

Full Length Research Paper

Effect of enzyme/substrate ratio on the antioxidant properties of hydrolysed African yam bean

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The use of natural antioxidant as compared with synthetic antioxidant in food processing is a growing trend as consumers prefer natural to synthetic antioxidant mainly on emotional ground. This study investigates the antioxidant activity of hydrolysed African yam bean (*Sphenostylis sternocarpa*) which is regarded as one of the neglected underutilized species (NUS) of crop in Africa and Nigeria especially to improve food security and boost the economic importance of the crop. The antioxidant properties of African yam bean hydrolysates (AYH) produced at different enzyme to substrate (E/S) ratios of 1: 100 and 3: 100 (W/V) using pepsin (pH 2.0, 37°C) were studied. 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity of the hydrolysates was significantly influenced by the E/S ratio as DPPH radical scavenging activity ranged from 56.1 to 75.8% in AYH_(1:100) and 33.3 to 58.8% in AYH_(3:100) with 1000 µg/ml having the highest DPPH radical scavenging activity. AYH_(1:100) and AYH_(3:100) had higher reducing activities of 0.42 and 0.23, respectively at a concentration of 1000 µg/ml. The phenolic content of hydrolysates followed similar trend as DPPH and reducing power activity. The usage of higher enzyme to substrate ratio reduces the antioxidant activity of the hydrolysates, hence reducing its potential health benefits.

Key words: African yam bean, hydrolysates, enzyme/substrate ratio, antioxidant activities.

INTRODUCTION

The rapidly growing food industry, which constantly demands new ingredients has drawn researchers to legume components, Clemente et al. (1998) and Adebowale et al. (2005), in a bid to finding antioxidants from natural sources, which may have less potential health hazard compared with synthetic antioxidants. African yam bean (*Sphenostylis sternocarpa* Hochst. Ex A. Rich), belongs to the family, Papilionaceae, it is sometimes classified in the sub family Leguminosae, and usually cultivated for its edible seeds and tuberous roots, it is an important crop in western, central and some parts of East Africa and it is cultivated for both its seeds and tubers. The seeds vary in size and colour (marbled, brown, dark brown, black and grey), with protein content (21.0 to 29.0%) lower than soybean (38%), and 50%

carbohydrate mainly starch (Eromosele et al., 2008). Its amino acid spectrum indicated that lysine and methionine which are limiting amino acids in most vegetable proteins are better than compared to other legumes including soybean (Evans and Haismer, 1979). Apart from the use of soybean as an animal-alternative protein source, the exploitation of protein from other legumes is rare (Smart, 1989). Protein isolates are a good substrate for the production of hydrolysates with improved functional and nutritional properties which facilitates the revalorization of numerous oilseeds and grain legumes (Vioque et al., 2002). The presence of antinutritional factors is reduced in the production of isolates from legumes; they are also sources of bioactive peptides, which are short chain peptides with beneficial biological activities which are released from food proteins during hydrolysis (Vioque et al., 2000). Protein hydrolysates from different sources, such as whey, soy protein (Pena-Ramos and Xiong, 2003), egg-yolk (Sakanaka et al., 2004), prawn

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(Suetsuna, 2000), tuna cooking juice (Jao and Ko, 2002), yellow - fin sole frame (Jun et al., 2004), Alaska Pollack frame (Je et al., 2005) herring (Sathivel et al., 2003), mackerel (Wu et al., 2003) and capelin (Amarowicz and Shahidi, 1997) have been found to possess antioxidant activities capable of scavenging free radicals. Free radicals are highly reactive species due to their single unpaired unbalanced electrons; they are involved in the occurrences of many chronic diseases such as cardiovascular diseases, neurodegenerative disorders and cancer (Dong et al., 2008), it is therefore important to eliminate them from the body. Thus, antioxidant peptides generated by hydrolysis of food proteins constitute a new source of functional components that could inhibit deleterious oxidative processes both *in vivo* and in foods.

With the acknowledgment of the nutritional potentials of AYB, the crop may well contribute to solving food security problems in Africa, until now; there is dearth information on the antioxidant properties of hydrolysates from AYB with respect to the enzyme/substrate ratio. The aim of the present study was to investigate if the antioxidant properties of the hydrolysates from AYB will be influenced by the enzyme/substrate ratio.

