



Anti-glycation Properties and Antioxidant Activities of *Moringa oleifera* Seed Protein Hydrolysate Fractions Obtained from Trypsin Proteolysis

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Abstract

Keywords:

Moringa oleifera, Hydrolysate, Peptides, Glycation, Antioxidant

Proteins from *Moringa oleifera* were extracted via alkaline solubilization followed by acid precipitation. They were then subjected to hydrolysis using trypsin and the resulting hydrolysates separated into <1kD, 1-3kD and 3-5kD peptide fractions by means of size exclusion chromatography. The hydrolysate and the peptide fractions were evaluated for their effects on hemoglobin glycation and four free radical systems – DPPH radicals, ferric ions, hydroxyl radicals and superoxide radicals. Results revealed that the peptides inhibited hemoglobin glycation in a synergistic manner, as the unfractionated hydrolysate slowed down the formation of glycated hemoglobin better than the constituent fractions ($IC_{50} = 0.163 \pm 0.028$ mg/mL). Peptide fraction 3, Ft₃, demonstrated the best activities against DPPH radical, ferric ions and superoxide radicals ($35.764 \pm 1.933\%$, 44.709 ± 0.676 mM Fe^{2+} and $92.223 \pm 1.794\%$ respectively) whereas fraction 2, Ft₂, scavenged hydroxyl radicals better than other fractions ($82.000 \pm 1.155\%$). This suggested a direct relationship between average molecular weights of these peptide fractions and their antioxidant abilities, such that peptide fractions with higher molecular weights had better capacities to neutralize free radicals. It is concluded that *M. oleifera* seed proteins may encode potentially bioactive peptides, which could be optimized for the development of food additives and peptide-based therapeutic alternatives in the management of diabetes mellitus.

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INTRODUCTION

Diabetes mellitus, a devastating metabolic disorder, results from disruptions in insulin production and/or signaling. This leads to the attendant deregulation of the metabolism of fuel molecules, causing severe hyperglycemia, glycosuria and other symptoms characteristic of the disease (Arise *et al.*, 2016). The excess blood glucose combines with blood proteins (especially albumin in plasma and hemoglobin in erythrocytes) in a non-enzymatic fashion, known as glycation. This represents one of the hallmarks of poorly treated diabetes mellitus (Ramasamy *et al.*, 2005; Singh *et al.*, 2014). These adducts, referred to

as advanced glycated end products, or AGEs, are known to activate a plethora of pro-inflammatory pathways, through the generation of reactive oxygen species, ultimately causing oxidative stress (Olusola and Ekun, 2019). These AGEs bind and activate the receptor for advanced glycated end products, (RAGE), which also potentiate downstream signaling cascades which are vital to the pathogenesis of diabetic nephropathy, neuropathy and retinopathy (Perrone *et al.*, 2020).

Oxidative stress generally describes a state of imbalance between the production of free radicals and the capability of a biological system (cells, tissues) to quickly neutralize these compounds and/or repair the

resulting damage caused (Chandra *et al.*, 2015). These pro-oxidants are produced from the oxidation of biomolecules in the body through metabolism *in vivo* or from exposure to environmental pollutants, toxicants and other xenobiotics (Voet *et al.*, 2016). When normal cellular redox states are impaired, toxic responses such as erythrocyte lysis, DNA strand breakage, nucleotide base damage, among other deleterious effects may occur as a result (Chandra *et al.*, 2015). This is thought to occur through the production of free radicals such as singlet oxygen, peroxides, superoxides, that damage cellular components such as lipids, proteins and DNA (Urribari *et al.*, 2010). In addition, some reactive oxygen species have been known to act as intracellular messengers in redox signaling; and as a result, oxidative stress can potentially cause disruptions in normal mechanisms of cellular signaling (Han *et al.*, 2014). Oxidative stress is highly related to glycation, since the depletion of glutathione also reduces the activity of glyoxalase-I, thus increasing concentrations of glyoxal and methylglyoxal, formed nonenzymatically as products, mainly in glycolysis, and leads to the formation of AGEs (Shangari and O'Brien, 2004). In addition, the binding AGE/RAGE increases the levels of ROS (Urribari *et al.*, 2010) through activation of NADPH oxidase and mitochondrial pathways (Wautier *et al.*, 2001). Consequently, the activity of superoxide dismutase (SOD), catalase and indirectly other endogenous antioxidant defense is decreased, such as glutathione (GSH) and ascorbic acid (Chen *et al.*, 2018). Oxidative stress is also involved directly and/or indirectly in the progression of other diseases such as cancer (Halliwell, 2007), Parkinson's disease, Alzheimer's disease (Pohanka, 2013) atherosclerosis, myocardial infarction (Dean *et al.*, 2011; Ramond *et al.*, 2011) among other disease conditions.

