EVALUATION OF PROXIMATE, FUNCTIONAL AND ANTIOXIDATIVE PROPERTIES OF ENZYMATIC HYDROLYSATES FROM FERMENTED *KARIYA* (*HILDERGARDIA BARTERII*) SEED PROTEIN ISOLATES

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ABSTRACT
Background: *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).*
Method:*The degree of hydrolysis (DH) proximate, functional properties and some anti-oxidative characteristics of enzymatic protein hydrolysates were evaluated.*
Results*: The results showed that pancreatin hydrolysates (FPcKPH; 65.20% and UPcKPH; 50.50%) had higher degree of hydrolysis 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 that 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).*
Conclusion:*The study concluded that fermented kariya seeds hydrolysates could find applications as potential natural anti-oxidant in food.*

Keywords: Hydrolysates, Antioxidant, Fermented, Pepsin, Pancreatin, *Kariya*

Submitted: 27.02.2016 Reviewed: 09.05.2016 Accepted: 07.06.2016

1. INTRODUCTION

In recent years, bioactive peptides derived from natural food sources have been widely researched due to their potential health benefits associated with high bioactivity, low molecular weight, easy absorption and less toxicity effects (Vioque *et al.*, 2000). 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 it into peptides with desired size, charges and surface properties (Rafik, 2010). The peptides produced by hydrolysis have been reported to exist smaller molecular masses and less secondary structure than intact protein. This has been found to lead to improved functional properties of the proteins (Molina Ortiz and Wagner, 2002). However, the usage of plant proteins, especially from cereals and oilseeds has been growing intensively over the last decades. They have been used as alternative for animal proteins in human nutrition, functional agents and bioactive components in food (Ogunwolu *et al*., 2009). Moreover, research carried out in last few years showed that many oil seeds have seeds have antioxidant effects. *Kariya* seeds (*Hildergardia barterii*) as oil seeds are consumed mostly in West African countries as raw or roasted nuts having a flavour like that of peanuts. It is grown for the ornamental nature of its flowers which are conspicuous during dry season. Theseed kernels have been reported to contain 17.5 % protein (Ogunsina *et al.*, 2011). Moure (2006) 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 using hydrolysis, particular functional and anti-oxidative properties are obtained.Fermentation as an old 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 (Oyarekua, 2012). 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 (Nenadis *et al.*, 2004). 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 (Vioque *et al.*, 2000). 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 restricts 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* seeds as oil seeds have good proportion of protein (17.5%) and this level protein represents prospects for obtaining functional peptides. Research efforts on *kariya* seeds have focused mainly on the whole and defatted flours. Gbadamosi and Famuwagun (2015) worked on the fermented isolates of these seeds. The chemical and fatty acids compositions of the seeds have also been studied. Recently, there is a remarkable report on the physical, functional and nutritional properties of *kariya* seeds defatted flours as well as its isolates. The properties of the oils extracted from its kernel has also been reported by Adebayo *et al.* (2013; 2015). Availability of the information on *kariya* seeds protein hydrolysates could offer *kariya* as a potential source of functional and natural bioactive peptides and therefore increase its utilization as food ingredients.

2. MATERIALS AND METHODS

Collection and Preparation of plant 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.

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 50^0C and the water was changed every 6 h interval for 4 times. The soaked seeds were then transferred into different calabash pots, lined uniformly with banana leaves (up to 5 layers) and allowed to ferment for 96 h inside the incubator (30°C). The second portion was neither soaked nor fermented. The fermented sample was oven dried at 60 °C 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 sohxlet extraction apparatus. The defatted flours were desolventized 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. *Kariya* protein isolate was prepared by a method described by (Gbadamosi *et al.*, 2012). A known weight (100 g) of the defatted flours (fermented and unfermented) was dispersed in 1000 ml of distilled water to give final flour to liquid ratio of 1:10 in separate containers. The suspension was gently stirred on a magnetic stirrer for 10 min. The pH of the resultant

slurry was adjusted to the point at which the protein was 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 0.1N 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 1M NaOH prior to freeze-drying. The freeze-dried protein was later stored in air-tight plastic container at room temperature.