MATERIALS AND METHODS

Sources of materials

African yam bean (SSSWN90) was obtained from the Institute of Agricultural Research and Training (IART) Ibadan, Nigeria and all other chemicals and reagents used in this work were of food- grade or reagent - grade.

Protein isolate was prepared from defatted African yam bean (AYB) flour (Chuan et al., 2009).

Preparation of African yam bean hydrolysates

The protein hydrolysates were prepared from the isolate as described by Chuan et al. (2009). Four gram of African yam bean protein isolate (AYBI) was dispersed in 200 ml of distilled water at room temperature. The mixture of protein and enzyme (Pepsin) at two enzyme to substrate (E/S) ratios of 1:100 and 3:100 (V/W) were incubated at temperature and pH optimal for the enzyme (pH 2.0; 37°C) in a water bath. The pH of the mixture was kept constant during hydrolysis by addition of 0.5 N NaOH. The change in degree of hydrolysis (DH) during the enzymatic hydrolysis was followed by pH- stat method (Adler - Nissen, 1986).

The percent DH was calculated according to the following equation:

$$\text{DH (\%)} = (\text{B} \cdot \text{N}_b \times 100) / \text{Mp} \cdot \alpha \cdot h_{\text{tot}}$$

Where, B is the amount of the alkali consumed in (ml); N_b is the normality of the alkali; Mp is the mass of the protein being hydrolysed (protein in grammes, %N \times 6.25); $1/\alpha$ is the calibration factor for pH - stat and the h_{tot} is the content of peptide bonds.

After 90 min hydrolysis time, aliquots of the digested mixture was taken out, and heated at 70°C for 15 min and then cooled immediately in ice water to room temperature. The resulting digest was centrifuged at 4000 g for 20 min to remove insoluble residues. The supernatants was then adjusted to pH 7.0, and lyophilised to

produce the hydrolysates samples, which were stored at -20°C before further analysis.

Chemical analyses

The 1,1-diphenyl- 2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was determined as described by Shimada et al. (1992). Two millilitres of the sample solution with various solid concentrations (0 to 1.0 mg/ml) were fully mixed with 2 ml of 2.0×10^{-4} M DPPH solutions in methanol (freshly prepared). The resulting solution was then left to stand for 30 min, prior to being spectrophotometrically measured at 517 nm. A low absorbance at 517 nm indicates a high DPPH radical scavenging activity. The methanol was used as the blank.

The DPPH radical inhibition as a percentage is calculated by $[1 - (\text{test sample absorbance}/\text{blank sample absorbance})] \times 100$.

Reducing power

The ferric reducing power of the fruit extracts was determined by using potassium ferricyanide–ferric chloride method (Oyaizu, 1986). Different dilutions of the samples amounting to 1 ml were added to 2.5 ml 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1%). The mixtures were incubated at 50°C for 20 min, after which 2.5 ml trichloroacetic acid (10%) was added, two and one half milliliters of the mixture was taken and mixed with 2.5 ml water and 0.5 ml 1% FeCl_3 . The absorbance at 700 nm was measured after allowing the solution to stand for 30 min. A graph of absorbance vs. hydrolysate concentration was plotted to observe the reducing power.

Total phenolic content

The total phenolic content of the samples were determined using Folin - Ciocalteu's reagent (FCR) as described by Waterman and Mole (1994). A calibration curve with different concentration of gallic acid in methanol, in the range of 0.5 to 1.3 mg /100 ml was obtained with an R^2 value of 0.990. The results were expressed as milligrams of gallic acid equivalents (GAE) per ml of hydrolysates. AYH samples were diluted in 10 ml of methanol. 250 μl of the resulting solution was thoroughly mixed with 10 ml of water and 1.25 ml of Folin - Ciocalteu's reagent. After 5 min incubation, 3.75 ml of 20% sodium carbonate was added and volume adjusted with water to 25 ml. After 2 h incubation the absorbance was read at 750 nm using a Cary UV 50 spectrophotometer, Agilent Technologies Canada Inc.

Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and means separated using Duncan's multiples range test using Statistical Package for Social Sciences (SPSS) version 17 .0 computer software.

