, extended processing time, and high operational cost (Kantakas & Wiriyacharee, 2023). Three-phase partitioning (TPP) has emerged as an alternative mild technique for biomolecule recovery (Dennison, 2012). This method preserves protein structure and is considered environmentally friendly with potential for industrial application due to the use of relatively inexpensive chemicals (Gagaoua & Hafid, 2016). Nevertheless, studies on TPP for okara protein extraction remain limited (Senso, 2018).

Black soybean has long been consumed worldwide, particularly in Asian countries, and has recently gained attention due to its high levels of bioactive compounds, especially phenolics and anthocyanins, which contribute to antioxidant activity and are generally higher than those in yellow soybean (Bhartiya *et al.*, 2020). The Thai black soybean cultivar Sukhothai 3 is a promising local genotype with high protein content and strong potential for value-added processing. However, research on protein extraction from black soybean okara remains limited, particularly regarding systematic comparisons between green extraction methods (Kantakas & Wiriyacharee, 2023). To the best of our knowledge, no study has directly compared protease-assisted extraction and three-phase partitioning for protein recovery from Sukhothai 3 black soybean okara.

Therefore, this study aimed to characterize the biochemical composition of Sukhothai 3 black soybean and its okara and to compare protease-assisted extraction (Protease-P6SD and Protease-A2SD) with three-phase partitioning (TPP) for protein extraction. The study evaluated extraction yield, amino acid profile, color characteristics, total phenolic content, total flavonoid content, and antioxidant activity, and also characterized the residual carbohydrate and lipid fractions before protein extraction. This work provides a comprehensive comparison of green and conventional extraction strategies and offers scientific evidence for the valorization of black soybean okara for functional food applications.

MATERIALS AND METHODS

Defatted dried black soybean okara

Fresh black soybean okara (residue) was obtained from the Institute of Food Research and Product Development (IFRPD), Kasetsart University (Thailand). The okara was dried in a redLine laboratory oven (BINDER GmbH, Germany) at 50℃ until the moisture content was less than 5% and finely processed using a household blender (HR2221/00, Philips, The Netherlands). Lipids were extracted according to the method of Liengprayoon *et al.* (2013) with modifications in sample weight and extraction solvent volume. Ten grams of dried okara powder were extracted with 100 mL of chloroform: methanol (2:1, v/v) and shaken at 200 rpm for 6 h using a Stuart SO1 orbital shaker (Bibby Scientific Ltd., UK) at 25℃. The extract was filtered to separate the okara from the solvent using Whatman No. 1 filter paper. Then, 0.9% (w/v) sodium chloride solution was added at a 1:5 ratio (v/v), and the mixture was gently shaken and allowed to stand for phase separation. The bottom phase containing lipids was collected and evaporated using a rotary evaporator (BÜCHI Rotavapor R-200, BÜCHI, Switzerland) at 40℃. The extracted lipids were weighed, and the extraction yield was calculated based on the dry weight of okara. The lipid extracts were kept at −20℃ for fatty acid analysis.

After phase separation, the defatted okara was washed with the same solvent three times (5 mL each time). The defatted okara was then re-dried in a BINDER redLine oven (BINDER GmbH, Germany) at 50℃ and stored in a tightly sealed container for subsequent protein extraction. This step was performed as a pre-treatment to eliminate residual lipids prior to protein extraction.

Okara protein extraction using protease enzyme

The defatted sample was mixed with water at a 1:9 (w/v) ratio. Two commercial protease enzymes were used in this study: Protease-A2SD (a fungal neutral protease with high protease & peptidase activities) and Protease-P6SD (a semi-alkaline protease with high proteolytic activity), both kindly provided by Amano Enzyme Asia Pacific Co., Ltd. (Pathum Thani, Thailand).

To evaluate the effect of enzyme concentration on protein extraction efficiency, enzymes were added at concentrations ranging from 0.5 to 2% (w/w) of dried black soybean okara. The optimal enzyme concentration was selected based on the highest protein extraction yield obtained in preliminary experiments. The extraction temperature (50℃) and incubation time (5 hr) were selected according to the recommended activity ranges provided in the enzyme manufacturer's datasheets. After incubation, enzyme activity was terminated by heating at 90℃ for 10 min, followed by centrifugation at 8,000 rpm for 20 min at 4℃ using a Sorvall ST16R refrigerated centrifuge (Thermo Fisher Scientific, USA). The supernatant was collected and freeze-dried using a CRYOTEC Crios freeze dryer (Cryotec Co., Ltd., France). The resulting extracts were stored at −20℃ until further analysis.

