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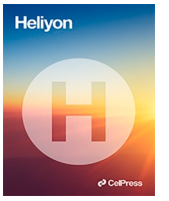

Research article

# Comparison of characteristics and antioxidant activities of sesame protein hydrolysates and their fractions

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ABSTRACT

Sesame meal is a by-product obtained from oil extraction. We investigated the characteristics and antioxidant activities of a sesame protein hydrolysate (SPH–B), as well as its peptide fractions. Four peptide fractions (F1; >100 kDa, F2; 10–100 kDa, F3; 1–10 kDa, and F4; <1 kDa) of SPH-B were prepared. The characteristics and antioxidant properties of SPH-B and its peptide fractions were evaluated. Sesame protein (SP) contained protein fractions with molecular weights ranging from 10 to 44 kDa, whereas SPH-B had peptide fractions ranging from 8 to 44 kDa. The peptide fractions had molecular weight ranging from 7 to 10 kDa. The four peptide fractions had a higher α-helix content and lower surface hydrophobicity than SPH-B and SP. They exhibited better antioxidant properties, with higher ABTS and DPPH radical scavenging activities, higher metal chelating activity, and greater inhibition of linoleic acid peroxidation, suggesting that sesame peptide fractions can use as plant-based functional ingredients and potentially health-promoting properties.

## 1. Introduction

In recent times, the exploration into antioxidant peptides sourced from plant proteins has garnered significant attention for their potential application as preservatives or nutraceuticals in foods and beverage industry [1]. These plant-based antioxidants present a promising alternative to animal-derived or synthetic counterparts for incorporation as functional food ingredients. Concerns regarding animal-derived ingredients stem from their environmental footprint and implications on animal welfare associated with livestock production [2]. There is also concern about the use of some types of synthetic antioxidant because of their potentially adverse health effects [3]. Plant-derived antioxidant peptides may be able to overcome some of these challenges. In recent years, food by-products and waste streams have been investigated as potential sources of plant proteins for producing antioxidant peptides, with the aim of reducing food waste, decreasing pollution, and increasing the economic value of the food supply chain [4].

Sesame (*Sesamum indicum*) is widely grown in tropical regions around the world, including India, Sudan, China, Burma, and Thailand. The annual global production of sesame seeds in 2021 was reported to be over 6.3 million metric tons, with the major

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production areas being Africa (3.8 million metric tons) and Asia (2.3 million metric tons). Sesame consumption is steadily increasing because it is a good source of valuable nutrients, including lipids, proteins, vitamins, minerals, and dietary fibers [5]. Sesame has one of the highest oil contents of any seed (48–55%) and is therefore primarily used commercially for oil production. However, it is also a good source of plant protein, with levels varying between about 20 and 25% [6]. Sesame meal is obtained as the primary by-product of sesame oil processing, which is generally used as animal feed due to its high protein content (>30%) and low cost [7]. In addition, the essential amino acid content of sesame meal accounts for around 30% of total amino acids. Sesame protein is rich in sulfur-containing amino acids (methionine and cysteine), which are believed to play an important role in its antioxidant activity [5,8].

Antioxidant peptides can be produced by controlled enzymatic hydrolysis of proteins extracted from plants [9]. A wide range of proteases are available for this purpose, including alcalase, flavourzyme, chymotrypsin, pepsin, papain, bromelain, pancreatin, and trypsin [8]. The types, concentrations, and activities of the antioxidant peptides produced depend on the nature of the protein source, enzyme type, and hydrolysis conditions [10]. Peptide sequences with high levels of phenolic, acidic, basic, and/or hydrophobic amino acids have been reported to exhibit good antioxidant activities, which is attributed to their proton/hydrogen donor properties [4,11]. In addition, the antioxidant properties of peptides have been linked to their molecular weights (MWs), with lower MW peptides (<10 kDa) exhibiting stronger antioxidant activities [3,10]. Ultrafiltration has been widely used to obtain low-MW antioxidant peptides (<10 kDa) from plant protein hydrolysates, including those obtained from sesame [3] and rice bran [10]. Moreover, some studies suggest that smaller peptides (<1 kDa) exhibit even better antioxidant properties, including those obtained from mushrooms [8], peas [12], and watermelon seed [4].

Subtilisin A is a protease obtained from *Bacillus licheniformis* that can be used to produce plant protein hydrolysates. It cleaves cleaving internal peptide bonds in polypeptide chains near amino acids with aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl-containing (Ser), and basic (Lys) residues [13]. According to our previous studies, low-MW peptides (<1 kDa) isolated using ultrafiltration exhibited the strongest antioxidant activity. However, there have been no previous studies on the characteristics and antioxidant activities of low-MW peptides (<1 kDa) produced from sesame protein hydrolysates. Therefore, the objective of this study was to investigate the characteristics and antioxidant activities of peptide fractions obtained from sesame meal protein hydrolysate (SMPH) produced by Subtilisin A treatment. This study provides valuable information for the development of plant-derived functional food ingredients with enhanced antioxidant properties.

## 2. Materials and methods

### 2.1. Materials

Sesame meal (SM) was obtained from the Learning Organization and Development Centre of Sesame for Sustainable Agro-Household Industry (Faculty of Agriculture, Ubon Ratchatani University, Thailand). Subtilisin A from *Bacillus licheniformis* (Lot I30602b, E.C.3.4.21.14,>8 tyrosine-equivalent Unites/mg) was purchased from the Magazyme Company (Wicklow, Ireland). All chemical reagents used in the determination of the antioxidant activity of the protein hydrolysates were of analytical grade. Commercial grade hexane was used as a solvent for oil extraction from sesame meal.

### 2.2. Preparation of sesame protein (SP)

Sesame protein (SP) was prepared from sesame meal (SM) using an alkaline-acid extraction method described previously Noptana and Onsaard [14], with some modifications. Briefly, the SM was defatted using hexane (1:5 %w/v) by stirring at room temperature (25 °C) for 1 h. This process was repeated 3 times to improve the efficiency of oil removal. The defatted sesame meal (DSM) was dispersed in distilled water at a ratio of 1:20 (w/v). The resulting DSM dispersion was then adjusted to pH 9 using 1 M or 0.5 M NaOH, stirred for 1 h, and then centrifuged at 28,313×*g* at 4 °C for 15 min to collect the supernatant. The supernatant was adjusted to pH 4.5 with 1 M or 0.5 M HCl, stirred for 30 min, and then centrifuged at 28,313×*g* at 4 °C for 15 min. The precipitate was neutralized to pH 7. The dialyzed proteins were freeze-dried to obtain sesame protein (SP) powder. The chemical compositions of SM, DSM, and SP were determined for the content of protein (6.25 factor), fat, moisture, and ash using the AOAC methods (1999) [15]. The percentage of carbohydrate was calculated as follows: carbohydrate (%) = 100 – (protein + fat + moisture + ash).