RESULTS AND DISCUSSION

Degree of hydrolysis (DH)

The DH obtained after 90 min hydrolysis time was 15%

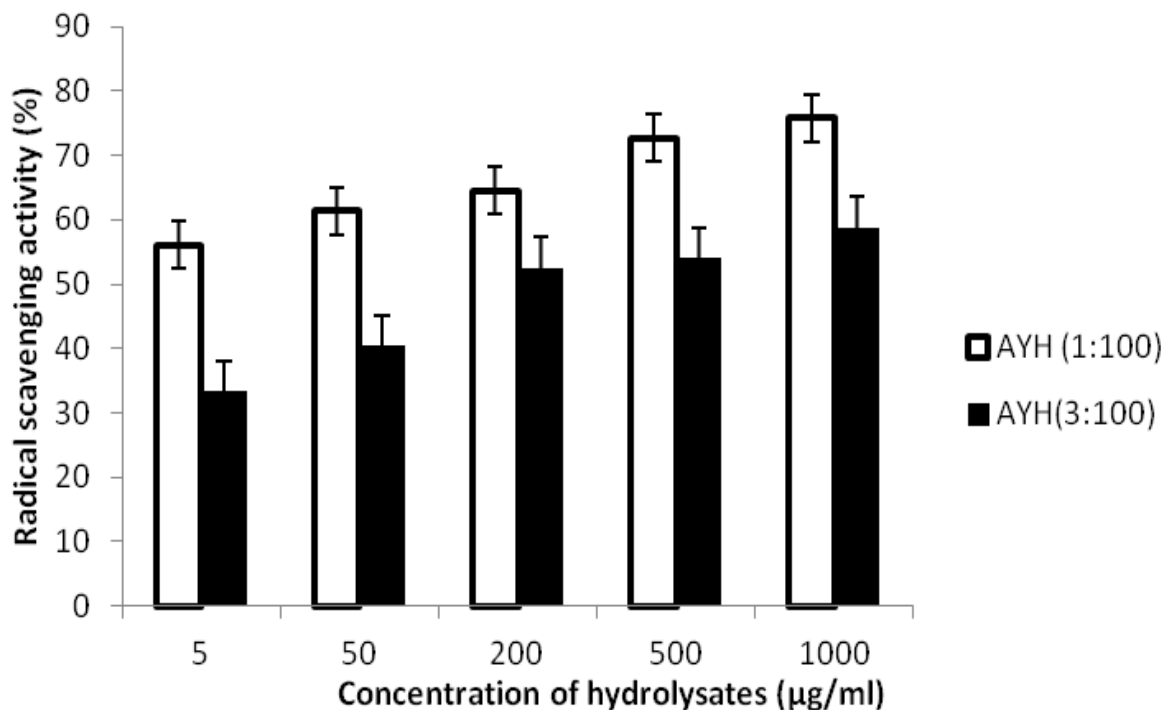


Figure 1. Effect of different concentrations of pepsin hydrolysates of African Yam Bean on their DPPH radical scavenging activity in relation to enzyme-to-substrate ratio. Bars represent the standard error from triplicate determinations. AYH_(1:100) – Africa Yam Bean hydrolysate produced at enzyme – to – substrate ratio 1:100. AYH_(3:100) – Africa Yam Bean hydrolysate produced at enzyme – to – substrate ratio 3:100.

for both concentration under study, results obtained fairly compares with results of previous workers. Gwiazda et al. (1994), using Alcalase and Neutrase for rapeseed proteins reported DH values in the range of 8 to 10%, while Pouliot et al. (1995) reported that milk proteins digested by Trypsin and Chymotrypsin, gave a DH ranged from 6.5 to 7.5% and from 5.5 to 6.0%, respectively. Kamau and Lu (2011) reported a significant increase in DH when the E/S ratio was doubled to 2%, the variation in this result compared to our observation could be attributed to the difference in the enzymes used. The DH is a measure of the extent of hydrolytic degradation of proteins and it is the most used indicator for comparison among different proteolytic processes. The principle of the pH stat technique involves the release of dissociated protons from the free amino groups when hydrolysis is carried out at neutral or alkaline conditions (Adler - Nissen, 1986). The liberation of protons into the surrounding medium leads to a reduction in the pH of the reaction mixture. The number of peptide bonds cleaved was estimated from the amount of base required to maintain a constant pH during the reaction (Adler - Nissen, 1986). All hydrolytic curves showed a high initial rate of reaction in the first 20 min but proteolysis rate decreased gradually in the following time although the data was not shown. According to Adler - Nissen (1986), the reduction in hydrolysis rate have been

attributed to the competition between unhydrolyzed protein and the peptides being constantly formed during hydrolysis, the rate of reaction of the enzyme, levelled out after one hour owing to reduced number of susceptible peptide bonds.

