

Peptide Fractions from Pepsin-digested *Moringa oleifera* Seed Proteins Inhibit Hemoglobin Glycation and Carbohydrate-hydrolyzing Enzymes

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Abstract

The multidirectional abilities of peptide digests and fractions obtained from the hydrolysis of food-based proteins have been investigated in recent times. This study aims to evaluate the effects of pepsin-derived *Moringa oleifera* seed protein hydrolysates and fractions on hemoglobin glycation and the carbohydrases - α -amylase and α -glucosidase. Proteins were extracted from *M. oleifera* seeds and consequently digested using pepsin. The hydrolysates obtained were separated into fractions of <1 kD, 1-3 kD, and 3-5 kD ranges using size-exclusion chromatography and comparison with elution volumes of known standards. The activities of the hydrolysates and peptide fractions against both the non-enzymatic glycation of hemoglobin and the carbohydrases were determined *in vitro*. Results revealed that the hydrolysate and its peptide fractions demonstrated varying abilities against the glycation of hemoglobin, with the unfractionated hydrolysate showing better activities (78.230 ± 0.774 % at a maximum concentration of 1.0 mg/ml) than its peptide fractions. Also, the hydrolysates and fractions demonstrated higher inhibitory effects on α -amylase (with all fractions displaying above 50% inhibition at a final concentration of 1.0 mg/mL) than against α -glucosidase. Kinetic analysis of a selected fraction showed that it inhibited α -amylase via a mixed mechanism ($K_i = 0.029$ mg/mL) but displayed an uncompetitive mode for α -glucosidase inhibition ($K_i = 0.333$ mg/mL). Therefore, it is inferred that *M. oleifera* seed proteins encode potentially therapeutic peptide sequences that could be further processed to formulate potential antidiabetic agents.

Keywords: *Moringa oleifera*; Peptide; Pepsin; Hemoglobin; α -Amylase; α -Glucosidase.

INTRODUCTION

Diabetes mellitus is an endocrine and metabolic disease, which is characterized by elevated plasma and urinary glucose levels as a direct result of inadequate insulin production and/or action (Desmukh and Jain, 2015). In its various forms, diabetes mellitus causes a plethora of derangements in the metabolisms of carbohydrates, proteins, and lipids (Olusola and Ekun 2019a). These cause a wide range of complications such as diabetic ketoacidosis, neuropathy, nephropathy, and cardiovascular and multiple organ damage at later stages of the disease (Piero *et al.*, 2014; Olusola *et al.*, 2018). One particular aspect in the pathogenesis of diabetes mellitus is the fact that persistent hyperglycemia leads to the formation of advanced glycated end products (AGEs), as glucose complexes with proteins, and to a relatively lesser extent, lipids in a non-enzymatic, covalent fashion (Ramasamy *et al.*, 2005). Proteins such as hemoglobin and albumin are frequently glycated (Singh *et al.*, 2014). These glycated end products are known to potentiate certain pro-inflammatory pathways via the production of reactive oxygen species. These AGEs bind to, and activate the receptor for advanced

glycated end products, (RAGE), which in turn activate downstream signaling cascades which play important roles in the progression of diabetic nephropathy, neuropathy, and retinopathy (Singh *et al.*, 2014; Perrone *et al.*, 2020). Current strategies employed in the management of diabetes mellitus have been aimed at controlling postprandial glucose levels (Arise *et al.*, 2016). These include insulin preparations (for type 1 diabetes mellitus) and a combination of lifestyle changes and/or the use of oral hypoglycemic drugs (in the case of type 2 diabetes mellitus), which act by modulating the activities of key proteins and enzymes involved in carbohydrate hydrolysis and transport (Olusola and Ekun, 2019a). Enzymes such as α -amylase, α -glucosidase, and dipeptidyl peptidase (iv) have been key pharmacologic targets of oral antidiabetic agents (Rosenstock and Zimmerman, 2007, Arise *et al.*, 2016, Olusola and Ekun, 2019b). However, owing to certain deleterious effects and cost of procurement on the part of these synthetic drugs, attention has since turned to products from natural sources with the purpose of exploring them as possible alternatives in the management of diabetes mellitus (Oboh *et al.*, 2011, Parveen *et al.*, 2017). In recent times, peptide products

and protein hydrolysate preparations from enzymatic hydrolysis of plant and animal proteins are increasingly exploited and investigated for their numerous bioactivities (Lopez-Barrios *et al.*, 2014; Majumder and Wu, 2015; Siow and Gan, 2016). Peptides differing in lengths, sizes, and derivation have been widely used for a number of therapeutic purposes (Kumar *et al.*, 2015, Vilcacundo *et al.*, 2019). One plant whose proteins contain potentially biologically active peptides is *Moringa oleifera*.