### 2.3. Preparation of sesame protein hydrolysate (SPH)

The SPH was prepared using Subtilisin A from *Bacillus licheniformis* (Lot I30602b, E.C.3.4.21.14,>8 tyrosine-equivalent Units/mg) as described by Onsaard et al. [13] with slight modifications. Briefly, SP was dispersed in 50 mM phosphate buffer (pH 8) at a ratio of 1:20 (w/v). The hydrolysis conditions were as follows; an enzyme-to-substrate (E/S) ratio of 2.88 U/g protein; incubation temperature at 60 °C; and incubation time from 30 min to 12 h. The enzyme was inactivated by placing the container in boiling water for 15 min, followed by cooling by immersion in cold tap water. The hydrolysate samples were centrifuged at 28,313×*g* for 15 min at 4 °C. The supernatant was collected and stored at −20 °C until further use.

### 2.4. Fractionation of protein hydrolysates

The sequential fractionation of SPH was prepared using a series of ultrafiltration membranes (KrosFlo® KR2i Tangential Flow Filtration System, Spectrum Labs, USA) with 100, 10, and 1 kDa molecular weight cut-off (MWCO) values at 20 mL/min feed rate. Four

sesame protein hydrolysate fractions (SPHF) with molecular weights >100 kDa (F1), 10–100 kDa (F2), 1–10 kDa (F3), and <1 kDa (F4) were prepared and then freeze dried. The SPHFs were stored at −20 °C before further analysis.

### 2.5. Characteristics of protein hydrolysates and their fractions

#### 2.5.1. Protein solubility

The protein solubility of the sesame protein hydrolysate (SPH–B) was determined using a procedure described by Noptana and Onsaard [14], with some modifications. Protein concentrations were determined using the Lowry method [16] with bovine serum albumin (BSA) as a protein standard. For the total protein, the protein hydrolysate samples were solubilized in 4 M NaOH and stirred for 1 h before the determination of the protein content. The protein solubility of SPH-B was then calculated as follows:

Protein solubility (%) = (Protein content of soluble SPH-B/Total protein) × 100.

#### 2.5.2. Degree of hydrolysis

The degree of hydrolysis (DH) was determined by measuring the soluble protein content after addition of 10% trichloroacetic acid (TCA) to promote protein precipitation, as described by Qi et al. [17]. A SPH-B solution was mixed with an equal amount of 20% TCA solution to obtain a final concentration of 10% TCA. The resulting mixture was then vortexed for 1 min, and then centrifuged at 7600×*g* for 15 min at 25 °C. The concentration of soluble protein (or peptides) in the supernatant was determined using the Lowry method [16] with bovine serum albumin (BSA) as a protein standard. For the total protein, the protein hydrolysate samples were first solubilized in 4 M NaOH and stirred for 1 h before the determination of the protein concentration. The degree of hydrolysis (DH) of the SPH-B samples was calculated as follows:

$$\mathrm{DH}\ (\%) = (\text{soluble protein content after TCA precipitation}\ /\ \text{Total protein content}) \times 100.$$

#### 2.5.3. Molecular weight

The molecular weights of SP, SPH-B, and its peptide fractions were characterized using SDS-PAGE analysis according to Laemmli [18]. A 12% separating gel and 4% stacking gel was prepared in a Mini-Protein 3 cell (Bio-Rad Laboratories Inc, Richmond, CA, USA.). Protein solutions (8 mg/mL) were mixed at a 1:1 (v/v) ratio with sample buffers (0.5 M Tris-HCl pH 6.8, 20% glycerol, 10%SDS, and 0.2% (w/v) bromophenol blue without 2-mercaptoethanol) and boiled for 3 min. Ten microliters of protein solution and 3 μL of a molecular weight marker (BIO-HELIX-PM008-0500, GenScript, Keelung, Taiwan) were loaded in a well and electrophoresis was conducted at a constant current of 200 voltages. Then, the gel was stained overnight in 0.3% (w/v) Coomassie brilliant blue R-250 mixed with 45% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained with 50% (v/v) ethanol and 7.5% (v/v) acetic acid for 2 h, and then again by 5% (v/v) ethanol and 7.5% (v/v) acetic acid for 3 h before drying. The dried gel was then scanned to determine the location and intensity of the protein bands, which was used to calculate the MW.

#### 2.5.4. Color

Color measurements of the samples were made using a colorimeter (UltraScan® Vis, HunterLab, USA). The colorimeter was calibrated using a light trap and a white-surface calibration plate. The measurement conditions used were: reflectance specular excluded (RSEX), area view 0.375 in, and nominal UV filter position. The CIELab color values were recorded as L* (lightness to darkness, 100 to 0), a* (redness to greenness, 0 to +100 = red; −80 to 0 = green), and b* (yellowness to blueness, 0 to +70 = yellow; −100 to 0 = blue). The total color difference (ΔE) between the SP (control) and treated samples was calculated using the equation:

$$\Delta E = \left(\Delta L*^2 + \Delta a*^2 + \Delta b*^2\right)^{1/2}$$

#### 2.5.5. Protein secondary structures

Fourier transform infrared (FT-IR) spectroscopy (Nicolet 6700, Thermo Scientific, USA) was used to provide information about the type and interactions of the functional groups present in the protein samples, according to the method of Onsaard et al. [13], with some modifications. Briefly, FT-IR spectra were measured range from 4000 to 400 $cm^{-1}$ with 32 scans per sample and a resolution of 6 $cm^{-1}$ at ambient conditions. The amine I band (1600-1700 $cm^{-1}$) of the RBPHFs and SPHFs were analyzed using Peakfit software to calculate the proportions of secondary structures.

#### 2.5.6. Surface hydrophobicity

The surface hydrophobicity ($SH_o$) was determined according to the method described by Singh et al. [19]. Briefly, 4 mL of protein fraction samples with different protein concentrations (0.02–0.1 mg/ml) were prepared in 0.1 M phosphate buffer (pH 7). Then, 20 μL of 8 mM 1-anilino-8-naphthalenesulfonate (ANS) solution in the same buffer was added. Then the fluorescence intensity was measured using a spectrofluorometer (LS-55, PerkinElmer, UK) at 390 nm (excitation) and 470 nm (emission) wavelengths. The initial slope of the fluorescence intensity against protein concentration (mg/ml) was calculated and considered to be the $SH_o$.

### 2.6. Determination of antioxidant properties

The antioxidant properties of the proteins and peptides were characterized using a number of different assays.