Moringa oleifera, a perennial plant, is a member of the *moringaceae* family. It was previously endemic to the Indian Himalayas in Asia, but is now prevalent in many parts of sub-Saharan Africa and the Middle East (Olusola *et al.*, 2018). *M. oleifera* is cultivated by locals for its nutritional and numerous health promoting benefits of its stem bark, roots, seeds and leaves (Leone *et al.*, 2016). *Moringa oleifera* seeds have a considerably high protein content (33–36%) despite being an oil-based seed, which makes it a good source of potentially bioactive peptides upon proteolysis (Gopalakrishnan *et al.*, 2016). In addition, Okereke and Akaninwor (2013) found that *M. oleifera* seeds contain high amounts of essential amino acids and it is also abundant in aspartate, lysine, arginine and glutamate. The stem bark and seeds of the plant have been demonstrated to exhibit anti-inflammatory, anti-ulcerative, antihypertensive and antineoplastic properties (Siddhuraju and Beck, 2003; Anwar *et al.*, 2007) just as its leaves have been reported to elicit antimicrobial and hypoglycemic activities (Divi *et al.*, 2012).

It is important that natural agents are utilized as additives and supplements for lowering the occurrence of glycated products and reduction of oxidative stress due to disease progression in diabetes mellitus (Bains and Hall, 2012). As a result, there is increased interest in investigating newer and previously underutilized plant sources for their multidirectional potentials. Specifically, protein hydrolysate preparations and peptides from previously underutilized plants are being investigated for their biological activities (Arise *et al.*, 2016; Famuwagun *et al.*, 2020; Ekun *et al.*, 2022). Peptides obtained from hydrolysis of plant and animal proteins have been identified as safer alternatives to synthetic supplements. These peptides are obtained from their parent proteins either by enzymatic hydrolysis or microbial fermentations (Ulagesan *et al.*, 2018; Ekun *et al.*, 2022). As a result, they possess simpler structures than their parent proteins, thus enhancing greater stability in different situations such as heat and protease activities. Furthermore, they often elicit no immunoreactions and often exhibit enhanced nutritional and other functional properties (Xie, *et al.*, 2008). For most peptides, their bioactivities are mostly dependent on the peptide size, and composition of aminoacyl residues (Li and Li, 2013; Alashi *et al.*, 2014).

MATERIALS AND METHODS

Collection of *M. oleifera* Seeds

M. oleifera seeds were collected from a farmstead in Akungba Akoko, Ondo State. They were identified and voucher samples were deposited at the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba Akoko.

Chemicals and Reagents

Trypsin (from bovine pancreas), 1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, trichloroacetic acid (TCA), potassium ferricyanide, ferric chloride, pyrogallol, ethylene diamine tetraacetic acid (EDTA) and hemoglobin were products of Sigma-Aldrich laboratories, Co-Artrim, United Kingdom. All chemicals and reagents used were of analytical grade.

Equipment

Magnetic stirrer, vacuum oven, Spectramax Plus 385 microplate spectrophotometer, photoelectric colorimeter, freeze drier, water bath and a bench centrifuge.

Isolation of *M. oleifera* Seed Protein

The seeds were dried, blended into powder and kept in an airtight container at 10°C. This was subsequently defatted using n-hexane as was previously described by Arise *et al.*, (2016) with slight modifications. Ten (10) grams of the meal was extracted three times with three hundred (300) mL of n-hexane. The meal was then dried at 40°C in a vacuum oven and ground again

to obtain a fine powder, termed defatted seed meal, which was stored at -20°C.

The protein component of the defatted meal was extracted using the method described by Alashi *et al.* (2014). The defatted seed meal was suspended in 0.5 M NaOH pH 12.0 at a ratio of 1:10, and stirred for one hour to enhance solubilization in alkaline medium. This was centrifuged at 20°C and 3000 g for 10 min. Two additional extractions of the residue from the centrifugation process were performed with the same volume of 0.5 M NaOH and the supernatants were then pooled. The pH of the supernatant was adjusted to 4.0 to facilitate acid-induced protein precipitation using 0.5 M HCl solution; the precipitate formed was recovered by centrifugation. The precipitate was washed with distilled water, adjusted to pH 7.0 using 0.1 M NaOH, freeze-dried and the protein isolate was then stored at -20°C until required for further analysis.