Okara protein extraction using three-phase partitioning

Protein extraction using the three-phase partitioning technique was carried out following the procedure described by Senso (2018). A 10 g aliquot of defatted dried black soybean okara was mixed with ammonium sulfate $(NH_4)_2SO_4$ solution at 30% (w/v) in a 1:9 ratio (w/v). This concentration was selected based on the optimization study of Senso (2018), which reported that 30% (w/v) ammonium sulfate provided the highest protein recovery and efficient phase separation in the three-phase partitioning process. Ethanol (90 mL) was then added. The extraction tube was shaken at 150 rpm in an incubator for 30 min, and then the mixture was filtered through Whatman No. 1 filter paper to separate the solution from the okara. The filtered okara was mixed with DI water at a 1:10 (w/v) ratio, and the residue was then removed using filter paper. The liquid was dried using a freeze dryer (CRYOTEC Crios, Cryotec Co., Ltd., France) for 48 hr or until completely dry. The dried sample was kept in a dark, sealed chamber at −20℃ until further analysis.

Chemical composition

Proximate composition, including moisture, protein, ash, fat, and crude fiber, of black soybean and dried black soybean okara was performed according to AOAC methods (1995). Soluble protein content was determined using the Lowry method (Lowry *et al.*, 1951) because of its high sensitivity for soluble protein determination. All determinations were performed in triplicate. The carbohydrate content was determined by subtracting the sum of moisture, protein, ash, fat and crude fiber from 100%.

Fatty acid composition

The lipid extracts from okara were analyzed for their fatty acid composition using gas chromatography with flame ionization detection (GC-FID) after conversion to the corresponding fatty acid methyl esters (FAMEs). All samples were prepared as described by Liengprayoon *et al.* (2013). The chromatographic separation was performed using a Shimadzu GC17A gas chromatograph (Shimadzu, Japan) fitted with a BPX70 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, SGE, Victoria, Australia) and a Shimadzu AOC20i automatic injector (Shimadzu, Japan) with an injected volume of 1 μL. Helium was used as the carrier gas at an initial linear velocity of 1.25 mL/min. The temperatures of the

split injector (split ratio 1:15) and the flame ionization detector were 250℃. The oven temperature was programmed at 160℃ for 0.5 min, increased to 200℃ at 10℃/min, and then kept constant for 4 min. The total analysis run time was 9.1 min. Data acquisition was performed using GCsolution software version 2.32 (Shimadzu, Japan). Fatty acids were identified and quantified by comparing their peak retention times with those of standard compounds. The results were expressed as a percentage of the total fatty acids.

Amino acid composition

The amino acid compositions of the okara protein extracts were analyzed following the method of Dai *et al.* (2014) with modifications to the sample amount and hydrolysis solution volume. In this study, 20 ± 2 mg of extract powder was hydrolyzed with 2 mL of 6 M hydrochloric acid (HCl) in a glass tube and heated in an ES-60 block heater (Hangzhou MIU Instruments Co., Ltd., China) at 110℃ for 24 hr. During hydrolysis, the tubes were gently shaken at 2 and 19 hr to ensure complete suspension of the samples. After 24 hr, the solution volume was brought up to 100 mL with distilled water and mixed thoroughly. The resulting solution was further diluted 10-fold to obtain amino acid concentrations within the range of 2.5-50 µM. A 2.5 µL aliquot of solution was derivatized with 10 µL of borate buffer, 2.5 µL of 0.04 M o-phthaldialdehyde (OPA), and 1 µL of 3-mercaptopropionic acid for 5 min before HPLC analysis.

For tryptophan analysis, 20 ± 2 mg of sample was digested with 2 mL of 4 M sodium hydroxide (NaOH) at 110℃ for 24 hr before HPLC analysis. During digestion, the tubes were gently shaken at 2 and 19 hr. After 24 hr, the solution was transferred to a volumetric flask, and the final volume was adjusted to 50 mL with distilled water to achieve a tryptophan concentration in the range of 2.5-50 µM. Five milliliters of the solution were centrifuged at 600 × g for 10 min using a Sorvall ST16R refrigerated centrifuge (Thermo Fisher Scientific, USA). Subsequently, 15 µL of the supernatant was injected into an HPLC system (1260 Infinity, Agilent Technologies, USA). Amino acid separation was performed using an Eclipse Plus C18 column (3.5 × 100 mm) with fluorescence detection (Ex 340 nm, Em 450 nm). The HPLC conditions were set as follows: column temperature 30℃ and mobile phase flow rate 0.7 mL/min.