#### 2.6.1. ABTS radical scavenging activity assay

The ABTS assay was carried out according to the method described previously Re et al. and Thammarathip et al. [11,20]. An $ABTS^{+}$ solution was prepared by mixing 7 mM of ABTS and 2.45 mM of potassium persulfate solutions at a ratio of 1:0.5, and then storing the mixture in the dark at room temperature for 12–16 h. This solution was then diluted with phosphate buffer saline (pH 7.4) to obtain an absorbance of $0.700 \pm 0.02\ cm^{-1}$ at 734 nm. After dilution, 25 μL of peptide fractions (0.5–6 mg/ml) or distilled water (control) were mixed with 2.5 mL of $ABTS^{+}$ solutions followed by incubating at room temperature for 6 min. The positive control was used at concentrations of about 0.01–0.4 mg/ml of ascorbic acid. The absorbance was then measured at 734 nm using a UV–visible spectrophotometer (PG Instruments Limited, T60UV/Visible, USA). The ABTS radical scavenging activity of the samples was expressed as the percentage of inhibition:

$$\text{ABTS radical scavenging}\ (\%) = \left[\left(A_{control}\text{-}A_{sample}\right) \,/\, A_{control}\right] \times 100$$

Here, $A_{control}$ and $A_{sample}$ are the measured absorbances of the control and sample, respectively.

#### 2.6.2. DPPH radical scavenging assay

The DPPH radical scavenging activity of the protein fractions was measured according to a method described previously Girgih et al. and Lu et al. [3,21]. Initially, peptide samples were dissolved in 0.1 M sodium phosphate buffer (pH 7). Then these peptide solutions (1 mL, 0.1–1 mg/mL) and positive control (1 mL, 0.5–50 μg/mL of ascorbic acid) were mixed with 1 mL of 100 μM DPPH in 99.9% methanol. The mixture was incubated in the dark at room temperature for 30 min and the absorbance at 517 nm was then measured using a UV-VIS spectrometer (PG Instruments Limited, T60UV/Visible, USA). The control sample consisted of sodium phosphate buffer and DPPH in place of the peptide fraction samples. Peptide fraction samples mixed with sodium phosphate buffer were used as backgrounds. The DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging}\ (\%) = \left[\left(A_{control}\text{-}\left(A_{sample}\text{-}A_{background}\right)\right) \,/\, A_{control}\right] \times 100$$

Here, $A_{control}$, $A_{sample}$ and $A_{background}$ are the measured absorbances of the control, sample, and background, respectively.

#### 2.6.3. Metal chelating activity

The ability of the peptide fraction samples to chelate ferrous ions was determined according to the method described by Phongthai et al. [22]. Peptide fractions solutions (1 mL, 1 mg/ml) or positive control (1 mL, 1 mg/ml of ascorbic acid) were mixed with 3.7 mL of distilled water, and then 0.1 mL of 2 mM ferrous chloride ($FeCl_2$) solution and 0.2 mL of 5 mM ferrozine solution were added and the sample was vigorously mixed using a vortex device. The mixtures were then incubated at room temperature for 10 min before measuring their absorbance at 562 nm using a UV–visible spectrophotometer. Ethylenediaminetetraacetic acid (EDTA) was used as a standard. The values are expressed as mmol EDTA equivalent/g sample.

#### 2.6.4. Linoleic peroxidation inhibition activity

A linoleic acid peroxidation inhibition assay was performed according to a method described by de Castro and Sato [9]. Protein fractions and ascorbic acid (positive control) were diluted in distilled water to obtain a final volume of 10 mL at a concentration of 2 mg/ml. The peptide fraction solution or distilled water (control) or positive control were combined with 130 μL of linoleic acid and 10 mL ethanol, and then the total volume was adjusted to 25 mL using distilled water. The resulting mixture was then incubated at 42 °C for 24 h in the dark. Afterwards, an antioxidant activity assay was carried out by adding 0.1 mL of sample to 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride. This mixture was then allowed to stand at room temperature (25 °C) for 3 min. The absorbance was read at 500 nm and the lipid peroxidation inhibition activity of fraction samples was calculated as follows:

$$\text{Inhibition}\ (\%) = \left[\left(A_{control}\text{-}A_{sample}\right) \,/\, A_{control}\right] \times 100$$

Here, $A_{control}$ and $A_{sample}$ are the measured absorbances of the control and sample, respectively.

### 2.7. Statistical analysis

All experiments were conducted in three replicates. Averages and standard deviations were reported. Data were analyzed by analysis variance (ANOVA) and the mean comparison was used Duncan's multiple range test at $p < 0.05$ using SPSS software version 15.

## 3. Results and discussions

### 3.1. Chemical composition of sesame meal, defatted sesame meal, and sesame protein

The chemical compositions of SM, DSM, and SP are shown in Table 1. The sesame meal mainly consisted of fat and protein. After defatting, the fat content decreased, and the protein content increased. The sesame protein extracted from the DSM using the alkaline-acid approach had a higher protein content than the DSM. The protein content of the sesame protein was similar to that reported for

black sesame protein (80.4%), red sesame protein (79.9%), and white sesame protein (80.9%) using the alkali-acid precipitation extraction method [6].

### 3.2. Protein solubility

The effect of reaction time (0–12 h) on the protein solubility of the SPH-B samples was measured (Fig. 1A). The protein solubility increased with increasing reaction time, being 32, 36, 38, and 45% after 0.5, 1, 3, and 6 h, respectively. The protein solubility did not increase further when the reaction time was raised from 6 to 12 h, which suggests that all the large insoluble protein matter had been removed by hydrolysis. In general, the increased protein solubility can be related to the conversion of large protein molecules into smaller peptide molecules by Subtilisin A. Singh et al. [19] also reported that the solubility of rice bran protein hydrolysate treated with papain increased when the reaction time was raised from 30 to 150 min. Furthermore, Shahbal et al. [23] reported that the solubility of pea, hemp, oat, and rice protein hydrolysates treated with alcalase increased when the reaction time was raised from 0 to 300 min. From these results, a reaction time at 6 h was selected to produce protein fractions in the remainder of the study.

### 3.3. Degree of hydrolysis

The effect of reaction time (0–12 h) on the degree of hydrolysis of the sesame protein hydrolysate is shown in Fig. 1B. The DH value increased from around 6.81 to 13.00% when the reaction time was increased from 0 to 12 h. The degree of hydrolysis increased rapidly during the initial stages of hydrolysis (0–1 h) but then increased more slowly during the later stages (from 1 to 6 h). Like the protein solubility, the DH was not significantly different between 6 and 12 h, the change in degree of protein hydrolysis with time followed a similar trend to the change in protein solubility. The reason that the DH did not increase after 6 h may have been because all of the susceptible peptide bonds had already been cleaved. The proteases produced by *Bacillus licheniformis* can cleave internal peptide bonds in polypeptide chains near specific amino acids: Phe, Trp, Tyr, Glu, Lys, Met, Leu, Ala, and Ser [13]. Once all of these bonds have been cleaved, then the hydrolysis reaction stops.