Influence of enzyme to substrate (E/S) on 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) of African yam bean hydrolysates (AYH)

DPPH radical scavenging activities at different E/S ratio of AYH_(1:100) and AYH_(3:100), decreases as the ratio of enzyme to substrate increases as presented in Figure 1. This low activity was probably due to extensive hydrolysis resulting to free amino acids that have been reported to have low antioxidant activity compared to peptides (Decker et al., 2001; Hernandez-Ledesma et al., 2005). DPPH is a stable free radical that shows maximum absorbance at 517 nm, when DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged, and the absorbance is reduced, the decrease in absorbance is taken as a measure for radical-scavenging. Thus, the DPPH radicals could be widely used to investigate the scavenging activity of some natural compounds (Shimada et al., 1992). The DPPH radical scavenging activity of the AYH

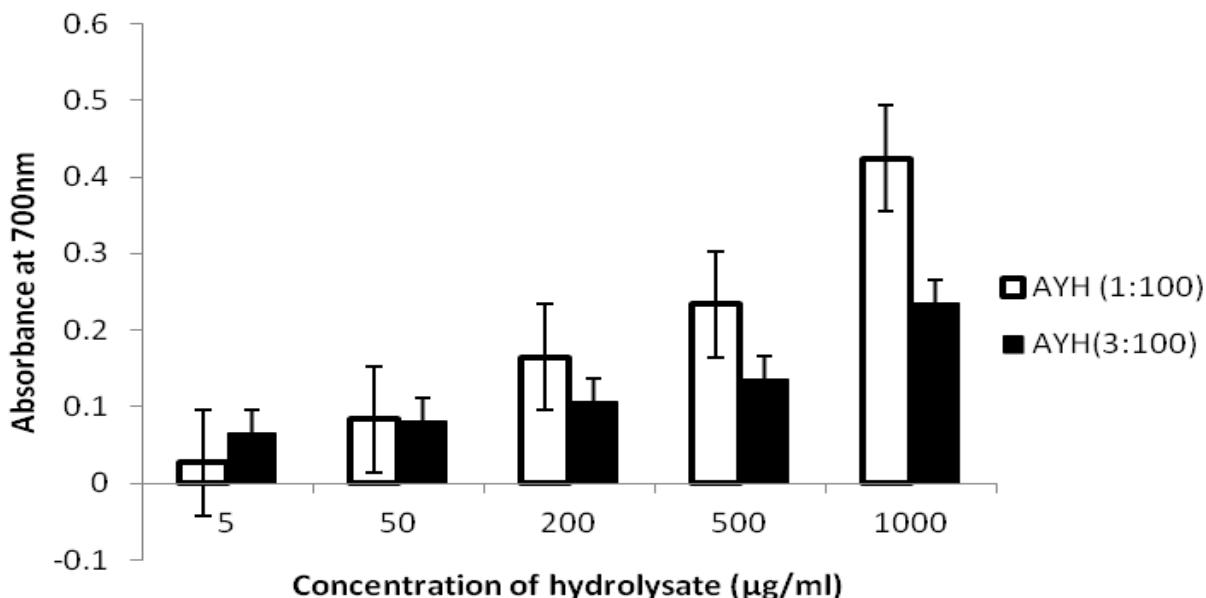


Figure 2. Effect of different concentrations of pepsin hydrolysates of African Yam Bean on their reducing power in relation to enzyme-to-substrate ratio. Bars represent the standard error from triplicate determinations. AYH_(1:100) – Africa Yam Bean hydrolysate produced at enzyme – to – substrate ratio 1:100. AYH_(3:100) - Africa Yam Bean hydrolysate produced at enzyme – to – substrate ratio 3:100.

Table 1. Correlation between DPPH radical scavenging activity and Reducing Power at the different enzyme to substrate ratio.