Total phenolic content quantification (TPC)

Total phenolic content (TPC) of okara protein isolates was determined as described by Ainsworth and Gillespie (2007). Briefly, 0.5 mL of distilled water and 125 µL of okara protein isolates or the standard were added to the test tubes. Folin-Ciocalteu reagent (125 µL) was added to the solution and allowed to react for 6 min. Then, 1.25 mL of 7% sodium carbonate (Na_2CO_3) in distilled water and 1 mL of distilled water were added to the test tubes. Each test tube was gently mixed and left to stand at room temperature (25℃) for 90 min to allow the reaction to complete. Absorbance was recorded at 760 nm using a UV-1800 spectrophotometer (Shimadzu, Japan). Measurements were performed in triplicate and compared against a gallic acid standard curve at different concentrations (0–100 µg/mL). The calibration curve was linear with the equation y = 0.0047x - 0.1019 and a coefficient of determination (R^2) of 0.9993. TPC was expressed as milligrams of gallic acid equivalent (mg GAE) per gram of dry extract.

Total flavonoid content quantification (TFC)

Total flavonoid content (TFC) in okara protein isolates was quantified using the procedure described by Wolfe *et al.* (2003). Two hundred fifty-five microliters of the protein isolates were mixed with 75 µL of 5% sodium nitrite ($NaNO_2$) and 1.25 mL of distilled water, then left for 5 min at room temperature. Next, 150 µL of 10% aluminum chloride ($AlCl_3$) was added, followed by an incubation for 6 min. The mixture was then treated with 500 µL of 1 M sodium hydroxide (NaOH) and 275 µL of distilled water and mixed thoroughly. A catechin standard curve (30–300 µg/mL) was prepared with distilled water as the blank. Absorbance was measured at 510 nm using a UV-1800 spectrophotometer (Shimadzu, Japan). All measurements were conducted in triplicate. The calibration curve showed good linearity with the equation y = 0.0034x - 0.0146 and a coefficient of determination (R^2) of 0.9996. TFC values were expressed as milligrams of catechin equivalent (mg CE) per gram of dry extract.

Color measurement

The color of the protein extract samples was determined in triplicate using a spectrophotometer (CM 3500 d, Minolta Camera Co., Ltd., Japan). The CIE color values were recorded as L* (lightness, 0 = black, 100 = white), a* (-a* = green, +a* = red), and b* (-b* = blue, +b* = yellow).

DPPH radical scavenging activity assay

The free radical scavenging capacity of the okara protein isolates was measured by the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay, as described by Zhu *et al.* (2006), with slight modifications to the sample and DPPH solution volumes and reaction time. Specifically, 0.1 mL of each sample was mixed with 0.1 mL of DPPH solution (0.1 mM) prepared in 95% ethanol. All samples were prepared in microplates, and the absorbance was measured at 517 nm using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, USA) after a 30-min reaction time at room temperature in the dark. Ascorbic acid, α-tocopherol, and butylated hydroxytoluene (BHT) were used as positive controls. A lower absorbance represented a higher DPPH scavenging activity. The percentage of free radical scavenging was calculated from triplicate tests according to Equation 1.

$$\text{DPPH scavenging activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

Equation 1

Where A_0 and A_1 are the absorbance of the blank and sample/standard, respectively.

ABTS radical scavenging activity assay

The antioxidant activity of the samples was measured using the 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) assay (ABTS assay), a widely applied method for assessing free radical scavenging capacity. The measurement followed the method of Re *et al.* (1999) with conditions comparable to those used for the DPPH assay. ABTS reagent solution was freshly prepared by combining 7 mM ABTS solution with 2.45 mM potassium persulfate in a 2:1 (v/v) ratio and incubating the mixture in the dark at ambient temperature for 16 h. The dark-blue solution was diluted with ethanol until the absorbance reading reached 0.70 ± 0.02 at 734 nm. Subsequently, 20 µL of each sample or standard (ascorbic acid, α-tocopherol, and BHT) was added to 2 mL of ABTS reagent. After incubation for 6 min, the absorbance was measured at 734 nm using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, USA). Analyses of all samples and standards were performed in triplicate, and the ABTS radical scavenging activity (%) was calculated using Equation 2.

$$\text{ABTS scavenging activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

Equation 2

Where A_0 and A_1 are the absorbance of the blank and sample/standard, respectively.