Preparation of *M. oleifera* Seed Protein Hydrolysates

The protein isolate was hydrolysed using the methods described by Onuh *et al.* (2015). Hydrolysis was carried out using trypsin (pH 8.0, 37°C). The protein isolate (5% w/v, based on the protein content of the isolate) was dissolved in phosphate buffer, pH 8.0. The enzyme was added to the slurry at an enzyme-substrate (E:S) ratio of 1:50. Digestion was performed at the specified conditions for 6 hours with continuous stirring. The enzyme was then inactivated by boiling in water bath (95–100°C) for 15 min and undigested proteins were precipitated by adjusting the pH to 4.0 with 2 M HCl/2 M NaOH followed by centrifugation at 7000 g for 30 min. The supernatant containing target peptides were then collected. Protein content of samples were determined using biuret assay method with bovine serum albumin (BSA) as standard.

Fractionation of *M. oleifera* Seed Protein Hydrolysates

The *Moringa oleifera* seed protein hydrolysates were separated into molecular weight fractions using gel filtration chromatography as described by Ekun *et al.*, (2022). Briefly, 5 mL of the clear supernatant resulting from protein hydrolysis, at a protein concentration of 10 mg/mL was filtered, suspended in 50 mM phosphate buffer of pH 7 and passed into a sephadex G25 chromatographic column of dimensions 30 cm x 4 cm which had earlier been equilibrated with the buffer. The same phosphate buffer was used to elute the separating fractions, and the elution peaks were monitored at 400 nm according to Prasad *et al.*, (2017). The separating fractions eluted under the same elution peak were collected, pooled and their molecular weights were determined by comparison with the graph of the logarithm of molecular weights against elution volumes of known standards. The eluates, according to their molecular weights, were

then sorted into <1 kD, 1-3 kD and 3-5 kD ranges. Peptide fractions of molecular weights higher than 5 kDa were removed and discarded. The collected peptide fractions were stored at -20°C for further analysis.

Inhibition of Hemoglobin Glycation

This was investigated by estimating the extent of hemoglobin glycation according to the method described by Venu, *et al.*, (2016) with modifications. Glucose solution (2%), 0.06% hemoglobin and Gentamycin (0.02%) solution were prepared in phosphate buffer 0.1 M, pH 7.4. 1ml each of above solution was mixed. 0.2 mg/ml - 1.0 mg/ml of hydrolysate was added to above mixture. Gallic acid was used as standard. The mixture was kept in dark at room temperature for incubation for 72 hours. At 520 nm, haemoglobin glycation was measured with a spectrophotometer and % inhibition was calculated thus:

$$\text{Percentage of hemoglobin glycation} = \frac{\text{Abs (sample)} - \text{Abs (control)}}{\text{Abs (sample)}} \times 100\%$$

Determination of DPPH Radical Scavenging Activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured by using the assay method described by Arise *et al.*, (2016) with slight modifications. 1 mL each sample at different concentrations (0.2– 1.0 mg/ml) was added to 1 mL 0.1 mM DPPH dissolved in 95 % ethanol. The mixture was shaken vigorously and incubated in the dark and at room temperature for 30 min. The absorbance was read at 517 nm. Ethanol (95 %) was used as a blank. The control solution consisted of 0.1 mL of 95 % ethanol and 2.9 mL of DPPH solution. Analyses were carried out in triplicates. Percentage inhibition of DPPH radical was calculated as follows: % DPPH inhibition = (Abs_{control} - Abs_{sample}) x 100 / (Abs_{control})

Determination of Ferric Reducing Antioxidant Property (FRAP)

The reducing power of *M. oleifera* seed protein hydrolysates was measured according to the method reported by Olusola *et al.*, (2018) with slight modification. An aliquot of 1 mL of different concentrations (0.2 – 1.0 mg/ml) of each sample (suspended in 0.2 M phosphate buffer, pH 6.6) was mixed with 1 mL of 1% potassium ferric cyanide solution. The mixture was then incubated at 50°C for 30 minutes followed by the addition of 1 ml 10% (w/v) TCA. 1 mL of the incubation mixture was added with 1 mL of distilled water and 0.2 ml of 0.1% (w/v) ferric chloride in test tubes. After a 10 min reaction time, the absorbance of resulting solution was read at 700 nm. Higher absorbance suggested stronger reducing power. Ascorbic acid was used as the reference antioxidant. An aqueous solution of known Fe(II) concentrations (FeSO₄·7H₂O; 2.5 2.0, 1.5 1.0

and 0.5 mM) were used for calibration. Results were expressed as mM Fe²⁺/mg hydrolysate. All the tests were performed in triplicate.