Analysis	Sample	Linear regression equation	R ² value
DPPH radical scavenging activity	AYH _(1:100)	Y = 0.018x + 59.93	0.867
Reducing power	AYH _(1:100)	Y = 0.000x + 0.056	0.975
DPPH radical scavenging activity	AYH _(1:300)	Y = 0.021x + 40.28	0.704
Reducing power	AYH _(1:300)	Y = 0.000x + 0.067	0.984

was dependent on enzyme used as well as the hydrolysis conditions (T, pH, E/S) that also had influence on DH. The AYH possibly contained amino acids and peptides that were electron and hydrogen donors, or possessed radical trapping ability. The response of peptides could therefore be influenced by the accessibility to the oxidant-antioxidant test systems, solubility in assay solvents as well as their purity, other antioxidant tests would therefore be required to better characterize the AYH.

Influence of enzyme to substrate (E/S) on reducing power of African yam bean hydrolysates (AYH)

The reducing power of AYH_(1:100) and AYH_(3:100) increased with increase in the sample concentration, hydrolysates (1000 µg) prepared using pepsin had the highest reducing power for the different E/S ratio (Figure 2). However, AYH_(1:100) had a stronger reducing power than AYH_(3:100) at the same DH for all the sample

concentration. The difference might be attributed to the specific peptide/amino acid composition, as reported by Wu et al. (2003), although this was not investigated in this study. This increase with reference to the concentration of protein hydrolysates had been reported by previous workers (Rajapakse et al., 2005b; Chuan et al., 2009). Reducing power in the protein hydrolysates was determined as the ability to reduce Fe³⁺ to Fe²⁺, which indicates the capacity to act as an antioxidant by donating electrons. It has been reported that plant protein hydrolysates produced using alcalase have metal-chelating activity that can inhibit oxidative damage due to reactions catalyzed by the transition metals iron and copper (Megias et al., 2007). Thus, the data indicate that the peptides generated after hydrolysis for 90 min for AYH_(1:100) might have a size, structure or sequence that translates into better reducing power, although sequencing was not carried out in this work.

Table 1 shows the correlations between DPPH radical scavenging activity and reducing power at the different

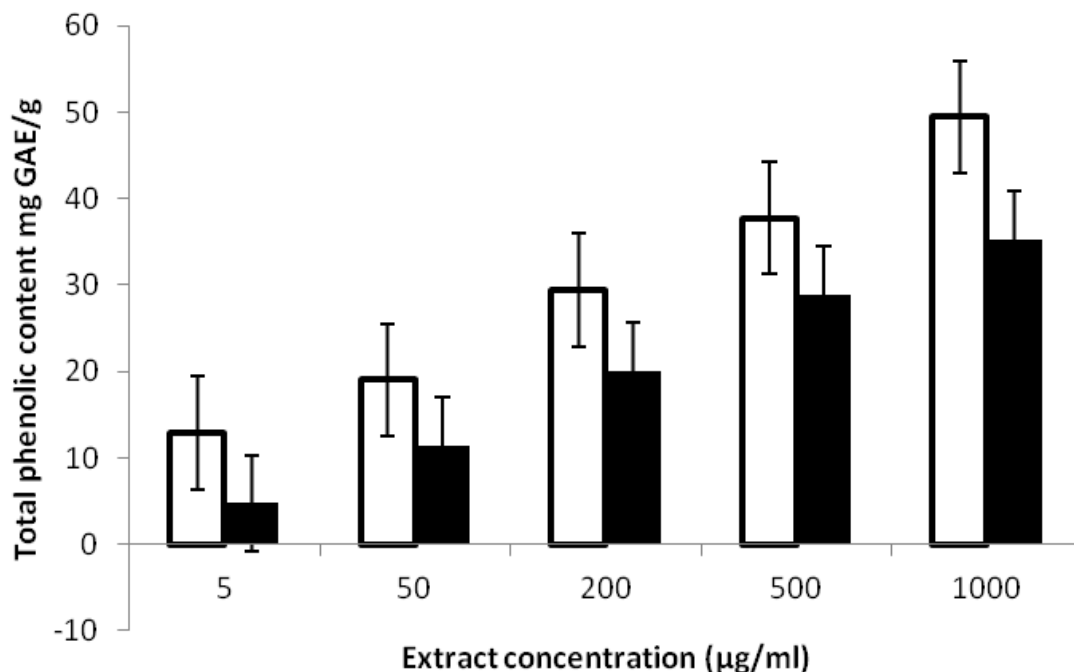


Figure 3. Effect of different concentrations of pepsin hydrolysates of African Yam Bean on their Total phenolic content in relation to enzyme-to-substrate ratio. Bars represent the standard error from triplicate determinations. AYH_(1:100) – Africa Yam Bean hydrolysate produced at enzyme – to – substrate ratio 1:100. AYH_(3:100) - Africa Yam Bean hydrolysate produced at enzyme – to – substrate ratio 3:100.

