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Full Length Research Article

Moringa oleifera Seed Protein Hydrolysates: Kinetics of α-amylase Inhibition and Antioxidant Potentials

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Proteins from n-hexane - treated *Moringa oleifera* seed flour were isolated using alkaline solubilization followed by acid-induced precipitation. Two proteolytic enzymes, pepsin and trypsin were used to hydrolyze the protein isolates. The resulting hydrolysates were then evaluated for α-amylase inhibitory properties and kinetics as well as antioxidant activities against superoxide radicals and ferric ions. With the use of starch as substrate, the hydrolysates demonstrated a concentration-dependent inhibition of α-amylase with peptic hydrolysates exhibiting 77.591±0.173% and tryptic hydrolysates demonstrating 84.183±1.670% inhibition (IC_{50} = 0.547 mg/ml to 0.591 mg/ml). Kinetic data showed an uncompetitive subtype of mixed inhibition for peptic hydrolysates and an uncompetitive mode for tryptic hydrolysates, with $k_{i,}$ of 0.166 mg/ml and 0.179mg/ml for peptic and tryptic hydrolysates respectively. Antioxidant assays using superoxide radicals and ferric ions indicated that tryptic hydrolysates had higher scavenging activitiy while peptic hydrolysates possessed higher ferric reducing power. These results suggest that *Moringa oleifera* seed proteins may contain biologically active peptide sequences which could be harnessed for the formulation of new additives to food and for development of novel anti-diabetic agents.

Keywords: *Moringa oleifera*, hydrolysates, pepsin, trypsin, α-amylase inhibition, antioxidant potentials.

INTRODUCTION

Peptides from plant and animal sources have continuously gained attention due to their multifunctional abilities ranging from uses as food additives to their utilization in the treatment of disease conditions (Arise *et al.,* 2016[b]). Studies have demonstrated the abilities of these peptides to inhibit key enzymes and scavenge free radicals involved in the pathophysiology of conditions such as diabetes mellitus, hypertension and oxidative stress (Girgih *et al.,* 2015, Arise *et al.,* 2016[a], Arise *et al.,* 2016[b]). One plant that has been extensively studied for its numerous bioactivities is *Moringa oleifera*.

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Moringa oleifera is a perennial plant belonging to the *moringaceae* family. It is native to the Western and sub-Himalayan regions in India, from which it has been introduced to other parts of the world such as the Middle East and Africa (Madubuike *et al.,* 2015). Parts of the plant (roots, leaves, stem and seeds) are edible and are used for nutritional purposes and as traditional medicine (Leone *et al.,* 2016). Various parts of *Moringa oleifera* have been reported for their numerous biological activities. Its leaves possess purgative, antimicrobial and hypoglycemic effects (Siddhuraju and Becker, 2003, Divi *et al.,* 2012). Its stem bark has been demonstrated to have anti-cancer, anti-ulcerative, and anti-inflammatory properties (Siddhuraju and Becker, 2003). *Moringa oleifera* seeds have considerably high protein content

(about 33-36% by weight) (Gopalakrishnan *et al.,* 2016, Mune Mune *et al.,* 2016). Amino acid analysis indicated that the seeds are a good source of essential amino acids (40% of total amino acid content) and it is especially rich in aspartic acid, glutamic acid, lysine and arginine (Okereke and Akaninwor, 2013). Solvent extracts of its seeds have been shown to possess antioxidant and antihypertensive activities (Anwar *et al.,* 2007).

Diabetes mellitus (DM) is a disorder of the endocrine system, which occurs as a result of absence of insulin, impaired insulin function or both (Piero *et al.,* 2014). Its characteristic symptoms include chronic hyperglycemia associated with impairments in the regulation of carbohydrate, fat and protein metabolism. These cause a myriad of metabolic derangements resulting in multiple organ damage at later stages of the disease (Arise *et al.,* 2016[b]). Current therapeutic strategies are aimed at controlling blood glucose levels by lifestyle changes, infusion of exogenous insulin or modulating the activities of key enzymes involved either directly or indirectly in glucose metabolism. Key enzymes such as α-amylase, α-glucosidase and dipeptidyl peptidase-4 are known pharmacologic targets for many hypoglycemic drugs (Yu *et al.,* 2012, Arise *et al.,* 2016[b]). However, most conventional chemotherapeutic strategies are expensive to procure and places an economic burden on patients. Also, many of these drugs are not without their deleterious side effects, causing eventual damage to vital organs such as liver and kidney (Wang, *et al.,* 2010, Liu, *et al.,* 2011).

Oxidative stress occurs due to imbalance between the systemic production of reactive oxygen species and the ability of the body to rapidly detoxify the reactive intermediates or to repair the resulting damage. Free radicals alter the normal redox state of cells and can cause deleterious effects by damaging cellular components such as lipids, DNA and proteins. (Chandra *et al.,* 2015). Diabetes mellitus has also been shown to be a cause and effect of oxidative stress, releasing free radicals leading to impairment of tissue function and their eventual damage in the long term (Rahimi-Madiseh *et al.,* 2016) and as such there is a growing need to identify newer, more effective, cost effective and considerably safer antioxidant agents and inhibitors of enzymes involved in diabetes mellitus from a number of natural materials (Wang, *et al.,* 2010, Liu, *et al.,* 2011). These include peptides and protein hydrolysates from a number of plant and animal sources in recent times (Yu *et al.,* 2012, Arise *et al.,* 2016[b]).

Despite several reports about the biofunctionalities of solvent extracts of different parts of *M. oleifera*, information concerning the antidiabetic potentials of protein hydrolysates of its seeds have been scarce and hence, the focus of this study is to evaluate the α-amylase inhibitory properties and kinetics as well as antioxidant activities of hydrolysates obtained from *Moringa oleifera* seed proteins.