Determination of Superoxide Radical Scavenging Activity (SRSA)

The method described by Xie *et al.*, (2008) was used to determine SRSA. Samples (1 mg/mL final concentration) were each dissolved in 50 mM Tris-HCl buffer, pH 8.3 containing 1 mM EDTA and 80 µL was transferred into a clear bottom microplate well; 80 µL of buffer was added to the blank well. This was followed by addition of 40 µL 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each well in the dark and the change in the rate of reaction was measured immediately at room temperature over a period of 4 min using a spectrophotometer at a wavelength of 420 nm. The superoxide scavenging activity was calculated using the following equation:

$$\text{Superoxide scavenging activity (\%)} = (\Delta\text{Abs}/\text{min}_b - \Delta\text{Abs}/\text{min}_s) / \Delta\text{Abs}/\text{min}_b \times 100$$

where b and s are blank and sample, respectively.

Determination of Hydroxyl Radical Scavenging Activity (HRSA)

Determination of the ability of the hydrolysates to prevent Fe²⁺/H₂O₂ induced deoxyribose decomposition was carried out using the method reported by Ogunwa *et al.*, (2016) with some modifications. The reaction mixture contained 120 µL of 20 mM deoxyribose, 400 µL of 0.1 M phosphate buffer pH 7.4, 40 µL of 20 mM hydrogen peroxide and 40 µL of 0.5M iron (II) tetraoxosulphate (VI). The hydrolysates were added to the mixture. Distilled water was then added to make the mixture volume up to 800 µL. This was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 0.5ml of 2.8% thiobarbituric acid, TBA. The reaction tubes were incubated in boiling water for 20 mins, after which they were centrifuged at 3000g for 10 minutes. The absorbance was taken at 532 nm using a spectrophotometer.

Percentage inhibition of the hydroxyl radical was calculated as follows:

$$\% \text{ OH inhibition} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) \times 100 / (\text{Abs}_{\text{control}})$$

Statistical Analysis

Results were expressed as mean of triplicate observations ± standard deviation. The data were statistically analyzed using One Way Analysis of Variance (ANOVA) and Duncan's multiple range tests. Differences were considered statistically significant at p<0.05 using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSIONS

Inhibition of Hemoglobin Glycation

The effects of *Moringa oleifera* seed protein hydrolysates and fractions obtained from tryptic proteolysis on non-enzymatic hemoglobin glycation, in comparison to gallic acid are illustrated in Figure 1 and their IC₅₀ values are displayed in Figure 2. All hydrolysates and fractions demonstrated significantly lower (p<0.05) inhibitory effects when compared to gallic acid (control) especially at higher concentrations. The percentage inhibition of hemoglobin glycation by tryptic hydrolysate and fractions indicated that the unfractionated hydrolysate displayed a concentration-dependent reduction in inhibitory activity. It also had higher (p<0.05) inhibitory effects than the fractions at all study concentrations except at 0.4 mg/ml and 1.0 mg/ml. Tryptic hydrolysate fraction Ft₁ had maximal inhibition of 54.246 ± 0.755 % at a concentration of 0.4mg/ml as there was a significant decline (p<0.05) in its activity as concentration increased. Fractions Ft₂ and Ft₃ had maximum inhibition of hemoglobin glycation of 33.048 ± 3.325 % and 56.577 ± 4.844 % respectively at a final concentration of 1.0 mg/ml. These results translated to IC₅₀ values of 0.163 ± 0.028 mg/mL for the unfractionated tryptic hydrolysate, which was significantly lower (p<0.05) than 0.582 ± 0.008 mg/mL, 1.092 ± 0.001 mg/mL and 0.975 mg/mL obtained for fractions Ft₁, Ft₂ and Ft₃ respectively. The formation of advanced glycation end products (AGEs) as a result of poorly controlled hyperglycemia in diabetes mellitus leads to a plethora of complications such as retinopathy, renal dysfunction atherosclerosis, among other devastating conditions (Ramasamy *et al.*, 2005; Singh *et al.*, 2014). These AGEs cause deleterious effects by promoting the generation of reactive oxygen species which activate a cascade of signaling pathways, leading to an increase in the production of pro-inflammatory mediators, invariably causing other complications such as the formation of atherosclerotic plaques and culminating in cardiovascular disease in diabetic patients (Han *et al.*, 2014). Certain plant extracts have been reported to inhibit hemoglobin glycation *in vitro* (Hosseini *et al.*, 2015; Venu *et al.*, 2016), but information has been scarce on the abilities of peptides and protein hydrolysates to inhibit hemoglobin glycation. In this study, the unfractionated hydrolysates had better inhibitory activities than their fractions at all study concentrations, and this indicates that fractionation in this case, may have reduced the inhibitory activities of the peptide fractions to prevent non-enzymatic glycation of hemoglobin. This result suggests that there could be synergistic effects among these peptides, which make them more effective as a mixture than being fractionated. Among the fractions in this study, the small sized fractions (Ft₁) inhibited the formation of glycated hemoglobin at lower

