Effect of hydrolysis time on nutritional, functional and antioxidant properties of protein hydrolysates prepared from gingerbread plum (*Neocarya macrophylla*) seeds

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Abstract

Nutritional, functional properties and antioxidant activities of enzymatic hydrolysates of gingerbread plum seed protein isolates (HGPSPIs) were investigated. HGPSPIs were prepared at different times (5 - 180 min) using two food-grade proteases. Pepsin was applied in the first hydrolysis stage followed by trypsin. The hydrolysis time showed a significant effect (p < 0.05) on nutritional parameters such as amino acid score, essential amino acid index, biological value and protein efficiency ratios. Emulsifying activity index and foaming capacity decreased as hydrolysis time increases. During the whole hydrolysis period of 180 min, the antioxidant activities (hydroxyl radical scavenging, ferrous chelating, reducing power and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging) of HGPSPIs increased as function of incubation time. Furthermore, within all samples a large amount of small-sized peptides (500 - 3000 Da) was observed with increasing hydrolysis time. The above results indicated that hydrolysis time has positively affected nutritional and antioxidant parameters of HGPSPIs but had a negative impact on the functional properties studied.

Introduction

Gingerbread plum (*Neocarya macrophylla*) is one of the sources of under-explored oilseeds found in the wild and/or semi-cultivated in various part of the World for its edible fruits (with a peculiar flavor sometimes likened to avocado) and peanut-like shaped kernels. The kernel is an excellent source of oil which is composed of oleic acid 40%, eleostearic acid 31%, linoleic acid 15%, palmitic acid 12% and stearic acid 2%. It also contains two phytosterols; parincerium sterol A and B (Burkill, 1995) and some protein.

The value of protein in gingerbread plum kernels (20%) (Amza *et al.*, 2010) is comparable to that found in various oilseeds such as groundnut, soybean, palm kernel, cotton seed, locust bean, melon seeds, conophor nut, castor bean, African oil bean, sunflower seed, rapeseed, sesame seed, linseed, safflower and other such seeds (Robellen *et al.*, 1989). Gingerbread plum kernel protein isolate can be efficiently obtained by alkali solubilization/acid precipitation from the defatted seed meal (Amza *et al.*, 2011b). It has also been demonstrated that upon enzymatic hydrolysis; functional and nutritional properties, antioxidant activities of proteins could be modified. Indeed, enzymatic hydrolysis has been reported to increase solubility, modify foaming, emulsifying and gelation properties as well as producing bioactive peptides from certain proteins (Spellman *et al.*, 2003).

Bioactive peptides from natural sources have gained interest in recent years due to consumers' preferences and health concerns associated to the use of synthetic food additives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG). As reported by Ajibola *et al.* (2011), a large range of seeds has been used as protein sources for antioxidant hydrolysates, of which soybean, canola, hemp seed, flaxseed, peanut, sesame seed, rapeseed, etc. Nevertheless, some of the seeds (e.g. gingerbread plum kernels) are at present not well known and thus may be grossly underutilized in relation to their potential.

Indeed, an overview of available literatures on the previous works carried out on gingerbread plum (Frederick, 1961; Cook *et al.*, 1998; Cook *et al.*, 2000; Audu *et al.*, 2005; Balla *et al.*, 2008; Mann *et al.*, 2009; Amza *et al.*, 2010, Amza et al., 2011a; Amza *et al.*, 2011b; Dan-Guimbo *et al.*, 2011; Warra, 2012) revealed that there is scanty information on the functional, nutritional and antioxidant properties of its kernel protein hydrolysates, suggesting the potential for development of value-added products

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from gingerbread plum seeds. Consequently, preparing hydrolysates from gingerbread plum seed proteins could be one way of producing high-value ingredients from this underutilized oilseed.

Therefore, the objective of this study is to demonstrate the potentiality of gingerbread plum kernel protein hydrolysates prepared at various incubation times by a combination of two food-grade proteases (pepsin and trypsin). Several *in-vitro* antioxidant assays such as diphenyl-1-picryhydrazyl (DPPH), metal chelation, hydroxyl radical and ferric reducing are used to evaluate the antioxidant activities. Functional properties (emulsifying and foaming properties) and nutritional characteristics are also evaluated. Additionally, changes in the molecular weight distribution and amino acid composition during hydrolysis are also investigated to assess their relations with antioxidant activities.

Materials and Methods

Raw material and chemicals

Gingerbread plum kernels were obtained from Birni N'Gaouré, southern region of Republic of Niger. The kernels were kept dried in a desiccator at room temperature until use. DPPH (2,2-diphenyl-1-picrylhydrazyl), Pyrocatechol violet, Pepsin (E.C. 3.4.23.1, 800-2500 units/mg) and trypsin (E.C. 3.4.21.4, 104 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of the highest grade commercially available.

Preparation of defatted gingerbread plum seed flour and isolate

Gingerbread plum seed protein flour and isolate were prepared as previously reported by Amza *et al.* (2011b).

