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Peptide Fractions from Chymotrypsin-hydrolyzed *Moringa oleifera* Seed Proteins Inhibit α-amylase and α-glucosidase *in vitro*

Oluwafemi Emmanuel Ekun*, Augustine Olusegun Olusola, Joseph Adaviruku Sanni, Feyisayo Ishola
Department of Biochemistry, Faculty of Science, Adekunle Ajasin University, Akungba Akoko, Ondo State, Nigeria.

Corresponding author*
oluwafemi.ekun@aaua.edu.ng

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Abstract

This study attempted to investigate the activities of chymotrypsin-digested *Moringa oleifera* seed proteins and their peptide fractions on carbohydrate-hydrolyzing enzymes**.** Proteins from *M. oleifera* seeds were isolated using isoelectric point precipitation and hydrolyzed using chymotrypsin. The hydrolysates obtained were fractionated into peptide fractions of <1 kD, 1-3 kD and 3-5 kD ranges by means of gel-filtration chromatography. The inhibitory effects of the hydrolysates and their fractions on α-amylase and α-glucosidase were evaluated, and kinetics of inhibition were also determined. Using starch and p-nitrophenyl glucopyranoside as substrates, the hydrolysate and fractions demonstrated concentration-dependent inhibition of α-amylase and α-glucosidase respectively (IC_{50} of 0.172 ± 0.005 mg mL^{-1} to 1.312 ± 0.267 mg mL^{-1}, for α-amylase inhibition and IC_{50} of 0.463 ± 0.008 mg mL^{-1} to 0.696 ± 0.051 mg mL^{-1} for α-glucosidase inhibition). Kinetic analysis revealed that selected hydrolysate fractions competitively inhibited α-amylase while displaying a mixed mode of inhibition of α-glucosidase. This study suggests that subjecting *M. oleifera* seed proteins to proteolysis could yield therapeutic peptide products having immense potentials that could be harnessed to develop novel anti-diabetic agents and additives to food, which could serve as cost effective alternatives to current therapies.

Keywords: *Moringa oleifera*; hydrolysate; peptide; chymotrypsin; α-amylase; α-glucosidase.

INTRODUCTION

Bioactive peptides and protein hydrolysate preparations obtained either by enzymatic hydrolysis or microbial fermentation of plant and animal proteins have been investigated for their therapeutic capacities and other health promoting benefits (Lopez-Barrios *et al*., 2014, Ulagesan *et al*., 2018). Peptide products have been reported to demonstrate numerous bioactivities against hypertension (Yamada *et al*., 2013; Majumder and Wu 2015), cancer (Vileghe *et al*., 2010; Thundimadathil 2012) oxidative stress (Olusola *et al*., 2018), pathogenic microorganisms (Ulagesan *et al*., 2018) and recently, diabetes mellitus (Olusola and Ekun 2019, Famuwagun *et al*., 2020), in various *in vitro* assays and/or animal models.

Diabetes mellitus is a metabolic disorder that is caused by an absolute or relative insulin deficiency (Rhoades and Bell 2013), and it is the fourth leading cause of health problem globally (IDF 2020). It is characterized by chronic hyperglycemia associated with derangements in the regulation of carbohydrate, fat and protein metabolism (Olusola and Ekun 2019). This results in the appearance of metabolites such as ketone bodies and advanced glycated end products in the blood, and these in turn cause oxidative tissue and organ damage, ketoacidosis among other complications at later stages of the disease (Arise *et al*., 2016). Carbohydrate – hydrolyzing enzymes (such as α-amylase and α-glucosidase) and incretin degrading enzymes (such as dipeptidyl peptidase IV) have been key pharmacologic targets for many hypoglycemic drugs (Arise *et al*., 2019) and as such have been used in the management of diabetes mellitus, in addition to lifestyle changes. However, owing to certain adverse effects caused by several of these drugs (Yu *et al*., 2012), in addition to high cost of procurement especially in third world countries, attention has turned to alternatives from natural sources, and this has included peptide products from proteins in seeds and leaves of plants.

