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Studies on the Proximate, Functional and Antioxidant Properties of Fermented and Unfermented *Kariya* (*Hildergardia barterii)* Seed Protein Hydrolysates Obtained by Enzymatic Hydrolysis

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Authors' contributions

This work was carried out in collaboration between both authors. Authors OSG and AAF designed the study. Author AAF performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OSG and AAF managed the analyses of the study. Authors OSG and AAF managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The degree of hydrolysis (DH), proximate composition, functional properties and anti-oxidative characteristics of enzymatic *kariya* seed hydrolysates were evaluated with a view to increasing the utilisation of the seed as food ingredient. Protein hydrolysates were obtained from fermented and unfermented *kariya* seed protein isolate using two proteolytic enzymes: Pepsin and pancreatin to obtain fermented pancreatin *kariya* protein hydrolysate (FPcKPH), unfermented pancreatin *kariya* protein hydrolysate (UPcKPH), fermented pepsin *kariya* protein hydrolysate (FPsKPH) and unfermented pepsin *kariya* protein hydrolysate (UPsKPH). The results showed that pancreatin

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hydrolysates had higher degree of hydrolysis (FPcKPH; 65.20% and UPcKPH; 50.50%) than pepsin hydrolysates (FPsKPH; 53.00% and UPsKPH; 33.93%) while the fermented hydrolysates showed higher DH than the unfermented hydrolysates both in the pancreatin and pepsin hydrolysates. The results also showed that fermented hydrolysates had better functional properties than the unfermented samples. The protein contents of the fermented hydrolysates were also improved. The antioxidant characteristics showed that fermented *kariya* protein hydrolysates exhibited higher DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging activity (FPcKPH, IC_{50}1.03; FPsKPH, IC_{50}1.40; UPcKPH, IC_{50} 1.51; UPsKPH, IC_{50} 6.97 mg extract/ml), metal chelating (FPcKPH, IC_{50} 0.95; FPsKPH, IC_{50}0.53; UPcKPH, IC_{50} 1.27; UPsKPH, IC_{50} 1.06 mg extract/ml) and ferric reducing antioxidant power (FPcKPH, 0.63; FPsKPH,0.50; UPcKPH 0.35; UPsKPH, 0.23 AAµg/g). The study concluded that fermented *kariya* seeds hydrolysates could find applications as potential source of natural anti-oxidants in food.

Keywords: Hydrolysates; antioxidant; fermented; pepsin; pancreatin; kariya.

1. INTRODUCTION

The use of plant proteins, especially from cereals and oilseeds has been growing intensively over the last decades. Plant proteins are being used as alternative for animal proteins in human nutrition, functional agents and bioactive components in food [1]. Moreover, research carried out in last few years showed that many oil seeds have antioxidant effects.

Kariya (*Hildergardia barterii*) seeds are consumed mostly in West African countries as raw or roasted nuts having a flavour like that of peanuts. It is grown for its ornamental nature. The seed kernels have been reported to contain 17.5% protein [2].

Fermentation as one of the oldest processing method is known to have added value to foods and has been reported to increase the functional and anti-oxidative capacity of some legumes and seed proteins [3].

Bioactive peptides derived from natural food sources have been studied widely due to their potential health benefits associated with high bioactivity, low molecular weight, easy absorption and less toxicity effects [4]. Enzymatic hydrolysis is one of the most important protein structure modification processes in the food industry, known to improve properties of dietary protein without affecting its nutritive value by converting the protein to peptides with desired size, charges and surface properties [5]. The peptides produced by hydrolysis of proteins have been reported to exert to improve functional properties of proteins [6]. [7] reported most native proteins do not show anti-oxidative properties desirable for food industries, hence the modification for improvement of these properties need to be addressed. The most important feature affecting the properties of a protein is its surface structure which has effects on the interaction of a protein with water or other proteins. Thus, by modifying the structure of the protein with hydrolysis, particular functional and anti-oxidative properties are obtained. Human body is damaged due to exposure or aggression of various substances which may cause undesired interactions. Some of these substances are free radicals which accept electrons to generate an electronic balance between neighbouring molecules, which leads to oxidative stress in body, resulting in condition such as aging, cardiovascular system problems, cancer, constipation and nervous system damage [8]. Food technology has shown that within the proteins are available molecules which can reduce or counteract free radicals. Among these inactive substances are bioactive peptides, which are amino acid sequences within the protein but when they are released after hydrolysis, have beneficial effects in the body [4].

Many synthetic antioxidants may be used to retard lipid peroxidation in a number of foods. However, the safety and negative consumer perception of synthetic antioxidants restrict their applications in food products. A number of studies are currently exploring the use of plant protein sources such as soybeans, chickpeas, almonds *etc.* for the production of bioactive peptides with different biological activities. *Kariya* seed as oil seed has high level of protein (17.5%) and this proportion presents prospects for obtaining functional peptides from the seed. Research efforts on *kariya* seed have focused seed flours. The chemical and fatty acids compositions of the seed have also been studied. The properties of oil extracted from *kariya* kernel have been reported by [9]. Recently, there is a remarkable report on the

physical, functional and nutritional properties of *kariya* seeds defatted flours as well as its isolates. [10] reported that the protein content of *kariya* defatted flour was 42.5%. [11] worked on the fermented isolates of the seed and reported that the protein content of the fermented and unfermented isolates from *kariya* seed ranged between 90 to 93%. Availability of the information on *kariya* seed protein hydrolysates could offer *kariya* as a potential source of functional and natural bioactive peptides and therefore increasing its utilization as food ingredients.

2. MATERIALS AND METHODS

2.1 Raw Materials

Dried *kariya* pods were gathered from ornamental *kariya* trees in Obafemi Awolowo University, Ile-Ife, Nigeria. The nuts extracted from the pods were sorted to remove extraneous materials such as stones and leaves. The kernels were obtained by shelling the nuts which were cleaned to remove chaff and immature kernels.