Enzymatic hydrolysis of gingerbread plum seed protein isolate

The lyophilized gingerbread plum kernel protein isolate was dissolved at 1% (w/v) protein in distilled water of pH 2. The pepsin solution was prepared at 0.1% (w/v) in distilled water of pH 2. The protein solutions were mixed with pepsin solution at the enzyme/substrate ratio of 1/100 (w/w). The mixtures were incubated at 37°C for 3 h. The pepsin was first inactivated by adjusting the pH to 7 and then the 0.1% (w/v) trypsin solution was added at the same enzyme/substrate ratio and further incubated at 37°C for 3 h. Subsequently, the protein solutions were boiled in a water bath at 95°C for 15 min to inactivate the enzymes and then centrifuged at 10,000 × g for 30 min (High-speed refrigerated centrifuge CR21 III,

Hitachi Koki Co., Ltd. Japan). The supernatants were lyophilized to obtain protein hydrolysate powders and kept at −20°C until use (Chanput *et al.*, 2009).

Degree of hydrolysis (DH)

The DH was defined as the percent ratio of the number of peptide bonds broken to the total number of peptide bonds in the substrate studied and was determined as previously described by Cao *et al.* (2008) and reported by Zhou *et al.* (2012).

Determination of molecular weight distribution

Gingerbread plum kernel protein fractions hydrolysates were analyzed for molecular weight distribution according to the procedure described by Li *et al.* (2008). A Waters TM 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA) was used. The hydrolysates were loaded onto TSK gel G2000 SWXL column (7.8 i.d. x 300 mm, Tosoh, Tokyo, Japan), eluted with 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 mL/ min and monitored at 220 nm. A molecular weight calibration curve was obtained from the following standards from Sigma: cytochrome C (12,500 Da), aprotinin (6500 Da), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da). Results were processed using Millennium 32 Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

Amino acid analysis

The lyophilized hydrolysates were digested with HCl (6 M) at 110°C for 24 h under nitrogen atmosphere. Reversed phase high performance liquid chromatography (RP-HPLC) analysis was carried out in an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) assembly system after precolumn derivatization with o-phthaldialdehyde (OPA) (Jarrett *et al.*, 1986).

Each sample (1 μl) was injected on a Zorbax 80 A C18 column (4.6 i.d. x180 mm, Agilent Technologies, Palo Alto, CA, USA) at 40°C with detection at 338 and 262 nm. Mobile phase A was 7.35 mmol/l sodium acetate/triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mmol/l sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as g of amino acid per 100 g of protein.

Determination of nutritional parameters

Nutritional features of enzymatic hydrolysates of gingerbread plum seed protein isolates (HGPSPIs) were determined on the basis of their amino acid profiles. Amino acid score (AAS) was calculated

using the FAO/WHO (1973) reference pattern. Essential Amino Acid Index (EAAI) was calculated according to Labuda *et al.* (1982) using the amino acid composition of the whole egg protein as standard. Biological values were calculated according to Oser (1959). Protein efficiency ratio (PER) values were calculated according to the equations developed by Alsmeyer *et al.* (1974) and Lee *et al.* (1978), as modified by Shahidi *et al.* (1991) and Shahidi *et al.* (1995) and reported by Ovissipour *et al.* (2009).

$$AAS = \frac{\text{mg of amino acid in 1g test protein}}{\text{mg of amino acid in requirement pattern}}$$

$$EAAI = \sqrt[9]{\frac{[Lys \times Thr \times Val \times Met \times Ile \times Leu \times Phe \times His \times Tryp]a*}{[Lys \times Thr \times Val \times Met \times Ile \times Leu \times Phe \times His \times Tryp]b*}}$$

Where $a*$ represents the content of amino acids in test sample and $b*$, the content of the same amino acids in standard protein (%) [Egg], respectively.

$$BV = 1.09 \times \text{Essential Amino Acid Index} [EAAI] - 11.7$$

$$PER \begin{cases} Eq.1 = -0.684 + 0.456[Leu] - 0.047[Pro] \\ Eq.2 = -0.468 + 0.453[Leu] - 0.04[Tyr] \\ Eq.3 = -1.816 + 0.435[Met] + 0.780[Leu] + 0.211[His] - 0.944[Tyr] \\ Eq.4 = 0.08084[Thr + val + Met + Ile + Leu + Phe + Lys] - 0.1094 \\ Eq.5 = 0.06320[Thr + Val + Met + Ile + Leu + Phe + Lys + His + Arg + Tyr] - 0.1539 \end{cases}$$

Determination of functional properties
Emulsifying properties

Emulsifying properties were determined according to the method reported by Jamdar *et al.* (2010) with slight modifications. Vegetable oil (10 ml) and 30 ml of 1% protein solution were mixed and the pH was adjusted to 7. The mixture was homogenized using a homogenizer (FA 25 model, Fluko, Shanghai, China) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10 min 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 a spectrophotometer (UVmini-1240; Shimadzu, Kyoto, Japan). The absorbance measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsifying activity index (EAI) and emulsion stability (ES) as follows:

$$EAI\,(m^2/g) = (2 \times 2.303 \times A_{500nm})/F \times \text{Protein weight (g)}$$