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 (Omoni and Aluko, 2006). A 5% (w/v) *kariya* protein isolate's slurry was adjusted to pH 2.0 and incubated at 37 ºC followed by addition of pepsin. Slurry was adjusted to pH 7.5 and incubated at 40 °C 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 at 4ºC) for 30 min. The resulting supernatants were used to measure the degree of hydrolysis. The 4-hour hydrolysates obtained were freeze-dried and then preserved at -18°C for subsequent analysis.

Measurement of Degree of Hydrolysis (DH)

The DH was determined by formaldehyde titration method according to method described by Song *et al*. (2013). Five millilitres of hydrolysates supernatant were diluted with 60 ml distilled water, while magnetically stirring, and titrated by 0.05M NaOH (standard titration solution) to pH 8.2 and volume 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:

$$DH = \frac{C \times (V1 - V2) X\, V/5}{m \times percentage\ of\ protein\ in\ the\ raw\ material\ x\ 8} \ x\ 10$$

Where:

C = the concentration of standard titration solution of NaOH (0.05M)

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 kariya protein hydrolysates

m = the mass of the raw material

Proximate Composition of the Freeze-dried Hydrolysates

Crude protein, Ash content, Crude fibre, Crude fat, Moisture and Carbohydrates were determined using the method of A.O.A.C (2000).

Functional Properties of the Freeze-dried Hydrolysates

Oil absorption capacity (OAC) and Water absorption capacity (WAC), swelling capacity foaming properties and emulsifying properties were determined using the method of A.O.A.C (2000).

ANTIOXIDANT PROPERTIES OF *KARIYA* HYDROLYSATES

DPPH (diphenyl-1-picryhydrazyl) radical scavenging activity assay

The free radical scavenging ability of the hydrolysates was determined using the stable radical DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) as described by (Pownall *et al.*, 2010). Samples concentrations of between 0.5-2.5

mg/ml was prepared. 1ml of 0.3Mm DPPH was added to the prepared samples to initiate the reaction. Ascorbic acid was used as positive control and the change in colour was measured spectrophotometrically at 517nm. The percent of inhibition was calculated from the following equation:

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

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 regression plot inhibition percentage against extract concentrations.

Metal chelating ability of the Hydrolysates

The metal-chelating activities of the samples was carried out according to the method described by (Singh and Rajini, 2004). Solutions of 2mM $FeCl_2 \cdot 4H_2O$ and 5mM ferrozine was diluted 20 times. Sample concentrations was made between 6.25-100mg/ml, EDTA was used as positive control and the change in colour was monitored at 562 nm after a period of 10 min incubation. The percent inhibition of ferrozine–Fe^{+2} complex formations was calculated using the formula:

$$Chelating\ effec = \frac{Acontrol - Asample}{Acontrol} \times 100$$

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 samples.

Determination of ferric reducing abilities of the hydrolysates

The ferric reducing abilities of the samples following the method described by Benzie and Strain (1999). Sample concentrations were prepared from 20-100µg/ml. Ascorbic acid was used as positive control was and standard FRAP reagent was added. Absorbance was read at 593nm after incubating for 10 minutes.

Statistical Analysis

Data obtained were subjected to analysis of variance. Significance of mean differences was determined. Significance was accepted at $p \leq 0.05$.