2.2 Preparation of Fermented Defatted *Kariya* Flour Samples

Kariya kernels were rinsed and divided into 2 portions: A portion was soaked for 24 h with warm water at 50℃ and the water was changed every 6 h interval. The fermentation was carried out by transferring the soaked seeds into calabash pots, lined uniformly with banana leaves (up to 5 layers) and allowed to ferment for 96 h inside the incubator (30℃). The second portion was neither soaked nor fermented. The fermented sample was oven dried at 60℃ for 6 h to terminate the fermentation process. The fermented and the unfermented samples were milled separately using Kenwood grinder and sieved through 200 µm sieve. The resulting flours of the two samples were subsequently defatted using n-hexane in a Soxhlet extraction apparatus. The defatted flours were de-solventized by drying in a fume hood and the dried flours finely ground in a kenwood grinder (PM-Y44B2, England) set at high speed to obtain homogenous defatted flours.

2.3 Preparation of *Kariya* Protein Isolates

Kariya protein isolate was prepared by a method described by [12]. Defatted flours (fermented and unfermented) were dispersed in 1000 mL of distilled water to give final flour to liquid ratio of 1:10 in separate containers. The suspensions were gently stirred on magnetic stirrers for 10 min. The pH of the resultant slurries was adjusted to the point at which the proteins were most soluble (pH 10.0). The extraction was allowed to proceed with gentle stirring for 4 h keeping the pH constant. Non-solubilized materials were removed by centrifugation at 3500 × g for 10 min. The proteins in the extracts were then precipitated by drop wise addition of 0.1 N HCl with constant stirring until the pH was adjusted to the point at which the protein was least soluble (pH 4.0). The mixture was centrifuged (Harrier 15/80 MSE) at 3500 × g for 10 min to recover the protein. After separation of proteins by centrifugation, the precipitate was washed twice with distilled water. The precipitated protein was re-suspended in distilled water and the pH was adjusted to 7.0 with 1 M NaOH prior to freeze-drying. The freeze-dried protein was later stored in air-tight plastic container at room temperature.

2.4 Preparation of *Kariya* Protein Hydrolysates

Kariya protein hydrolysates were prepared for the two samples i.e. fermented and unfermented protein isolates samples by two different proteolytic enzymes (pepsin and pancreatin) acting on each of the samples following the method reported by [13]. A 5% (w/v) *kariya* protein isolate’s slurry was adjusted to pH 2.0 and incubated at 37ºC followed by addition of pepsin. Slurry was adjusted to pH 7.5 and incubated at 40℃ followed by the addition of pancreatin (4% w/w, on the basis of protein content of *kariya* protein isolate) for the fermented sample and for the unfermented sample in the same manner. The digestion was carried out for 4 h and the pH was maintained by adding 1 M NaOH or HCl when necessary. The digestion was terminated by placing the mixtures in boiling water for 30 min to inactivate the enzymes, ensure complete denaturation of enzyme protein and coagulation of undigested proteins. The mixture was then allowed to cool to room temperature and centrifuged (7000 ×g) with centrifuge (MSE, Harrier 15/80) for 30 min. The 4-hour hydrolysates obtained were freeze-dried and then preserved at -18℃ for subsequent analysis.

2.5 Measurement of DH

The DH was determined by formaldehyde titration method according to method described by [14]. Five millilitres of hydrolysates

supernatant were diluted with 60 ml distilled water, while magnetically stirring, and titrated by 0.05 M NaOH (standard titration solution) to pH 8.2 and volume was recorded. Then, 10 ml formaldehyde (12%) were added into the beaker and continued for titration with 0.05 M NaOH to reach pH 9.2 and the titre of NaOH was also recorded. The value of DH was calculated according to the following equation:

$$DH = \frac{C \times (V1 - V2) X\, V/5}{m \times percentage\ of\ protein\ in\ the\ raw\ material\ x\ 8}\ x\ 100$$

Where:

C = The concentration of standard titration solution of NaOH (0.05 M)
V1 = The titre volume (mL) of 0.05 M NaOH titrating up to pH 9.2
V2 = The titre volume (mL) of 0.05 M NaOH titrating up to pH 8.2
V = The total volume (mL) of protein hydrolysates
m = The mass of the raw material

2.6 Moisture Content Determination

Moisture content was determined by the official method of [15]. About 1 g (W_1) of the samples in moisture cans and drying in a hot air-oven (Uniscope, SM9053, England) at 105±1ºC until constant weight (W_2) was obtained. The samples were removed from the oven, cooled in a desiccator and weighed. The results were expressed as percentage of dry matter as shown in the equation below:

$$Moisture\ content\ (\%) = \frac{W_1 - W_2}{W_1} \times 100$$

$W_1 = weight\ of\ flour\ before\ drying$,
$W_2 = weight\ of\ flour\ after\ drying$

2.7 Ash Content Determination

Ash content was determined by the official method of [15]. This was carried out using muffle furnace (Carbolite AAF1100, United Kingdom). Two grams (W_3) of the sample were weighed into already weighed (W_2) ashing crucible and placed in the Muffle furnace chambers at 700ºC until the samples turned into ashes within 3 h. The crucibles were removed, cooled in a desiccator and weighed (W_1). Ash content was expressed as the percentage of the weight of the original sample.

$$Ash\ content (\%) = \left(\frac{W_1 - W_2}{W_3}\right) \times 100$$

$W_1 = weight\ of\ crucible + ash$
$W_2 = weight\ of\ empty\ crucible$
$W_3 = weight\ of\ sample$