enzyme – substrate ratio. A linear correlation was observed, and a positive significant correlation was observed between DPPH and reducing power at the same extract concentration. Studies have shown that there is a correlation between antioxidative activities and reducing power of certain bioactive compounds. Pepsin hydrolysates prepared from Africa Yam Bean was found to exhibit antioxidant activity regardless of the enzyme – to substrate ratio used and the concentration used.

Influence of enzyme to substrate (E/S) on Total phenolic content of African yam bean hydrolysates (AYH)

Figure 3 shows the Total phenolic content (TPC) in mg GAE/g of the hydrolysates at different enzyme to substrate ratio. The TPC ranged from 12.86 to 49.44 mg GAE/g for AYH_(1:100) and 4.75 to 35.26 mg GAE/g for AYH_(3:100), a decrease in TPC with increase in the E/S ratio was observed. TPC was observed to increase with concentration of African yam bean hydrolysates produced from pepsin. According to Shahidi and Naezck (2004), natural phenolic exert beneficial effects mainly through their antioxidant activity. These compounds are capable of decreasing oxygen concentration, intercepting singlet oxygen, preventing 1st-chain initiation by scavenging initial radicals, such as hydroxyl radicals, chelating metal

ion catalysts, decomposing primary product of oxidation to non-radical species and breaking chains to prevent continued hydrogen abstraction from substances (Enujiugha, 2010). Phenolic compounds are known to contribute to the overall antioxidant activities of the plant foods.

Conclusion

The enzymatic hydrolysis by pepsin resulted in effective breakdown of the AYB isolate, showing excellent antioxidant properties, including DPPH radical scavenging ability, reducing power and total phenolic content. The antioxidant properties of AYH_(1:100) and AYH_(3:100) increased with increase in the sample concentration, hydrolysates (1000 µg/ml) prepared using pepsin had the highest antioxidant properties for the different E/S ratio. AYH_(1:100) produced at 1000 µg/ml displayed better properties than AYH_(3:100). Therefore, African yam bean protein hydrolysates (AYH) can be used in food systems as a natural additive possessing excellent antioxidative properties.

REFERENCES

Adebowale YA, Adeyemi IA, Oshodi AA (2005). Functional and Physicochemical properties of flours of six *Mucuna* species. Afr. J.