### 3.4. Characteristics of sesame meal protein hydrolysates and their fractions

#### 3.4.1. Molecular weight

The molecular weight (MW) profiles of SP, SPH-B, and the peptide fractions obtained through ultrafiltration are shown in Fig. 2A. Under non-reducing conditions, SP displayed protein bands with MWs ranging from 11 to 48 kDa, while SPH-B exhibited protein bands spanning from 5 to 45 kDa. The SPH-B fractions F1 toF3 revealed protein bands with the 5–11 kDa range, whereas the SPH-B-F4 fraction did not displayed any discernible protein bands. These finding indicate the enzyme in hydrolyzing sesame protein and demonstrate that ultrafiltration successfully fractionated the produced peptides based on their molecular dimensions. Singharaj and Onsaard [6] reported sesame protein bands, extracted in an alkaline solution at pH 10 and precipitated at pH 4.5, ranged from 42 to 83 kDa under non-reducing conditions and from 12 to 39 kDa under reducing conditions. Gul et al. [5] identified four strong bands of sesame protein (extracted in alkaline solution at pH 10 and then precipitated at pH 4.5) with MW ranges of 37–50, 25–37, 20, and <15 kDa under reducing conditions. According to the literature, 80–90% of sesame proteins are water-soluble 11S globulins (α-globulin) and 2S albumins (β-globulin), while 1–2% consists of 7S globulins [5]. Sesame seed 11S globulins are composed of acidic and basic subunits with MWs around 30.5–33.5 kDa and 20.0–24.5 kDa, respectively. The 2S albumins in sesame seed have MWs around 13–15 kDa and 7S globulins have MWs ranging from 12.4 to 65.5 kDa [5,24]. These finding suggest that sesame proteins consist of polypeptide chains linked by disulfide bonds. When hydrolyzed by the protease, only peptides with MWs below 12 kDa were observed, indicating cleavage of the 2S albumins. The ultrafiltration process influenced the molecular weight (MW) patterns due to the use of membranes with distinct molecular weight cut-offs. These membrane selectively permitted peptides below a certain size to pass through. Similar outcomes were reported by Wu et al. [25] in their study on soy protein hydrolysates subjected to different ultrafiltration membranes.

#### 3.4.2. Color

The tristimulus color coordinates of SP, SPH-B, and their peptide fractions are shown in Table 2. The L*, a*, and b* values of SP were 26.65, 1.61, and 2.36, respectively. After hydrolysis by protease (SPH–B), the L*, a*, and b* values changed, suggesting that the enzyme treatment increased the lightness, redness, and yellowness of the samples. The L*, a*, and b* values of the peptide fractions

**Table 1**
Chemical composition of sesame meal (SM), defatted sesame meal (DSM) and sesame protein (SP).

| Proximate composition | SM | DSM | SP |
|---|---|---|---|
| Moisture | $4.95 \pm 0.20^{a}$ | $4.93 \pm 0.12^{a}$ | $0.70 \pm 0.18^{b}$ |
| Protein | $31.85 \pm 0.43^{c}$ | $42.62 \pm 0.82^{b}$ | $81.07 \pm 0.57^{a}$ |
| Fat | $32.80 \pm 0.02^{a}$ | $3.38 \pm 0.12^{b}$ | $1.96 \pm 0.13^{c}$ |
| Ash | $8.03 \pm 0.17^{b}$ | $10.97 \pm 0.24^{a}$ | $2.81 \pm 0.07^{c}$ |
| Carbohydrate | $22.37 \pm 0.38^{b}$ | $38.10 \pm 0.85^{a}$ | $13.45 \pm 0.33^{c}$ |

The results are presented as the mean ($n = 3$) ± SD, and those with different letters are significantly different ($p < 0.05$) within the same row.

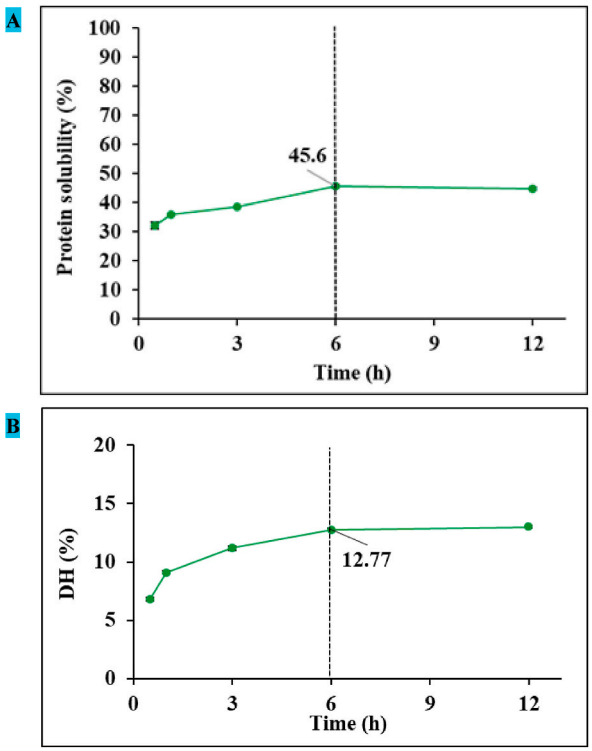


**Fig. 1.** Protein solubility (A) and degree of hydrolysate (B) of sesame protein using protease (Subtilisin A) from *Bacillus licheniformis* as follows; an enzyme-to-substrate (E/S) ratio of 2.88 U/g protein and incubation temperature at 60 °C for 30 min to 12 h at pH 8.

were even higher increasing. The smaller peptide (SPH–B–F4) fraction had the highest L*, a*, and b* values. The ΔE value of the SP was 70.82 and the ΔE value of SPH-B, and their peptide fractions decreased after hydrolysis and membrane ultrafiltration. The increase of L* values indicated that the brightness of the SP increased after hydrolysis and ultrafiltration, which may have been because smaller particles were formed in the powder after freeze drying, which led to stronger light scattering [24]. The increase in the a* (redness) and b* (yellowness) values after hydrolysis and ultrafiltration may have been due to greater susceptibility of the amino acids in the smaller peptides to chemical degradation (because they are more exposed to the surrounding environment), as well as changes in non-protein components. For instance, there may have been some brown pigments formed via the Maillard reaction [26], as well as due to degradation of flavonoids and other polyphenols [27]. Overall, these results showed that hydrolysis and ultrafiltration altered the color of the peptides produced, which may have important commercial implications for some food applications.