Moringa oleifera, is a plant that belongs to the family *moringaceae*. It is originally endemic to the Himalayan regions of India but is also abundant in the Middle East and cultivated in many regions of Sub-Saharan Africa (Madubiike *et al.*, 2015). It is fast-growing, and drought resistant and this may have accounted for its wide geographical spread (Madubiike *et al.*, 2015, Leone *et al.*, 2016, Abd-Rami *et al.*, 2018). Various parts of the plant have been utilized for numerous purposes such as water treatment, as a food source and supplements, and also in traditional medicine (Anwar *et al.*, 2007, Abd-Rani *et al.*, 2018). Its seeds and leaves are rich sources of nutrients and essential oils (Kwaambwa *et al.*, 2015), and proteins (Mune-Mune *et al.*, 2016). Its proteins consist of albumins, globulins, prolamins, and glutelins (Kwaambwa *et al.*, 2015) which could yield potentially bioactive peptide products on enzymatic hydrolysis. In addition, its seeds are abundant in certain amino acids such as glutamate, aspartate, arginine, proline, and threonine, but limited in sulfur-containing amino acids (Freire *et al.*, 2015; Mune-Mune *et al.*, 2016). Various extracts of its leaves and seeds have been reported to possess potent pharmacologic effects (Abd-Rani *et al.*, 2018). Solvent extracts of its leaves demonstrated antioxidant properties, anticancer potentials (Monera *et al.*, 2008; Al-Asmari *et al.*, 2015), and antimicrobial activities (Walter *et al.*, 2011; Abd-Rani *et al.*, 2018). In addition, *M.oleifera* leaf and seed extracts (aqueous and ethanolic) were found to demonstrate hypoglycemic, and hypolipidemic potentials in rat models, by reducing insulin resistance and increasing insulin sensitivity (Tuorkey, 2016); as well as inhibiting the formation of advanced glycated end products (Nunthanawanich *et al.*, 2016). Crude enzymatic digests of *M. oleifera* seed proteins demonstrated enzyme-inhibitory activities in earlier studies (Olusola *et al.*, 2018, Olusola and Ekun, 2019b). However, hydrolysate fractionation is essential to further characterize bioactive peptides (Awosika and Aluko, 2019). As a result, this study aims to evaluate the activities of fractionated peptides obtained from pepsin-assisted hydrolysis of *M. oleifera* seed protein on hemoglobin glycation and carbohydrate hydrolyzing enzymes, in a bid to identify novel peptides with antidiabetic potentials.

MATERIALS AND METHODS

Materials

Collection of Seeds

M. oleifera seeds were obtained from farms in Akungba Akoko, Ondo State and they were identified, and voucher samples were deposited at the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba Akoko.

Chemicals and Reagents

Pepsin (from porcine gastric mucosa), and α -amylase (fungal), α -glucosidase (human) were products of Sigma-Aldrich laboratories, Co-Artrim, United Kingdom. All other chemicals and reagents used were of analytical grade and were also products of Sigma-Aldrich laboratories, United Kingdom.

Methods

Isolation of *M. oleifera* Seed Proteins

The seeds were dried and pulverized before being kept in an air-tight container at 4°C. This was subsequently defatted using n-hexane as was previously described by Arise *et al.*, (2016^a) with slight modifications. The meal was extracted three times with n-hexane using a meal/solvent ratio of 1:10 (w/v). 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). Defatted watermelon seed meal was suspended in 0.1 M NaOH pH 12.0 at a ratio of 1:10, and stirred for one hour to facilitate alkaline solubilization. This was centrifuged at 18°C and 3000 g for 10 min. Two additional extractions of the residue from the centrifugation process were performed with the same volume of 0.1 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 0.1 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 prior to further analysis.

Preparation of *M. oleifera* Seed Protein Hydrolysates

The protein isolate was hydrolyzed using the methods described by Olusola and Ekun, (2019^b) with slight modifications. Hydrolysis was carried out using pepsin (pH 2.2, 37°C). The protein isolate (5% w/v, based on the protein content of the isolate) was dissolved in glycine buffer at pH 2.2. 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 6 hours with continuous stirring. The enzyme was then inactivated by boiling in a 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 6000 g for 30 min. The supernatant containing target peptides was then collected. Protein content of samples was determined using the 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 according to the method described by Ekun *et al.*, (2022). Summarily, 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 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. 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 (vitamin B12, tryptophan, aspartame, glycine, and bovine serum albumin). 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.