2.8 Protein Content Determination

The total protein content was determined using Kjeldahl method [15]. The freeze-dried hydrolysates (0.20 g) was weighed into a Kjeldahl flask. Ten milliliter of concentrated sulphuric acid was added followed by one Kjeltec tablet (Kjeltec-Auto 1030 Analyzer, USA). The mixture was digested on heating racket to obtain a clear solution. The digest was cooled, and made up to 75 ml with distilled water and transferred onto Kjeldahl distillation set up followed by 50 ml of 40% sodium hydroxide solution, the ammonia formed in the mixture was subsequently distilled into 25 ml, 2% boric acid solution containing 0.5 ml of the mixture of 100 ml of bromocresol green solution (prepared by dissolving 100 mg of bromocresol green in 100 ml of methanol) and 70 ml of methyl red solution (prepared by dissolving 100 mg of methyl red in 100 ml methanol) indicators. The distillate collected was then titrated with 0.05 M HCl. Blank determination was carried out by excluding the sample from the above procedure,

$$(\%)protein = \frac{1.401 \times M \times F\ (ml\ titrant - ml\ blank)}{sample\ weight}$$

$M = Molarity\ of\ acid\ used = 0.05$
$F = Kjeldahl\ factor = 6.25$

2.9 Carbohydrate

Crabohydrate content was expressed as a percentage of the difference between the addition of other proximate chemical components and 100% as shown in equation below;

Carboydrate content =100- (crude protein +crude fat+ash+fibre+moisture)

2.10 Functional Properties of the Freeze-dried Hydrolysates

2.10.1 Determination of water absorption capacity (WAC)

The WAC was determined at room temperature using method of [16]. Two grammes of the sample were dispersed in 20 ml of distilled water. The suspension was mixed for 30 s every 10 min using a glass rod and after mixing five times,

centrifuged (Hospibrand, 0502-1, USA) at 4000 × g for 20 min. The supernatant was carefully decanted and then the contents of the tube were allowed to drain at a 45° angle for 10 min and then weighed. The water absorption capacity was expressed as percentage increase of the sample weight.

$$Water\ absorption\ capacity\ (\%) = \left(\frac{W_3 - W_2}{W_1}\right) \times 100$$

$W_3 = weight\ of\ tube + sample\ after\ centrifuging\ and\ decanting$

$W_2 = weight\ of\ tube + sample\ before\ centrifuging$

$W_1 = weight\ of\ sample$

2.10.2 Determination of oil absorption capacity (OAC)

Oil absorption capacity of the flour samples was determined by the centrifugal method described by [17] with slight modifications. One gram of the sample was mixed with 10 ml of pure vegetable oil (Gino oil(R)) for 60 s, the mixture was allowed to stand for 10 min at room temperature, centrifuged at 4000 × g for 30 min and the oil that separated was carefully decanted and the tubes allowed to drain at an angle of 45°for 10 min and then weighed. Oil absorption was expressed as percentage increase of the sample weight.

$$Oil\ absorption\ capacity\ (\%) = \left(\frac{W_3 - W_2}{W_1}\right) \times 100$$

$W_3 = weight\ of\ tube + sample\ after\ centrifuging\ and\ decanting$

$W_2 = weight\ of\ tube + sample\ before\ centrifuging$

$W_1 = weight\ of\ sample$

2.10.3 Determination of foaming capacity (FC) and stability (FS)

Foam capacity and foam stability was determined by a modification of the method described by [17]. The protein hydrolysates sample (500 mg) was dispersed in 100 mL. The solution was homogenized for 2 min using blender (Kenwood, model HM430) set at high speed. The percentage ratio of the volume increase to that of the original volume of protein solution was calculated and expressed as foam capacity. Foam stability was expressed as percentage of the volume of foam remaining after 60 min of quiescent period.

$$Foaming\ capacity\ (\%) = \frac{V_2 - V_1}{V_1} \times 100$$

$$Foaming\ stability\ (\%) = \frac{V_3 - V_1}{V_1} \times 100$$

$V_1 = volume\ before\ whipping\ (mL)$

$V_2 = volume\ after\ whipping\ (mL)$,

$V_3 = volume\ after\ standing\ for\ 30\ min\ (mL)$

2.10.4 Determination of emulsifying activity index (EAI) and Emulsifying stability index (ESI)

The method of [12] was used. The protein sample (500 mg) was dispersed in 100 ml of distilled water and gently stirred to disperse the sample. The protein solution was mixed with 10 ml of pure Gino® oil and the mixture was homogenized using blender (Kenwood, model, HM430) set at speed 10 for 60 s. Five hundred microliters of the aliquot of the emulsion were transferred from the bottom of the blender after homogenization, and mixed with 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using spectrophotometer (Unicam Hexios α, UV- Visible Spectrophotometer England). The EAI was expressed as interfacial area per unit weight of protein (m^2g^-1). Emulsion stability determined by allowing the emulsions to stand for 10 min at room temperature and absorbance determined. The ESI was determined as described in the following formula [18].

Emulsion activity index (m^2/g) = $\frac{2 \times 2.303 \times \text{A}}{0.25 \times \text{protein weight (g)}}$

Emulsion stability index (%)= $\frac{\text{EAI at 10 min}}{\text{EAI at 0 min}} \times 100$

Where;

A= Absorbance at 0 min after homogenization

3. ANTIOXIDANT PROPERTIES OF *Kariya* HYDROLYSATES

3.1 The DPPH Radical Scavenging Activity Assay

The free radical scavenging ability of the extract was determined using the stable radical DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) as

described by [19]. To 1 mL of different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) of the extract or standard (Vitamin C) in a test tube 1 ml of 0.3 mM DPPH in methanol was added. The mixture was mixed and incubated in the dark for 30 min after which the absorbance was read at 517 nm against a DPPH control containing only 1 ml methanol in place of the extract.

The percent of inhibition was calculated from the following equation:

$$Inhibition(\%) = \frac{(\text{Acontrol}-\text{Asample})}{\text{Acontrol}}\ x\ 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Inhibition concentration leading to 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentrations.