3. RESULTS AND DISCUSSIONS

Degree of Hydrolysis of Fermented and Unfermented *Kariya* Hydrolysates

The percentage of peptide bonds cleaved during the hydrolysis of protein measured in terms of degree of hydrolysis (DH) for the pancreatin and pepsin hydrolysed *kariya* protein hydrolysates are presented in Table 1. The results showed 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%) yield and these values were significantly different at P<0.05 as shown in Table 1. According to Zheng *et al.* (2008), the degree of hydrolysis (DH) is significantly influenced by the hydrolysis conditions that included 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 this 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 hydrolytic effects of these enzymes. These results agreed with the reports of Leon *et al.* (2010) for degree of hydrolysis of lentil (*Lentil sculenta*) protein hydrolysates where pancreatin hydrolysed hydrolysates had higher yield than the pepsin hydrolysed hydrolysates. The results also showed that fermented hydrolysates had higher values of (DH) than unfermented hydrolysates, indicating that fermentation enhanced the production of more bioactive peptides with shorter chains (Nakajima *et al.*, 2005).The degree of hydrolysis (DH) is important because protein hydrolysates with high value of (DH) which correlates with presence of more short peptides

chain have been found to be responsible for the higher free radical scavenging ability of black soybeans (Ralison *et al*.,2013).

Proximate Composition of the Hydrolysates

The proximate composition of the hydrolysates is shown in Table 2. Samples hydrolysed with pancreatin enzymes was found to contain higher protein content than the pepsin hydrolysed samples both in the fermented and unfermented form. Sample FPsKPH contained 94.31% protein content and this value was higher than the value obtained for UPsKPH but lower than the value obtained for sample FPcKPH. Fermented hydrolysates produced using pancreatin enzyme contained the highest protein content. This value correlated with the trend obtained with the degree of hydrolysis of the samples. The protein contents obtained in this study were higher than the values reported by Gbadamosi and Famuwagun, (2015) for unhydrolysed fermented and unfermented *kariya* protein isolates. This showed that the hydrolysis was able to degrade more of the native proteins.

Table 1: Degree of hydrolysis (DH) of Fermented and Unfermented *Kariya* Protein Hydrolysates

Sample	Degree of Hydrolysis (%)
UPsKPH	33.93±0.94^{a}
UPcKPH	50.50±1.23^{b}
FPsKPH	53.00±0.82^{c}
FPcKPH	65.20±.91^{d}

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within 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

The ash content is an indication of the level of mineral elements available in a particular food samples. In this 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. 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. Crude fibre was not detected in any of the samples and the moisture content 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. Generally, the result obtained in this study for the proximate composition compared well with those reported by Muhamyankaka *et al.* (2013) on the composition of enzymatic hydrolysis of pumpkin meal protein. Similar trend was also reported by Zhao *et al*. (2012). The differences the result obtained in this study for all the hydrolysates.

Functional Properties of the Hydrolysates

The water absorption of the hydrolysates revealed that 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).

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 within 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

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 ability of the hydrolysates to swell as measured by swelling capacity is shown in Table 3. The swelling power of the samples ranged from 282.09% to 310.24%. The highest swelling capacity was obtained in sample FPcKPH while the lowest was recorded for FPcKPH and the values were significantly different ($p<0.05$). Just like the water absorption capacity, the fermented hydrolysates were higher than the unfermented hydrolysates. The pancreatin hydrolysed samples were found to swell better than the pepsin hydrolysed samples both in the fermented and unfermented form.

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 these values were significantly different ($p<0.05$) from one another. The hydrolysates produced using pancreatin enzymes was found to be 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 was 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
between 242-260 m^2/g. It was observed in this 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 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 functional properties of the hydrolysates produced in this study are different from one another. 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 this study showed that the samples are mostly favoured in this order:FPcKPH>FPsKPH>UPcKPH>UPsKPH.

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}
Swelling Capacity (%)	282.09 ± 1.13^{d}	290.00 ± 2.09^{c}	302.43 ± 1.69^{b}	310.24 ± 2.59^{a}

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within the row are significantly ($P < 0.05$) different.