Determination of Peptide Yield

The percentage peptide yield was determined using the method described by Girgih *et al.* (2011). The peptide yields (%) of *Moringa oleifera* seed protein hydrolysates and fractions, were calculated as the ratio of peptide content of lyophilized hydrolysate/fraction to the protein content of unhydrolysed protein isolate.

Inhibition of Hemoglobin Glycation

This was investigated by estimating the degree of non-enzymatic 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. 1 ml of each of the above solutions was mixed. 0.2 mg/ml - 1.0 mg/ml of hydrolysate was added to the above mixture. Gallic acid was used as standard. The mixture was kept in the dark at room temperature for incubation for 72 hours. At 520 nm, hemoglobin glycation was measured with a spectrophotometer, and % inhibition was calculated thus:

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

Determination of α -amylase Inhibition

An α -amylase-inhibitory assay was performed according to the method reported by Oboh *et al.* (2011). Briefly,

125 μ L of hydrolysate (0.5 to 2.0 mg mL⁻¹) was placed in test tubes and 125 μ L 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 125 μ L of 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9, with 6 mM NaCl) was added at regular intervals. The reaction mixtures were incubated at 25 °C for 10 min. The reaction was terminated by adding 250 μ L of 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 2.5 mL distilled water and the absorbance was measured at 540 nm. A control was also prepared using the same procedure except that the hydrolysate was replaced with distilled water. The α -amylase-inhibitory activity was calculated as shown:

$$\% \text{ Inhibition} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

Determination of Kinetic Parameters of α -amylase Inhibition

The kinetic study of α -amylase inhibition was conducted according to the method described by Olusola and Ekun (2019^a). 125 μ L of the hydrolysate was pre-incubated with 125 μ L of α -amylase solution for 10 min at 25°C in a set of tubes. In another set of tubes, 250 μ L of phosphate buffer (pH 6.9) was also pre-incubated with 125 μ L of α -amylase solution. Starch solution (125 μ L) of increasing concentrations (1.0 to 8.0 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 then boiled for 5 min after the addition of 250 μ L of 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:

$$\begin{aligned} \text{Specific Activity } ((\mu\text{mol mg protein}^{-1}) \text{ min}^{-1}) \\ = \text{Maltose released} / \text{Incubation time} \\ \times \text{ME} \end{aligned}$$

where maltose concentration is in μ mol/mL⁻¹; Incubation time = 10 min; ME= amount of enzyme (in mg) in the 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 (K_m , K'_m , V_{max} , V'_{max} , CE, and CE') of α -amylase inhibition by hydrolysates were determined by analysis of the double reciprocal plot. The inhibition constant (K_i) was determined using a secondary plot known as the Dixon plot (Palmer, 2007), by plotting a graph of the inverse of initial velocities on the x-axis

against inhibitor concentrations on the x-axis, at a fixed concentration of substrate.

Determination of α -Glucosidase Inhibition

The effect of the hydrolysates on α -glucosidase activity was determined according to the method described by Kim *et al.*, (2005) using α -glucosidase from *Saccharomyces cerevisiae*. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, and pH 6.9. 100 μ L of α -glucosidase (1.0 U/mL) was pre-incubated with 50 μ L of the different concentrations of the hydrolysates for 10 min. Then 50 μ L of 3.0 mM (pNPG) as a substrate dissolved in 20 mM phosphate buffer (pH 6.9) was added to start the reaction. The reaction mixture was incubated at 37°C for 20 min and stopped by adding 2 mL of 0.1 M Na₂CO₃ solution. The α -glucosidase activity was determined by measuring the yellow-colored para-nitrophenol released from p-NPG at 405 nm. The results were expressed as a percentage of the blank control. Percentage inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

Determination of Kinetics of α -Glucosidase Inhibition

The kinetic parameters of α -glucosidase by the hydrolysates were determined according to the method reported by Ali *et al.*, (2006). Briefly, 50 μ L of the (5 mg/mL) hydrolysate was pre-incubated with 100 μ L of α -glucosidase solution for 10 min at 25°C in one set of tubes. In another set of tubes, α -glucosidase was pre-incubated with 50 μ L of phosphate buffer (pH 6.9). 50 μ L of pNPG at increasing concentrations (0.63–2.0 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25°C, and 500 μ L of Na₂CO₃ solution was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically at 405 nm using a para nitrophenol standard curve and converted to reaction velocities. 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 of the hydrolysates on α -glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis Menten kinetics. The inhibition constant (K_i) was also determined using the Dixon plot

(Palmer, 2007), by plotting a graph of the inverse of initial velocities on the x-axis against inhibitor concentrations on the x-axis, at a fixed concentration of substrate.