3.2 Metal Chelating Ability Assay

The metal-chelating activity of the protein hydrolysates was carried out according to the method described by [20]. Solutions of 2 mM $FeCl_2{\cdot}4H_2O$ and 5 mM ferrozine was diluted 20 times (1 mL of each of the two solutions made up to 20 ml with distilled water separately). An aliquot (1 mL) of different concentrations (6.25, 12.5, 25.0, 50.0 and 100.0 mg/ml) of sample extract was mixed with 1 mL $FeCl_2{\cdot}4H_2O$. After 5 min incubation, the reaction was initiated by the addition of ferrozine (1 mL). The mixture was shaken vigorously and after a further 10 min incubation period, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine–Fe^{+2} complex formations was calculated using the formula:

$$\text{Chelating effect} = \frac{\text{Acontrol} - \text{Asamples}}{\text{Acontrol}} \times 100$$

Where $A_{control}$ = absorbance of control sample (the control contains 1 mL each of $FeCl_2$ and ferrozine, complex formation molecules) and A_{sample} = absorbance of a tested sample.

3.3 Determination of Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method with absorbance measured with a spectrophotometer [21]. The principle of this method is based on the reduction of a colourless ferric-tripyridyltriazine complex to its blue ferrous coloured form owing to the action of electron donating in the presence of antioxidants. A 300 mmol acetate buffer of pH 3.6, 10 mmol/L 2, 4, 6-tri-(2-pyridyl)-1, 3, 5-triazine and 20 mmol $FeCl_3.6H_2O$ was mixed together in the ratio of 10:1:1 respectively, to give the working FRAP reagent. A 50 µl aliquot of the extract at concentration (0.0, 0.2, 0.4, 0.0.6, 0.8 and 1 mg/mL) and 50 µl of standard solutions of ascorbic acid (20, 40, 60, 80, 100 µg/ml) were added to 1 mL of FRAP reagent. Absorbance was measured at 593 nm exactly 10 minutes after mixing against blank reagent containing 50 µl of distilled water and 1 ml of FRAP reagent.

The reducing power was expressed as equivalent concentration (EC) which is defined as the concentration of antioxidant that gave a ferric reducing ability equivalent to that of the ascorbic acid standard.

3.4 Statistical Analysis

All the analyses were conducted in triplicate and subjected to statistical analysis using analysis of variance (ANOVA). Differences among the treatment means were separated using Duncan's multiple range test [11].

4. RESULTS AND DISCUSSION

4.1 Degree of Hydrolysis of Fermented and Unfermented *Kariya* Hydrolysates

The results of the degree of hydrolysis of fermented and unfermented hydrolysates are presented in Table 1. It was found that fermented pancreatin *kariya* protein hydrolysates (FPcKPH) had the highest value (62.5%), followed by fermented pepsin *kariya* protein hydrolysates (FPsKPH) (53%), unfermented pancreatin *kariya* protein hydrolysates (UPcKPH) (50.5%) while the lowest value was obtained for unfermented pepsin *kariya* protein hydrolysates (UPsKPH) (33.93%) and these values were significantly different at $P<0.05$ as shown in Table 1. According to [22], the degree of hydrolysis (DH) is significantly influenced by the hydrolysis conditions that include time, temperature, pH of the substrate and the enzyme used in the hydrolysis as well as the concentration of the enzymes used. The results obtained in the present study revealed that pancreatin hydrolysed fermented sample had higher short peptides yield than the pepsin hydrolysed sample. Similar results were obtained for the

unfermented protein sample and this could be attributed to the differences in the specificity of these enzymes on peptides chains of *kariya* proteins. The result from the present study were in agreement with the reports of [23]. This indicated that fermentation enhanced the production of more bioactive peptides with shorter chains.

4.2 Proximate Composition of the Hydrolysates

The results of the proximate composition of the fermented and unfermented hydrolysates are shown in Table 2. It was found that samples hydrolysed with pancreatin enzymes contain higher protein content than the pepsin hydrolysed samples both in the fermented and unfermented form. Sample FPsKPH contained 94.31% protein and this value was higher than the value (92.19%) obtained for UPsKPH but lower than the value obtained for sample FPcKPH. Fermented hydrolysates produced using pancreatin enzyme contained the highest protein content. The protein contents obtained in the present study were higher than the values reported by [11] for unhydrolysed fermented and unfermented *kariya* protein isolates. This could be due to the removal of non-protein materials during enzymatic protein hydrolyses. The fat content of the fermented samples (FPsKPH and FPcKPH) were found to be higher than their corresponding unfermented hydrolysates (UPsKPH, UPcKPH) and these values were significant (P<0.05) from each other. The ash content is an indication of the level of mineral elements available in a particular food samples. In the present study, the level of ash obtained for sample UPcKPH (3.98%) was higher than the value obtained for UPsKPH and also higher than the two fermented hydrolysates.

Table 1. Degree of Hydrolysis (DH) of fermented and unfermented *kariya* protein hydrolysates

Sample	Degree of hydrolysis (%)
UPsKPH	33.93±0.94^{d}
UPcKPH	50.50±1.23^{c}
FPsKPH	53.00±0.82^{b}
FPcKPH	65.20±0.91^{a}

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript along the column are significantly (P < 0.05) different, FPcKPH: Fermented pancreatin Kariya protein hydrolysates, FPsKPH: Fermented pepsin Kariya protein hydrolysates, UPcKPH: unfermented pancreatin Kariya protein hydrolysates, UPsKPH: unfermented pepsin Kariya protein hydrolysates

Crude fibre was not detected in any of the samples and the moisture contents of the unfermented hydrolysates were higher than the fermented samples. The differences in the composition of the hydrolysates might be related to different processing method used, specificity of the enzymes used in releasing different sizes of the peptides having different composition. The moisture contents of the samples were found to be in the range 0.46-0.54%. It was found that the moisture content of unfermented hydrolysates was not significantly different from sample FPsKPH. The carbohydrates content of the samples was also found to be in the range of 0.91-3.82%. It was found that sample UPsKPH had the highest carbohydrates content while the least was recorded in sample FPcKPH. Generally, the result obtained in the present study for the proximate composition agreed with those reported by [24] on the composition of enzymatically hydrolysed pumpkin meal protein. Similar trend was also reported by [25].