### *3.4.3. Protein secondary structures*

Differences in the secondary structures of the SP, SPH-B, and peptide fractions were determined using FT-IR spectroscopy. Secondary structures like α-helix, β-sheet, and β-turn form in polypeptide chains due to localized hydrogen bonding between different amino acids. These hydrogen bonds form between peptide N–H donors and C═O acceptors [13]. The FT-IR spectra of SP, SPH-B, and their peptide fractions were measured at wavelengths ranging from 1000 to 2000 $cm^{-1}$ (Fig. 3A) The protein secondary structure of all the proteins showed an amide I band (1600-1700 $cm^{-1}$), amide II band (1500-1600 $cm^{-1}$), and amide III band (1200-1400 $cm^{-1}$). The Amide II band is due to the stretching vibrations of C–N (18–40%), while the amide III band is due to the bending stretching vibrations of N–H (4–60%) and stretching vibrations of C–N (18–40%) [13,26]. The amide II band of SP, SPH-B, and their peptide fractions (F1–F4) were observed at wavelengths of 1513.85, 1529.27, 1521.56, 1531.20, 1519.63, and 1531.20 $cm^{-1}$, respectively. Meanwhile, the amide III bands of SP, SPH-B, and their peptide fractions (F1–F4) were observed at 1394.28, 1390.42, 1386.57, 1388.50, 1388.50, and 1388.50 $cm^{-1}$ for the bending stretching vibrations of N–H and at 1234.22, 1234.22, 1230.26, 1240.00, 1234.22, and 1230.36 $cm^{-1}$ for the stretching vibration of C–N, respectively (Fig. 3A). The amide II and amide III regions of their peptide fractions had lower peaks than the SPH-B and SP, which may be due to changes in the conformation and chemistry of the peptide chains after hydrolysis, ultrafiltration, and dehydration.

The amide I band is the stretching vibration of C═O peptide linkages (~80%), which is sensitive to the formation of β-sheet, α-helix, β-turn, and random coils structures [13]. In general, C═O and N–H groups form intramolecular hydrogen bonds to form α-helix structures, while polypeptide chains form inter-chain hydrogen bonds to stabilize β-sheet structures. The β-turn structures are formed by weakly hydrogen-bonded structures, whereas the unfolded conformation corresponds to random coils and is related to protein flexibility [5]. As seen in Fig. 3B, the wavenumbers of the amide I peak of SP, SPH-B, and their peptide fractions (F1–F4) were observed at 1633.41, 1633.41, 1641.13, 1641.13, 1639.20, and 1637.27 $cm^{-1}$, respectively. The amide I peak in SPH-B was broader

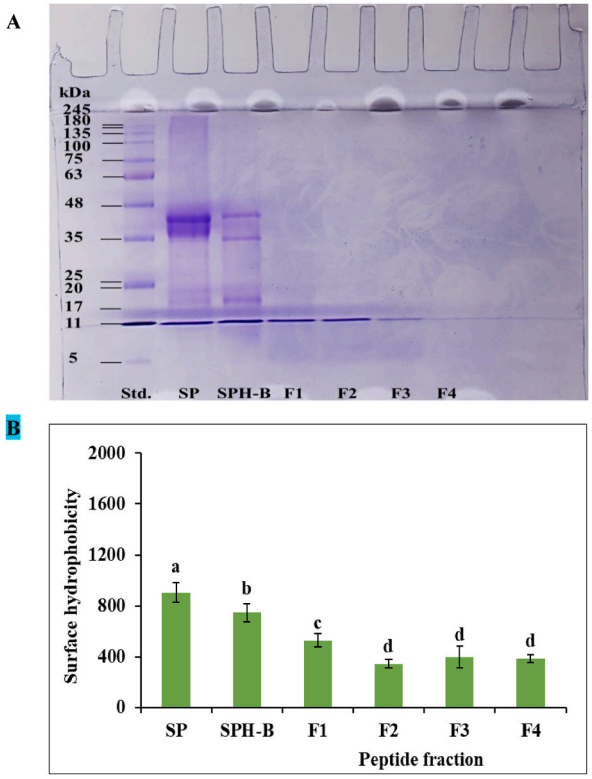


**Fig. 2.** (A) Molecular weight (kDa) and (B) Surface hydrophobicity ($SH_o$) of sesame protein (SP), sesame protein hydrolysate by *Bacillus licheniformis* (SPH–B), and their peptide fractions (F1–F4). The Molecular weight at under non-reducing condition as follows; lane 1: Protein standard, lane 2: SP, lane 3: SPH-B, lane 4: F1(>100 kDa), lane 5: F2 (10–100 kDa), lane 6: F3 (1–10 kDa), and lane 7: F4 (<1 kDa). The $SH_o$ at 0.02–0.1 mg/mL protein concentrate. The different letters mean that the variances in SHo of protein, protein hydrolysate, and peptide fractions from SP are significant ($P < 0.05$). Peptide fractions: F1; >100 kDa, F2; 10–100 kDa, F3; 1–10 kDa and F4; <1 kDa.

**Table 2**
Color of sesame protein (SP), sesame protein hydrolysates by *Bacillus licheniformis* (SPH–B), and their peptide fractions (F1–F4).

| Sample | L* | a* | b* | ΔE* |
|---|---|---|---|---|
| SP | $26.65 \pm 0.01^{e}$ | $1.61 \pm 0.04^{e}$ | $2.36 \pm 0.04^{e}$ | $70.82 \pm 0.00^{a}$ |
| SPH-B | $34.47 \pm 0.38^{d}$ | $4.18 \pm 0.25^{d}$ | $6.93 \pm 1.19^{d}$ | $63.45 \pm 0.53^{b}$ |
| SPH-B-F1 | $38.90 \pm 0.01^{c}$ | $5.44 \pm 0.04^{c}$ | $9.33 \pm 0.02^{c}$ | $59.47 \pm 0.01^{c}$ |
| SPH-B-F2 | $38.69 \pm 1.00^{c}$ | $5.43 \pm 0.05^{c}$ | $9.87 \pm 0.07^{c}$ | $59.76 \pm 0.99^{c}$ |
| SPH-B-F3 | $43.32 \pm 0.62^{b}$ | $6.97 \pm 0.63^{b}$ | $12.11 \pm 1.40^{b}$ | $55.84 \pm 0.21^{d}$ |
| SPH-B-F4 | $57.61 \pm 1.16^{a}$ | $11.69 \pm 0.21^{a}$ | $21.82 \pm 0.49^{a}$ | $46.77 \pm 0.72^{e}$ |

The results are presented as the mean ($n = 9$) ± SD, and those with different letters are significantly different ($p < 0.05$) within the same columns. Peptide fractions: F1; >100 kDa, F2; 10–100 kDa, F3; 1–10 kDa and F4; <1 kDa.