RESULTS

Peptide Yield

The peptide yields of the hydrolysate and its fractions are illustrated in Table 1. The unfractionated hydrolysate had the highest yield. However, among the fractions, the 1-3 kD fraction had significantly higher yields ($p < 0.05$) when compared to the other peptide fractions.

Table 1. Peptide Yields of *M. oleifera* Seed Protein Hydrolysate and its Fractions.

Sample	Yield
Unfractionated Hydrolysate	80.545 ± 4.949 ^a
Fraction 1 (<1 kD)	19.709 ± 1.096 ^c
Fraction 2 (1-3 kD)	25.835 ± 2.166 ^b
Fraction 3 (3-5 kD)	14.485 ± 0.968 ^d

Values are presented as means ± standard error of the mean of triplicate determinations. Yields carrying different symbols are significantly different ($p < 0.05$). Yields carrying the same letter or symbol are not significantly different ($p < 0.05$).

Inhibition of Hemoglobin Glycation

The effects of *Moringa oleifera* seed protein hydrolysates and fractions on non-enzymatic hemoglobin glycation, in comparison to gallic acid, at a concentration range of 0.2 mg/ml to 1.00 mg/ml is illustrated in Figure 1. The hydrolysate and fractions demonstrated significantly lower ($p < 0.05$) inhibitory effects when compared to gallic acid (control), especially at higher concentrations. The unfractionated peptic hydrolysate had higher ($p < 0.05$) inhibitory activities than its fractions, attaining a maximal value of 78.230 ± 0.774 % at 1.0 mg/ml. Considering the peptic hydrolysate fractions, Fraction F1 had a maximum inhibition of 66.667 ± 2.138 % at 0.8 mg/ml which was not significantly different ($p < 0.05$) from that of fraction F2, with activity of 62.667 ± 3.079 at a concentration of 0.2 mg/ml, but was higher ($p < 0.05$) than fraction F3 which had 58.730 ± 1.833% inhibition at a final concentration of 1.0 mg/ml.

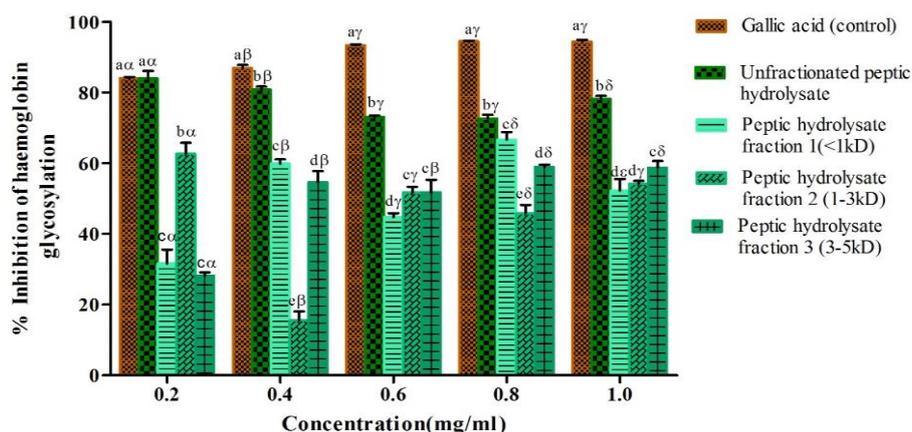


Figure 1. Percentage Inhibition of Hemoglobin Glycation by Peptic *Moringa oleifera* Seed Protein Hydrolysate and its Fractions.

Bars are expressed as means \pm standard error of the 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 ($p<0.05$).

Inhibition of α -amylase activity

The percentage α -amylase inhibitory activity of *Moringa oleifera* seed protein hydrolysate and their fractions in

relation to arabinose are illustrated in Figure 2. The inhibitory activities of peptic hydrolysates and fractions at all study concentrations were significantly ($p<0.05$) lower than those of arabinose. All hydrolysate fractions demonstrated higher ($p<0.05$) inhibitory activity than the unfractionated hydrolysate. Also, the fractions demonstrated percentage inhibitory activity above 50% at concentrations of 0.40 mg/ml to 1.0 mg/ml. Fractions 1 and 2 with 66.8% and 64.59% inhibition respectively at a maximum concentration of 1.0 mg/ml had higher ($p<0.05$) effects than the unfractionated hydrolysate and fraction 3.