Table 2. Proximate composition of fermented and unfermented *kariya* protein hydrolysates

	UPsKPH	UPcKPH	FPsKPH	FPcKPH
Protein (%)	92.19±0.49^{d}	93.01±0.29^{c}	94.31±0.38^{b}	95.11±2.89^{a}
Fat (%)	0.45±0.04^{b}	0.41±0.18^{b}	0.56±0.02^{a}	0.52±0.05^{a}
Ash (%)	3.01±0.99^{c}	3.98±1.02^{a}	3.21±0.89^{b}	3.20±0.20^{b}
Fibre (%)	ND	ND	ND	ND
Moisture (%)	0.53±0.02^{a}	0.50±0.07^{a}	0.54±0.11^{a}	0.46±0.11^{b}
Carbohydrate (%)	3.82±0.18^{a}	2.10±0.04^{b}	1.31±0.14^{c}	0.91±0.08^{d}

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript across the row are significantly (P < 0.05) different.
ND: Not determined, FPcKPH: Fermented pancreatin kariya protein hydrolysates, FPsKPH: Fermented pepsin kariya protein hydrolysates, UPcKPH: unfermented pancreatin kariya protein hydrolysates, UPsKPH: unfermented pepsin kariya protein hydrolysates

4.3 Functional Properties of the Hydrolysates

The water absorption of the hydrolysates revealed that sample UPsKPH had 231.8% and this value was lower than the 242.91% recorded for sample UPcKPH as shown in Table 3. The fermented hydrolysate produced using pancreatin enzyme (FPcKPH) was found to have 272.19% water absorption capacity as shown in Table 3 and this value was significantly ($P<0.05$) higher than fermented pepsin-hydrolysed hydrolysates (FPsKPH).

The oil absorption capacity of the samples ranged from 169.31-205.98%. The oil absorption capacity of sample UPsKPH was found to be 169.31%. This value was lower than the value obtained for UPcKPH. The value obtained for fermented pancreatin hydrolysed sample (FPcKPH) was found to be higher than the value recorded for FPsKPH.

The foaming properties of the samples are shown in Table 3. The results showed that fermented hydrolysates had lower foaming properties when compared with unfermented hydrolysates. The foaming capacity of the hydrolysates ranged from 22.19-34.20% with UPsKPH having the highest value. The lowest foaming capacity was obtained for sample FPsKPH and the values were significantly different ($P<0.05$) from one another. The hydrolysates produced using pancreatin enzymes higher than the hydrolysates produced with pepsin enzymes. The same trend observed for foaming capacity was also recorded for foaming stability where the fermented hydrolysates were found to have lower foaming stability than the unfermented hydrolysates.

The ability of the hydrolysates produced to bring together two immiscible phases as shown by its emulsifying properties is presented in Table 3. The ability of the samples to form emulsion ranged between 242-260 m^2/g. It was observed in present study that fermented hydrolysates formed better emulsion than the unfermented samples. Also, pancreatin hydrolysed hydrolysates were able to form emulsions better than the pepsin hydrolysed samples. The emulsions formed by these hydrolysates were relatively stable as shown by its emulsion stability index in Table 3. The results showed that the stability index of the hydrolysates ranged from 29.84-49.29%. Sample FPcKPH was found to be most stable among the samples while sample UPsKPH was least stable and stabilities of these samples varied significantly ($P<0.05$). The different in the degree of hydrolysis of the samples as shown in Table 1 might be a very important factor responsible for these differences. However, a look at the functional properties in the present study showed that the samples are favoured in this order: FPcKPH>FPsKPH>UPcKPH>UPsKPH.

It was obvious that fermentation improved most of the functional properties of the hydrolysates. Studies on fermentation of protein seeds have shown that the release of some micro-organisms and their activities play important role in this respect. Studies by [11] and [26] reported similar results on the fermented protein isolates of *kariya* seed and fermented coconut flours respectively.

Pancreatin hydrolysed hydrolysates appeared to have better functional properties than the pepsin hydrolysed samples. Studies have shown different enzymes exhibit distinct specificities of action. The different enzymes used with their different specific reactions might account for different functional properties of the samples. During hydrolysis, studies have shown that the native protein are degraded into different peptide sizes. Some of the hydrophobic chains which

Table 3. Functional properties of fermented and unfermented *kariya* protein hydrolysates

	UPsKPH	UPcKPH	FPsKPH	FPcKPH
Water absorption capacity (%)	231.81±1.80^d	242.91±2.43^c	258.91±0.09^b	272.19±1.19^a
Oil absorption capacity (%)	169.31±2.89^d	171.92±1.04^c	197.91±0.09^b	205.98±1.05^a
Foaming capacity (%)	28.91±0.99^b	34.20±0.39^a	22.19±0.09^d	23.10±0.32^c
Foaming stability (%)	23.81±0.79^b	29.19±1.09^a	16.80±0.69^d	17.50±0.44^c
Emulsion capacity (m^2/g)	242.91±1.05^d	245.12±2.01^c	258.21±2.99^b	260.18±0.59^a
Emulsion stability (%)	29.84±0.14^c	25.21±1.19^d	42.19±1.09^b	49.29±1.01^a

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript across the row are significantly ($P < 0.05$) different.
FPcKPH: Fermented pancreatin kariya protein hydrolysates, FPsKPH: Fermented pepsin kariya protein hydrolysates, UPcKPH: unfermented pancreatin kariya protein hydrolysates, UPsKPH: unfermented pepsin kariya protein hydrolysates

were inactive in the native protein are made active by this action. The possibility of pepsin and pancreatin producing different peptides sizes with different functional properties might account for the observations in the present study. Similar observation was made by [27] on the soybeans hydrolysates. [28] also observed similar results on the production of functional protein hydrolysates from soyabeans and Lupin seeds. Therefore, to improve the functional properties of *kariya* seeds, fermentation followed by enzymatic hydrolysis could be a good step.

