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Antioxidant activity of palm kernel meal protein hydrolysate and characterization of its peptide profile

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Abstract

Palm kernel meal (PKM) is a major by-product of the palm oil industry, and its high protein content is a potential source of value-added functional food or feed. In this study, the total protein from PKM was isolated and hydrolyzed with alcalase enzyme (pH 7.5, 55°C) to obtain palm kernel protein hydrolysate. The results showed that PKM protein hydrolysate after 60 min of hydrolysis exhibited strong radical scavenging activity with IC_{50} values of 5.73±0.23 and 7.84±0.90 μg/mL as determined by 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), respectively. Furthermore, PKM protein hydrolysate was not toxic to mouse L929 fibroblast cells while protecting cells from H_2O_2-induced oxidative damage. Analysis of its peptide profile by liquid chromatography-mass spectrometry (LC-MS/MS) revealed nine peptide sequences with hydrophobic and negatively-charged amino acids with molecular weights ranging from 1,085.13 to 1,292.32 Da. Taken together, alcalase hydrolysate of PKM was found to have potent antioxidant and cytoprotective properties justifying further study for potential development as a functional food/feed.

Keywords: palm kernel meal; protein hydrolysate; antioxidant activity; cytoprotective effect.

Practical application: Palm kernel meal (PKM) protein hydrolysate, produced by degrading PKM proteins into amino acids and peptides, has biological activities such as antioxidant activity. Using alcalase enzyme, PKM protein hydrolysate showed promising antioxidant activity *in vitro* with the ability to protect mouse L929 fibroblast cells from H_2O_2-induced oxidative damage. Antioxidant peptides were identified. These results revealed that PKM protein hydrolysate has potential application as a natural antioxidant compound.

1 INTRODUCTION

Palm (*Elaeis guineensis*) is one of the most abundant and low-cost agricultural plants for oil production in the tropical region (Onoja et al., 2019). During solvent extraction, palm kernel meal (PKM) is obtained as a major by-product and typically contains high amounts of crude protein (16–19%) together with fat (12–20%) and fiber (12–20%) (Ezieshi & Olomu, 2007). PKM has gained attention as a new source of biologically active ingredients with potential for development as a functional food or feed.

A huge number of free radicals can be produced through living metabolic processes. The excessive accumulation of these unstable molecules can lead to intracellular and tissue damage, contributing to a variety of diseases (Valko et al., 2007). In addition, free radical damage is linked to oxidative stress as it arises from an imbalance of reactive oxygen species (ROS) and the antioxidant defense system. Antioxidant compounds can decrease oxidative stress and protect against its harmful effects by directly scavenging these radicals (Lobo et al., 2010). The food and animal feed industries are interested in antioxidants derived from natural sources because of their safety and consumer concern over synthetic antioxidants.

Protein hydrolysates are a complex mixture of peptides and free amino acids of various molecular sizes produced by the biological or chemical degradation of proteins. An increasing number of protein hydrolysates that can alleviate the harmful effects of unpaired molecules have been reported. For example, the tree peony seed protein hydrolysate can reduce Cd-induced oxidative damage, inflammation, and apoptosis in zebrafish embryos (Li et al., 2022). Hydrolyzed whey protein at low concentrations has good antioxidant ability via the Fenton reaction (Vavrusova et al., 2015). Bioactive peptides, the main components of protein hydrolysates, have a wide range of biological activities including antioxidant, antimicrobial, and cytoprotective properties (Coelho et al., 2018; Mechmeche et al., 2017; Tonolo et al., 2020). Bioactive peptides from milk inhibit lipid peroxidation in Caco-2 cells through activation of the NrF2 pathway (Tonolo et al., 2020). Pentapeptides derived from hydrolysates of *Miichthys miiuy* swim bladder can reduce injury caused by H_2O_2-induced stress in HUVECs (Cai et al., 2019). A cytoprotective effect of synthesized peptides from soybean protein hydrolysates against oxidative stress in human intestinal Caco-2 cells was also demonstrated (Zhang et al., 2019).