Where, F is the oil volume fraction of 0.25. The {ES} (%) was calculated as

$$(A_0 - A_{10}/A_0) \times 100$$

Foaming properties

Foaming capacity (FC) and foaming stability (FS) of HGPKPIs were determined according to the method of Jamdar *et al.* (2010). An aliquot (20 ml) of 0.5% sample solution was adjusted to pH 7, followed by homogenization at a speed of 16,000 rpm, to incorporate air for 2 min at ambient temperature. The whipped sample was immediately transferred into a 100 ml cylinder and the total volume was read after 30 s. The FC was calculated according to the following equation:

$$FC\,(\%) = (A_0 - B) \times 100/B$$

Where A_0 is the volume after whipping (ml), B is the volume before whipping (ml). The whipped sample was allowed to stand at 25°C for 10 min and the volume of whipped sample was then recorded. Foam stability was calculated as follows:

$$FS\,(\%) = (A_t - B) \times 100/B$$

Where A_t is the volume after standing (ml) and B is the volume before whipping (ml).

Determination of antioxidant properties

The antioxidant properties were determined according to the following previously published methods: reducing power by Ebrahimzadeh *et al.* (2010); DPPH radical scavenging activity, Parthasarathy *et al.* (2009); hydroxyl radical-scavenging activity and metal chelating activity, Li *et al.* (2008).

Statistical analysis

All experiments were conducted at least in triplicate with SPSS Inc. software (version 17.0). One-way analysis of variance (ANOVA) was used to determine significant differences between means, with the significance level taken at a = 0.05. Duncan's test was used to perform multiple comparisons between means.

Results and Discussion

Degree of hydrolysis (DH) of gingerbread plum protein hydrolysates

The DH values of enzymatic hydrolysates of gingerbread plum seed protein isolates (HGPSPIs) showed a steady increased over 180 min period of incubation with slowing hydrolysis rate as the hydrolysis time increased (Figure 1). The same trend of DH versus incubation time was also observed for palm kernel (Zarei *et al.*, 2012), barley hordein

Table 1. Molecular weight distribution of HGPSPI* prepared with different hydrolysis times
(Results are presented as means ± standard deviations (n = 3))

Time of hydrolysis (min)	Percentage content of each peptide fraction (Da)					
	<500	500–1000	1000–3000	3000–5000	5000–10,000	>10,000
5	7.93 ± 0.08	5.38 ± 0.29	21.63 ± 0.17	24.95 ± 0.14	28.95 ± 0.29	11.08 ± 0.14
10	7.41 ± 0.16	6.01 ± 11	23.22 ± 0.12	23.55 ± 15	27.97 ± 0.12	11.73 ± 0.20
15	9.61 ± 0.09	7.02 ± 0.21	25.30 ± 0.09	22.09 ± 0.27	26.30 ± 0.66	9.62 ± 0.06
20	9.77 ± 0.24	7.00 ± 0.13	26.39 ± 0.29	22.38 ± 0.08	26.15 ± 0.36	8.22 ± 0.09
25	12.21 ± 0.20	7.48 ± 0.25	25.75 ± 0.11	20.82 ± 0.30	25.61 ± 0.08	7.96 ± 0.12
30	8.90 ± 0.23	9.64 ± 0.16	36.40 ± 0.46	21.49 ± 0.19	20.20 ± 0.33	3.35 ± 0.24
60	10.13 ± 0.14	11.28 ± 0.38	34.76 ± 0.47	20.96 ± 0.22	19.13 ± 0.31	3.71 ± 0.19
90	13.02 ± 0.17	15.79 ± 0.04	36.37 ± 0.48	17.85 ± 0.16	14.97 ± 0.20	1.58 ± 0.04
120	13.18 ± 0.29	18.91 ± 0.17	38.43 ± 0.42	15.72 ± 0.15	12.18 ± 0.15	1.18 ± 0.16
180	15.49 ± 0.60	21.27 ± 0.23	35.77 ± 0.41	13.82 ± 0.16	12.34 ± 0.13	1.08 ± 0.11

* HGPSPIs: Hydrolysates of gingerbread plum seed protein isolates.
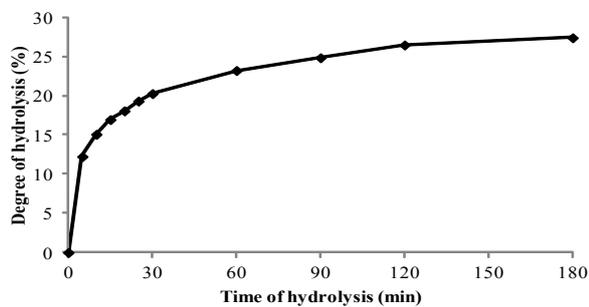
Figure 1. Kinetic curve of DH of HGPSPI (Results are presented as means ± standard deviations (n = 3)).