4.4 DPPH Radical Scavenging Activities

The results of DPPH free radical scavenging activities of fermented pancreatin *kariya* protein hydrolysates (FPcKPH) and unfermented pancreatin *kariya* protein hydrolysates (UPcKPH) and fermented pepsin *kariya* protein hydrolysates (FPsKPH) and unfermented pepsin *kariya* protein hydrolysates (UPsKPH) are presented in Fig. 1. The results showed that the DPPH free radical scavenging activities of all the extracts were concentration dependent. The free radical scavenging activities as measured by DPPH assay increased with increasing sample concentration for all the samples from 0.5-2.5 mg/ml. The increases were significant except at 0.5 mg/ml for FPsKPH and FPcKPH. The DPPH scavenging activities of the extracts followed the following order: FPcKPH>FPsKPH>UPcKPH>UPsKPH at all concentrations between 0.5-2.5 mg/ml. Among the different hydrolysates, FPcKPH exhibited the highest radical scavenging activity value (91.23% at 2.5 mg/ml) followed by FPsKPH (85.25% at 2.5 mg/ml) and UPcKPH (81.35% at 2.5 mg/ml), while the lowest DPPH radical-scavenging activity was obtained with UPsKPH (23.44%) at the same concentration. DPPH radical scavenging activity of these extracts reveal antioxidant potency based on IC_{50} values when compared with ascorbic acid as shown in Table 4. Lower value of IC_{50} indicates a higher antioxidant activity [29]. The results obtained present study revealed that fermented hydrolysates (FPcKPH and FPsKPH) with lower IC_{50} values (1.03 and 1.40 mg extract ml) had better scavenging than unfermented hydrolysates (UPcKPH and UPsKPH) with higher IC_{50} (1.51 and 6.97 mg extract ml). The fermented pancreatin *kariya* protein hydrolysates (FPcKPH) showed higher scavenging ability than its pepsin counterpart (FPsKPH). The same trend was also observed for the unfermented pancreatin and pepsin hydrolysates. Comparing the enzymes used for the hydrolysis, it was observed that the enzymes (pepsin and pancreatin) behaved differently probably due to differences in their catalytic activities and specificities towards the substrates. The differences in the radical scavenging ability found in the present study might be attributed to the differences in peptide chains of the hydrolysates due to fermentation, the specificities of enzymes used to hydrolyze the proteins and release of some bioactive peptides as a result of the actions of micro-organisms during fermentation [30]. These results agree with that reported by [31] where fermented soybeans showed higher radical scavenging capabilities than unfermented soybeans. Similarly, the results obtained in this study for fermented hydrolysates were comparable with that obtained for protein hydrolysates from germinated black Soybean (*Glycine max.*) (0.97 and 1.52 mg/ml) by [32] but higher than that of soybean and fermented soybean (IC_{50} values of 21.09 and 14.28 mg extract/ml respectively) as reported by [30] and also reported by [33] on oat flour protein isolates hydrolysed with alcalase and trypsin. The higher DPPH free radical scavenging properties of fermented *kariya* protein hydrolysates over the unfermented hydrolysates correlated with the results obtained for the degree of hydrolysis and also in line with the report of [34] and [35] on the free radical scavenging abilities of soyabean hydrolysates.

4.5 Metal Chelating Activity

The ability of fermented and unfermented *kariya* seed protein hydrolysates prepared under the influence of two proteolytic enzymes (pepsin and pancreatin) to chelate and deactivate transition metal is shown in Fig. 2. The metal chelating ability of the extracts followed the following order: FPsKPH>FPcKPH>UPsKPH>UPcKPH at all concentrations between 6.25-100 µg/ml. At a concentration of 100 µg/ml, FPsKPH exhibited the highest ferrous ion-chelating ability value (92.81%) followed by FPcKPH and UPsKPH (85.24% and 75.62%, respectively), while the lowest chelating ability was obtained with UPcKPH (50.77%). The results also showed the metal chelating potency based on IC_{50} when compared with EDTA. The highest IC_{50} value was obtained for UPcKPH (1.27 µg extract/ml) followed by UPsKPH and FPcKPH (1.06 and 0.95 µg respectively) while FPsKPH (0.53 µg extract/ml) had the lowest and therefore highest chelating activity. The values were lower when compared to that of EDTA (0.05 µg extract/ml) as presented in Table 4. The results obtained in the

present study showed that fermentation significantly increased the metal chelating ability of the samples when compared with the unfermented extracts. Also, the enzymes reacted differently with the extracts as pepsin digest had better metal chelating abilities than pancreatin digest in the fermented extracts (FPsKPH>FPcKPH) and similar results were also observed in the unfermented extracts (UPsKPH>UPcKPH).The trend observed in metal chelating ability with respect to the activities of the pepsin and pancreatin hydrolysed hydrolysates was opposite to the trend observed in the reactions of these enzymes with DPPH where pancreatin hydrolysates had better free radical scavenging capabilities than pepsin hydrolysates. This is in agreement with the result obtained for African yam bean seed protein hydrolysates reported by [34]. [36] also observed similar results on fermented soy hydrolysates.