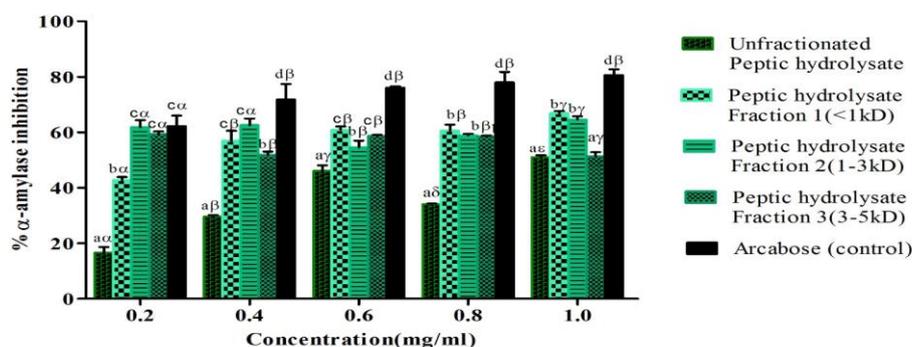


Figure 2. Percentage α -amylase Inhibitory Activity of Peptic *Moringa oleifera* Seed Protein Hydrolysates.

Bars are expressed as means \pm standard error of the 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 ($p<0.05$).

Kinetics of α -amylase Inhibition

The effect of a selected peptide fraction – fractions F1, (<1 kD), – on the catalytic activity of α -amylase in converting starch to maltose is presented in Figure 3.

Kinetic parameters determined from the Lineweaver-Burk plot in the absence and presence of two different concentrations of the peptide fraction were summarized in Table 2. In the absence of the hydrolysate fraction, the Michaelis constant, K_m , of α -amylase for its substrate was found to be 0.552 mg/ml of starch while maximal velocity, V_{max} was 3.890 mM/mg/min. Inhibition of α -amylase activity increased with increasing concentrations of the peptide fraction, such that the K_m of the enzyme was increased while V_{max} and catalytic efficiency, CE, of α -amylase were reduced in the presence of the inhibitory fraction. The enzyme-inhibitor dissociation constant, K_i ,

of α -amylase inhibition by peptic hydrolysate fraction F1 was determined to be 0.029 mg/ml.

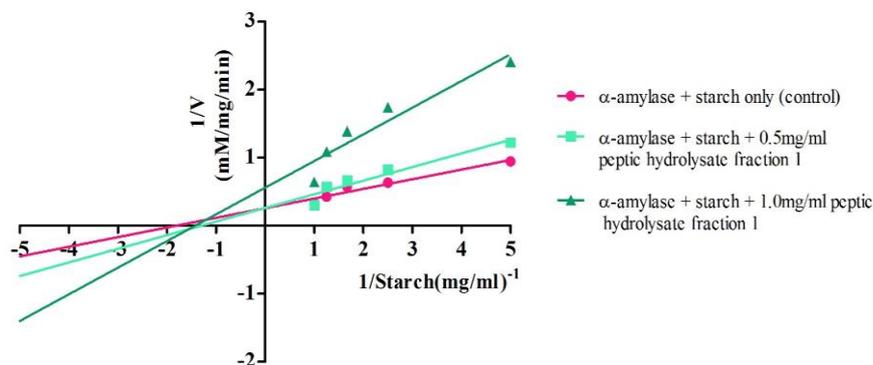


Figure 3. Lineweaver-Burk plot of α -amylase inhibition by *Moringa oleifera* seed protein hydrolysate fraction 1 (<1kD) obtained from peptic proteolysis.

Table 2. Kinetics of α -Amylase-catalyzed Reactions in the Presence and Absence of *M. oleifera* Seed Protein Hydrolysate Fraction 1.

Kinetic parameters	No inhibitor	Peptic hydrolysate fraction 1 (mg/ml)	
		0.5	1.0
k_m or k'_m (mg/ml)	0.552	0.768	0.701
V_{max} or V'_{max} (mM/mg/min)	3.890	3.833	1.789
CE (mmol/ml/min)	7.053	4.993	2.551
K_i (mg/ml)	-	0.029	

K_m or K'_m : Michaelis constant in the absence or presence of inhibitory hydrolysate fractions; V_{max} or V'_{max} : Maximum velocity in the absence or presence of inhibitory hydrolysate fractions; CE: Catalytic Efficiency; K_i : Enzyme-Inhibitor dissociation constant.