Statistical analysis

Experiments were performed in triplicate unless otherwise stated. Results are expressed as mean ± standard deviation (SD). Amino acid composition was determined by a single HPLC analysis for each sample; therefore, these data are reported as individual values without statistical comparison. Statistical analysis for all other experiments was performed using one-way analysis of vari-

ance (ANOVA), followed by the least significant difference (LSD) post-hoc test. Statistical significance was defined at $p<0.05$. No correlation analysis was performed in this study. All analyses were conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Biochemical components of Sukhothai 3 black soybean and okara

Chemical composition

The chemical compositions of black soybean and okara are presented in Table 1. Black soybean contained 44.38 ± 0.62% protein, 20.97 ± 0.26% fat, 5.75 ± 0.02% crude fiber, 5.27 ± 0.11% ash, and 23.63 ± 0.59% carbohydrate. In contrast, okara contained 36.21 ± 0.06% protein, 7.75 ± 0.00% fat, 22.35 ± 0.03% crude fiber, 3.66 ± 0.01% ash, and 30.03 ± 0.09% carbohydrate. Statistical analysis using an independent t-test indicated significant differences in protein, fat, crude fiber, and ash contents between black soybean and okara ($p<0.05$), whereas carbohydrate content did not differ significantly ($p>0.05$).

Compared with black soybean, okara was characterized by lower protein, fat, and ash content but a higher crude fiber content. This compositional change is consistent with the loss of soluble components during soybean processing and the retention of insoluble fractions in okara. Nevertheless, protein remained the major component of okara, consistent with previous findings for Sukhothai 1 black soybean (Kantakas & Wiriyacharee, 2023).

The carbohydrate content of okara (approx. 30%) was higher than previously reported values (3.8–5.3%) (Li *et al.*, 2012). This discrepancy may be attributed to carbohydrate being calculated by difference, as well as variations in cultivar and processing conditions. Despite the reduction after soybean processing, okara retained a relatively high protein content (36.21%), supporting its potential as a promising raw material for protein extraction and amino acid analysis.

Fatty acid composition

After soybean processing, okara retained a considerable fat content (7.75 ± 0.00%, w/w) (Table 1), which could interfere with subsequent protein extraction. Therefore, defatting was performed before protein extraction. The total lipid content significantly decreased ($p<0.05$) from 17.54 ± 0.25% in black soybean to 14.28 ± 1.22% in okara (Table 2).

A total of nine fatty acids were identified (Table 2), with linoleic acid (C18:2) being the predominant fatty acid in both samples (52.37 ± 0.17% and 54.61 ± 0.45% for black soybean and okara, respectively), followed by oleic acid (C18:1), palmitic acid (C16:0), and stearic acid (C18:0). Significant differences ($p<0.05$) were observed for all fatty acids except C14:0 between black soybean and okara. In par-

Table 1. Chemical compositions of black soybean and okara (% dry weight basis).

Chemical composition	Black soybean	Okara
Protein	44.38 ± 0.62^{a}	36.21 ± 0.06^{b}
Crude fiber	5.75 ± 0.02^{b}	22.35 ± 0.03^{a}
Fat	20.97 ± 0.26^{a}	7.75 ± 0.00^{b}
Ash	5.27 ± 0.11^{a}	3.66 ± 0.01^{b}
Carbohydrate[1]	23.63 ± 0.59^{a}	30.03 ± 0.09^{a}

Values are expressed as mean ± standard deviation (SD) of triplicate measurements ($n=3$). Different lowercase letters (a–b) within the same row indicate significant differences between black soybean and okara ($p<0.05$), as determined by an independent t-test. [1] Carbohydrate was calculated by difference.

Table 2. Fatty acid profiles of black soybean and okara (%total fatty acids).

Fatty acid	Black soybean	Okara
C14:0	0.14 ± 0.00^{ns}	0.14 ± 0.01^{ns}
C16:0	12.44 ± 0.10^{a}	11.99 ± 0.23^{b}
C18:0	4.83 ± 0.01^{a}	4.59 ± 0.10^{b}
C18:1	23.26 ± 0.49^{a}	20.87 ± 0.18^{b}
C18:2	52.37 ± 0.17^{b}	54.61 ± 0.45^{a}
C18:3	6.05 ± 0.24^{b}	7.13 ± 0.05^{a}
C20:0	0.36 ± 0.01^{a}	0.34 ± 0.01^{b}
C22:0	0.31 ± 0.01^{a}	0.26 ± 0.00^{b}
C24:0	0.15 ± 0.01^{a}	0.12 ± 0.01^{b}
Total SAFA*	18.68 ± 0.10^{a}	17.43 ± 0.32^{b}
Total MUFA**	23.26 ± 0.49^{a}	20.87 ± 0.18^{b}
Total PUFA***	60.76 ± 0.40^{a}	61.73 ± 0.48^{b}
MUFA/SAFA	1.17 ± 0.03^{a}	1.20 ± 0.01^{b}
PUFA/SAFA	3.25 ± 0.01^{b}	3.54 ± 0.09^{a}
Total lipid	17.54 ± 0.25^{a}	14.28 ± 1.22^{b}