concentrations while the larger sized fractions (Ft₃) prevented hemoglobin glycation at higher concentrations. However, more investigation is required in further studies to determine the nature of the specific aminoacyl residues that may be involved in preventing glycation of proteins in both *in vitro* and *in vivo* models, thus charting novel courses in the search for new, peptide-based food additives in the management of diabetes mellitus.

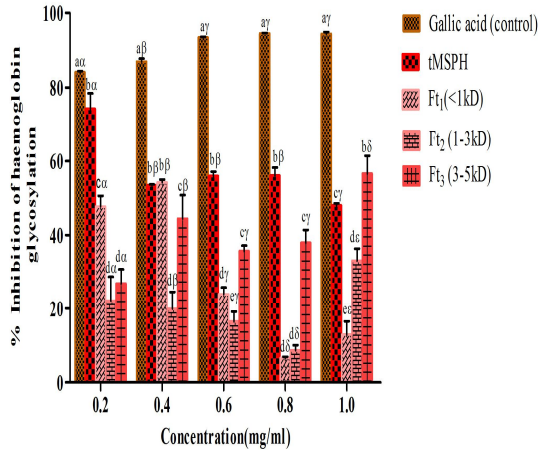


Figure 1. Percentage Hemoglobin Glycation Inhibitory Activities of Tryptic *Moringa oleifera* Seed Protein Hydrolysate and its Fractions

Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values within the same concentration but with different letters are significantly different (p<0.05). Values at different concentrations of the same hydrolysate with different symbols are also significantly different (p<0.05). Bars carrying the same letter or symbol are not significantly different from one another (p<0.05). Abbreviations: tMSPH: tryptic *M. oleifera* Seed Protein Hydrolysate, Ft₁: Tryptic fraction 1(< 1kD), Ft₂: Tryptic fraction 2(1-3 kD) Ft₃: Tryptic fraction 3(3-5 kD).

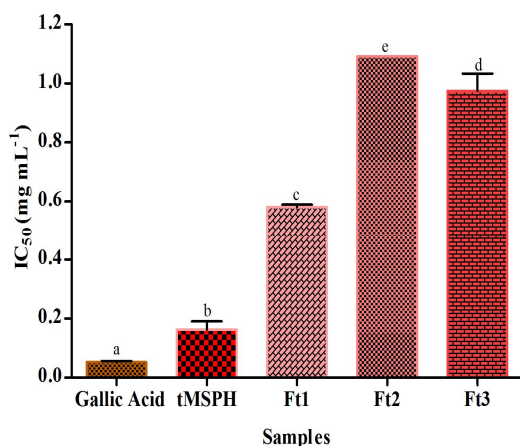


Figure 2. Fifty Percent (50%) Hemoglobin Glycation Inhibitory Concentration Values (IC₅₀) of *Moringa oleifera* Seed Protein Hydrolysate and its Fractions

Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the

same letters do not differ significantly while bars with different letters are significantly different (p<0.05) from one another. Abbreviations: tMSPH: tryptic *M. oleifera* Seed Protein Hydrolysate, Ft₁: Tryptic fraction 1(< 1kD), Ft₂: Tryptic fraction 2(1-3 kD) Ft₃: Tryptic fraction 3(3-5 kD).

DPPH Radical Scavenging Activity

The abilities of *M. oleifera* seed protein hydrolysates and their fractions to scavenge the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical in comparison to ascorbic acid are depicted in Figure 3. The hydrolysate and its fractions demonstrated significantly lower (p<0.05) DPPH scavenging activities when compared to ascorbic acid. The unfractionated hydrolysate showed a concentration-dependent increase in DPPH radical, attaining a maximal activity of 46.069 ± 1.029 % at a concentration of 1.0 mg/ml, which was significantly higher (p<0.05) than those of its fractions, especially at higher concentrations. This was comparable to 47.10 ± 1.33 % obtained for unfractionated tryptic hydrolysates of watermelon seed proteins (Arise *et al.*, 2016) Tryptic hydrolysate fraction Ft₃, however displayed reduced activity as concentration increased, declining from 35.764 ± 1.933 % at 0.2 mg/ml to 30.903 ± 1.513 % at 1.0 mg/ml. Fractions Ft₁ and Ft₂ had lower (p<0.05) DPPH scavenging activities when compared to F3, but at 1.0mg/ml Ft₂ had higher (p<0.05) activity than Ft₁ at 1.0mg/ml. Thus, the unfractionated tryptic hydrolysate, tMSPH, had increased DPPH inhibitory effects than the fractions, and this is suggestive of an inverse correlation between average molecular weights of these peptide fractions and their DPPH scavenging activities. This could imply that peptides in unfractionated digests showed additive or synergistic effects in the neutralization of the DPPH radical, and as such the fractionation of these hydrolysates reduced their scavenging activities. This is consistent with earlier reports that high molecular weight peptides were found to significantly inhibit DPPH radical (Siow and Gan, 2016).