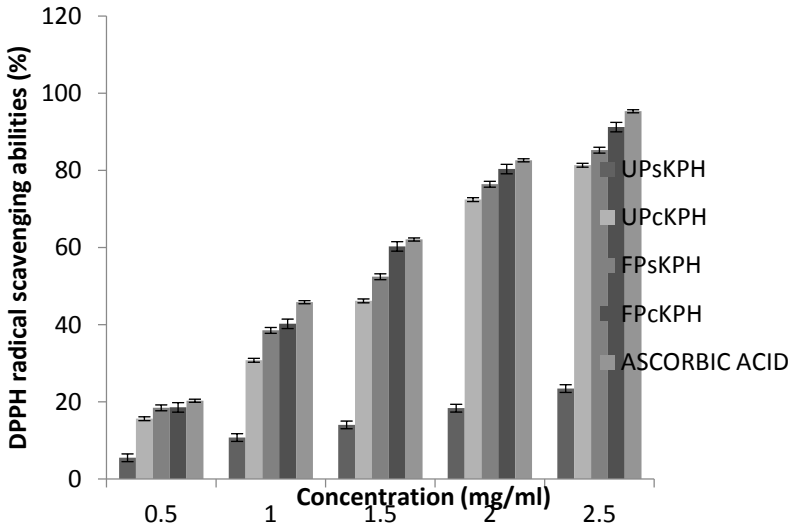
FPcKPH: Fermented pancreatin *kariya* protein hydrolysates**, FPsKPH:** Fermented pepsin *kariya* protein hydrolysates, **UPcKPH:** unfermented pancreatin *kariya* protein hydrolysates, **UPsKPH:** unfermented pepsin *kariya* protein hydrolysatesranged

In this study, it was clearly shown 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. Gbadamosi and Famuwagun (2015) reported similar results on the fermented protein isolates of *kariya* seed. Igbabul *et al* (2014) reported similar results on the functional properties of fermented coconut flours. In producing the hydrolysates in this study, the kariya seeds were fermented even getting to hydrolysing them, the micro-organism already acted and improved these functional properties at the pre-hydrolysing stage. Also, pancreatin hydrolysed hydrolysates appeared to have better functional properties than the pepsin hydrolysed samples. In this case, studies have shown different enzymes have 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 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 this study. Similar observation was made by Ferial *et al*. (2013) on the soyabeans hydrolysates. Ashraf*et al*. (2006) 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, enzymatic hydrolysis with fermentation is a good step.

ANTIOXIDANT PROPERTIES OF THE HYDROLYSATES

DPPH radical scavenging activities

The 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 as presented in the Figure 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 0.5-2.5mg/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 0.5-2.5mg/ml.



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

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 (Kang *et al.*, 2009). The results obtained in this study revealed that fermented hydrolysates (FPcKPH and FPsKPH) with lowerIC_{50} values (1.03 and 1.40 mg extract ml)had better capabilities 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 this study might be attributed to the differences in peptide chains 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 (Nakajima *et al*., 2005). These results agree with that reported by Samruan *et al*. (2012) 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 Ralison *et al.*(2013) 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 Samruan *et al*.(2012) and also reported by Tsopmo *et al*.(2009) 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 Elias *et al*.(2008) and Samruan *et al*. (2012) on the free radical scavenging abilities of soyabeans hydrolysates.

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 Figure 3. The metal chelating ability of the extracts followed the following order: FPsKPH>FPcKPH>UPsKPH>UPcKPH at all concentrations between 6.25-100µg/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 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 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 hadbetter free radical scavenging capabilities than pepsin hydrolysates. This is in agreement with the result obtained for African yam bean seed protein hydrolysates reported by Ajibola *et al.* (2011). Chen *et al*., 1998 also observed similar results on fermented soy hydrolysates.