When palm kernels are mechanically expeller-processed, the resulting palm kernel cake (PKC) contains 5–12% oil, whereas solvent extraction produces PKM which contains 0.5–3% oil (Okeudo et al., 2005). There are several reports of the antioxidant

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activity of PKC protein hydrolysates but fewer on PKM protein hydrolysates. The PKC protein hydrolysate fermented by *Paenibacillus polymyxa* can promote poultry growth without causing side effects (Alshelmani et al., 2016). Trypsin-digested PKC protein hydrolysates exhibit potent antiradical properties (Ng et al., 2013). PKC protein hydrolysate generated using alcalase exhibits both antioxidant and angiotensin-converting enzyme (ACE) inhibitory activity (Ng et al., 2022). This study aimed to assess the antioxidant and cytoprotective activities of PKM protein hydrolysate and analyze its peptide profile.

2 MATERIALS AND METHODS

2.1 Materials

PKM was obtained from a palm oil company in Nakhon Pathom province, Thailand. Protease inhibitor was purchased from Cell Signaling, USA; β-mercaptoethanol was purchased from Bio-rad, USA. Alcalase was purchased from Merck, USA. OPA (Phthaldialdehyde), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reagents were obtained from Sigma Aldrich, USA. Thiazolyl blue tetrazolium bromide BioChemica (MTT) was purchased from Applichem, Germany. Dulbecco's modified eagle medium (DMEM), FBS, and 0.25% trypsin EDTA were obtained from Gibco, UK. HiTrapTM DEAE Sepharose Fast Flow anion exchange column was purchased from Cytiva, Sweden. All other chemicals and reagents were of analytical grade and commercially available.

2.2 Isolation of PKM protein

The PKM was defatted using n-hexane at a 1:30 (w/v) ratio, and its protein was isolated according to the method described by Zarei et al. (2012) with modifications. Briefly, the PKM was dissolved in 0.03 N NaOH solution at a 1:10 (w/v) ratio (pH 11) and stirred at room temperature for 1 h. After centrifugation at 8,820×*g*, the pH of the supernatant was adjusted to 3.5 using 1 M HCl. The precipitate was then obtained by centrifugation at 10,000×g for 10 min. It was immediately dissolved in deionized water (DI water), and the pH was adjusted to 7 using 1 M NaOH. Concurrently, the protease inhibitor was added and the solution was kept at -20°C for further experimentation.

2.3 Preparation of PKM protein hydrolysate

PKM protein hydrolysates were prepared according to the method described by Zarei et al. (2014) with some modifications. First, pH of the PKM protein was adjusted with 1 M NaOH to 7.5, hydrolyzed with alcalase enzyme (1.5 U of enzyme/g protein), and incubated at 55°C for 15, 30, 60, or 120 min. The reaction was heat at 100°C for 10 min to stop and centrifuged at 4,000×*g* for 20 min to collect supernatants and PKM protein hydrolysates.

2.4 Determination of degree of hydrolysis

The degree of hydrolysis (%DH) was estimated according to a previously described method (Nielsen et al., 2001) with minor modifications. Briefly, 36 µL of sample was mixed with 215 µL of freshly prepared O-pthyaldyaldehyde (OPA) reagent. The OPA reagent was composed of 25 ml of 100 mM sodium tetraborate, 2.5 mL of 20% (w/v) SDS solution, 1.0 mL of 40 mg/mL of OPA dissolved in methanol, and 100 µL of β-mercaptoethanol. Subsequently, the prepared solution was incubated for 2 min at room temperature. Absorbance was measured at 340 nm and calculated with Equation 1:

$$\%DH = \frac{A_{sample} - A_{control} - A_{standard}}{A_{sample}} \times 100 \quad (1)$$

2.5 Determination of free radical scavenging activity

2.5.1 DPPH radical scavenging activity

DPPH radical scavenging properties were evaluated according to a previous method (Memarpoor et al., 2013) with minor modification. A volume of 100 µL of sample (concentration of 0.97–500 µg/mL) was mixed with 100 µL of freshly prepared DPPH reagent and incubated for 30 min in the dark. Then the absorbance was measured with a microplate reader at 517 nm. The % decolorization (or % DPPH scavenging) was calculated by using the following formula and IC_{50} was calculated using the GraphPad Prism 7.00 program. L-Glutathione and DI water were used as positive and negative controls, respectively (Equation 2):

$$\%Decolorization = 1 - \left(\frac{A_{sample}}{A_{control}}\right) \times 100 \quad (2)$$