α -Glucosidase Inhibitory Activity

The inhibitory activities of the *M. oleifera* seed protein hydrolysates and their fractions on α -glucosidase – catalyzed hydrolysis of p-nitrophenyl glucopyranoside at varying concentrations in comparison to arabinose (control) are presented in Figure 5. Peptic hydrolysate and its fractions displayed lower ($p < 0.05$) inhibitory activities when compared to the standard, arcabose. However, peptic hydrolysate fraction 1 (<1 kD) attained

maximum inhibitory activity of 46.09 ± 3.331 % at a concentration of 0.6mg/ml, while fraction 2, PF2, attained a maximum inhibitory extent of 59.449 ± 2.342 % at a concentration of 0.8 mg/ml. Fraction 3 had significantly lower ($p < 0.05$) α -glucosidase inhibitory activity when compared to the unfractionated peptic hydrolysate and the other low molecular weight fractions obtained from peptic digestion.

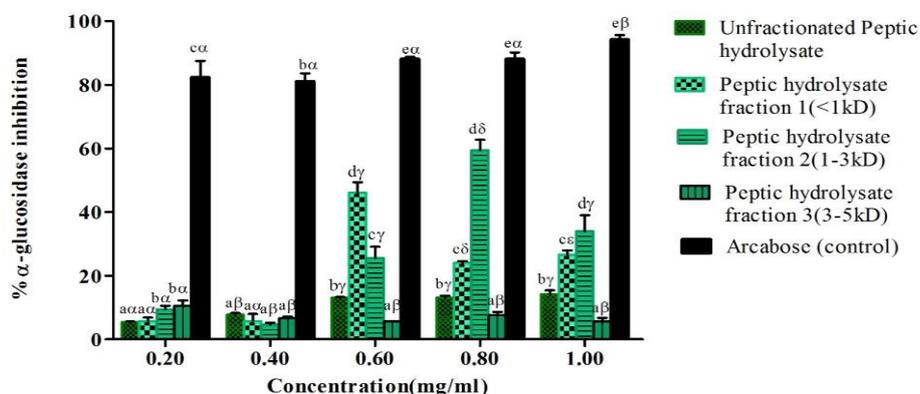


Figure 4. Percentage α -glucosidase Inhibitory Activity of Peptic *Moringa oleifera* Seed Protein Hydrolysates.

Bars are expressed as means \pm standard error of the 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 ($p < 0.05$).

Kinetics of α -glucosidase Inhibition

The effect of a selected *M. oleifera* seed protein hydrolysate fraction (fraction F1, (<1 kD) on the kinetics of α glucosidase – catalyzed hydrolysis of p-nitrophenyl glucopyranoside, p-NPG, to p-nitrophenol are illustrated in Figure 5. The kinetic parameters from the resulting Lineweaver Burk plots are summarized in Table 3. In the

absence of an inhibitor, the Michaelis constant, K_m of α -glucosidase for its substrate was determined to be 0.297 mg/mL p-NPG, while maximum velocity, V_{max} , was 270.27 mM/mg/min. The hydrolysate fraction also caused reductions in the V_{max} and catalytic efficiency, CE of the enzyme at concentrations of 0.5 mg/mL and 1.0 mg/mL.

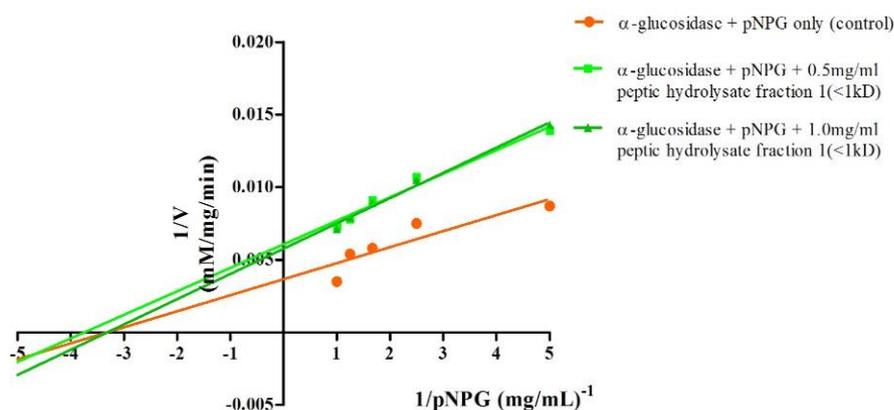


Figure 5. Lineweaver-Burk plot of α -glucosidase inhibition by *Moringa oleifera* seed protein hydrolysate fraction 1 (< 1kD) obtained from peptic proteolysis.