Ferric Reducing Activity (FRAP)

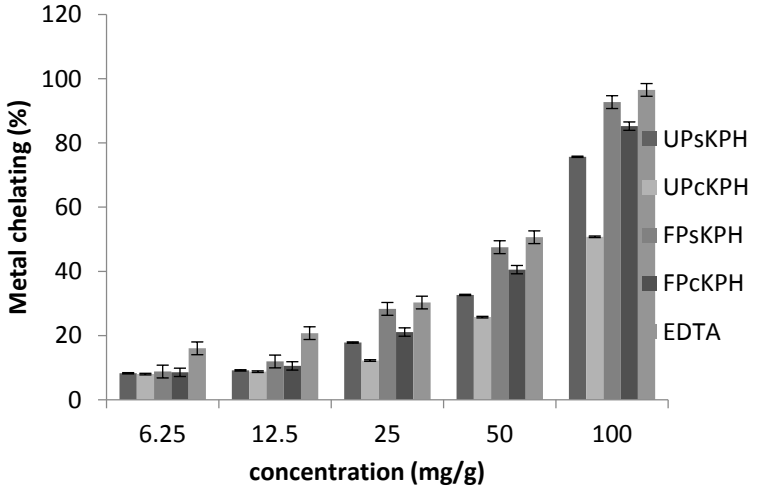
This reducing abilities of fermented and unfermented *kariya* protein hydrolysates produced from two different enzymes, pancreatin (FPcKPH and UPcKPH) and pepsin (FPsKPH and UPsKPH) are shown in Figure 3. The reducing ability of all the extracts was influenced by concentration between 0.0-1.0mg/ml. The radical scavenging activities increased significantly ($p \leq 0.05$) 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 (0.63µg/g at 1.0 mg/ml) followed by FPsKPH (0.50µg/g at 1.0 mg/ml) and UPcKPH (0.35µg/g at 1.0 mg/ml), while the lowest ferric reducing activity was obtained with UPsKPH 0.23µg/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).

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}	

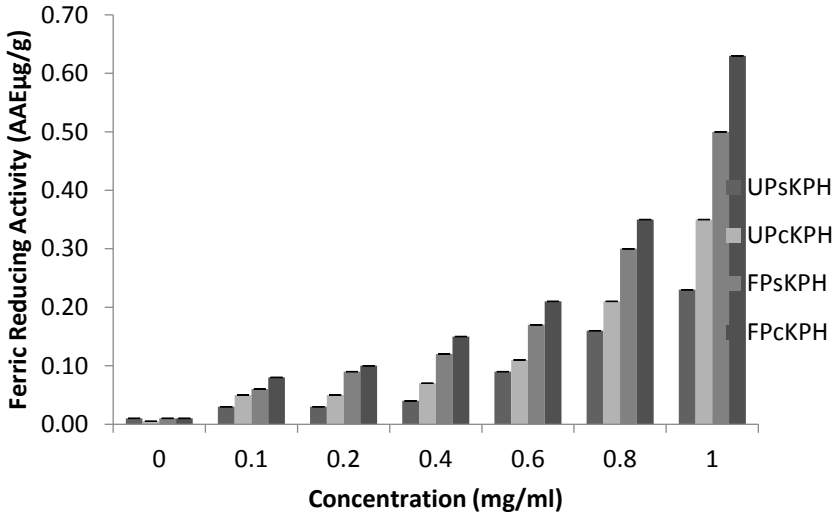
Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within same column are significantly ($P < 0.05$) different.

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.



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. 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)

This same trend was also observed with DPPH free radicals of the extracts. The result obtained is similar to that of defatted palm kernel cake protein hydrolysate as reported by Zarei *et al*. (2012), which showed differences in the reducing power activity of the hydrolysates produced using different enzymes: pepsin hydrolysed cake (1.34µg/g) and pancreatin hydrolysed cake (0.74µg/g). Juntachote (2005) 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: Zhang *et al*. (2009) rapeseed fractions and You *et al*. (2009) loach peptide hydrolysates, all pointed out of the release of some hydrophobic fractions with special reducing abilities during fermentations could be responsible for the trend.

4. CONCLUSION

The 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 led to significant increase in the proximate and functional properties. DPPH free radical scavenging abilities, ferric reducing abilities and metal chelating capabilities of the samples were also improved. The study concluded that *kariya* seed extracts could serve as functional foodand as natural source of antioxidant in the food industry to prevent lipid oxidation and maintain the wholesomeness and freshness of food products during production and storage.

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