(Bamdad *et al*., 2011), peanut (Dong *et al*., 2011) and rapeseed (Chabanon *et al*., 2007). As stated by Guerard *et al.* (2002), the reduction in the reaction rate may be explained by the formation of reaction products, the decrease in concentration of peptide bonds available for hydrolysis, enzyme inhibition and/or enzyme deactivation. In this study, the low range of DH values (12.23% - 27.37%) obtained for HGPSPIs when compared with other hydrolysates: 19 % - 87 % for palm kernel cake protein hydrolysates produced using different enzymes (Zarei *et al*., 2012); 26.8% to 44.7% (with Alcalase) and 23.84 to 43.14% (with papain) for raw herring press cake, may be due to the type of enzymes used. Indeed, Hoyle and Merritt (1994) indicated that alkaline proteases like Alcalase exhibit higher activities than neutral or acid ones such as papain or pepsin. Also, differences in DH values could be explained on the basis of protein composition. Zhou *et al.* (2012) have reported that after being digested with several commercial proteases, the DH values obtained for cuttlefish were higher than those obtained for sardine; they speculated that the differences may be due to a difference in protein composition of the tissues.

Molecular weight distribution (MWD) of gingerbread plum protein hydrolysates

The results on MWD of HGPSPIs are presented in Table 1. During the incubation process and as the hydrolysis time increased (5 – 180 min), the peptides of high molecular size (>10,000 Da) gradually decomposed into small-sized peptides (11.08% – 1.08%). The peptides with molecular sizes between 3,000 and 10,000 Da decreased by half at the end of hydrolysis (53.9% - 26.16%). On the other hand, the last proportion of peptides (<3,000 Da) showed a progressive increased. After 5 min of hydrolysis, the content was 34.94% while the value increased to 72.53% at the end of the hydrolysis (180 min). The above results correlated well with findings from rice dreg protein hydrolysates (Qiang *et al.*, 2012) and loach protein hydrolysates (You *et al.*, 2009). Therefore, the hydrolysis seemed to follow a behavior of ''zipper'' type mechanism, as already reported for scallop (*Patinopecten yessoensis*) and abalone (*Haliotis discus hannai Ino*) muscle hydrolysates (Zhou *et al*., 2012).

Amino acid composition of gingerbread plum protein hydrolysates

Amino acid contents of HGPSPIs are given in Tables 2a and b. The hydrolysis time did not have an appreciable impact on the mole percents of most amino acids in the hydrolysates. Our results are in agreement with those reported by Kong and Xiong (2006) who indicated that hydrolysis does not change the amino acid composition of soluble fractions of protein hydrolysates. Amza *et al.* (2011b) have investigated the amino acid composition of gingerbread plum seed flour and protein isolates and suggested that glutamic acid is the major amino acid, followed by arginine

Table 2a. Total amino acid composition of HGPSPI* prepared with different hydrolysis times
(Results are presented as means ± standard deviations (n = 3))

Amino acid	Symbol	Isolate	Time of hydrolysis (min)				
			5	10	15	20	25
Aspartic	Asp	9.32 ± 0.05	8.09 ± 0.06	8.19 ± 0.08	8.15 ± 0.26	8.29 ± 0.06	8.37 ± 0.08
Glutamic	Glu	22.45 ± 0.34	31.01 ± 0.13	29.31 ± 0.12	29.81 ± 0.34	28.92 ± 0.13	28.52 ± 0.12
Serine	Ser	5.37 ± 0.07	4.60 ± 0.15	4.66 ± 0.08	4.65 ± 0.03	4.60 ± 0.11	4.71 ± 0.10
Histidine	His	2.27 ± 0.01	1.53 ± 0.13	1.64 ± 0.06	1.69 ± 0.10	1.67 ± 0.06	1.76 ± 0.11
Glycine	Gly	4.32 ± 0.05	3.32 ± 0.05	3.52 ± 0.12	3.55 ± 0.07	3.5 ± 0.07	3.57 ± 0.07
Threonine	Thr	2.61 ± 0.13	1.59 ± 0.05	1.79 ± 0.06	1.79 ± 0.07	1.82 ± 0.06	1.90 ± 0.12
Arginine	Arg	11.94 ± 0.07	13.55 ± 0.10	13.00 ± 0.19	12.93 ± 0.09	12.84 ± 0.04	12.87 ± 0.07
Alanine	Ala	4.45 ± 0.10	3.06 ± 0.16	3.36 ± 0.07	3.42 ± 0.07	3.47 ± 0.10	3.58 ± 0.11
Tyrosine	Tyr	3.27 ± 0.17	2.59 ± 0.06	2.58 ± 0.18	2.67 ± 0.07	2.57 ± 0.02	2.69 ± 0.10
Cysteine-s	Cys-s	1.28 ± 0.06	1.93 ± 0.03	1.79 ± 0.06	1.79 ± 0.05	1.70 ± 0.06	1.68 ± 0.06
Valine	Val	6.16 ± 0.06	4.38 ± 0.12	4.63 ± 0.11	4.78 ± 0.06	4.78 ± 0.06	4.84 ± 0.12
Methionine	Met	1.33 ± 0.03	2.44 ± 0.06	2.42 ± 0.12	2.49 ± 0.03	2.42 ± 0.03	2.42 ± 0.02
Phenylalanine	Phe	5.38 ± 0.15	4.47 ± 0.06	4.60 ± 0.15	4.73 ± 0.07	4.71 ± 0.12	4.81 ± 0.02
Isoleucine	Ile	4.34 ± 0.12	3.56 ± 0.08	3.73 ± 0.05	3.76 ± 0.13	3.81 ± 0.06	3.87 ± 0.10
Leucine	Leu	7.95 ± 0.07	7.04 ± 0.15	7.18 ± 0.06	7.17 ± 0.11	7.28 ± 0.09	7.33 ± 0.03
Lysine	Lys	4..15 ± 0.12	3.84 ± 0.11	4.04 ± 0.13	3.96 ± 0.06	4.01 ± 0.13	4.02 ± 0.12
Proline	Pro	3.75 ± 0.08	2.98 ± 0.11	3.39 ± 0.11	2.58 ± 0.06	3.49 ± 0.07	2.97 ± 0.07