2.5.2 ABTS radical scavenging activity

The scavenging reaction was determined following the protocol described by Re et al. (1999) with some modifications. ABTS radical cations (ABTS+) were generated by reacting ABTS+ stock solution with potassium persulfate. The mixture was incubated in the dark at 4°C for 12–16 h to obtain an absorbance of 0.7 at 734 nm. Before use, the stock solution was diluted with PBS buffer. Subsequently, 50 µL of sample (concentration of 0.97–500 µg/mL) was mixed with 200 µl of ABTS solution, and the absorbance was measured at 734 nm using a microplate reader within 2 min. The %decolorization was calculated using Equation 3, and IC_{50} was calculated using the GraphPad Prism 7.00 program:

$$\%Decolorization = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \quad (3)$$

2.6 Cytotoxicity determination

Mouse L929 fibroblast cells were cultured in DMEM containing 10% FBS and 1% of 10,000 unit/ml penicillin–streptomycin at 37°C with 5% CO_2. The toxicity of each sample on mouse L929 fibroblast cells was estimated by MTT assay as previously described (Ciapetti et al., 1993) with modifications. Briefly, mouse L929 fibroblast cells (1×10^4 cells/well) were seeded in 96-well plates and incubated at 37°C with 5% CO_2

overnight. Subsequently, the medium was discarded, and cells were treated with samples (concentration of 0.97–500 μg/mL) and then incubated for 24 h at 37°C with 5% CO_2. Thereafter, 100 μL of 0.4 mg/mL stock solution of MTT reagent in PBS was added and incubated for 3 h. After that, the MTT solution was removed and 100 μl of 100% DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Cell viability (%) was calculated using Equation 4:

$$\%\text{Cell viability} = \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \tag{4}$$

2.7 Cytoprotective effect determination

After mouse L929 fibroblast cells (1×10^4 cells/well) were grown in DMEM containing 10% FBS and 1% of 10,000 unit/ml penicillin–streptomycin at 37°C and under 5% CO_2 atmosphere, they were incubated with various concentrations of a sample (concentration of 0.97–500 μg/mL) for 24 h. After incubation, the supernatant was removed and cells were exposed to 1,000 μM of H_2O_2 for 3 h. H_2O_2 was then removed, and cells were incubated with 100 μl of 0.4 mg/ml stock MTT solution in PBS at 37°C, 5% CO_2 for 3 h. Subsequently, the MTT solution was removed and crystals were dissolved with 100% DMSO (100 μL). Absorbance was measured at 570 nm, and %cell viability was calculated using Equation 5:

$$\%\text{Cell viability} = \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \tag{5}$$

2.8 Partial purification and identification of peptide profiles

Fast protein liquid chromatography (FPLC) (GE Healthcare, Sweden) was used to fractionate the antioxidant peptides of the PKM protein hydrolysate according to the method described by Kapel et al. (2006) with some modifications. PKM protein hydrolysate (1.25 mg/mL) was injected into a DEAE Sepharose Fast Flow anion exchange column equilibrated with 2.5 mM Tris-HCl buffer (pH 8.0). Peptides were then eluted using a linear gradient of NaCl (0–1 M) in 2.5 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.5 mL/min. Fractions of 1 mL were collected, and protein concentrations were determined by Bradford assay; antioxidant activity was measured by DPPH and ABTS methods. The fraction that showed the strongest antioxidant activity was selected to identify the peptide sequence profile by liquid chromatography-mass spectrometry (LC-MS/MS). The peptide sequence profile of this antioxidant fraction was identified using a Thermo ScientificTM UltiMateTM 3000 RSLC nano system coupled to a SCIEX TripleTOF 6600+ Q-TOF equipped with an OptiFlow Turbo V Ion Source. In brief, the sample was loaded into the electrospray source and subsequently dissolved in solvent A (i.e., 2% acetonitrile and 0.1% formic acid) and solvent B (i.e., 80% acetonitrile and 0.1% formic acid) with a flow rate of 0.3 μl/min. Spectra were analyzed in positive ion reflector mode with mass/charge (*m/z*) ranges of 350–1,500 Da in MS mode and 350–1,000 Da in MS/MS mode. All analyses were performed using the PEAK studio database to obtain peptide sequencing. The net charge and water solubility of identified peptides were determined via the website https://pepcalc.com/peptide-solubility-calculator.php. The primary structure of the peptide was drawn by the website https://pepdraw.com/, and the hydrophobicity (%) and protein family were analyzed by the website https://aps.unmc.edu/prediction.

2.9 Statistical analysis

All measurements were done in triplicate; the results are expressed as the mean±SD performed in three replications. Significant differences between mean values were determined by an analysis of variance using the GraphPad Prism 7.00 software and accepted at $p<0.05$.