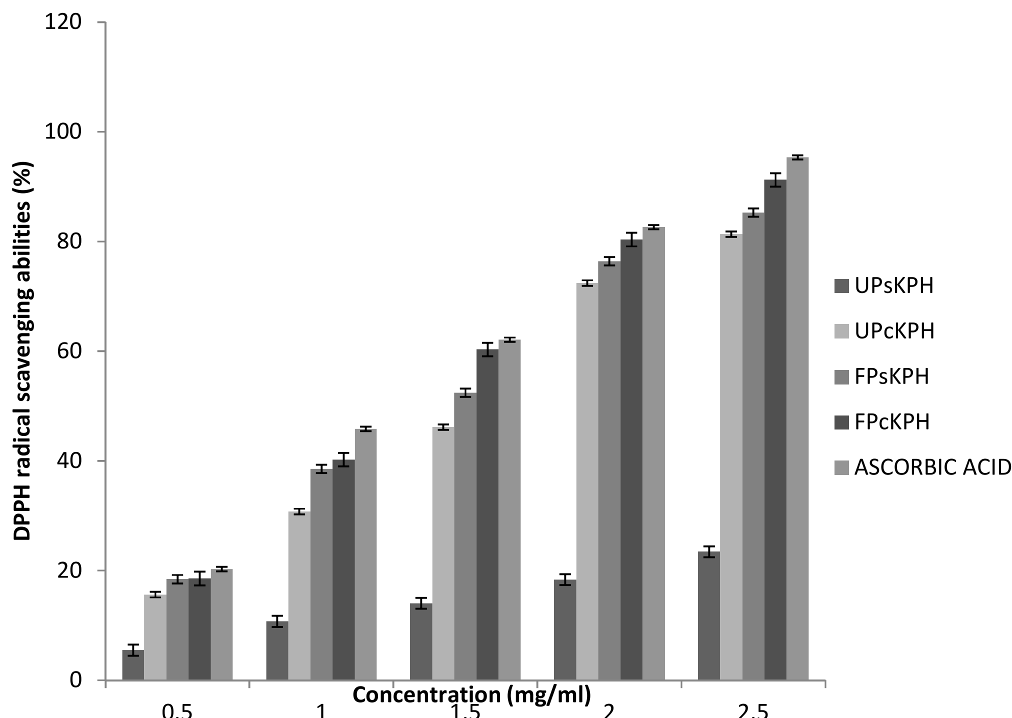

Fig. 1. DPPH radical scavenging activities of fermented and unfermented *kariya* protein hydrolysates at different concentrations. Error bars showing the standard deviation (n=3)

FPcKPH: Fermented pancreatin kariya protein hydrolysates, FPsKPH: Fermented pepsin kariya protein hydrolysates, UPcKPH: unfermented pancreatin kariya protein hydrolysates, UPsKPH: unfermented pepsin kariya protein hydrolysates

Table 4. Antioxidant properties of fermented and unfermented *kariya* seed protein hydrolysates

Sample	DPPH IC_{50} (mg/ml)	MC IC_{50} (µg/ml)	FRAP (AAEµg/g)
FPcKPH	$1.03±0.01^{a}$	$0.95±0.17^{a}$	$0.63±0.01^{a}$
FPsKPH	$1.40±0.07^{b}$	$0.53±0.24^{b}$	$0.50±0.02^{b}$
UPcKPH	$1.51±0.16^{c}$	$1.27±0.23^{c}$	$0.35±0.12^{c}$
UPsKPH	$6.97±0.04^{d}$	$1.06±0.23^{d}$	$0.23±0.11^{d}$
Ascorbic acid	$0.08±0.03^{e}$	-	
EDTA	-	$0.05±0.06^{e}$	

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript along the same column are significantly (P < 0.05) different

EDTA: Ethylene diamine tetra-acetate; DPPH: (diphenyl-1-picryhydrazyl) radical scavenging activity; MC: Metal chelating activity; FRAP: Ferric reducing power assay, FPcKPH: fermented pancreatin kariya protein hydrolysates, FPsKPH: fermented pepsin Kariya protein hydrolysates, UPcKPH: unfermented pancreatin kariya protein hydrolysates, UPsKPH: unfermented pepsin Kariya protein hydrolysates

4.6 Ferric Reducing Activity (FRAP)

The reducing abilities of fermented and unfermented *kariya* protein hydrolysates produced from two different enzymes, pancreatin (FPcKPH and UPcKPH) and pepsin (FPsKPH and UPsKPH) are shown in Fig. 3. The reducing ability of all the extracts was influenced by concentration between 0.0-1.0 mg/mL. The radical scavenging activities increased significantly (P≤0.05) for all samples. The Ferric reducing abilities of the extracts followed the following order: FPcKPH>FPsKPH>UPcKPH>UPsKPH at all concentrations between 0.0-1.0 mg/ml. Among the different hydrolysates, FPcKPH exhibited the highest ferric reducing ability value (0.63 µg/g at 1.0 mg/ml) followed by FPsKPH (0.50 µg/g at 1.0 mg/ml) and UPcKPH (0.35 µg/g at 1.0 mg/ml), while the lowest ferric reducing activity was obtained with UPsKPH (0.23 µg/g) sample at the same concentration as shown in Table 3. Also the enzymes reacted differently with the extracts as pancreatin digest had better ferric reducing abilities than pepsin digest in the fermented extracts (FPcKPH>FPsKPH) and similar results were also observed in the unfermented extracts (UPcKPH>UPsKPH). This same trend was also observed with DPPH free radicals scavenging assay of the extracts. The result obtained is similar to that of defatted palm kernel cake protein hydrolysate as reported by [37] which showed differences in the reducing power activities of the hydrolysates produced using different enzymes: pepsin hydrolysed cake (1.34 µg/g) and pancreatin hydrolysed cake (0.74 µg/g). [38] revealed that samples with higher reducing power have better abilities to donate electron and free radicals to form stable substances, thereby interrupting the free radical chain reactions. Similar results were observed by many authors: [36] and [39] all pointed out that the release of some hydrophobic fractions with special reducing abilities during fermentations could be responsible for the increased reducing activities of rapeseeds peptide reactions and loach peptide hydrolysates, respectively.