Table 3. Kinetic Parameters of α -Glucosidase Inhibition by *M. oleifera* Seed Protein Hydrolysate Fraction 1.

Kinetic parameters	No inhibitor	Peptic hydrolysate fraction 1 (mg/ml)	
		0.5	1.0
k_m or k'_m (mg/ml)	0.297	0.262	0.293
V_{max} or V'_{max} (mM/mg/min)	270.270	163.934	172.414
CE (mmol/ml/min)	910.001	625.702	588.443
K_i (mg/ml)	-	0.333	

K_m or K'_m : Michaelis constant in the absence or presence of inhibitory hydrolysate fractions; V_{max} or V'_{max} : Maximum velocity in the absence or presence of inhibitory hydrolysate fractions; CE: Catalytic Efficiency; K_i : Enzyme-Inhibitor dissociation constant.

DISCUSSION

Peptide Yield

Peptide yield gives an estimate of the amount, in percentage, of peptides generated relative to the whole protein subjected to enzymatic proteolysis; thus it represents an important index in determining the efficiency of the overall process (Alashi *et al.*, 2014), as these enzymes degrade the proteins into several peptides of varying lengths and sizes. It, therefore, follows that a high peptide yield is indicative of increased proteolysis and resultant peptide release (Girgih *et al.*, 2011). The peptide yield of the unfractionated hydrolysates was higher than those of their corresponding fractions put together, and this could be due to peptide loss during the process of chromatography and the removal of peptides whose molecular weights were higher than 5 kDa. This is consistent with the reports of Awosika and Aluko (2019) in their work with yellowfield pea protein digests and peptide fractions. The yield of peptic hydrolysates obtained in this study was higher than $32.33 \pm 1.046\%$

and $68.90 \pm 1.00\%$ determined for peptic digests of *Arachis hypogea* (Olusola and Ekun, 2018) and watermelon seed proteins (Arise *et al.*, 2016^b) respectively. This implies that *M.oleifera* seed proteins also had a lot of hydrophobic amino acid residues, making them susceptible to cleavage by pepsin, as this enzyme, though relatively non-specific but has a preference for hydrolyzing peptide linkages at the C-terminal ends of hydrophobic aminoacyl residues (Mune-Mune *et al.*, 2016; Voet *et al.*, 2016).

Inhibition of Hemoglobin Glycation

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. *M. oleifera* seed protein digests and their fractions exhibited lower inhibitory effects on hemoglobin glycation than gallic acid. However, 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, peptic fractions 1 and 2, exhibited inhibitory effects above 60%. Pepsin is relatively non-specific in its cleavage specificities (Naik, 2012), releasing peptides that have hydrophobic and aromatic side chains that could significantly slow down the glycation of hemoglobin. This is consistent with the reports of Han *et al.*, (2014), that Asn-Trp dipeptides inhibited the formation of AGEs in mice models. However, more investigation is required in further studies to determine the nature of other 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.

α -Amylase Inhibitory Activity and Kinetics of Inhibition

The enzyme α -amylase in mammals is an important component of both saliva and pancreatic juice, and it catalyzes the hydrolysis of α -(1-4) glycosidic bonds of polysaccharides, releasing glucose and maltose in the process (Voet *et al.*, 2016). In recent times, protein hydrolysate preparations and peptides from some plant and animal sources have been demonstrated to inhibit α -amylase activity, with potential implications for alternate therapies to the management of diabetes mellitus (Arise *et al.*, 2016^b, Olusola and Ekun, 2018, Awosika and Aluko, 2019). In this study, all hydrolysates and peptide fractions demonstrated lower α -amylase inhibitory activities when compared to arabinose, and this is not unexpected because arabinose is a synthetic inhibitor of α -amylase. Also, all peptide fractions exhibited better α -amylase inhibition than the unfractionated hydrolysates, and this could be that the fractionation process improves bioactivity, by allowing for more bioactive peptides to gain access to the enzyme active site, causing inhibition of the enzyme. This is also consistent with the reports of Malomo and Aluko, (2016) that unfractionated

hydrolysates do contain large molecular weight peptides that possess antagonistic effects to enzyme inhibition.