*HGPSPIs: Hydrolysates of gingerbread plum seed protein isolates

Table 2b. Total amino acid composition of HGPSPI* prepared with different hydrolysis times (Results
are presented as means ± standard deviations (n = 3))

Amino acid	Symbol	Isolate	Time of hydrolysis (min)				
			30	60	90	120	180
Aspartic	Asp	9.32 ± 0.05	8.23 ± 0.09	8.42 ± 0.16	8.28 ± 0.05	8.11 ± 0.02	8.43 ± 0.04
Glutamic	Glu	22.45 ± 0.34	28.05 ± 0.06	27.38 ± 0.10	27.04 ± 0.09	26.63 ± 0.08	26.62 ± 0.11
Serine	Ser	5.37 ± 0.07	4.79 ± 0.08	4.86 ± 0.06	4.94 ± 0.19	4.90 ± 0.06	4.87 ± 0.10
Histidine	His	2.27 ± 0.01	1.70 ± 0.11	1.81 ± 0.09	1.73 ± 0.05	1.78 ± 0.04	1.80 ± 0.05
Glycine	Gly	4.32 ± 0.05	3.55 ± 0.05	3.67 ± 0.06	3.74 ± 0.09	3.86 ± 0.11	3.81 ± 0.12
Threonine	Thr	2.61 ± 0.13	1.89 ± 0.06	2.00 ± 0.02	2.01 ± 0.03	2.01 ± 0.05	2.06 ± 0.05
Arginine	Arg	11.94 ± 0.07	12.73 ± 0.06	12.57 ± 0.09	12.58 ± 0.09	12.63 ± 0.16	12.50 ± 0.12
Alanine	Ala	4.45 ± 0.10	3.55 ± 0.05	3.76 ± 0.09	3.82 ± 0.18	3.88 ± 0.09	3.91 ± 0.04
Tyrosine	Tyr	3.27 ± 0.17	2.58 ± 0.05	2.67 ± 0.11	2.58 ± 0.06	2.59 ± 0.11	2.65 ± 0.12
Cysteine-s	Cys-s	1.28 ± 0.06	1.70 ± 10	1.62 ± 0.02	1.57 ± 0.09	1.45 ± 0.12	1.55 ± 0.06
Valine	Val	6.16 ± 0.06	4.78 ± 0.09	4.96 ± 0.08	5.07 ± 0.05	5.0 3 ± 0.07	5.09 ± 0.12
Methionine	Met	1.33 ± 0.03	2.51 ± 0.12	2.44 ± 0.11	2.38 ± 0.10	2.43 ± 0.09	2.33 ± 0.05
Phenylalanine	Phe	5.38 ± 0.15	4.74 ± 0.06	4.90 ± 0.02	4.83 ± 0.06	4.84 ± 0.03	4.98 ± 0.12
Isoleucine	Ile	4.34 ± 0.12	3.81 ± 0.05	3.98 ± 0.11	3.94 ± 0.06	3.99 ± 0.05	4.01 ± 0.06
Leucine	Leu	7.95 ± 0.07	7.32 ± 0.06	7.45 ± 0.09	7.41 ± 0.05	7.51 ± 0.11	7.42 ± 0.2
Lysine	Lys	4..15 ± 0.12	4.05 ± 0.15	4.12 ± 0.14	4.20 ± 0.06	4.30 ± 0.06	4.19 ± 0.2
Proline	Pro	3.75 ± 0.08	3.80 ± 0.04	3.19 ± 0.03	3.82 ± 0.06	4.03 ± 0.05	3.67 ± 0.03