SAFA* = saturated fatty acids; MUFA** = monounsaturated fatty acids; PUFA*** = polyunsaturated fatty acids. Values were expressed as mean ± SD ($n=3$). Values in the same row with different letters are significantly different ($p<0.05$). ns: Not significantly different ($p>0.05$).

ticular, linoleic acid (C18:2) and linolenic acid (C18:3) were higher in okara, whereas oleic acid (C18:1) and palmitic acid (C16:0) were lower. These findings are consistent with previous studies reporting linoleic acid as the dominant fatty acid in soybean and its by-products (Bhartiya *et al*., 2020; Kumar *et al*., 2016).

Unsaturated fatty acids (MUFA and PUFA) accounted for more than 80% of total fatty acids in both samples, indicating that okara remained a rich source of unsaturated fatty acids after processing. The high PUFA proportion further suggests that black soybean okara remains a nutritionally valuable source of essential fatty acids, particularly linoleic acid (Bishehkolaei & Pathak, 2024; Li *et al*., 2024).

Protein extraction of defatted black soybean okara

Optimization of enzymatic extraction conditions

The Brix value (°Brix), representing total soluble solids in a solution, was used as a preliminary indicator for selecting appropriate enzymes and extraction conditions due to its rapid measurement and cost-effectiveness. However, it should be noted that °Brix is an indirect measure and does not specifically reflect protein content, as it includes all soluble components, such as sugars, peptides, and other low-molecular-weight compounds. Nevertheless, previous studies have demonstrated that higher °Brix values correlate with increased concentrations of extractable solids, thereby supporting its use in the preliminary optimization of extraction processes (Aydin *et al*., 2025).

Preliminary results showed that among the six enzymes tested (ProteaseMSD, ProteaseP6SD, ProteaseA2SD, ProteAx, ProtinSD-NY10, and Umamizyme), ProteaseP6SD and ProteaseA2SD produced the highest °Brix values (data not shown) and were therefore selected for further study. The °Brix values of extracts obtained using these enzymes at different concentrations (0.5–2.0% w/w) are presented in Table 3. Increasing enzyme concentration from 0.5% to 1.0% improved °Brix values; however, further increase to 2.0% resulted in only a slight improvement. For example, ProEx-P6SD increased from 4.1 to 4.5, while ProEx-A2SD showed minimal change (4.0 to 4.1). This suggests a diminishing return at higher enzyme concentrations. The limited increase at higher concentrations may be attributed to interactions between proteins and other released compounds from soybean cells, leading to the formation of insoluble complexes (Kasai *et al*., 2004). Considering both extraction efficiency and enzyme usage, 1.0% was selected as the optimal concentration for subsequent experiments.

Extraction yield of enzyme extraction and three-phase partitioning methods

The protein extraction yields obtained from two extraction methods are presented in Table 4. The three-phase partitioning technique (ProEx-TPP) showed a significantly higher yield (26.61 ± 2.24%) compared to protease-assisted extraction, which ranged from 15.36 ± 1.63% (ProEx-A2SD) to 16.49 ± 0.95% (ProEx-P6SD) (p<0.05).

The lower yield observed in enzyme-assisted extraction could be attributed to the complex structure of soybean okara, as proteins are located within intact plant cells. Disruption of the okara cell wall could therefore enhance protein extraction efficiency (Preece *et al*., 2015). Additionally, the high content of insoluble fiber further limits enzyme accessibility, thereby reducing extraction efficiency (Kasai *et al*., 2004). In contrast, the TPP method enhances protein separation through phase partitioning, leading to improved extraction efficiency. Similar findings have been reported in previous studies, where TPP was shown to be an effective and rapid technique for protein recovery from plant-based materials (Yan *et al*., 2017). Senso (2018) also reported that TPP was an efficient one-step extraction technique for obtaining proteins and phenolics from yellow soybean okara separately in different solvent phases.

To further evaluate extraction performance, the amino acid composition of the obtained protein extracts from both methods was analysed as discussed in the following section on "Amino acid profiles".