- Biotechnol. 4: 1461-1468.
- Adler - Nissen J (1986). Enzymic hydrolysis of food proteins. Elsevier Applied Science Publishers.
- Amarowicz R, Shahidi, F (1997). Antioxidant activity of peptide fractions of capelin protein hydrolysates, *Food Chem.*, 58: 355-359.
- Chuan HT, Jing P, Da-Wen Z. Zhong, C (2009). Physicochemical and antioxidant properties of buckwheat (*Fagopyrum esculentum* Moench) protein hydrolysates. *Food Chem.*, 115: 672-678.
- Clemente AR, Sanchez-Vioque J, Vioque Bautista J, Millan, F (1998). Effect of cooking on the protein quality of chickpea (*Cicer atietinum*) seeds. *Food Chem.*, 62: 1-6.
- Decker EA, Ivanov V, Zhu B, Frei, B. (2001). Inhibition of Low-Density Lipoprotein Oxidation by Carnosine and Histidine. *J. Agric. Food Chem.*, 49(1): 511-516
- Dong SY, Zeng MY, Wang DF, Liu ZY, Zhao YH, Yang, HC (2008). Antioxidant and biochemical properties of protein hydrolysates prepared from Silver carp (*Hypophthalmichthys molitrix*). *Food Chem.*, 107: 1485-1493.
- Enujiugha, VN (2010). The antioxidant and free radical scavenging of phenolics from African locust bean seeds (*Parkia biglobosa*). *Adv. in Food Sci.*, 32(2): 88 -93
- Eromosele CO, Arogundade LA, Eromosele IC, Ademuyiwa O (2008). Extractability of African yam bean (*Sphenostylis sternocarpa*) protein in acid, salt and alkaline aqueous media. *Food Hydrocol.*, 22: 1622-1628.
- Evans ID, Haismer, DR (1979). Rheology of gelatinized starch suspension. *J. Texture Stud.*, 10 : 347-370.
- Gwiazda S, Wyrzykowska A, Wasyluk K (1994). Comparison of selected method for determination the degree of hydrolysis of rapeseed proteins. *Pol. J. Food Nutr. Sci.*, 3/44: 165-173
- Hernandez-Ledesma B, Davalos A, Bartolome B, Amigo L. (2005). Preparation of antioxidant enzymatic hydrolysates from α -lactalbumin and β -lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J. Agric. Food Chem.*, 53: 588-593.
- Jao CL, Ko WC (2002). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolysates from tuna cooking juice, *Fish. Sci.*, 68: 430-435.
- Je JY, Park PJ, Kim SK (2005). Antioxidant activity of peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate, *Food Res. Int.*, 38: 45-50.
- Jun SY, Park PJ, Jung WK Kim SK (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein, *Eur. Food Res. Technol.*, 219: 20-26.
- Kamau SM, Lu RR (2011). The effect of enzymes and hydrolysis conditions on degree of hydrolysis and DPPH radical scavenging activity of whey protein hydrolysates. *Curr. Res. Dairy Sci.*, 3: 25-35.
- Megias C, Pedroche J, Yust MM, Gir'on-Calle J, Alaiz M, Mill'an, F (2007). Affinity purification of copper chelating peptides from Chickpea protein hydrolysates. *J. Agric. Food Chem.*, 55: 3949-3954
- Oyaizu M (1986). Studies on product of browning reaction prepared from glucose amine, *JPN J. Nutr.*, 44: 307-315.
- Pena-Ramos EA, Xiong YL (2003). Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties, *Meat Sci.*, 64: 259-263.
- Pouliot Y, Gauthier SF, Bard C (1995). Skim milk solids as substrate for the preparation of casein enzymatic hydrolysates. *J. Food Sci.*, 60: 111-116.
- Rajapakse N, Mendis E, Jung WK, Je, JY, Kim SK (2005b), "Purification of radical scavenging peptide from fermented mussel sauce and its antioxidant properties," *Food Res. Int.*, 38: 175-182.
- Sakanaka S, Tachibana Y, Ishihara N, Juneja, LR (2004). Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system, *Food Chem.*, 86: 99-103
- Sathivel S, Bechtel PJ, Babbitt J, Smiley S, Crapo C, Reppond KD (2003) . Biochemical and functional properties of herring (*Clupea harengus*) byproduct hydrolysates, *J. Food Sci.*, 68: 2196 -2200.
- Shahidi F, Naezki, M (2004). Antioxidant properties of Food phenolics. Phenolics in Foods and Nutraceuticals. Boca Raton, Fla. CRC presentation. 1: 403.
- Shimada K, Fujikawa K, Yahar K, Nakamura, T (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion, *J. Agric. Food Chem.*, 40: 945-948.
- Smart J (1989). The Potential Value of Under – exploited Plants in Soil Conservation and Land Reclamation. In: Wickens, G.E., Hag, N, and Day, P. (Ed.). *New Crops for Food and Industry*. Chapman and Hall, London. pp.93-100.
- Suetsuna K, (2000). Antioxidant peptides from the protease digest of prawn (*Penaeus Japonicus*) muscle, *Mar. Biotechnol.*, 2: 5-10.
- Vioque J, Sanchez-Vioque R, Clemente A, Pedroche J. Milla'n, F (2002). Partially hydrolyzed rapeseed protein isolates with improved functional properties. *J. Am. Oil Chem. Soc.*, 77: 1-4.
- Vioque J, Torres A, Quiles J (2000). Time spent watching television, sleep duration and obesity in adults living in Valencia, Spain. *Int. J. Obes. Relat. Metab. Disord.*, 24: 1683-1688.
- Waterman PG, Mole S (1994). Analysis of phenolic plant metabolites, Blackwell Scientific Publications, Oxford, U. K.
- Wu HC, Chen HM, Shiau CY (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*), *Food Res. Int.*, 36: 949-957.