3 RESULTS AND DISCUSSION

3.1 Preparation and characterization of PKM protein and protein hydrolysate

PKM protein was isolated by alkaline extraction and acid precipitation, a traditional method of extracting protein from plant seeds and leaves. This technique is affordable and well-suited for mass production (Amagliani et al., 2017). The results showed that %yield of total PKM protein was 0.58±0.01%, and the protein concentration was 2.15±0.05 mg/mL. The protein was then hydrolyzed with alcalase enzyme at 55°C, pH 7 for 15–120 min. The specificity of the enzyme significantly influences the bioactivity and production of protein hydrolysate (He et al., 2019). The alcalase-hydrolyzed proteins have higher protein recovery and lower lipid content than those of hydrolyzed with other enzymes (Kong et al., 2008). As shown in Table 1, the protein concentration of the PKM protein hydrolysate significantly decreased when compared with that of non-hydrolyzed (0 min). Linder et al. (1995) discovered that the most important parameters associated with recovery after hydrolysis of veal bone protein are duration and enzyme concentration. In our study, the decrease in PKM protein hydrolysate concentration was associated with the presence of short-chain peptides and the composition of amino acids. As a result, the amino acid composition and sample matrix components may have a significant impact on reliably detecting protein concentration using the Bradford assay (Noble et al., 2007; Reinmuth-Selzle et al., 2022).

Table 1. Protein concentration of palm kernel meal protein hydrolysate by duration of hydrolysis.

Time of hydrolysis (min)	Protein concentration (mg/mL)
0	2.15±0.11
15	1.80±0.10*
30	1.77±0.01*
60	1.77±0.01*
120	1.75±0.08*

*Statistically significant differences when compared with that of 0 min (p<0.05).

3.2 Degree of hydrolysis

Protease enzymes hydrolyze proteins into short-chain peptides and amino acid residues (Jamdar et al., 2010). In this study, alcalase enzyme was used to hydrolyze PKM protein at optimal pH (at 7.5) and temperature (at 55°C) conditions for 0, 15, 30, 60, or 120 min. The degree of hydrolysis of PKM protein hydrolysate had a range of 0–89.48±0.67% (Figure 1). The degree of hydrolysis increased significantly during the first 15 min; afterward, there was no further significant increase. This could have been due to a drop in enzyme concentration per substrate proportion, a change in temperature of hydrolysis, and the gathering of peptides in the products, including the competitive inhibition between un-hydrolyzed proteins and the peptides that are continually formed (Verma et al., 2017). The results implied that the time of hydrolysis could cause a significant change in the degree of hydrolysis. The degree of hydrolysis remained constant because it reached the plateau indicating complete hydrolysis (Zarei et al., 2012). The time curve may also be due to very fast peptide bond cleavage in the initial period, followed by slowing as the hydrolysis time passed (Doucet et al., 2003).

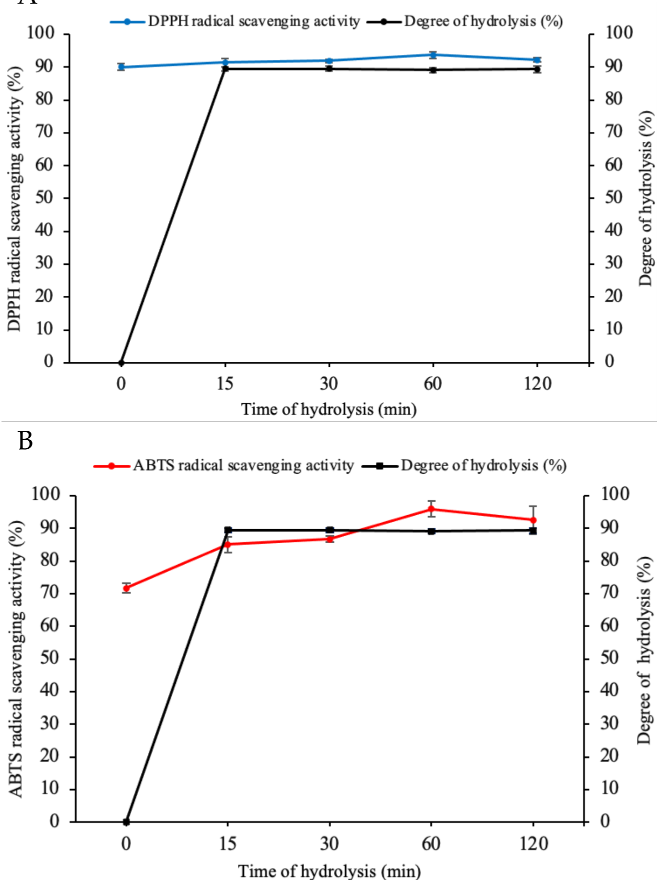


Figure 1. The degree of hydrolysis and antioxidant activity of palm kernel meal protein hydrolysate as determined by (A) 2,2-diphenyl-1-picrylhydrazyl and (B) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assays.