*HGPSPIs: Hydrolysates of gingerbread plum seed protein isolates

and aspartic acid. This is consistent with the results obtained in this work. Indeed, HGPSPIs are mainly composed of aspartic acid, glutamic acid, arginine and leucine. Their percentages ranged from 8.09 to 8.43 %, 26.62 – 31.01%, 12.50 – 13.55% and 7.04 – 7.51% of the total amino acids, respectively. These data were higher than those reported by Chabanon *et al.* (2007) for total amino acids of rapeseed protein

hydrolysates. However, the contents of cysteine, histidine and methionine were relatively lower than other amino acids. On the other hand, HGPSPIs showed lower amounts of aromatic amino acids (histidine, tyrosine and phenylalanine) compared to the isolate used as raw material. This lowering in the content of the mentioned amino acids could be useful for the treatment of congenital illness such as

Table 3a. Nutritional evaluation of HGPSPI* prepared with different hydrolysis times

Time of hydrolysis (min)	AAS (%)	EAAI (%)	BV (%)	Limiting amino acids	
				First	**Second**
5	39.83 ± 1.37e	57.83 ± 1.69d	51.34 ± 1.85e	Threonine	Lysine (69.88 ± 2.01)d
10	44.67 ± 1.62d	60.30 ± 1.60c	54.02 ± .75d	Threonine	Lysine (73.45 ± 2.44)bcd
15	44.75 ± 1.80d	60.95 ± 1.39ab	54.73 ± 1.52cd	Threonine	Lysine (72.12 ± 1.09)cd
20	45.42 ± 1.63d	61.13 ± 1.27ab	54.93 ± 1.38cd	Threonine	Lysine (72.90 ± 2.46)bcd
25	47.42 ± 2.89bcd	62.15 ± 1.56abc	56.04 ± 1.70abc	Threonine	Lysine (72.97 ± 2.22)bcd
30	47.25 ± 1.50cd	61.92 ± 1.64abc	55.79 ± 1.79abc	Threonine	Lysine (73.76 ± 2.81)cd
60	50.08 ± 0.72abc	63.58 ± 1.46ab	57.60 ± 1.58ab	Threonine	Lysine (75.12 ± 2.63)abc
90	50.25 ± 0.86abc	63.23 ± 1.05ab	57.22 ± 1.14ab	Threonine	Lysine (76.48 ± 1.11)ab
120	50.42 ± 1.37ab	63.86 ± 1.06a	57.95 ± 1.15a	Threonine	Lysine (78.12 ± 1.10)a
180	51.67 ± 1.37a	63.85 ± 1.08a	57.91 ± 1.18a	Threonine	Lysine (76.12 ± 0.96)ab

Means of three determinations ±SD; Mean values in columns with different letters (a, b, c, d or e) were significantly different
(Duncan's test); significance at ($p < 0.05$) (analysis of variance).
* HGPSPIs: Hydrolysates of gingerbread plum seed protein isolates
AAS: amino acid score
EAAI: essential amino acid index
BV: Biological value

Table 3b. Nutritional evaluation of HGPSPI* prepared with different hydrolysis times

Time of hydrolysis (min)	Protein efficiency ratio (PER) values				
	Eq.1	**Eq.2**	**Eq.3**	**Eq.4**	**Eq.5**
5	2.39 ± 0.06e	2.46 ± 0.06d	2.62 ± 0.11e	2.10 ± 0.05c	2.68 ± 0.06b
10	2.43 ± 0.02de	2.52 ± 0.01cd	2.74 ± 0.06de	2.18 ± 0.05b	2.73 ± 0.07ab
15	2.45 ± 0.07cde	2.49 ± 0.07d	2.65 ± 0.10de	2.21 ± 0.04b	2.75 ± 0.04ab
20	2.47 ± 0.04cd	2.57 ± 0.04bc	2.83 ± 0.12bc	2.22 ± 0.04b	2.75 ± 0.04ab
25	2.52 ± 0.02bc	2.58 ± 0.01bc	2.79 ± 0.04cd	2.25 ± 0.04ab	2.78 ± 0.05ab
30	2.47 ± 0.03bcd	2.58 ± 0.02bc	2.91 ± 0.08ab	2.24 ± 0.05ab	2.75 ± 0.05ab
60	2.56 ± 0.04a	2.64 ± 0.03ab	2.92 ± 0.03ab	2.30 ± 0.05a	2.81 ± 0.05a
90	2.52 ± 0.02bc	2.63 ± 0.02ab	2.93 ± 0.04ab	2.31 ± 0.03a	2.80 ± 0.05a
120	2.55 ± 0.05ab	2.67 ± 0.04a	3.03 ± 0.04a	2.32 ± 0.04a	2.82 ± 0.05a
180	2.52 ± 0.01bc	2.63 ± 0.01ab	2.86 ± 0.05bc	2.32 ± 0.04a	2.81 ± 0.05a

Means of three determinations ±SD; Mean values in columns with different letters (a, b, c, d or e) were significantly different
(Duncan's test); significance at ($p < 0.05$) (analysis of variance).
* HGPSPIs: Hydrolysates of gingerbread plum seed protein isolates
Eq.1 = −0.684 + 0.456[Leu] − 0.047[Pro]
Eq.2 = −0.468 + 0.453[Leu] − 0.04[Tyr]
Eq.3 = −1.816 + 0.435[Met] + 0.780[Leu] + 0.211[His] − 0.944[Tyr]
Eq.4 = 0.08084[Thr + val + Met + Ile + Leu + Phe + Lys] − 0.1094
Eq.5 = 0.06320[Thr + Val + Met + Ile + Leu + Phe + Lys + His + Arg + Tyr] − 0.1539

phenylketonuria or tyrosinamie, in which diets low in these amino acids are recommended.