Color measurement

Color parameters of the protein extracts were measured following the CIE (International Commission on Illumination) color specification system (L*, a* & b*) and are presented in Table 4 and Figure 1. The CIE color scale expresses color as three values; L* represents lightness, a* indicates the red-green axis, and b* indicates the yellow-blue axis. The ProEx-TPP sample showed a significantly higher L* value (72.26 ± 0.01) compared to ProEx-P6SD (59.94 ± 0.02) and ProEx-A2SD (53.79 ± 0.14) (p<0.05), indicating a lighter color. All

Table 3. Optimization of enzymatic hydrolysis conditions of black soybean okara.

Treatment	Protease concentration (% w/w)	Total soluble solids (°Brix)
Control	0	1.9 ± 0.02
ProEx-P6SD	0.5	3.7 ± 0.05
ProEx-P6SD	1.0	4.1 ± 0.00
ProEx-P6SD	2.0	4.5 ± 0.03
ProEx-A2SD	0.5	3.5 ± 0.02
ProEx-A2SD	1.0	4.0 ± 0.03
ProEx-A2SD	2.0	4.1 ± 0.07

Mean values ± SD.

Table 4. Percentage yield, soluble protein and color of black soybean okara protein isolate under different extraction conditions.

Okara protein extract	%Yield	L*	a*	b*
ProEx-P6SD	16.49 ± 0.95^{b}	59.94 ± 0.02^{c}	3.51 ± 0.00^{b}	15.84 ± 0.01^{b}
ProEx-A2SD	15.36 ± 1.63^{b}	53.79 ± 0.14^{b}	4.48 ± 0.01^{a}	20.24 ± 0.14^{a}
ProEx-TPP	26.61 ± 2.24^{a}	72.26 ± 0.01^{a}	0.39 ± 0.00^{c}	6.23 ± 0.01^{c}

Mean values in the same column with different letters are significantly different (p<0.05).

samples exhibited positive a* and b* values, suggesting reddish and yellowish tones, respectively.

The lighter color observed in ProEx-TPP may be attributed to the removal of pigment compounds from the seed coat during the extraction process. In enzyme-assisted extraction, hydrophilic pigments such as anthocyanins may remain in the aqueous phase, contributing to darker coloration (Bhartiya *et al.*, 2020). In contrast, the TPP method facilitates the separation of pigments into the solvent phase, resulting in a lighter protein extract. Further analysis of phenolic and flavonoid contents in the extracts is discussed in the following section.

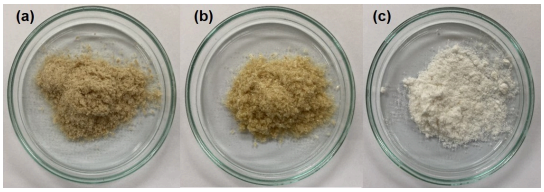

Fig. 1. Black soybean okara protein extract (after freeze-drying) obtained from (a) ProteaseP6SD, (b) ProteaseA2SD and (c) the three-phase partitioning technique.

Amino acid profiles

Eighteen amino acids were detected in all black soybean okara protein isolate extracts as shown in Table 5. The highest total essential amino acid content was observed in ProEx-P6SD (207.18 mg/g of extract), followed by ProEx-A2SD (196.84 mg/g of extract), whereas the TPP extract contained only 14.73 mg/g of extract. Both enzymatic extracts showed similar amino acid profiles, while the TPP extract differed markedly. Leucine was the most abundant in all samples, particularly in ProEx-P6SD (41.37 mg/g of extract), followed by ProEx-A2SD (37.97 mg/g of extract). Arginine and lysine were also present in relatively high levels in enzymatic extracts, whereas only trace amounts were detected in the TPP extract (Table 5).

Glutamic acid and aspartic acid were the predominant non-essential amino acids across all extracts. Because the amino acid composition was determined from a single analysis performed by an accredited external laboratory, statistical analysis could not be performed for these data. Therefore, the amino acid composition results are presented descriptively and should be interpreted accordingly.

In addition, protein solubility analysis revealed significant differences among the extraction methods. The TPP extract showed the lowest water-soluble protein content (2.39 ± 0.10%), whereas the enzymatic extracts showed higher values (43.20 ± 0.02% for ProEx-P6SD and 37.29 ± 0.41% for ProEx-A2SD, respectively). Enzymatic hydrolysis enhances solubility by producing smaller, more hydrophilic peptides, whereas TPP relies on phase separation, which is less effective in releasing soluble protein fractions from complex matrices (Zhang *et al.*, 2021). Previous studies support these findings. Zhao *et al.* (2022) reported that hydrophilic and

Table 5. Amino acid compositions of black soybean okara protein isolates under different extraction conditions.