3.3 In vitro antioxidant activity

The antioxidation capability of PKM protein and protein hydrolysate was reported as IC_{50}. It was measured as the concentration of antioxidants required to reduce the initial DPPH concentration by 50%. Accordingly, lower IC_{50} values indicated higher antioxidant properties. As shown in Table 2, PKM protein and protein hydrolysate at various time points (0, 15, 30, 60, and 120 min) exhibited IC_{50} values ranging from 5.73±0.23 to 7.43±0.22 µg/mL. These were significantly lower than that of L-glutathione (IC_{50} value of 28.25±0.97 µg/mL), indicating higher DPPH radical scavenging activity ($p<0.05$). By comparing IC_{50} values after hydrolysis, it was found that PKM protein hydrolysate after 60 min hydrolysis showed the highest antioxidant activity [as shown by its lowest IC_{50} value (5.73±0.23 µg/mL) when compared with that of the control group ($p<0.05$)]. It was observed that, whenever the concentration of PKM protein hydrolysate was increased, its antioxidant capacity increased as well. Sonklin et al. (2018) evaluated the DPPH antioxidant properties of the mung bean meal protein hydrolysate. They found that the greatest ability to quench DPPH radicals occurred after bromelain hydrolysis for 12 h. Alcalase-treated chickpea hydrolysate has a significant increase in DPPH scavenging properties (Xu et al., 2020).

As determined by ABTS radical scavenging activity, PKM protein and its hydrolysate with alcalase enzyme for 0, 15, 30, 60, or 120 min showed IC_{50} values ranging from 7.84±0.90 to 12.31±0.53 µg/mL which were significantly lower than that of L-glutathione (IC_{50} value of 14.03±0.64 µg/mL) (Table 2). PKM protein hydrolysate, after 60 min of hydrolysis, had the highest antioxidant efficiency. This might be due to the peptides

Table 2. IC_{50} values of palm kernel meal (PKM) protein and its hydrolysates at various times determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays.

Sample	IC_{50} (µg/mL)	
	DPPH assay	ABTS assay
PKM protein	7.43±0.22*	11.74±0.27*
PKM protein hydrolysate (15 min hydrolysis)	5.77±0.16*	12.31±0.53*
PKM protein hydrolysate (30 min hydrolysis)	5.83±0.33*	10.58±0.07*
PKM protein hydrolysate (60 min hydrolysis)	5.73±0.23*	7.84±0.90*
PKM protein hydrolysate (120 min hydrolysis)	6.00±0.89*	7.93±0.88*
L-glutathione (GSH)	28.25±0.97	14.03±0.64

Values are presented as the mean±standard deviation (SD); *statistically significant differences when compared with that of GSH ($p<0.05$).

produced during hydrolysis reacting with unstable electrons and being converted into more stable molecules than the parent protein (Pazinatto et al., 2013). Alcalase-treated rice bran protein hydrolysate has an effective ABTS scavenging property of 7.57 mg/L (Thamnarathip et al., 2016). The ability of hydrolysates to scavenge ABTS radicals is influenced by several considerations, including degree of hydrolysis, enzyme type, protein solubility, peptide group, and existence of free amino acids (de Oliveira et al., 2014).

In general, DPPH and ABTS assays are mechanically based on either a single electron transfer or a hydrogen atom transfer (HAT) reaction between an oxidant molecule and a free radical (Sonklin et al., 2018). The radicals measured can be stabilized not only by reduction via electron transfers but also by quenching via HAT mechanisms (Esfandi et al., 2019; Jiménez et al., 2004). Plant protein hydrolysate is recognized as a valuable source of antioxidants. Chen et al. (2021) reported that rice-derived protein hydrolysates exhibit dose-dependent antioxidation activity as determined by DPPH and ABTS assays with IC_{50} values of 42.58±2.1 and 2.11±0.88 mg/g, respectively. The DPPH and ABTS radical-scavenging activities of *Cardamine violifolia* protein hydrolysate demonstrate antiradical activity with IC_{50} of 0.58 and 0.193 mg/ml, respectively (Zhu et al., 2019). In this study, PKM protein hydrolysate showed promising antioxidant activity and it exhibited higher antioxidant activity when compared with that of PKC protein hydrolysate or PKCPH. The PKC-derived alcalase hydrolysate exhibited DPPH radical scavenging activity with an EC_{50} value of 199.17±0.72 mg/mL (Ng et al., 2022).