MATERIALS AND METHODS

Materials

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

Enzymes: Pepsin (from porcine gastric mucosa), trypsin (from bovine pancreas), α-amylase (from *Saccharomyces cerevisiae*), were products of Kem Light Laboratories, Mumbai, India.

Other Reagents: Ascorbic acid, trichloroacetic acid (TCA), potassium ferricyanide, ferric chloride, pyrogallol, starch, maltose, ethylene diamine tetraacetic acid(EDTA). These were products of Sigma-Aldrich laboratories, Co-Artrim, United Kingdom. All chemicals and reagents used were of analytical grade.

Equipment

Magnetic stirrer, soxhlet extractor, uv-visible spectrophotometer (Spectrumlab 752S), freeze drier, water bath and a bench centrifuge.

Methods

Isolation of *M. Oleifera* Seed Proteins

The seeds were dried, pulverized and stored in an air-tight container at 4°C. These were defatted using n-hexane as according to the method described by Wani *et al.* (2011). The meal was extracted four times with n-hexane (60–80°C) using a meal/solvent ratio of 1:10 (w/v), after which it was 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 facilitate solubilization in alkali. The slurry was then centrifuged at 18°C and 3000 g for 10 min. Two additional extractions of the residue from the centrifugation process was carried out with the same volume of 0.1 M NaOH and the supernatants were pooled. The pH of the supernatant

was adjusted to pH 4.0 to facilitate acid-induced protein precipitation using 3 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 stored at -20°C until required for further analysis.

Preparation of *M. oleifera* Seed Protein Hydrolysates

The protein isolate was hydrolyzed using the method described by Udenigwe *et al.* (2009) with slight modifications. The conditions for hydrolysis was specified for each enzyme in order to ensure optimal activity. Hydrolysis was carried out using each of pepsin (pH 2.2, 37ºC) and trypsin (pH 8.0, 37ºC). The protein isolate (5% w/v, based on the protein content of the isolate) was dissolved in the appropriate buffer (glycine buffer, pH 2.2 for pepsin and phosphate buffer, pH 8.0 for trypsin). The enzyme was added to the slurry at an enzyme-substrate ratio (E:S) of 2:100. Digestion was performed at the specified conditions for 8 hours with continuous stirring. The enzyme was inactivated by boiling in water bath (95–100^{o}C) for 15 min and undigested proteins were precipitated by adjusting the pH to 4.0 with 2M HCl/2M NaOH followed by centrifugation at 3500g for one hour. The supernatant containing target peptides were then collected. Protein content of samples was determined using biuret assay method of Gornall *et al*. (1949) with bovine serum albumin (BSA) as standard.

Determination of Degree of Hydrolysis

Degree of hydrolysis (DH) was determined by calculating the percentage of soluble protein in 10% trichloroacetic acid (TCA) in relation to total protein content of the protein isolate according to the method described by Hoyle and Merritt (1994) with slight modifications. 1 ml of protein hydrolysate was added to 1 ml of 20% TCA to produce 10% TCA soluble material. The mixtures were left to stand for 30 minutes for precipitation, followed by centrifugation at 4000 g for 20 min. The supernatants were analyzed for protein content using Biuret method of Gornall *et al*. (1949) with bovine serum albumin (BSA) as standard. The degree of hydrolysis (DH) was computed as shown below:

$$DH = \frac{Soluble\ peptide\ in\ 10\%\ TCA\ (mg)}{Total\ protein\ content\ of\ isolate\ (mg)} \times 100\%$$

Determination of Peptide Yield

The percentage peptide yield was determined using the method reported by Arise *et al.* (2016[a]). The peptide yields (%) was calculated as the ratio of peptide content of lyophilized hydrolysate to the protein content of unhydrolysed protein isolate.

Determination of α-amylase Inhibition

An α-amylase-inhibitory assay was carried out according to the method of Bernfield (1951) and described by Arise *et al.* (2016[b]) with slight modifications. Briefly, 250 µL of hydrolysate (0.2 to 1.0 mg mL–1) was placed in test tubes and 250 µL of 20 mM sodium phosphate buffer (pH 6.9, with 6mM NaCl) containing α-amylase solution (0.5 mg/mL) added. The content of each tube was pre-incubated at 25 °C for 10 min, after which 250 µL of 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9, with 6 mM NaCl) was added at timed intervals. The reaction mixtures was incubated at 25 °C for 10 min. The reaction was terminated by adding 250µL of 1% dinitrosalicylic acid (DNS) colour reagent and further incubated in boiling water for 5 min and cooled to room temperature. The content of each test tube was diluted with 5.0 mL distilled water and the absorbance measured at 540 nm. A control was prepared using the same procedure except that the hydrolysate was replaced with distilled water. The α-amylase-inhibitory activity was determined as shown:

% Inhibition = (Acontrol – Asample) / Acontrol × 100.

The concentration of hydrolysate resulting in 50% inhibition of enzyme activity (IC_{50}) was computed from a plot of percentage inhibition versus hydrolysate concentrations using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

Determination of Kinetic Parameters of α-amylase Inhibition

The kinetic study of α-amylase inhibition was conducted according to the modified method described by Ali *et al.* (2006). 250 µL of the hydrolysate was pre-incubated with 250 µL of α-amylase solution for 10 min at 25 °C in a set of tubes. In another set of tubes, 0.5ml of phosphate buffer (pH 6.9) was also pre-incubated with 250 µL of α-amylase solution. Starch solution (250 µL) of increasing concentrations (0.2 - 1.0 mg mL–1) was added to both sets of reaction mixtures to initiate the reaction. The mixture was then incubated for 10 min at 25 °C, and boiled for 5 min after the addition of 0.5ml of 1% dinitrosalicylic acid (DNS) reagent to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically from a maltose standard curve and converted to reaction velocities as shown below:

Specific Activity (mM /mg protein)/min) = Maltose released / Incubation time × ME, where maltose concentration is in mM/mL; Incubation time = 10 min; ME= amount of enzyme (in mg) in reaction mixture

A double reciprocal plot (1/V versus 1/[S]), where V is reaction velocity and [S] is substrate concentration was plotted. The mode of inhibition and the kinetic parameters (Km, K´m, V'max, V´max, CE and CE´) of α-amylase

inhibition by hydrolysates was determined by analysis of the double reciprocal plot. The inhibition constant (Ki) was determined using a web-server tool (http:// botdb.abcc.ncifcrf.gov/toxin/kiConverter.jsp) described by Cer *et al.,* (2009) which computes Ki values from experimentally determined IC_{50} for inhibitors of enzymes and of binding reactions between ligands and macromolecules.