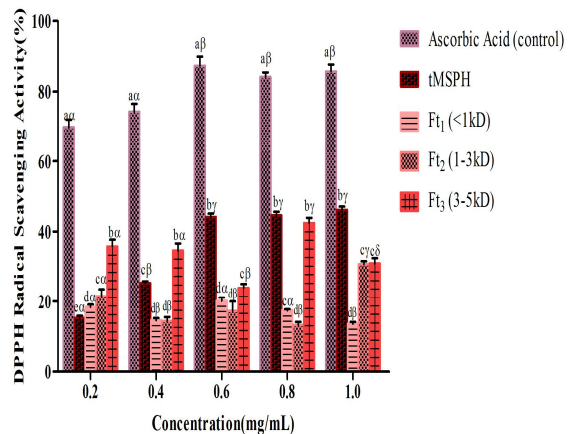


Figure 3. Percentage DPPH Radical Scavenging Activities of Tryptic *Moringa oleifera* Seed Protein Hydrolysate and its Fractions

Bars are expressed as means \pm standard error of mean of triplicate determinations ($n=3$). Values within the same concentration but with different letters are significantly different ($p<0.05$). Values at different concentrations of the same hydrolysate with different symbols are also significantly different ($p<0.05$). Bars carrying the same letter or symbol are not significantly different from one another ($p<0.05$). Abbreviations: tMSPH: tryptic *M. oleifera* Seed Protein Hydrolysate, Ft₁: Tryptic fraction 1(< 1kD), Ft₂: Tryptic fraction 2(1-3 kD) Ft₃: Tryptic fraction 3(3-5 kD).

Ferric Reducing Antioxidant Property

Ferric reducing antioxidant power assay is often utilized in evaluating the ability of natural antioxidants such as flavonoids, polyphenols and protein hydrolysates to donate protons (Olusola and Ekun, 2019). The abilities of *M. oleifera* seed protein hydrolysate and its fractions to reduce Fe (III) ions to Fe(II) in comparison to ascorbic acid, are displayed in Figure 4. The unfractionated hydrolysate and its peptide fractions demonstrated lower ferric reducing abilities than ascorbic acid. However, fractions Ft₃ and Ft₁ had higher reducing power than the rest as they reduced 44.71 ± 0.66 mM and 29.74 ± 0.93 of Fe³⁺ to Fe²⁺ respectively. This is consistent with previous studies on unfractionated hydrolysates of watermelon seed proteins (Arise *et al.*, 2016) and *Arachis hypogea* seed proteins (Olusola and Ekun, 2018) respectively, in that tryptic digests exhibited better reducing power. The mechanisms of the antioxidant property of peptides are associated with their ability to donate a proton or hydrogen atoms in terminating the radical chain reaction (Siow and Gan, 2016), and it suggests that the presence of positively charged amino acid residues on these peptides might confer increased ferric reducing activities (Li and Li, 2013).

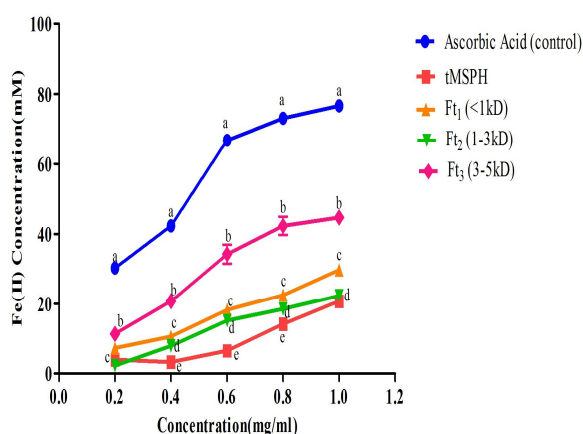


Figure 4. Ferric Reducing Antioxidant Properties of *Moringa oleifera* Seed Protein Hydrolysate and its Fractions obtained from Tryptic Digestion

Dots on each line graph are expressed as means \pm standard error of mean (SEM) of triplicate determinations ($n=3$). Dots belonging to different hydrolysate/fractions with different letters at the same concentration are significantly

different ($p<0.05$) from one another. Abbreviations: tMSPH: tryptic *M. oleifera* Seed Protein Hydrolysate, Ft₁: Tryptic fraction 1(< 1kD), Ft₂: Tryptic fraction 2(1-3 kD) Ft₃: Tryptic fraction 3(3-5 kD).