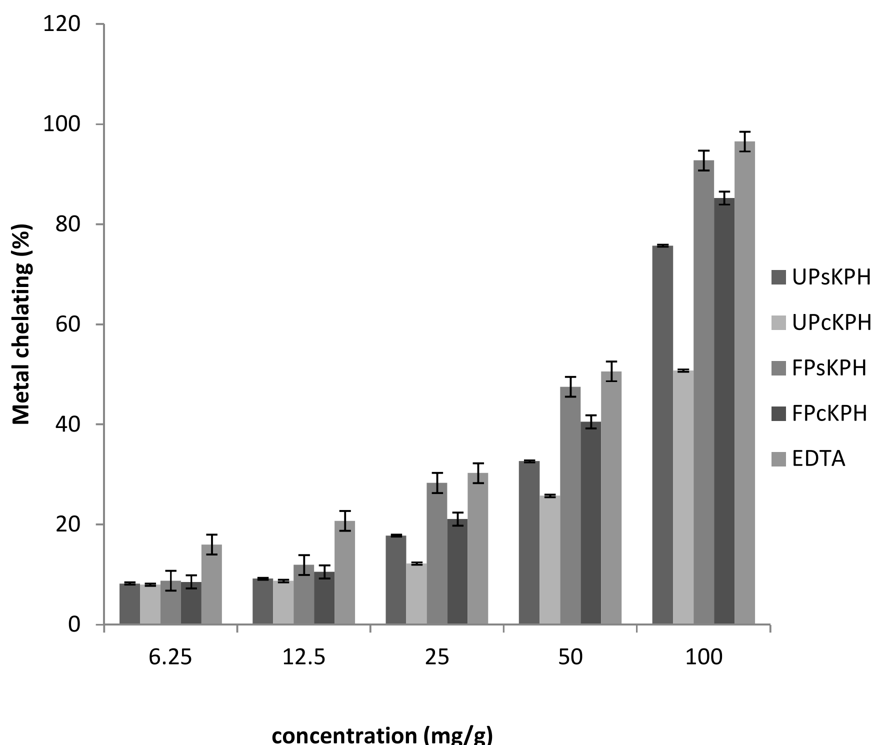

Fig. 2. Metal chelating ability of fermented and unfermented *kariya* protein hydrolysates at different concentration. Error bars showing the standard deviation (n=3)

FPcKPH: Fermented pancreatin kariya protein hydrolysates, FPsKPH: Fermented pepsin kariya protein hydrolysates, UPcKPH: unfermented pancreatin kariya protein hydrolysates, UPsKPH: unfermented pepsin kariya protein hydrolysates

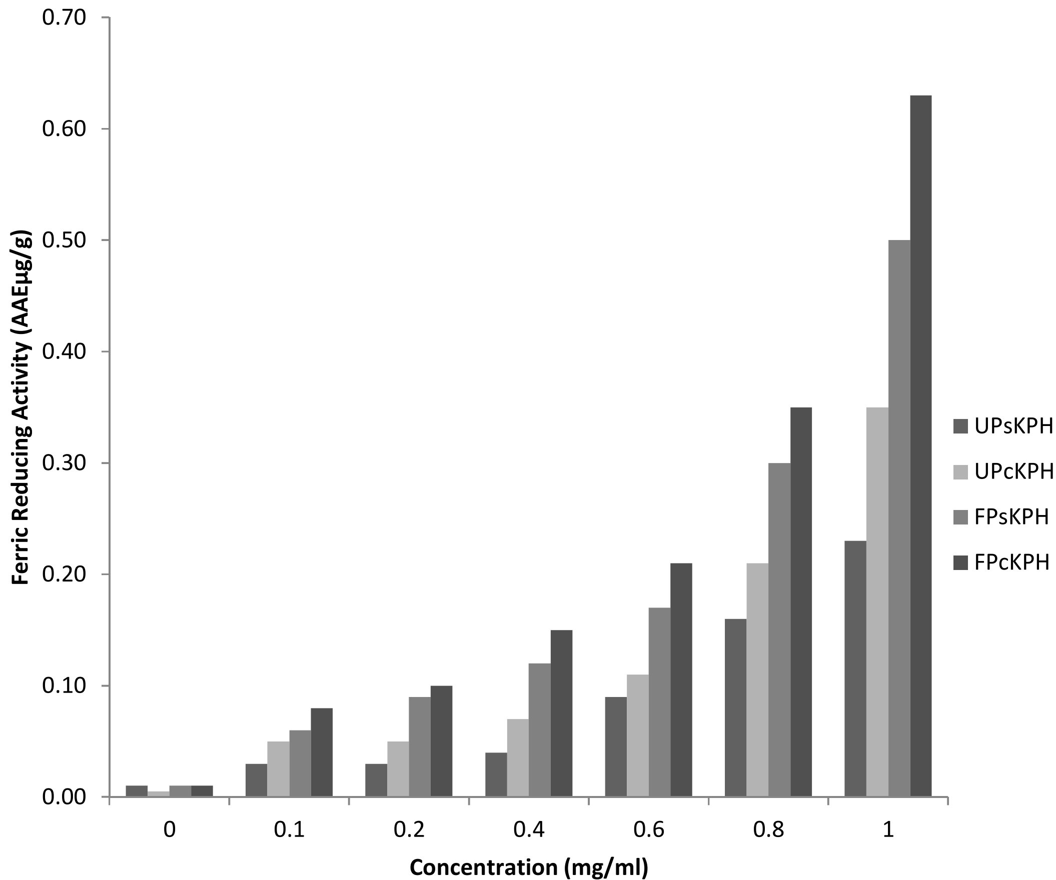

Fig. 3. Ferric Reducing Activity (FRAP) of fermented and unfermented *kariya* protein hydrolysates at different concentrations. Error bars showing the standard deviation (n=3)

FPcKPH: Fermented pancreatin kariya protein hydrolysates, FPsKPH: Fermented pepsin kariya protein hydrolysates, UPcKPH: unfermented pancreatin kariya protein hydrolysates, UPsKPH: unfermented pepsin kariya protein hydrolysates

5. CONCLUSION

The present study revealed that peptides in fermented hydrolysates were more easily hydrolysed than unfermented hydrolysates. The hydrolysates were also more easily hydrolysed by pancreatin than pepsin. The hydrolytic nature of enzymes used and fermentation significantly influence the proximate and functional properties of the hydrolysates. The DPPH free radical scavenging abilities, ferric reducing abilities and metal chelating capabilities of the samples were found to improve with fermentation and hydrolysis. The study concluded that *kariya* seed extracts could serve as a functional food and as natural source of antioxidant in the food industry to prevent lipid oxidation and maintain wholesomeness and freshness of food products during production and storage.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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