The Lineweaver-Burk plot was used to determine the mode of α -amylase inhibition by varying concentrations of selected peptide fractions in this study. In addition, the kinetic parameters determined from the double-reciprocal plots were summarized in Table 2, and it showed that the Michaelis constant, k_m of α -amylase (from *Saccharomyces cerevisiae*) in the absence of inhibitory hydrolysates is 0.552 mg/ml of starch, which is lower than 1.4 mg/ml (Acharya *et al.*, 2014) for α -amylase obtained from *Aspergillus oryzae*. The presence of increasing amounts of the hydrolysate fractions increased the apparent k_m of the enzyme for its substrate, while also reducing both of maximal velocity, V_{max} , and catalytic efficiency, CE, of α -amylase. The peptide fraction exhibited a mixed type of inhibition and this suggests that these peptides are capable of binding α -amylase in both its free form and in its starch-bound forms, creating dead-end complexes on both occasions. Arise *et al.*, (2016^b) reported a mixed type of inhibition of α -amylase for peptic, tryptic, and alcalde hydrolysates of *Citrullus lanatus* seed protein hydrolysates.

Inhibition of α -Glucosidase Activity and Kinetics of Inhibition

The enzyme α -glucosidase commonly resides on the brush border membranes of the intestinal mucosa and it is involved in carbohydrate digestion by hydrolyzing glucose residues from oligosaccharides (Voet *et al.*, 2016). Thus, the modulation of the activity of this enzyme represents one of the key strategies in the control of blood glucose levels in the management of diabetes mellitus (Qaisar *et al.*, 2014). The hydrolysates and their fractions demonstrated lower α -glucosidase inhibitory activities than the control (acarbose). This is because acarbose happens to be a dual inhibitor of α -amylase and α -glucosidase, with a higher binding affinity for α -glucosidase (Katzung *et al.*, 2012). The fractionation of peptic digests proved to improve the bioactivities of its fractions, with fractions F1 and F2 also demonstrating inhibitory effects, with maximal activity obtained at 0.6 mg/ml and 0.8 mg/mL respectively for the fractions, but these were higher than those of fraction 3. This also suggests that lower molecular weight peptides demonstrate better α -glucosidase inhibitory activities. However, at higher concentrations, these peptides could have antagonistic effects on one another, which could in turn, result in reduced inhibition of α -glucosidase activity (Awosika and Aluko, 2019). In contrast to the kinetics of α -amylase inhibition, there is limited data available in the literature regarding the kinetic analysis of α -glucosidase inhibition by protein hydrolysate fractions. The kinetic parameters obtained from the double-reciprocal plots of α -glucosidase inhibition by selected peptide fractions of *M. oleifera* seed proteins in Figure 5 were summarized in Table 3. The Michaelis constant, k_m , of α -glucosidase for

p-nitrophenyl glucopyranoside in the absence of inhibitor determined to be 0.297 mg/ml p-NPG in this study was slightly higher than 0.211 mg/ml (0.7mM) p-NPG obtained by Awosika and Aluko (2019) but lower than 6.31mg/ml reported by Arise *et al.* (2019). V_{max} , in the absence of inhibitory hydrolysates, was 270.27 mM/mg/ml. Peptic hydrolysate fraction 1 comprising peptides of molecular weight less than 1 kD demonstrated an uncompetitive inhibition mechanism, and this indicates that these peptides largely bind to other sites different from the substrate binding site on α -glucosidase. The enzyme-inhibitor binding constant, K_i value of 0.333 mg/mL obtained for peptic hydrolysate fraction 1 in this study was slightly higher than 0.305 mg/mL determined for unfractionated peptic hydrolysates of *M. oleifera* seed protein in another study (Olusola and Ekun, 2019). In addition, fraction 1 demonstrated a lesser binding affinity for α -glucosidase when compared with its binding to α -amylase. This could indicate that these peptides could perform better as α -amylase inhibitors.

CONCLUSION

The proteolysis of *M. oleifera* seed proteins using pepsin and their subsequent fractionation yielded peptide fractions that slowed the process of hemoglobin glycation and also inhibited carbohydrate-hydrolyzing enzymes *in vitro*. Fraction F1 containing the smallest-sized peptides demonstrated the strongest inhibitory activity against hemoglobin glycation and carbohydrates. Thus, peptide products from *M. oleifera* seed proteins possess bioactivities that could be harnessed for the development of novel anti-diabetic agents.

Competing Interests: The author declare that there are no competing interests.

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