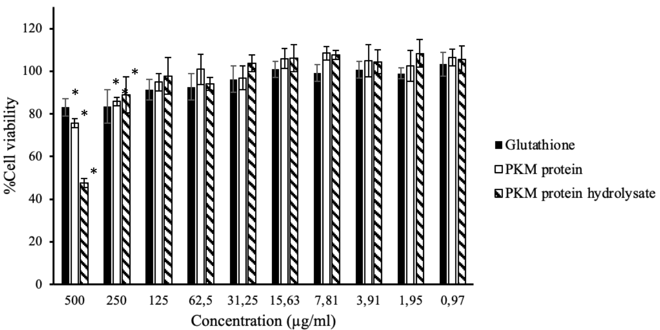
The degree of hydrolysis was obviously influenced by enzymatic hydrolysis conditions and the proportion (or percentage) of cleaved peptide bonds. The degree of hydrolysis is proportional to the number of cleaved peptide chains. A higher degree (%) of hydrolysis indicates the presence of a large number of smaller peptides, which are bioactive peptides with 2–20 amino acids (Karami & Akbari-Adergani, 2019). Figure 1 reveals that PKM protein hydrolysate at 60 min reflects the strongest antioxidant ability. A similar result was demonstrated by You et al. (2009), in which the maximum trolox equivalent antioxidant capacity is at 23% degree of hydrolysis and it subsequently decreases with increasing degree of hydrolysis.

3.3 Cytotoxicity of PKM protein and protein hydrolysate

The MTT technique was used to measure the toxicity of PKM protein and its hydrolysates (60 min) on mouse L929 fibroblast cells. This cell line is widely used in cytotoxicity testing, mainly in regard to toxicity toward cellular viability and proliferation. Cell viabilities when incubated with PKM protein and PKM protein hydrolysate at concentrations less than or equal to 125 µg/ml, were high (up to 91%) (Figure 2). It can be inferred that PKM protein and protein hydrolysate at concentrations less than or equal to 125 µg/ml were not toxic for mouse L929 fibroblast cells. At higher concentrations, more than or equal to 250 µg/mL, they showed moderate toxicity to mouse L929 fibroblast cells as there were significant reductions in cell viability (47.61±2.06% to 88.99±8.49%, respectively). Similar results were also observed in cells treated with high concentrations of L-glutathione (250–500 µg/mL). Chang et al. (2014) reported that, even at high concentrations, oil palm kernel protein hydrolysate produced by pepsin and pancreatin enzymes has no toxicity to the HepG2 cell line. The differences could be due to the specific factors and conditions used to extract proteins, such as the solvent, temperature, and source of palm oil, as well as the type of cell lines used.

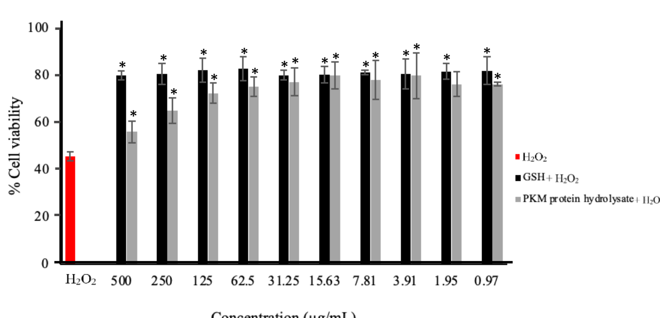
3.4 Cytoprotective effect of PKM protein and PKM protein hydrolysate

Excessive ROS can cause oxidative stress due to their damage to biomolecules including DNA, proteins, and lipids; this damage is thought to be important in the pathogenesis of some chronic diseases. Some studies have reported that ROS increase the chance of various diseases through genetic mutation, inflammation, and neurodegeneration (Lambeth, 2007; Simpson & Oliver, 2020). Hydrogen peroxide (H_2O_2) was identified as a ROS, capable of contributing damage to a wide range of cellular targets. Thus, it has been used in oxidative stress-induced cell damage models for assessing cytoprotective capacity (Linley et al., 2012). The mouse L929 fibroblast cells were treated with PKM protein hydrolysate at various concentrations and incubated for 24 h, followed by treatment with 1,000 µM H_2O_2 for 3 h. The results demonstrated the ability of PKM protein hydrolysate to protect these cells from H_2O_2-induced oxidative stress (Figure 3). The PKM protein hydrolysate at concentrations



*Statistically significant difference (p<0.05) when compared with that of the negative control group.