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) was each dissolved in 50 mM Tris–HCl buffer, pH 8.3 containing 1 mM EDTA and 80 µL was transferred into a clean test-tubes and 80 µL of buffer was added to the blank tube. This was followed by addition of 40 µL 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each tube 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 equation:

Superoxide scavenging activity (%) = $(\Delta Abs/min_b - \Delta Abs/min_s)/\Delta Abs/min_b$ x 100 where b and s are blank and sample, respectively.

Determination of Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing power of the hydrolysates was measured according to a slightly modified method of Oyaizu (1986). An aliquot of 1 ml of different hydrolysate concentrations (0.2 – 0.8 mg/ml), (0.2 M PBS, pH 6.6) was mixed with 1 ml of 1% potassium ferric cyanide solution. The mixture was 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 indicated stronger reducing power. Ascorbic acid was used as the reference antioxidant. An aqueous solution of known Fe (II) concentrations ($FeSO_4 \cdot 7H_2O$; 2.0, 1.0, 0.5, 0.25 and 0.125mM) was used for calibration. Results were expressed as mM Fe^{2+}/mg hydrolysate. All tests were performed in triplicate.

Statistical Analysis

Results were expressed as mean of replicates ± standard error of mean. 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) and Microsoft Excel version 2013.

RESULTS

Protein Yield of Isolate, Peptide Yield and Degree of Hydrolysis

The protein yield of isolation, peptide yield and degree of hydrolysis are presented in Table 1. The yield of isolation of *M. oleifera* seed proteins was 18.60%. Peptide yield of hydrolysates obtained by pepsin and trypsin treatment were 77.720±1.045% and 75.979±1.393% respectively, while the degree of hydrolysis of hydrolysates obtained from peptic and tryptic digestion were found to be 26.933±0.668% and 10.305±0.072% respectively. Peptide yield of both hydrolysates were not significantly different ($p<0.05$) from each other while the degree of hydrolysis of peptic hydrolysates were significantly higher ($p<0.05$) than those of tryptic hydrolysates.

Alpha-amylase inhibitory Activity

The α-amylase inhibitory activities of *M .oleifera* seed protein hydrolysates are shown in Figure 1. Both hydrolysates demonstrated a concentration-dependent inhibition of α-amylase, except for peptic hydrolysates showing a reduction at 0.8mg/ml. However, both hydrolysates showed percentage inhibitory activities above 50% at concentrations of 0.60mg/ml to 1.0mg/ml. Tryptic hydrolysates showed significantly higher ($p<0.05$) inhibitory activity at 0.2mg/ml, 0.8mg/ml and 1.0mg/ml, while peptic hydrolysates displayed a significantly higher($p<0.05$) inhibitory activity at a concentration of 0.6mg/ml. The 50% α-amylase inhibitory concentrations(IC_{50}) of *M. oleifera* seed protein hydrolysates are shown in Figure 2. The IC_{50} of peptic hydrolysates (0.547±0.074mg/ml) was not significantly different from that of tryptic hydrolysates (0.591±0.025mg/ml).

Table 1. Yields of *M. oleifera* seed protein isolate, hydrolysates and degree of hydrolysis. Values are presented as means ± standard error of mean (SEM) of triplicate determinations. Values bearing different superscripts are significantly different at p<0.05.

Parameter/Samples	Yield of Isolation (%)	Peptide Yield (%)	Degree of Hydrolysis (%)
Protein Isolate	18.60%	-	-
Peptic Hydrolysates	-	77.720±1.045%[a]	26.933±0.668%[a]
Tryptic Hydrolysates	-	75.979±1.393%[a]	10.305±0.072%[b]

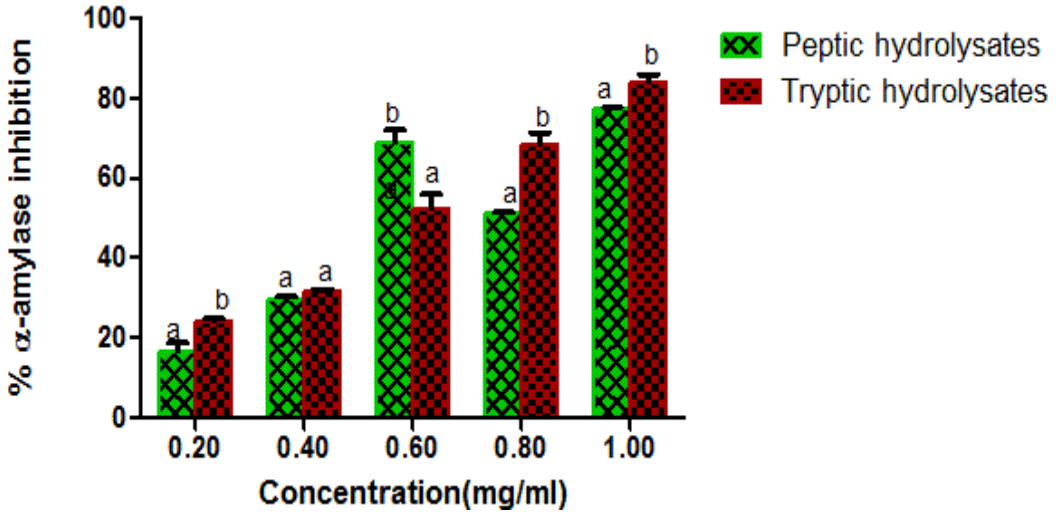

Figure 1. Percentage α-amylase inhibition by *M. oleifera* seed protein hydrolysates. Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Comparison is strictly within the same concentration value. Bars with the same letters do not differ significantly while values with different letters are significantly different from one another at p<0.05.