than that in SP. Similar findings were reported for rice bran protein hydrolysates [19]. Moreover, the amide I peak was even broader for the peptide fractions, which may have been due to progressive disruption of hydrogen bond formation with increasing hydrolysis. Comparison between the peptide fractions, showed that the highest peak was observed for the SPH-B-F4 fraction. Presumably, some of the ordered secondary structure in the protein was converted into disordered structures in the peptides [19]. The secondary structure of protein can be evaluated from the relative area of peaks at different absorption bands, which are β-sheet (1600-1640 $cm^{-1}$), random coil (1640-1650 $cm^{-1}$), α-helix (1650-1660 $cm^{-1}$) and β-turn (1660-1700 $cm^{-1}$) (Fig. 3C). Therefore, the different relative proportions of the secondary structure components in the proteins and peptides were determined. The β-sheet content of SP (83.06%) was higher than SPH-B (79.55%), SPH-B-F1 (68.65%), SPH-B-F2 (66.98%), SPH-B-F3 (59.72%) and SPH-B-F4 (58.92%). The α-helix content of the SPH-B-F4 (23.98%) and SPH-B-F3 (23.69%) were higher than that of the SPH-B-F2 (15.70%) and SPH-B-F1 (15.11%). Interestingly, no α-helix structure was observed in the SPH-B and SP samples. There were also differences in the β-turn contents of the proteins and peptides: SPH-B (20.45%) > SPH-B-F2 (17.32%) ≈ SPH-B-F4 (17.11%) > SP (16.94%) ≈ SPH-B-F3 (16.59%) ≈ SPH-B-F1 (16.24%). Surprisingly, random coil structures were not found in any of the proteins.

In summary, the majority of the secondary structure in the SP, SPH-B, and peptide fractions was β-sheet. The application of

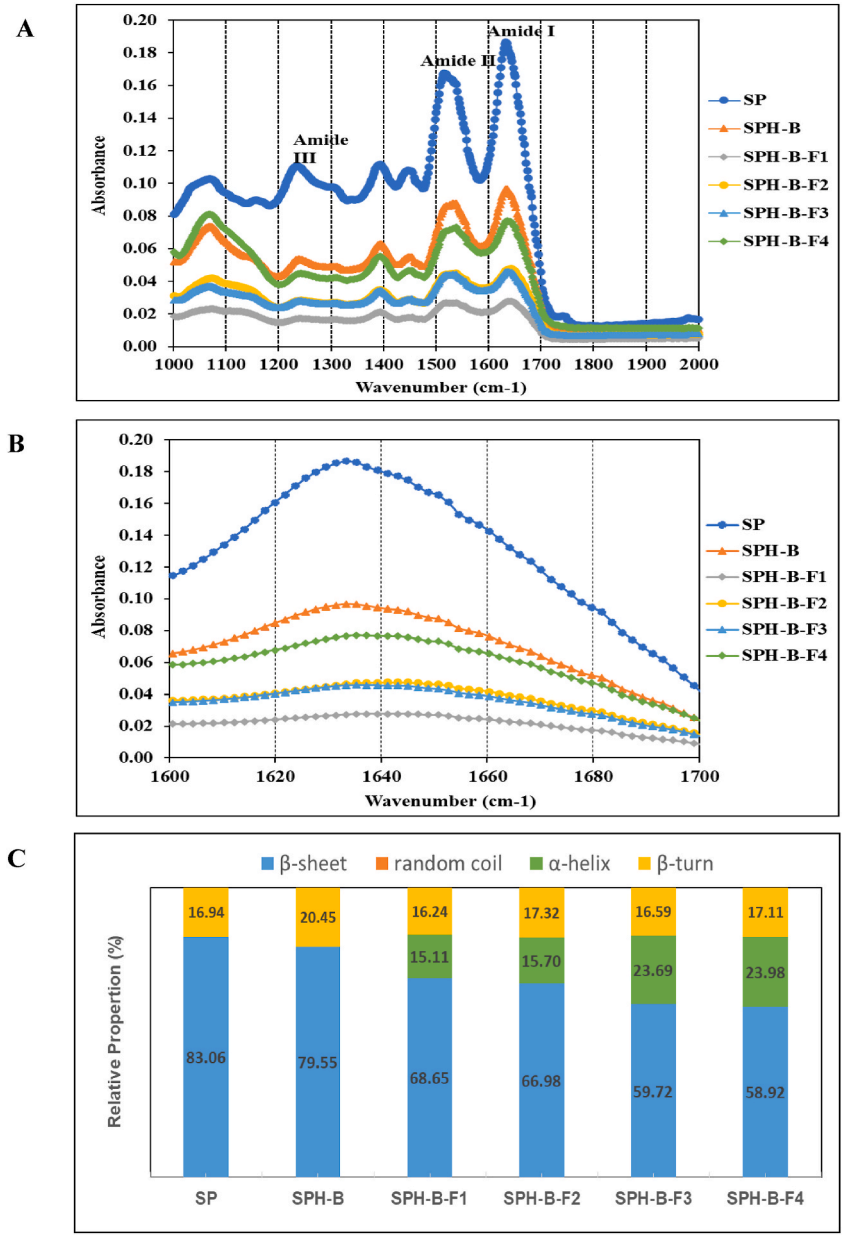


**Fig. 3.** Fourier transform infrared (FT-IR) spectroscopy of sesame protein (SP), sesame protein hydrolysate by *Bacillus licheniformis* (SPH–B), and their peptide fractions (F1–F4). (A) showing the amide I, II and II bands at 1000–2000 $cm^{-1,}$ (B) showing the amide I bands at 1600–1700 $cm^{-1}$, and (C) and relative proportion of secondary structure components [β-sheet, α-helix, β-turn and random coil]. Peptide fractions: F1; >100 kDa, F2; 10–100 kDa, F3; 1–10 kDa and F4; <1 kDa.

ultrafiltration decreased the β-sheet content and increased the α-helix content, suggesting that the smaller peptides had different secondary structures to the larger ones. In contrast to us, Gul et al. [5] reported the secondary structure of sesame protein to be β-sheet (20.45%), α-helix (45.58%), β-turn (17.65%), and random coil (16.32%). This difference may be due to differences in protein extraction methods, or due to differences in the methods used to measure and calculate the secondary structure content of the proteins. Similar to us, Singh et al. [19] reported that the β-sheet content of rice bran protein decreased after hydrolysis. Moreover, Zang et al. [28] reported that enzymatic hydrolysis decreased the β-sheet content and increased the β-turn content of rice bran protein.