Amino acids* (mg/g extract)	ProEx-P6SD	ProEx-A2SD	ProEx-TPP
Essential amino acids			
Histidine	7.80	7.12	0.35
Leucine	41.37	37.97	1.13
Isoleucine	24.66	22.94	0.50
Lysine	28.22	28.24	0.99
Methionine	1.34	3.48	0.00
Arginine	30.83	29.66	1.49
Phenylalanine	22.75	20.75	0.57
Threonine	17.81	16.14	0.60
Tryptophan	6.44	5.53	4.84
Valine	25.96	25.01	4.27
Non-essential amino acids			
Proline	34.84	32.63	3.89
Alanine	24.31	25.64	0.65
Cystine	44.64	104.31	0.66
Glycine	18.75	17.61	0.69
Glutamic acid	120.08	116.99	4.29
Serine	22.34	19.93	0.95
Aspartic acid	69.16	66.22	2.68
Tyrosine	13.55	12.63	0.42
Total amino acids	**554.85**	**592.80**	**28.95**
Total essential amino acids	**207.18**	**196.84**	**14.73**
Soluble protein (%)**	43.20 ± 0.02^{a}	37.29 ± 0.41^{a}	2.39 ± 0.10^{b}

*Amino acid composition was determined from a single analysis for each sample; therefore, no statistical analysis was performed for amino acid composition data.
**Soluble protein values are expressed as mean ± SD (n=3). Mean values in the same column with different letters are significantly different (p<0.05).

acidic amino acids in black bean proteins contribute to improved solubility, which may also be associated with antioxidant activity (Farvin *et al*., 2016). Enzymatic extraction may also preserve amino acid integrity and promote the formation of bioactive peptides with potential health benefits (Gagaoua & Hafid, 2016; Wei *et al*., 2018; Figueiredo *et al*., 2018).

In conclusion, the amino acid composition analysis indicated that enzymatic extraction yielded black soybean okara protein isolates with a more favorable essential amino acid profile, improved solubility, and better functional properties than TPP. Although TPP is simpler and potentially more cost-effective, its lower protein recovery and less favorable amino acid profile may limit its nutritional value. Therefore, enzymatic extraction shows strong potential for developing high-quality protein ingredients for functional foods and nutraceutical applications. Further optimization is needed to improve efficiency and scalability for industrial use.

Total phenolic content (TPC) and total flavonoid content (TFC)

Phenolic compounds are of interest because of their beneficial effects on human health, particularly their antioxidant properties (Colletti *et al*., 2020). Black soybean seeds are rich in polyphenols, including anthocyanins, which contribute to their characteristic dark color (Bhartiya *et al*., 2020).

As mentioned previously, the darker color of the enzymatic protein extracts was attributed to pigment compounds retained from the seed coats. Therefore, total phenolic content (TPC) and total flavonoid content (TFC) were determined (Table 6). The ProEx-TPP extract showed significantly lower TPC and TFC values (0.79 ± 0.04 mg GAE/g & 0.03 ± 0.01 mg CE/g, respectively; $p<0.05$) than the enzymatic extracts, suggesting that phenolic compounds likely contributed to the observed color differences among the extracts. No significant difference in TPC was observed between ProEx-P6SD and ProEx-A2SD, indicating that enzyme type had minimal influence on total phenolic recovery. In contrast, TFC differed significantly among all extraction treatments, indicating that flavonoid recovery was affected by both enzyme type and extraction method.

The TPC values reported for black soybean okara vary depending on soybean variety and extraction conditions. For example, Kantakas and Wiriyacharee (2023) reported a maximum TPC of 15.81 mg GAE/g sample from Sukhothai 1 black soybean okara extracted using 0.25–0.75% alcalase enzyme. The higher TPC values observed in our study may reflect genetic differences among soybean cultivars, environmental conditions, extraction methods, as well as analytical conditions (Li *et al*., 2024).

Although the Folin–Ciocalteu method is widely used for total phenolic quantification, it may be affected by interference from other compounds (Mikulić *et al*., 2022). Therefore, further characterization techniques such as high-performance liquid chromatography (HPLC) could be used to identify individual phenolic compounds. Overall, these findings highlight the potential utilization of black soybean okara as a source of natural antioxidant compounds for functional food and nutraceutical applications.

Antioxidant activity (DPPH and ABTS radical scavenging assays)

The antioxidant properties of soybeans contribute to reducing cancer risk, regulating cholesterol levels, and preventing cardiovascular diseases (Fan *et al*., 2022). Black soybeans contain various antioxidant compounds, including anthocyanins, which belong to the polyphenol group (Li *et al*., 2024).