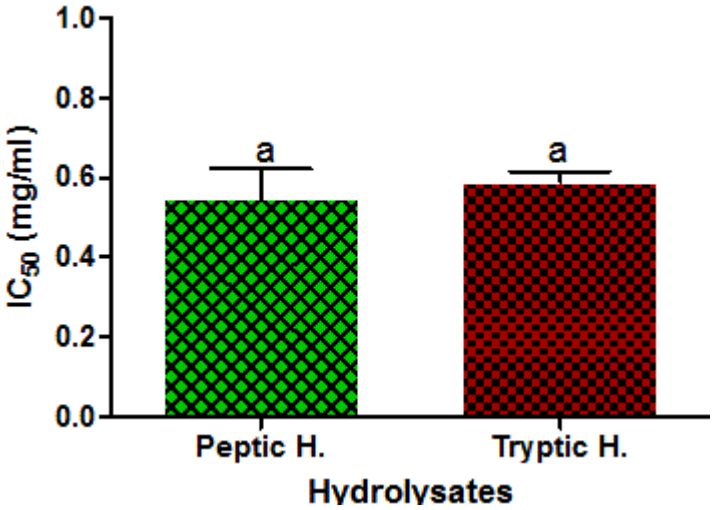

Figure 2. IC_{50} values of α-amylase inhibition by *M. oleifera* seed protein hydrolysates. Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while values with different letters are significantly different from one another at p<0.05.

Table 2. Kinetic parameters of α-amylase catalyzed hydrolysis of starch in the presence and absence of *Moringa oleifera* seed protein hydrolysates.

Kinetic Parameter	No inhibitor	Peptic Hydrolysates (mg/ml)		Tryptic hydrolysates (mg/ml)	
		0.5	1.0	0.5	1.0
K_m or K'_m (mg/ml)	2.301	1.044	1.377	0.7644	0.456
V_{max} or V'_{max} (mM/mg/min)	25.974	9.930	9.497	8.123	4.733
CE (mmol/ml/min)	11.288	9.515	6.897	10.627	10.379
K_i (mg/ml)	-	0.166		0.179	

Km/K'm – Michaelis constant in the absence/presence of inhibitory hydrolysates**; Vmax/V'max –** Maximum velocity in the absence/presence of inhibitory hydrolysates; **CE** – Catalytic Efficiency; **Ki** – Enzyme-inhibitor dissociation constant.

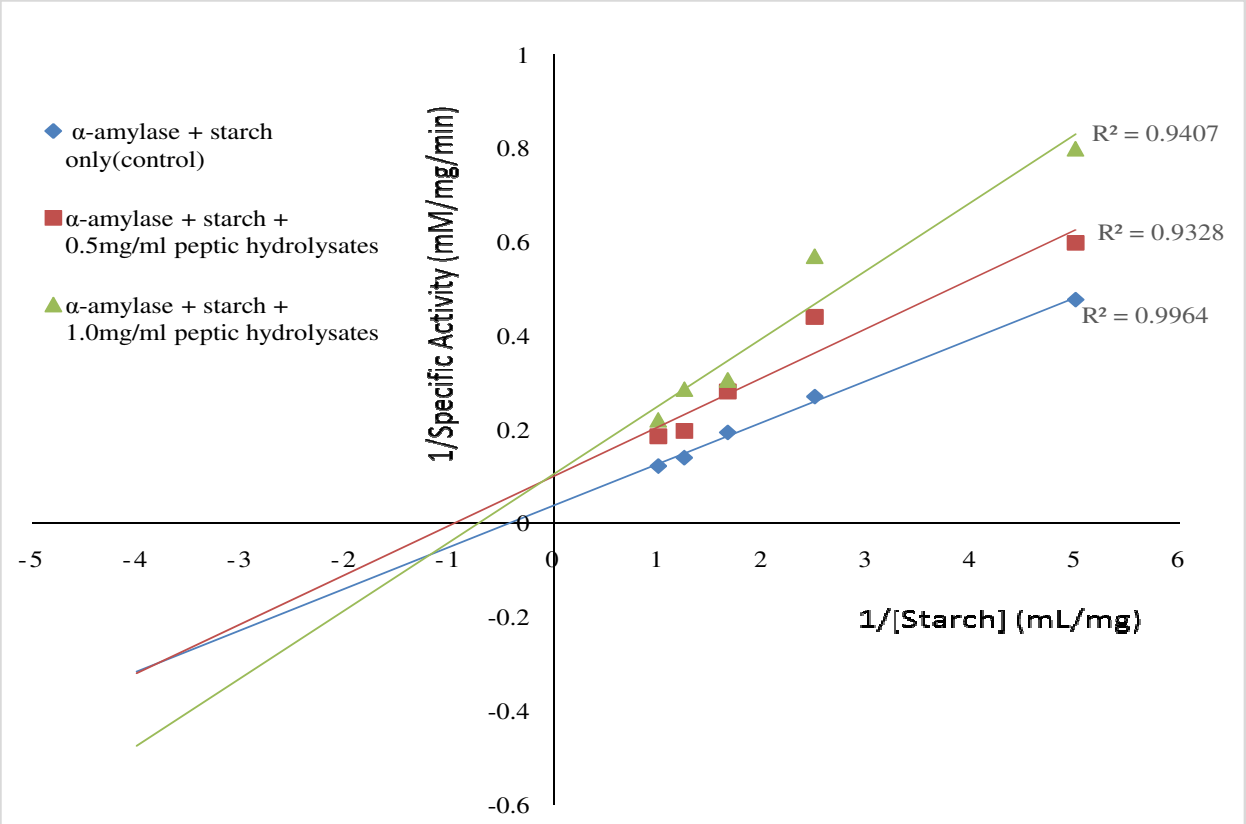

Figure 3. Lineweaver-Burk plot of α-amylase inhibition by *Moringa oleifera* seed protein hydrolysates derived from peptic proteolysis. R^2 – Coefficient of determination