Hydroxyl Radical Scavenging Activity

The relative abilities of the protein hydrolysate and its fractions to scavenge the hydroxyl radical in comparison to the control, ascorbic acid, are illustrated in Figure 5. Both the hydrolysate and its fractions displayed lower ($p<0.05$) OH[•] radical scavenging activities when compared to ascorbic acid. Fractions Ft₂ and Ft₃ demonstrated concentration-dependent increases in OH[•] radical scavenging activity, attaining maximum scavenging abilities of 82.000 ± 1.155 % and 65.667 ± 3.657 % respectively at a final concentration of 1.0 mg/ml. These were significantly higher ($p<0.05$) than the activities of the unfractionated hydrolysate and fraction Ft₁, both of which displayed activities less than 35% at all concentrations in this study. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Lee *et al.*, 2002, Sun *et al.*, 2013). This radical has the capacity to cause strand breakages in DNA, which contributes to carcinogenesis, mutagenesis and cytotoxicity (Spencer *et al.*, 1994). There is relative paucity of information relating to the activities of protein hydrolysates and/or peptide fractions on the hydroxyl radical, when compared to reports on their effects on other free radical systems. The peptide fractions were more efficient in scavenging the OH[•] radical than their unfractionated counterparts. This implies that fractionation of the hydrolysates released more bioactive peptides that contributed to the quenching of OH[•] radicals. These fractions showed concentration-dependent hydroxyl radical scavenging activities, with fractions Ft₂ and Ft₃ having OH[•] radical scavenging activity higher than 80%. Udenigwe and Aluko (2011) suggested that peptides with aminoacyl residues such as lysine, proline, phenylalanine and tyrosine could contribute significantly to the neutralization of free radical systems. Going by the cleavage specificities of the enzyme trypsin, used in the hydrolysis and consequent production of these peptide fractions, it could be inferred that these hydrolysate fractions contained peptides with these aminoacyl residues which could scavenge the hydroxyl radical, either by virtue of proton donation and/or by their high hydrophobicity.

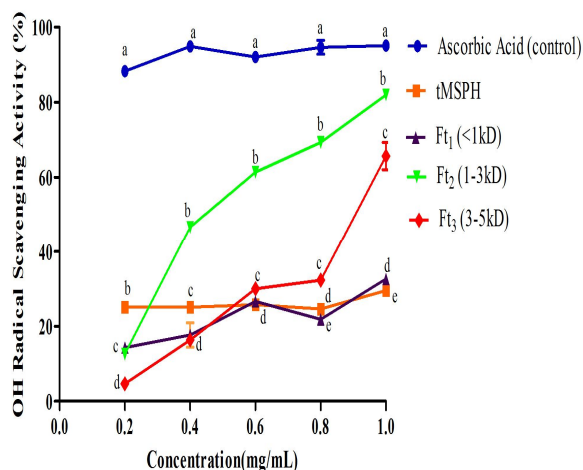


Figure 5. Hydroxyl Radical Scavenging Properties of Tryptic *Moringa oleifera* Seed Protein Hydrolysate and its Fractions

Dots on each line graph are expressed as means \pm standard error of mean (SEM) of triplicate determinations ($n=3$). Dots belonging to different hydrolysate/fractions with different letters at the same concentration are significantly different ($p<0.05$) from one another. Abbreviations: tMSPH: tryptic *M. oleifera* Seed Protein Hydrolysate, Ft₁: Tryptic fraction 1 (< 1kD), Ft₂: Tryptic fraction 2 (1-3 kD) Ft₃: Tryptic fraction 3 (3-5 kD).