Nutritional values of gingerbread plum protein hydrolysates

Nutritional parameters of gingerbread plum protein hydrolysates were computed and results are reported in Tables 3a and b. Chemical scores for essential amino acids of HGPSPIs were calculated with comparison to the FAO pattern (FAO/WHO, 1973). Threonine and lysine were found to be respectively the first and second limiting amino acids. The scores were significantly different (p < 0.05) and ranged from 39.83 to 51.67% (Table 3a). From the results, it was obvious that the differences in the score were due to amino acid composition among samples. Lysine, leucine, isoleucine, theonine, methionine, tryptophan and valine were also found to

be the limiting amino acids in many oilseed proteins (Baldwin, 1986).

Essential amino acid indexes (EAAIs) of HGPSPIs were 57.83 – 63.86% while the biological values (BV) were in the range of 51.34 to 57.95% (Table 3a). These values were higher than those reported for some complementary foods from popcorn, African locust bean and Bambara groundnut (Ijarotimi and Keshinro, 2012). However, the BV and EAAI values in this study were relatively low compared to the values reported by Oser (1959).

Generally, a protein with BV between 70 - 100% and EAAI above 90% is considered to be of good nutritional quality and to be useful as food when the value is around 80% and inadequate for food material below 70% (Oser, 1959; Ijarotimi and Keshinro, 2012). However, based on Friedman's (1996) ranking
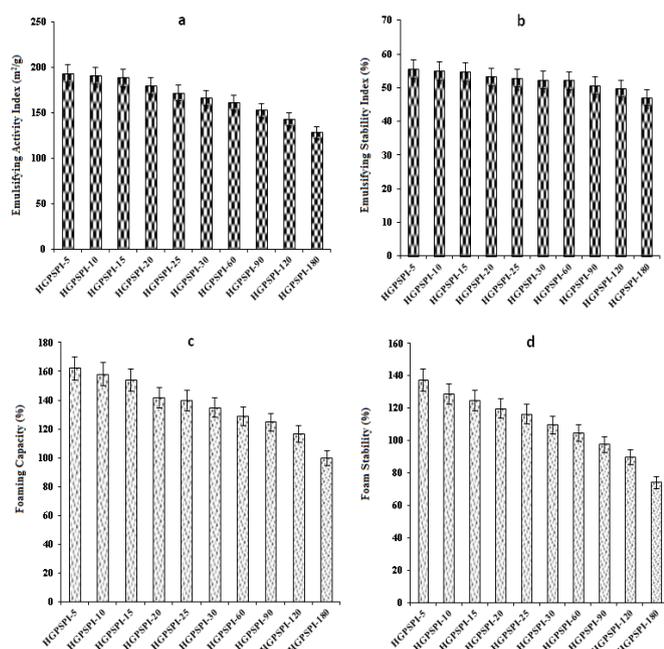
Figure 2. Functional properties of HGPSPI (at pH 7.0) prepared with different hydrolysis time: (a)) Emulsifying activity index (EAI); (b) Emulsifying stability index (ESI); (c) Foaming capacity; (d) Foam stability (Results are presented as means ± standard deviations (n = 3)).

of protein quality, HGPSPIs could be considered of good to high quality. Indeed, protein efficiency ratio (PER) below 1.5 implies a protein of low or poor quality; whereas PER between 1.5 and 2.0 indicates an intermediate protein quality; and PER above 2.0 means protein of good to high quality (Friedman, 1996). HGPSPIs protein efficiency ratios values were significantly different ($p < 0.05$) and variable according to the equation used. Overall, the PER values were in the range of 2.10 to 3.03 (Table 3b).

Functional properties of gingerbread plum protein hydrolysates

Figure 2 shows the influence of hydrolysis time on the emulsifying activity index (EAI), emulsion stability index (ESI) (Figures 2a and b), foaming capacity (FC) and foam stability (FS) (Figures 2c and d) of HGPSPIs. EAI and ESI of HGPSPIs decreased ($p < 0.05$) with increasing hydrolysis time. This trend was expected because as hydrolysis time increased, the peptides of high molecular size gradually decomposed into small-sized peptides (Table 1) which are less effective in stabilizing emulsions. Indeed, improvement in emulsifying property upon very limited hydrolysis could be attributed to exposure of the hydrophobic protein interior which enhances adsorption at the interface, forming a cohesive interfacial film, with the hydrophobic residues interacting with oil and hydrophilic residues with water (Phillips and Beuchat, 1981; Taha and Ibrahim,

2002). Thus, the peptides with low molecular weight may not be amphiphilic enough to exhibit good emulsifying properties. According to Lee *et al.* (1987) and Amiza *et al.* (2012), a peptide is required to have a minimum length of about 20 residues to possess good emulsifying and interfacial properties.