Kinetics of α-amylase Inhibition

The effects of peptic and tryptic hydrolysates of *M. oleifera* seed proteins on the catalytic activity of α-amylase in converting starch to maltose are shown in Figures 2 and 3. Kinetic parameters determined from Lineweaver-Burk plots in the absence and presence of two different concentrations of each of peptic and tryptic hydrolysates are summarized in Table 1. The km of the enzyme for its substrate was determined to be 2.301mg/ml of starch, while Vmax was 25.974 mmol/mg/min. The presence of increasing concentrations of the hydrolysates, appeared to have no effect on the Km of the enzyme, while maximal velocity, Vmax and catalytic efficiency, CE of α-amylase were reduced in the presence of the hydrolysates. Tryptic hydrolysates showed a more reduced Vmax while the catalytic efficiency of the enzyme was more decreased in the presence of peptic hydrolysates.

The enzyme-inhibitor dissociation constant, ki, of α-amylase inhibition by peptic hydrolysates (0.166mg/ml) was lower than that obtained for tryptic hydrolysates (0.179mg/ml). The mode of inhibition of peptic hydrolysates was the uncompetitive type of mixed inhibition while tryptic hydrolysates displayed a simple uncompetitive inhibition mechanism.

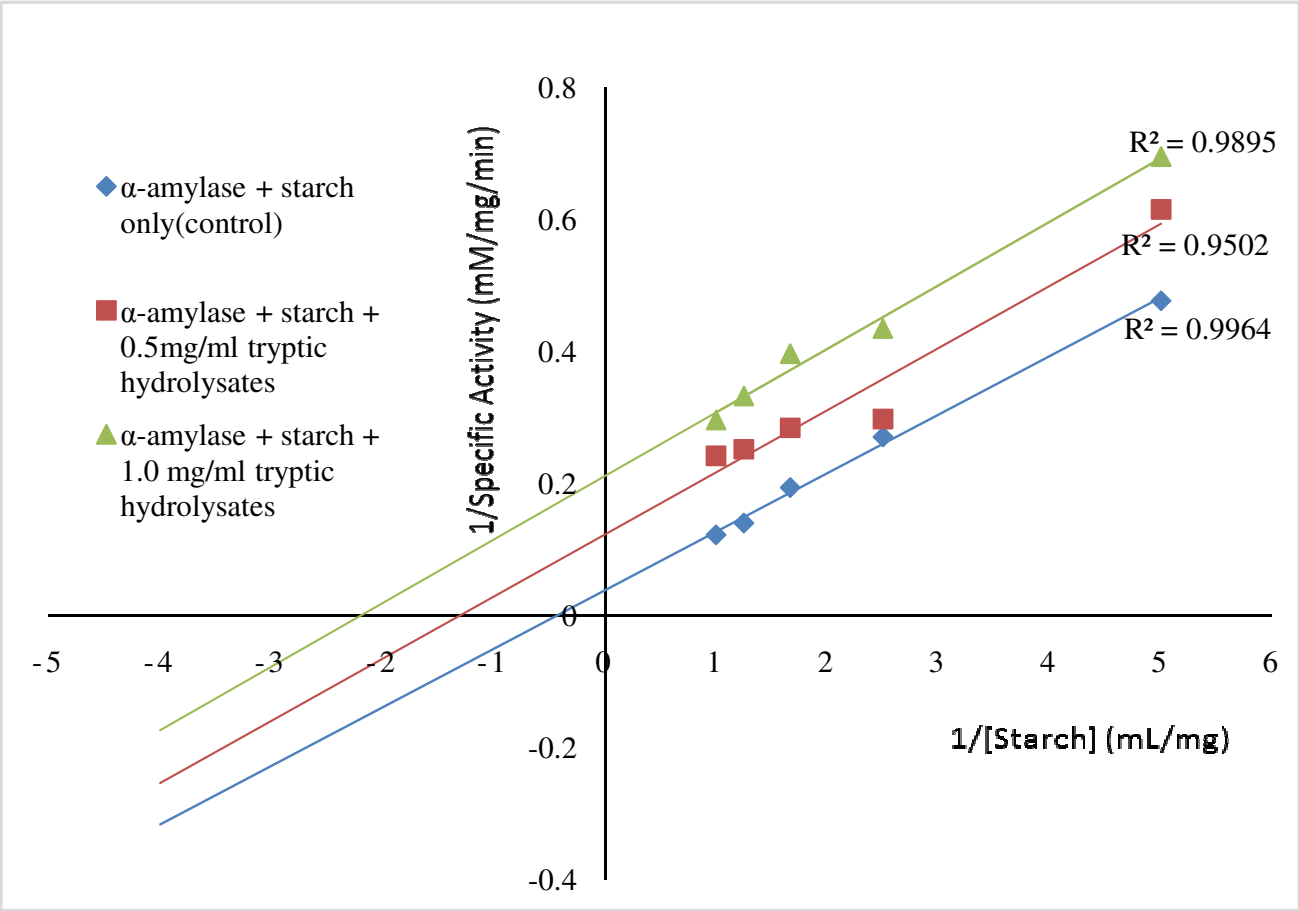

Figure 4. Lineweaver-Burk plot of α-amylase inhibition by *Moringa oleifera* seed protein hydrolysates derived from tryptic proteolysis.
R^2 – Coefficient of determination

Ferric Reducing Antioxidant Power

The ferric reducing antioxidant properties of ascorbate (control) and *Moringa oleifera* seed protein hydrolysates are illustrated in Figure 5. All samples displayed a concentration-dependent increase in ferric reducing power. All hydrolysates had significantly ($p<0.05$) reduced antioxidant activities at different concentrations when compared to ascorbate. However, peptic hydrolysates had significantly ($p<0.05$) higher antioxidant activities than tryptic hydrolysates at all concentrations.