Figure 2. Cytotoxicity of palm kernel meal protein and its hydrolysate on mouse L929 fibroblast cells. L-glutathione was used as a positive control.



*Statistically significant difference when compared with the H_2O_2-treated group (p<0.05).

Figure 3. Cytoprotective activity of palm kernel meal protein hydrolysate on the viability of mouse L929 fibroblast cells. L-glutathione was used as a positive control.

of 0.97–500 µg/mL significantly promoted cell survival, with increases in % cell viability when compared with that of an H_2O_2-treated group ($p<0.05$). These concentrations of PKM protein hydrolysate also protected cells from oxidative damage by H_2O_2. There are several studies that report the cytoprotective capability of protein hydrolysates from various sources. Hazelnut protein hydrolysate can inhibit ROS accumulation in HUVEC cells, implying that they have a strong protective effect on these cells (Liu et al., 2018). Zhang et al. (2019) also demonstrated that soybean protein hydrolysate, hydrolyzed by alcalase enzyme, strongly enhances the anti-oxidative capacity of human intestinal Caco-2 cells. At very high concentrations of PKM protein hydrolysate (250–500 µg/mL), only a relatively small percentage of cells survived. This was likely due to the toxicity which PKM protein has at high concentrations on mouse L929 fibroblast cells.

3.5 Partial purification and identification of peptide profile

FPLC using a HiPrep DEAE Sepharose Fast Flow anion exchange column was employed to partially purify and identify the peptide profile of the PKM protein hydrolysate. Using a linear gradient of NaCl (0–1 M) at a flow rate of 0.5 mL/min, 33 fractions were obtained (Figure 4). Fraction 15 showed the highest antioxidant activity as determined by DPPH and ABTS assays. Its peptide sequence profile was identified using LC-MS/MS. Table 3 shows the molecular weight, amino acid sequence, structure, hydrophobicity, and water solubility of these nine peptides from the PKM protein hydrolysate: VDEVLNAPREE, FFDEESFLH, AGITDYFDED, EADRTDYPE, ISDETIDAIH, LRPPSEEEE, VLRPPSEEEE, READSDDYPE, and SFDQPAREVDE. The peptide sequences were derived from a group of known proteins such as globulin, vicilin-like antimicrobial

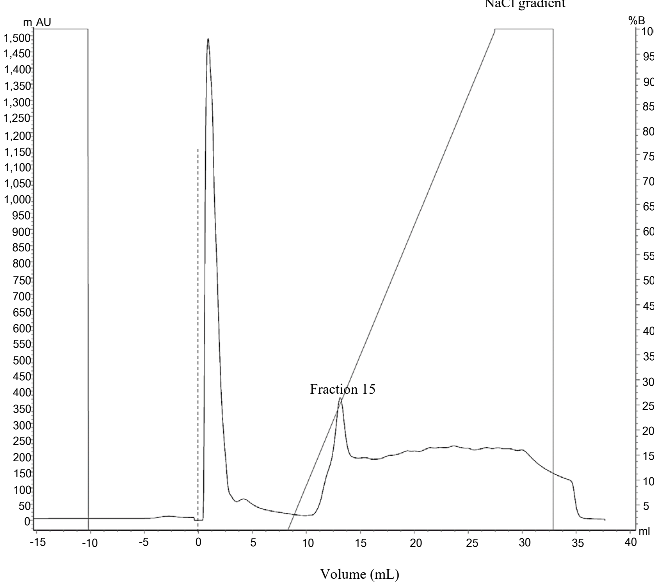


Figure 4. Chromatogram of palm kernel meal protein hydrolysate partially separated by FPLC.

Table 3. Peptide sequences of fraction 15 from partially purified palm kernel meal protein hydrolysate.