### *3.4.4. Surface hydrophobicity*

The surface hydrophobicity ($SH_0$) of a protein or peptide is related to the number of hydrophobic groups present at their surfaces [8]. An ANS probe that binds to non-polar surface groups was used as a fluorescent probe to measure $SH_0$ (Fig. 2B). The surface hydrophobicity of SPH-B and its peptide fractions was significantly lower than that of the sesame protein. This result suggests that

enzyme hydrolysis reduced the number of exposed hydrophobic groups on the surfaces of the peptides, thereby decreasing the binding of the ANS probe [28]. Other researchers have also reported that the surface hydrophobicity of proteins decreases with increasing degree of hydrolysis [19,29]. The surface hydrophobicity of the peptide fractions obtained by ultrafiltration (SPH–B–F1 to SPH-B-F4) was significantly ($p < 0.05$) lower than that of SPH and SP. However, the surface hydrophobicities of the SPH-B-F2 to SPH-B-F4 fractions were not significantly different.

Overall, these experiments show that the surface hydrophobicity of the smaller peptides was lower than that of the larger ones, which is consistent with previous studies [25,30]. This effect may have been because larger polypeptide chains can adopt conformations where clusters of non-polar amino acids are formed, leading to a larger hydrophobic region for the ANS to bind to Mundi and Aluko [30] reported a decrease in surface hydrophobicity for kidney bean peptides as their molecular weight decreased, whereas Wu et al. [25] reported a similar effect for soybean protein. In contrast, Kaprasob et al. [8] reported that smaller king boletus mushrooms peptides (<1 kDa) had higher surface hydrophobicities than larger ones (>10 kDa). These results suggest that the specific peptides formed by hydrolysis influence the surface hydrophobicity, which is probably because they have different non-polar amino acid sequences and polypeptide conformations.

### 3.5. Antioxidant properties

In our comprehensive analysis of the antioxidant capabilities of sesame proteins and their derived peptides, various assays were employed to elucidate their potential.

#### 3.5.1. ABTS radical scavenging activity

The ABTS radical scavenging assay, a method predicated on the transfer of hydrogen atoms and electrons between the ABTS + radical and the test substances, served as a cornerstone for evaluating the antioxidant prowess of both lipophilic and hydrophilic peptides [8,10]. Through this assay, we assessed the efficacy of SPH-B and its peptide fractions against ascorbic acid, a known antioxidant benchmark. The findings, summarized in Table 3, illustrate a clear hierarchy in antioxidant activity, with ascorbic acid leading due to its minimal $EC_{50}$ value, signifying unparalleled ABTS + scavenging ability.

The results from Table 3 indicate that ascorbic acid, used as a standard, has a significantly lower $EC_{50}$ value for $ABTS^{+}$ scavenging activity, suggesting it possesses a much higher antioxidant capability compared to the sesame protein hydrolysates by *Bacillus licheniformis* (SPH–B) and its peptide fractions (F1–F4). The decreasing $EC_{50}$ values from SPH-B through to SPH-B-F4 (SPH–B–F4 (<1 kDa, EC50 = 1.48 mg/mL) > SPH-B-F3 (1–10 kDa, EC50 = 1.67 mg/mL), > SPH-B-F2 (10–100 kDa, EC50 = 1.73 mg/mL) > SPH-B-F1 (>100 kDa, EC50 = 1.89 mg/mL) > SPH-B (EC50 = 2.36 mg/ml) demonstrate an inverse relationship between peptide size and antioxidant activity, with smaller peptides showing greater efficacy. This pattern highlights the importance of molecular size in the antioxidant potential of peptides, where smaller peptides more effectively neutralize free radicals. These observed results might be attributed to the smaller peptides ability to expose more antioxidant amino acids in the aqueous phase, enhancing their interaction with the ABTS + probe. Such findings align with previous studies, which have substantiated the superior free radical scavenging capabilities of smaller peptides, as evidenced in studies involving mushroom protein hydrolysates [8], pea protein hydrolysates [12], and watermelon seed protein hydrolysates [4].

Additionally, the observed results could be partially explained by the potential synergistic interplay between polyphenols and peptides, or due to the presence of peptides with phenolic groups. Extensive research has identified sesame as a rich source of bioactive compounds, notably polyphenols and bioactive peptides. These polyphenols, including sesamin, sesamolin, and sesamol, are renowned for their potent antioxidant properties, which confer cellular protection against oxidative stress, while also manifesting anti-inflammatory and anticancer effects [27,31]. In parallel, bioactive peptides derived from sesame protein hydrolysates have been shown to exhibit a range of beneficial activities, encompassing antioxidant effects, ACE inhibition, and anti-inflammatory properties [32]. Consequently, it remains challenging to definitively ascertain whether the antioxidant activity is solely attributable to peptides, arises from a synergistic effect between polyphenols and peptides, or is due to peptides possessing phenolic groups.

In summary, these experiments underscore the potential of ultrafiltration as a means to enhance the antioxidant activity of peptides. The results suggest that smaller peptides are more proficient in scavenging free radicals compared to their larger counterparts, and their synergistic effect with polyphenols, along with specific peptides released during the process, significantly bolster antioxidant

**Table 3**
The antioxidant activity of sesame protein hydrolysates by *Bacillus licheniformis* (SPH–B) and their peptide fractions (F1–F4).

| Fraction | $EC_{50}$ | | Metal chelating activity (mmol EDTA/g sample) | Linoleic peroxidation inhibition (%) |
|---|---|---|---|---|
| | $ABTS^{+}$ (mg/mL) | DPPH (μg/mL) | | |
| Ascorbic acid | $0.19 \pm 0.00^{e}$ | $2.42 \pm 0.15^{f}$ | $0.04 \pm 0.00^{f}$ | $18.99 \pm 1.21^{f}$ |
| SPH-B | $2.36 \pm 0.04^{a}$ | $704.813 \pm 9.16^{a}$ | $0.21 \pm 0.01^{e}$ | $32.99 \pm 0.29^{e}$ |
| SPH-B-F1 | $1.89 \pm 0.02^{b}$ | $511.12 \pm 18.52^{b}$ | $0.72 \pm 0.01^{d}$ | $34.55 \pm 0.28^{d}$ |
| SPH-B-F2 | $1.73 \pm 0.07^{c}$ | $210.48 \pm 9.62^{c}$ | $0.98 \pm 0.03^{c}$ | $42.38 \pm 0.60^{c}$ |
| SPH-B-F3 | $1.67 \pm 0.02^{c}$ | $160.20 \pm 6.52^{d}$ | $1.32 \pm 0.01^{b}$ | $46.29 \pm 1.01^{b}$ |
| SPH-B-F4 | $1.48 \pm 0.04^{d}$ | $125.62 \pm 3.25^{e}$ | $1.74 \pm 0.02^{a}$ | $54.53 \pm 0.99^{a}$ |

The results are presented as the mean (n = 9) ± SD, and those with different letters are significantly different ($p < 0.05$) within the same columns. Peptide fractions: F1; >100 kDa, F2; 10–100 kDa, F3; 1–10 kDa and F4; <1 kDa.

activities.

### 3.5.2. DPPH radical scavenging activity

The DPPH radical scavenging activity assay, which assesses the electron transfer from peptides to the DPPH radical, illustrates the antioxidant capabilities of SPH-B and its peptide fractions in comparison to ascorbic acid, as detailed in Table 3.