Superoxide Radical Scavenging Activity

The superoxide radical scavenging activities of ascorbate and *M. oleifera* seed protein hydrolysates are presented in Figure 6. Tryptic hydrolysates had significantly ($p<0.05$) higher scavenging activity at a concentration range of 0.2-1.0mg/ml when compared to peptic hydrolysates. Figure 7 shows EC_{50} values of *M. oleifera* seed protein hydrolysates in scavenging superoxide radical, as compared to ascorbate (control). Peptic hydrolysates scavenged the radical to a 50% inhibition at

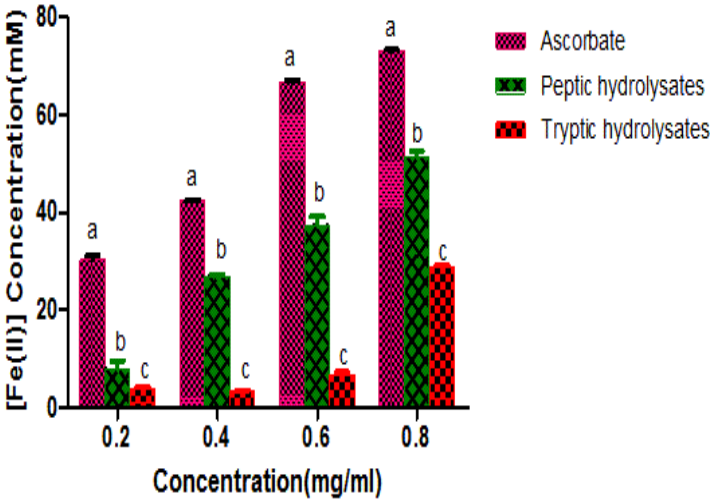

Figure 5. Ferric Reducing Antioxidant Properties of *M. oleifera* Seed Protein Hydrolysates.
Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Comparison is strictly within the same concentration value. Bars with the same letters do not differ significantly while values with different letters are significantly different from one another at $p<0.05$.

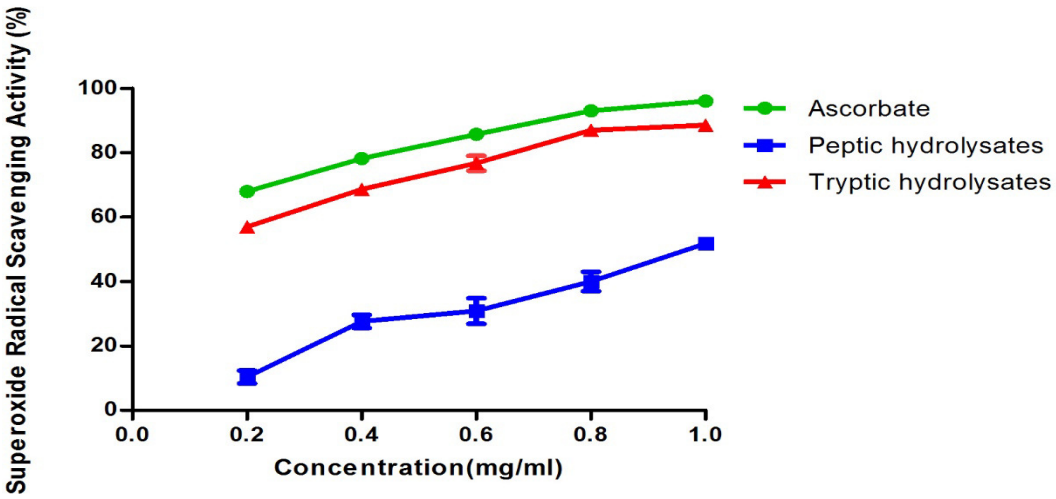

Figure 6. Superoxide radical scavenging activities of *M. oleifera* seed protein hydrolysates.
Points on the graph are expressed as means± standard error of mean (SEM) of triplicate determinations (n=3).

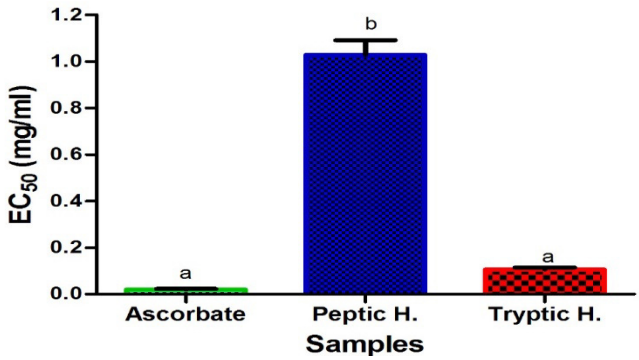

Figure 7. EC_{50} Values of *M. oleifera* seed protein hydrolysates in scavenging superoxide radical.
Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while values with different letters are significantly different from one another at $p<0.05$.

a concentration of 1.027±0.037mg/ml, while tryptic hydrolysates had 50% scavenging activities at a concentration of 0.106±0.005mg/ml. Tryptic hydrolysates had IC_{50} values comparable to ascorbate, while peptic hydrolysates had a significantly ($p<0.05$) higher IC_{50} value when compared to those of control and tryptic hydrolysates.