Peptide sequence	Length	Molecular weight (Da)	Structure of peptides	Net charge	Hydrophobic (%)	Water solubility	Charged amino acids	Protein family
VDEVLNAPREE	11	1,270.36		-3.00	36	Good	Aspartic acid, D glutamic acid, E arginine, R	7S globulin, vicilin-like antimicrobial peptides
FFDEESFLH	9	1,170.24		-2.90	44	Good	Aspartic acid, D glutamic acid, E histidine, H	7S globulin, vicilin-like antimicrobial peptides 2-2
AGITDYFDED	10	1,145.14		-4.00	30	Good	Aspartic acid, D glutamic acid, E	Cocosin 1-like
EADRTDYPE	9	1,095.09		-3.00	11	Good	Aspartic acid, D glutamic acid, E arginine, R	63 kDa globulin-like protein
ISDETIDAIH	10	1,113.19		-2.90	40	Good	Aspartic acid, D glutamic acid, E histidine, H	Serine carboxypeptidase II-3, carboxypeptidase
LRPPSEEEE	9	1,085.13		-3.00	11	Good	Glutamic acid, E arginine, R	Cocosin 1, cocosin 1 isoform X1 and X2
VLRPPSEEEE	10	1,184.27		-3.00	20	Good	Glutamic acid, E arginine, R	Cocosin 1, cocosin 1 isoform X1 and X2
READSDDYPE	10	1,196.15		-4.00	10	Good	Aspartic acid, D glutamic acid, E arginine, R	7S globulin, vicilin-like antimicrobial peptides
SFDQPAREVDE	11	1,292.32		-3.00	27	Good	Aspartic acid, D glutamic acid, E arginine, R	63 kDa globulin-like protein

peptides, cocosin, or carboxypeptidase. In addition, they were soluble and made up of 9–11 free amino acid residues with molecular masses ranging from 1,085.13 to 1,292.32 Da. All peptide sequences contained hydrophobic amino acids, including Ala(A), Val(V), Ile(I), and Leu(L) with moderate amounts of hydrophobic (25.0%) and negatively charged amino acids such as Glu(E) and Asp(D). Previous research revealed that PKC protein hydrolysate fraction 1 has the highest antioxidant activity with moderate hydrophobicity (23.60%) and contains Asp, Glu, His, Lys, Met, and Tyr (Ng et al., 2022). Zarei et al. (2014) reported that PKC protein antioxidant peptides WAF, YLLLK, WAFS, and AWFS exhibit the highest percentages of hydrophobic amino acid residues with 100, 80, 75, and 75%, respectively.

Sabeena Farvin et al. (2016) reported that hydrolyzed cod protein has a molecular weight of less than 3 kDa with free amino acids potentially promoting high antioxidant activity. In addition, peptides with high levels of Arg, Tyr, and Phe demonstrate significantly greater reducing power. Girgih et al. (2013) discovered that some hydrophobic amino acids (particularly Pro, Leu, and Ile) in hemp seed protein hydrolysate fractions enhance the antioxidant activity of the peptides, thereby providing a reservoir of electrons that can be donated to neutralize or reduce the toxic effects of ROS. The greater hydrophobicity leads to increased lipid solubility, contributing to the resulting total activity of ROS removal and improved antioxidant capability (Rajapakse et al., 2005). In addition, the correlation between hydrophobicity and antioxidant capability suggests that, according to the polar paradox, hydrophobic antioxidants are more active in emulsions than their hydrophilic counterparts (Laguerre et al., 2010). Nile tilapia protein hydrolysates containing negatively charged amino acids in their peptide sequences are associated with strong antioxidant activity in the RAW 264.7 cell model (Ngo et al., 2010).

CONCLUSION

In this study, protein was extracted from PKM and a protein hydrolysate was generated using alcalase enzyme. The protein hydrolysates produced with a hydrolysis time of 60 min had the highest antioxidant activity as determined by DPPH and ABTS assays. Both the PKM protein and its hydrolysate at low concentrations were not toxic to the mouse L929 fibroblast cells, while, at high concentrations, they were moderately toxic to the cells. At concentrations ranging from 0.97 to 500 µg/mL, PKM protein hydrolysate protected mouse L929 fibroblast cells from H_2O_2-induced damage. Nine antioxidant peptides, rich in hydrophobic and negatively charged amino acids, were identified. This study revealed the significant antioxidant activity of both PKM protein and the PKM protein hydrolysate. We suggested that they might be further studied for potential development as natural antioxidant sources or food/feed supplements.

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