Superoxide Radical Scavenging Activity

The effects of the tryptic digests of *M. oleifera* seed protein hydrolysate, and its fractions on the superoxide radical are illustrated in Figure 6. This showed that fraction Ft₃ demonstrated better ($p<0.05$) scavenging activities than ascorbic acid at concentrations of 0.4 mg/ml and 0.6 mg/ml, and reached an activity peak of 92.223 ± 1.794 % at a concentration of 0.8 mg/ml, which was comparable to 92.988 ± 0.304 % achieved by control at the same concentration. Fraction Ft₁ also showed a concentration-dependent superoxide scavenging activity, reaching a maximum of 91.094 ± 0.170 % at 0.8 mg/ml, which was lower ($p<0.05$) than the control, but significantly higher ($p<0.05$) than those of fraction Ft₂ and the unfractionated tryptic hydrolysate. Fraction Ft₂ also had increased activity as concentration increased, reaching a peak of 65.000 ± 2.375 mg/ml at a final concentration of 1.0 mg/ml, and it was higher ($p<0.05$) than that obtained by the unfractionated tryptic hydrolysate, which had less than an activity of 50% at all study concentrations. The superoxide anion radical is released during normal enzymatic redox reactions in living systems (Alashi *et al.*, 2014) and its overproduction has proven to be very harmful to cellular components such as cell membranes, DNA and proteins, and can damage cells and tissues, leading to disease conditions (Sun *et al.*, 2013). Although superoxide anion cannot directly initiate lipid oxidation, they are essential precursors of other highly reactive species, such as the highly reactive hydroxyl radical (Li *et al.*, 2008).

Thus, this assay is widely used in evaluating the antioxidative effects of naturally occurring compounds (Alashi *et al.*, 2014). The result showed that the peptide fractions showed better scavenging activities than the unfractionated hydrolysate, showing the influence of fractionation on bioactivity of these peptides, and this is corroborated by Famuwagun *et al.*, (2020) in their work with peptide fractions obtained from fluted pumpkin leaf proteins. In addition, higher molecular weight fractions scavenged the superoxide radical than peptide fractions with low molecular weight. The peptide fractions digestion exhibited higher superoxide quenching capacities comparable to ascorbic acid. The presence of cationic amino acid residues within the protein molecule and on the terminal ends of peptides obtained as a result of trypsin hydrolysis may have contributed to the observed high superoxide radical scavenging properties of tryptic peptide fractions (Udenigwe and Aluko, 2011, Li and Li, 2013, Voet *et al.*, 2016). However, their activities might have been antagonized by longer chain peptides in the solution, which might have accounted for the reduced scavenging activity seen with the unfractionated enzymatic digest.

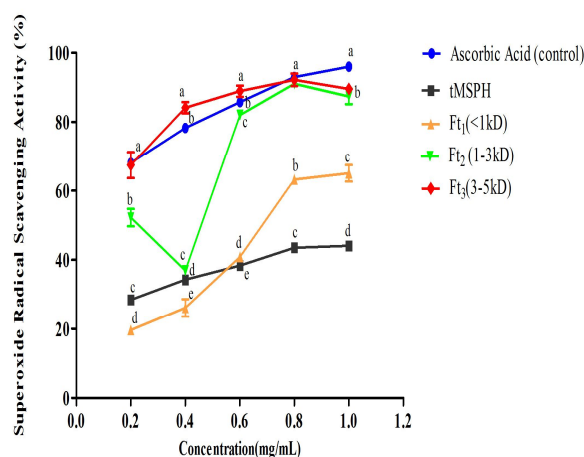


Figure 6. Superoxide Radical Scavenging Activities of Tryptic *Moringa oleifera* Seed Protein Hydrolysate and its Fractions

Dots on each line graph are expressed as means \pm standard error of mean (SEM) of triplicate determinations ($n=3$). Dots belonging to different hydrolysate/fractions with different letters at the same concentration are significantly different ($p<0.05$) from one another. Abbreviations: tMSPH: tryptic *M. oleifera* Seed Protein Hydrolysate, Ft₁: Tryptic fraction 1 (< 1kD), Ft₂: Tryptic fraction 2 (1-3 kD) Ft₃: Tryptic fraction 3 (3-5 kD).

CONCLUSION

The trypsin-assisted proteolysis of *M. oleifera* seed proteins and subsequent fractionation yielded hydrolysates and peptide fractions, which slowed down the rate of formation of glycosylated hemoglobin *in vitro*. In addition, they also exhibited

differing antioxidative abilities against DPPH, ferric ions, hydroxyl radicals and superoxide anions. Analysis of the peptide fractions revealed a direct proportion between average molecular weight of these peptides and their relative abilities to inhibit hemoglobin glycation and scavenge free radicals. Higher molecular weight peptide fractions displayed better bioactivities than fractions of low molecular weight. This not only justifies the use of *M. oleifera* seeds for alternative therapeutic purposes, but may also indicate that the proteins from the seed could be potential sources of biologically active peptides, which could be optimized to formulate new and potent anti-diabetic agents and food additives.

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