DISCUSSION

Protein Yield of Isolate, Peptide Yield and Degree of Hydrolysis

Several methods exist for isolation of proteins, such that alkaline extraction and isoelectric precipitation has been reported to be the most efficient (Pedroche *et al.,* 2004). Despite studies suggesting that protein content of *M. oleifera* seeds ranges between 33% and 36% (Gopalakrishnan *et al.,* 2016, Mune Mune *et al.,* 2016), the percentage protein yield of isolation obtained for *M.oleifera* seed proteins was 18.60%. This is slightly lower than 19.80% and 18.91% recorded for another study on *M.oleifera* seed proteins (Garza *et al.,* 2017) and *Citrullus lanatus* seed proteins (Arise *et al.,* 2016[b]) respectively. The obviously low value may be as a result of the nature of proteins in the seed. Oil seeds are known to contain proteins such as globulins, albumins and glutelins (Salunkhe *et al.,* 1992, Wani *et al.,* 2011) and as a result, proteins which are soluble in dilute acid, such as albumin may have been lost, leaving behind the insoluble globulins to constitute the major portion of the protein isolate.

Peptide yield is a useful parameter that can be utilized to determine the efficiency of the overall process of hydrolysis (Alashi *et al.,* 2014). Peptic hydrolysates had a

higher peptide yield of 77.72% and this is significantly higher than 68.90% and 55.0% obtained by Arise *et al.,* (2016[b]) and Alashi *et al.,* (2014) for watermelon seed protein hydrolysates and canola seed meal protein hydrolysates respectively. The relatively high yield obtained indicate that most of the proteins in *M.oleifera* seeds were susceptible to enzymatic hydrolysis by pepsin and could be converted into peptide products, which would be beneficial economically for industrial purposes. In addition, *M.oleifera* seeds are particularly rich in hydrophobic amino acid residues (Mune Mune *et al.,* 2016), and pepsin is an endoprotease which cleaves peptide bonds at C-terminal residues of hydrophobic amino acids (Voet and Voet, 2004). This may further explain the reason why peptic hydrolysates has slightly higher peptide yield when compared to tryptic hydrolysates. Tryptic hydrolysates have a yield of 75.98%, which is significantly higher than 41.38% obtained from watermelon seed protein hydrolysates. Trypsin is known to have specificity for lysine and arginine (Voet and Voet, 2004)., and *M.oleifera* seeds have a considerable amount of these amino acids (Mune Mune *et al.,* 2016), hence this may account for the high peptide yield obtained.

The degree of hydrolysis (DH) is a measure of the number of cleaved peptide bonds in a protein hydrolysate. DH can affect the molecular sizes and amino acid compositions of the peptides and thereby affect the biological activities of the peptides formed during hydrolysis. Therefore, the DH is an important parameter in determining the functional properties of protein hydrolysate preparations (Jamdar *et al.,* 2010). The degree of hydrolysis obtained for peptic hydrolysates (26.93±0.67%) at an enzyme-substrate ratio of 2:100 was higher than 19.38±0.86% (E:S of 1:100) obtained for watermelon seed protein hydrolysates (Arise *et al.,* 2016[b]) and 8% (E:S of 4:100) for hemp seed peptic protein hydrolysates (Malomo *et al.* 2015). However, the degree of tryptic hydrolysis (10.31±0.07%, E:S 2:100) was lower than 26.26 ±0.27% obtained for tryptic hydrolysates of watermelon seed protein hydrolysates (Arise *et al.,* 2016[b]). This could be as a result of conditions such as nature of the protein source, enzyme-substrate ratio and the conditions of hydrolysis.

Alpha-amylase Inhibitory Activity and Kinetics of Inhibition

Although there are several reports about α-amylase inhibitory activities of various plant extracts, there is limited information on protein hydrolysates and peptides with α-amylase inhibitory activities. The results showed that the hydrolysates demonstrated a concentration dependent increase in percentage α-amylase-inhibitory activity, with tryptic hydrolysates having a stronger inhibitory effect (84.18%) than peptic hydrolysates (77.59%) at a final concentration of 1.0mg/ml, but their IC_{50} values were not significantly different from each other. The 84.18% inhibition of α-amylase by tryptic hydrolysates is slightly higher than 82.97% inhibition of the same enzyme obtained for tryptic hydrolysates of *Citrullus lanatus* seed proteins(Arise *et al.,* 2016[b]), although that was obtained at a concentration of 2.0mg/ml. In the same vein, the extent of inhibition by peptic hydrolysates in this study is comparatively higher than 70.19% reported by Arise *et al* (2016[b]) for peptic hydrolysates of *Citrullus lanatus* seed proteins. This may indicate that oil seed proteins could release bioactive peptides with potent α-amylase-inhibitory potentials when digested with trypsin. According to Sumitani *et al.,* (2000), tyrosine, arginine and tryptophan residues are required for the inhibition of α-amylase. Arise and co-workers (2016[b]) also suggested that α-amylase binds to peptides containing cationic and branched chain residues such as Phe, Tyr, Trp and Lys. This could, in part, explain the reason why peptic hydrolysates also have a high α-amylase-inhibitory activity, since pepsin is known to cleave at C-terminals of hydrophobic amino acid residues.