The DPPH assay outcomes demonstrate that both sesame protein hydrolysates (SPH–B) and their peptide fractions exhibit an ascending scale of antioxidant activity inversely proportional to peptide size, in comparison with ascorbic acid. Ascorbic acid, showcasing the most potent antioxidant efficacy, is marked by the lowest $EC_{50}$ value, while SPH-B and its subdivisions display a spectrum of activity levels, reinforcing the correlation between reduced molecular size and augmented antioxidant capability. The smallest peptides, specifically SPH-B-F4, demonstrate markedly superior antioxidant activity in neutralizing DPPH radicals compared to their larger counterparts, highlighting the efficacy of smaller molecules; SPH-B-F4 (<1 kDa, $EC_{50}$ = 0.13 mg/mL) > SPH-B-F3 (1–10 kDa, $EC_{50}$ = 0.16 mg/mL) > SPH-B-F2 (10–100 kDa, $EC_{50}$ = 0.21 mg/mL) > SPH-B-F1 (>100 kDa, $EC_{50}$ = 0.51 mg/mL) > SPH-B ($EC_{50}$ = 0.70 mg/ml). The superior activity of F4 can be attributed to its smaller peptide size, which may facilitate easier interaction with and neutralization of free radicals. This aligns with existing research suggesting that peptide size and composition are critical factors influencing antioxidant activity. Furthermore, the significantly higher activity of ascorbic acid highlights the robustness of natural antioxidants but also underscores the potential of sesame protein hydrolysates as a complementary natural antioxidant source. Such findings are consistent with reports on other protein hydrolysates, like those from mushroom proteins and pigeon pea [8,33].

### 3.5.3. Metal chelating activity

The metal chelating activity assay evaluates the ability of various peptides to chelate iron, a known potent pro-oxidant. Binding of ferrous ions by peptides results in a decrease in the red color associated with ferrozine-$Fe2^{+}$ complexes [34]. The metal chelating activity of SPH-B and the other peptide fractions compared with ascorbic acid is shown in Table 3. The metal chelating activity results indicate the SPH-B-F4 fraction exhibits the highest activity.

The peptides iron-binding properties decrease with an increase in molecular weight: SPH-B-F4 (<1 kDa, 1.74 mmol EDTA/g sample) > SPH-B-F3 (1–10 kDa, 1.32 mmol EDTA/g sample) > SPH-B-F2 (10–100 kDa, 0.98 mmol EDTA/g sample) > SPH-B-F1 (>100 kDa, 0.72 mmol EDTA/g sample) > SPH-B (0.21 mmol EDTA/g sample), suggesting a superior ability to bind metals, which is a crucial antioxidant mechanism. This suggests that SPH-B-F4 peptides are particularly effective, likely due to their specific composition or size.

The analysis underscores the importance of understanding the relationship between peptide structure and antioxidant activity, suggesting further research into these peptides could uncover new natural antioxidants for food and pharmaceutical applications. Thus, the metal chelating activity of the peptides followed a similar trend to the ABTS and DPPH radical scavenging activities. Our results align with those of Foh et al. [34] who reported that low-MW peptides (<1 kDa) from tilapia had a higher metal chelating activity than higher MW peptides. Conversely, studies on protein hydrolysates from peanut kernels [35], kidney beans [30], and rice brans [10] found that smaller peptides were less effective in metal binding. This result may be because the metal binding activity of peptides depends on their amino acid sequence, chain length, and conformation, which depends on protein source, protease, and hydrolysis conditions. For instance, peptides containing high levels of glutamic acid, aspartic acid, histidine, lysine, and arginine are known to interact strongly with metal ions [4,10].

### 3.5.4. Lipid peroxidation inhibition activity

Lipid peroxidation is a major problem in the food industry since it adversely affects the quality, shelf life, and safety of foods [8,33].

For this reason, we examined the ability of the different peptides to inhibit lipid peroxidation using linoleic acid as a model unsaturated fatty acid.

The lipid peroxidation inhibition activity showcased in Table 3 reveals that the peptides derived from sesame protein hydrolysates, particularly SPH-B-F4, exhibit significant antioxidant capabilities: SPH-B-F4 (54.5%) > SPH-B-F3 (46.3%) > SPH-B-F2 (42.4%) > SPH-B-F1 (34.6%) > SPH-B (33.0%). This suggests a correlation between peptide composition or size and their efficacy in preventing lipid peroxidation, a key factor in oxidative stress. The ascending activity from SPH-B to SPH-B-F4 underlines the potential of smaller peptides in antioxidant applications, necessitating further investigation into their structural properties and functional mechanisms for potential use in food and pharmaceutical industries. Our results agreed with those of Kaprasob et al. [8] who reported that the low-MW peptide (<1 kDa) of mushroom protein hydrolysate exhibited the highest inhibitory activity on linoleic acid oxidation. Moreover, Mundi & Aluko [30] reported that the low-MW peptide (<1 kDa) of kidney bean protein hydrolysate showed the highest inhibition of linoleic acid oxidation as well. In contrast, our results differed from those of Olagunju et al. [33] who reported that pigeon pea hydrolysates with MW < 5 kDa exhibited stronger linoleic acid oxidation inhibition than those with MW < 1 kDa.

Overall, the antioxidant activity of the sesame peptides increased with decreasing molecular weight. This effect may have been because more antioxidant amino acids were exposed for the smaller peptides, as well as because smaller peptides could diffuse more rapidly and therefore interact with free radicals and other reactants more readily.

## 4. Conclusion

This research elucidates the potential of sesame protein hydrolysates (SPHs) derived from sesame meal, a by-product of sesame oil production, through enzymatic hydrolysis and ultrafiltration, to enhance the functional properties of proteins. Protein fractions within sesame protein (SP) exhibited a molecular weight range from 10 to 44 kDa, and peptide fractions within SPH-B ranged from 8 to 44

kDa, demonstrating significant improvements in solubility and antioxidant activities post-hydrolysis. Notably, SPHs showcased superior antioxidant capability, including ABTS and DPPH radical scavenging activities, increased metal chelating activity, and more effective inhibition of linoleic acid peroxidation. Peptide fractions, particularly those within the 7–10 kDa range, revealed enhanced α-helix content and reduced surface hydrophobicity compared to their precursors. These findings not only underscore the enhanced functional characteristics of sesame protein hydrolysates but also their promising for health-promoting applications in the food industry.

## CRediT authorship contribution statement

**Rodjana Noptana:** Writing – original draft, Methodology. **David Julian McClements:** Writing – review & editing, Resources, Methodology, Conceptualization. **Lynne A. McLandsborough:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Ekasit Onsaard:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## 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.

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