DPPH and ABTS *in vitro* radical scavenging assays were conducted to evaluate the antioxidant activities of the protein extracts. The enzymatic extracts (ProEx-P6SD and ProEx-A2SD) exhibited stronger antioxidant activity than the TPP extract, as indicated by their significantly lower DPPH IC_{50} values ($p<0.05$) (Table 6). For the DPPH assay, the IC_{50} values of ProEx-P6SD and ProEx-A2SD were 1.44 ± 0.01 and 1.12 ± 0.20 mg/mL, respectively, compared to 11.83 ± 0.19 mg/mL for ProEx-TPP. Comparable results were obtained in the ABTS assay, where ProEx-P6SD and ProEx-A2SD showed IC_{50} values of 9.34 ± 0.04 and 9.61 ± 0.11 mg/mL, respectively, with no significant difference between the two enzymatic extracts ($p>0.05$). In contrast, the ABTS radical scavenging activity of the ProEx-TPP extract did not reach 50% inhibition within the tested concentration range; therefore, the IC_{50} value could not be determined. At the highest tested concentration (100 mg/mL), the extract showed 18.41 ± 0.45% inhibition, indicating relatively weak antioxidant activity compared to the enzymatic extracts. This lower activity is consistent with its lower TPC and TFC values.

The enzymatic extracts exhibited stronger antioxidant activity together with higher TPC and TFC values, suggesting a possible contribution of phenolic and flavonoid compounds to radical scavenging activity. Previous studies have similarly reported that black soybean products with higher phenolic and flavonoid contents tend to exhibit greater antioxidant activity (Furuta *et al*., 2003). In addition, soybeans contain bioactive peptides composed of 2–20 amino acids, which contribute to antioxidant activity (Hsin *et al*., 2005). Similarly, the higher amino acid content observed in both enzymatic extracts may also contribute to their enhanced antioxidant capacity, which is consistent with previous findings reported by Bhartiya *et al*. (2020).

The differences observed between DPPH and ABTS assay results may be attributed to the distinct mechanisms of each assay. The DPPH assay is typically conducted in organic solvents and primarily reflects the activity of lipophilic antioxidants, whereas the ABTS

Table 6. Antioxidant capacity, total phenolic content (TPC) and total flavonoid content (TFC) in black soybean okara protein isolates under different extraction conditions.

Okara protein isolate	TPC, mg GAE/g sample	TFC, mg CE/g sample	DPPH (IC_{50}), mg/mL	ABTS (IC_{50}), mg/mL	ABTS inhibition (%) at 100 mg/mL
ProEx-P6SD	27.93 ± 0.13^{a}	1.73 ± 0.00^{a}	1.44 ± 0.01^{b}	9.34 ± 0.04^{a}	–
ProEx-A2SD	27.80 ± 0.20^{a}	1.21 ± 0.01^{b}	1.12 ± 0.20^{b}	9.61 ± 0.11^{a}	–
ProEx-TPP	0.79 ± 0.04^{b}	0.03 ± 0.01^{c}	11.83 ± 0.19^{a}	n.d.	18.41 ± 0.45
Ascorbic acid	–	–	0.004 ± 0.00^{c}	0.22 ± 0.00^{c}	–
α-tocopherol	–	–	0.02 ± 0.00^{c}	0.65 ± 0.01^{b}	–
BHT	–	–	0.12 ± 0.00^{c}	0.48 ± 0.00^{bc}	–

Mean values in the same column with different letters are significantly different ($p<0.05$); n.d.: IC_{50} could not be determined as the sample did not reach 50% inhibition within the tested concentration range.

assay is applicable in both aqueous and organic systems and can detect both hydrophilic and lipophilic compounds (Winitchai *et al.*, 2021). Therefore, further analysis is required to identify the specific antioxidant compounds present in Sukhothai 3 black soybean okara extracts.

CONCLUSION

This study evaluated Sukhothai 3 black soybean okara as a source of functional proteins using protease-assisted extraction and three-phase partitioning (TPP). Although TPP achieved a higher extraction yield, protease-assisted extraction produced protein isolates with higher essential amino acid content, protein solubility, and antioxidant activity. These findings suggest that protease-assisted extraction is a more suitable approach for producing functional protein isolates from black soybean okara. The results support the utilization of black soybean okara as a value-added ingredient for food and nutraceutical applications. Further optimization of the extraction process is recommended to improve industrial applicability.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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