The kinetic parameters determined from the double-reciprocal plots in Figures 3 and 4, were summarized in Table 1; and they suggest that the km of α-amylase in the absence of inhibiting hydrolysates is 2.301 mg/ml of starch which is comparatively higher than 1.3 mg/ml (Irshad *et al.,* 2012) and 1.4 mg/ml (Acharya *et al.,* 2014**)** for α-amylases obtained from *Ganoderma tsugae* and *Aspergillus oryzae* respectively, but lower than 6.639mg/ml reported by Arise *et al,* (2016[b]) for *Bacillus licheniformis* α-amylase. The peptic hydrolysates demonstrated uncompetitive subtype of mixed inhibition of α-amylase, even as tryptic hydrolysates showed a simple uncompetitive inhibition mechanism. These were reflected in the catalytic parameters obtained at different concentrations. For peptic hydrolysates, this indicates that the peptides that made up the hydrolysates could bind α-amylase in both its free and starch bound forms, but having higher affinity for the enzyme in its starch-bound form than in its free form. Tryptic hydrolysates displayed an uncompetitive inhibition mechanism, implying that they bind α-amylase in its starch-bound form and not in its free form. This could mean that the hydrolysates might bind to other sites distinct from the catalytic sites, resulting in progressive reduction or loss of activity with increasing concentrations. The kinetic data in Table 2 showed a concentration-dependent reduction in Vmax and CE of α-amylase for both hydrolysates. Ki values suggest that peptic hydrolysates had higher binding affinity for α-amylase than tryptic hydrolysates. The ki of 0.166mg/ml obtained for peptic hydrolysates was relatively higher than 0.042mg/ml reported for *Citrullus lanatus* seed protein hydrolysates derived from peptic digestion (Arise *et al,* 2016[b]), while the binding constant of 0.179mg/ml determined for tryptic hydrolysates was lower than 0.449mg/ml reported by

Arise *et al,* (2016[b]) for tryptic digests of *Citrullus lanatus* seed protein hydrolysates. This lends credence to the position of Sumitani *et al.,* (2000) that specific amino acid residues on peptides are required for α-amylase inhibition.

Ferric Reducing Antioxidant Power

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 (Yildrim *et al.,* 2000). The ability of bioactive compounds to reduce ferric ions has a strong correlation with their antioxidative properties. In this study, both hydrolysates demonstrated a concentration-dependent increase in Fe^{3+} -reducing property, but these were low when compared with ascorbate. However, Fe^{3+} -reducing activities of *M. oleifera* seed protein hydrolysates was higher than what was reported by Razali *et al.,*(2015) and Arise *et al.,* (2016[b]) for cobia skin gelatin hydrolysates and *Citrullus lanatus* seed protein hydrolysates respectively. The relatively low ferric reducing power of these hydrolysates when compared to ascorbate may be attributed to the relatively low amount of sulfur-containing aminoacyl residues in the hydrolysates, which would have otherwise contributed positively to antioxidative activity by donating protons to ferric ions in the reaction medium (Udenigwe and Aluko, 2011, Lopez-Barrios *et al.,* 2014). The results also showed that peptic hydrolysates showed better ferric reducing properties than tryptic hydrolysates at all concentrations. Similar trend was also observed for *Citrullus lanatus* seed protein hydrolysates (Arise *et al.,* 2016[b]) A number of reports have shown that there may be a direct correlation between degree of hydrolysis and ferric-reducing power, indicating that smaller peptides often exhibit higher reducing power (Vastag *et al.* 2011) and since peptic hydrolysates have a higher degree of hydrolysis than tryptic hydrolysates, this could be responsible for the comparatively higher Fe^{3+} -reducing property demonstrated by peptic hydrolysates. In addition, Udenigwe and Aluko (2011) had reported that sulfur-containing and acidic amino acid residues are positive contributors to ferric reducing properties of peptides while positively charged amino acid residues such as lysine and arginine have very weak effects. Okereke and Akaninwor (2013) reported that *M. oleifera* seeds are abundant in acidic amino acids. Pepsin cleaves at C-terminal residues of hydrophobic amino acids, and to a lesser extent acidic amino acids (Voet and Voet, 2004), yielding peptides with acidic aminoacyl residues that could donate protons to ferric ions, reducing them in the process, thus accounting for better reducing properties than tryptic hydrolysates. Trypsin on the other hand, cleaves peptides at C-terminals of arginine and lysine (Voet and Voet, 2004), producing peptides with positively charged residues, which in turn contribute weakly to the reduction of ferric ions (Udenigwe and Aluko, 2011).

Superoxide Radical Scavenging Activity

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 (Sun *et al.,* 2013). It is widely used in evaluating the antioxidative effects of naturally occurring compounds (Alashi *et al.,* 2014). In this study, both hydrolysates demonstrated significantly different superoxide radical scavenging properties. Hydrolysates from tryptic digestion showed better superoxide scavenging activity than peptic hydrolysates, and in a manner comparable to ascorbic acid. This is also reflected in their IC_{50} values, such that tryptic hydrolysates had lower scavenged the radical to a 50% extent at a concentration comparable to that obtained by ascorbic acid, but higher than that of peptic hydrolysates. This result correlates with earlier reports (Alashi *et al.,* 2014), which indicated that tryptic hydrolysates had better superoxide scavenging activities than peptic hydrolysates of canola seed proteins. A previous work has shown that acidic amino acids are negative contributors to superoxide scavenging activity of protein hydrolysates while lysine and leucine residues have positive effects in neutralizing superoxide radicals (Udenigwe and Aluko, 2011). Also, Li and Li (2013) reported that the C-terminal aminoacyl residue of a peptide plays an important role in antioxidant activity. The release of peptides with C-terminal lysine residues by trypsin may explain the increased superoxide scavenging properties of tryptic hydrolysates, whereas pepsin being less specific, likely yielded peptides with acidic residues that resulted in low superoxide scavenging effects. Also, the IC_{50} values suggest that a higher concentration of peptic hydrolysates would be needed to achieve the same level of inhibition as tryptic hydrolysates and ascorbic acid.

CONCLUSION

The subjection of *M.oleifera* seed proteins to proteolytic digestion by pepsin and trypsin yielded hydrolysates which exhibited potent alpha-amylase inhibitory activities and antioxidant effects. Peptic hydrolysates showed better α-amylase inhibitory properties and ferric reducing activities, while tryptic hydrolysates demonstrated more effective superoxide scavenging activities. These results indicate that both hydrolysates may have immense potentials as sources of novel antidiabetic peptides and food additives which could serve as cost-effective alternatives to current therapies. Fractionation of the hydrolysates and their characterization of resulting peptides are required to further elucidate the various mechanisms by which the peptides elicit their effects.

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