In this study, HGPSPIs showed the highest foaming capacity and stability; 162.5% and 137.5% respectively after 5 min of hydrolysis (Figures 2c and d). This is in line with previous reports showing that a good cohesiveness of foams is only reached with high molecular weight peptides or partially hydrolyzed proteins (Chabanon *et al.*, 2007; Jamdar *et al.*, 2010; Amiza *et al.*, 2012). Indeed, hydrolysis may improve foaming capacity but generally led to a decrease of the foam stability. This could be due to the fact that although smaller peptides (hydrophilic and/or charged) are able to incorporate air into the solution, they do not have enough strength to give stable foam (Kristinsson and Rasco, 2000; Jamdar *et al.*, 2010). It can be concluded that, limited protein hydrolysis may improve its functional properties. So, a compromise must be reached between hydrolysis time and functional properties.

Antioxidant activities of gingerbread plum protein hydrolysates

The IC_{50} values of hydroxyl radical scavenging activity, Fe^{2+} chelating activity, DPPH radical scavenging activity and the $AC_{0.5}$ values for reducing power of HGPSPIs are shown in Figure 3. Preliminary antioxidant tests on HGPSPIs (data not shown), revealed hydroxyl radical scavenging activity, Fe^{2+} chelating activity, DPPH radical scavenging activity and reducing power in a dose-dependent manner. Moreover, during the whole hydrolysis period (180 min), the antioxidant activities increased gradually with increasing incubation time, except for reducing power which showed an increase at initial stage of hydrolysis, declined at 30 min and then gradually increased thereafter (Figures 3a, b, c and d). The highest IC_{50} ($AC0.5$) values of hydroxyl radical scavenging activity, Fe^{2+} chelating activity, DPPH radical scavenging activity and reducing power were 4.31, 0.39, 2.10 and 15.39 mg/mL, respectively. In this study, the IC_{50} values for hydroxyl radical scavenging activity, Fe^{2+} chelating activity, DPPH radical scavenging activity and reducing power were higher than those reported by Zhou *et al.* (2012) for scallop and abalone muscle hydrolysates. Sun *et al.* (2012) reported chicken breast protein hydrolysate showing a reducing power with EC_{50} ranging from 0.50 to 2.37 mg/mL and DPPH radical scavenging ability with EC_{50} of 1.28 mg/mL. Results
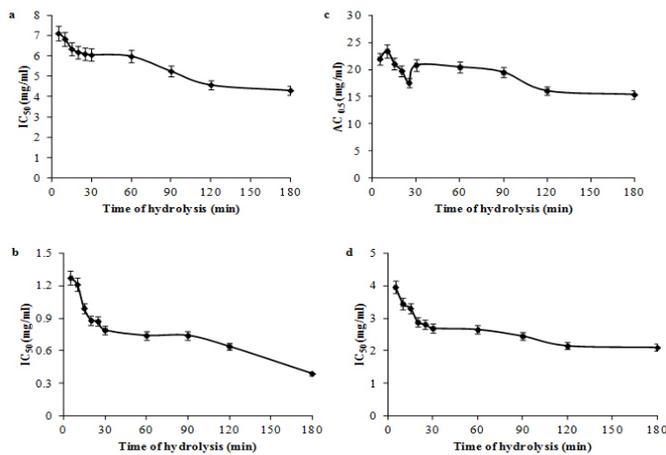
Figure 3. Antioxidant activities of HGPSPI prepared with different hydrolysis time: (a) hydroxyl radical scavenging activity; (b) Fe^{2+} chelating activity; (c) reducing power; (d) DPPH radical scavenging activity (Results are presented as means ± standard deviations (n = 3)).

on walnut (*Juglans regia* L.) protein hydrolysates (Chen *et al.*, 2012) showed a good hydroxyl radical scavenging activity with an IC_{50} value of 5.04 mg/ml. EC_{50} values for loach peptide (You *et al.*, 2011; Zhou *et al.*, 2012) were 17.0 mg/mL and 2.64 mg/mL for hydroxyl radical scavenging ability and DPPH radical scavenging ability respectively. Thus, compared to various protein hydrolysates reported in literatures, HGPSPIs could be considered as potent antioxidants.

Conclusions

This study revealed that gingerbread plum seed protein hydrolysates (HGPSPIs) were affected by the hydrolysis time in term of their nutritional quality, functional and antioxidant properties. Long hydrolysis time (180 min) has produced small-sized peptide, which lead to a decrease in HGPSPIs functional properties and an inverse effect on antioxidant activities. Thus, to obtain HGPSPIs with both strong antioxidant activity and acceptable functional properties, a controlled hydrolysis is necessary. On the other hand, according to FAO/WHO (1973), gingerbread plum seed protein hydrolysates fulfilled the normal requirements of all the essential amino acids except for threonine and lysine. Therefore, these results suggest possible use of HGPSPIs as nutrient supplements and as functional ingredients in food systems.

Acknowledgments

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