Moringa oleifera is a fast growing, drought – resistant and perennial plant, belonging to the genus *moringaceae* (Anwar *et al*., 2007). It is widely known as the "horseradish" family and native to India, especially in the Himalayan regions. It is now known to be cultivated in tropical and subtropical areas such as in tropical Africa and in South West Asia, where its young seed pods and leaves are used as vegetables as well as in herbal medicine (Leone *et al*., 2015; Abd-Rani *et al*., 2018). *M. oleifera* is a plant whose leaves and seeds have been excellent sources of essential oils and other nutrients (Texeira *et al*., 2014). Texeira *et al*. (2014)

found that whole *Moringa* leaf flour contained 28.7% crude protein, 7.1% fat, 10.9% ash, 44.4% carbohydrates, in addition to 3.0 mg/100g of calcium and 103.1 mg/100g iron. Also, another study reported that proximate analysis of its seeds indicated that the percentage nutrient composition of its protein, lipid, ash, fiber, and carbohydrate were about 35.5, 29.3, 4.6, 11.5, and 19.6% respectively (Kwaambwa *et al*., 2015; Mune-Mune *et al*., 2016). Proteins in *M. oleifera* leaves and seeds consist of albumin, prolamins, globulins and glutelins (Mune-Mune *et al*., 2016) which is mostly the case with many oil seeds (Wani *et al*., 2011). Its relatively high protein content makes it an excellent source of potential biologically active peptides (Olusola *et al*., 2018). Furthermore, Freire *et al*., (2015) and Mune-Mune *et al*., (2016) reported that *M. oleifera* leaves and seeds are especially rich in glycine, isoleucine, glutamate, aspartate, leucine, arginine, proline, threonine among other amino acids. Parts of the plant (roots, leaves, stem bark and seeds) have been used for nutritional purposes and as traditional medicine (Leone *et al*., 2015; Abd-Rami *et al*., 2018). Various parts of *M. oleifera* have been demonstrated to possess a myriad of bioactivities such as purgative, antimicrobial as well as normoglycemic effects (Siddhuraju and Beck, 2003; Divi *et al*., 2012). Its stem bark has been determined to have anti-proliferative, anti-ulcerative, as well as anti-inflammatory properties (Mahajan *et al*., 2009). The presence of certain polyphenols as well as other bioactive secondary metabolites in *M. oleifera* leaf extracts were reported to exert antihypertensive (Ndong *et al*., 2007), hypolipidemic and hypoglycemic effects (Anwar *et al*., 2007). Recently, crude enzymatic hydrolysates of *M. oleifera* seed proteins have been reported to possess enzyme-inhibitory activities *in vitro* (Olusola *et al*., 2018, Olusola and Ekun 2019). However, hydrolysate fractionation is essential in reducing peptide aggregation, liberating peptides in solution, which in turn leads to increased peptide bioactivities in the process (Awosika and Aluko 2019). Therefore, this study aims to examine the carbohydrase – inhibitory activities of peptide fractions obtained from chymoytrpsin – digested *M. oleifera* seed proteins in order to harness them as possible sources of novel antidiabetic peptides, and to further justify the value added uses of *M. oleifera* seed proteins.

MATERIALS AND METHODS

Materials

Collection of M. oleifera Seeds

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

Chemicals and Reagents

Chymotrypsin (from bovine pancreas), 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, Co-Artrim, United Kingdom.

Methods

Isolation of M. oleifera Seed Proteins

The seeds were dried and pulverized before being kept in an air-tight container at 4ºC. This was subsequently defatted using n-hexane as was previously described by Arise *et al*., (2016) 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 40ºC in a vacuum oven and ground again to obtain a fine powder, termed defatted seed meal, which was stored at -20ºC. The protein component of the defatted meal was extracted using the method described by Alashi *et al*., (2014). 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 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 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) and Olusola and Ekun (2019[b]) with slight modifications. The conditions for hydrolysis was tailored for each enzyme in order to optimize its activity. Hydrolysis was carried out using chymotrypsin (pH 8.0, 37ºC). The protein isolate was dissolved in 0.2M phosphate buffer, pH 8.0. 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 then inactivated by boiling in water bath (95–100ºC) for 15 minutes 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 minutes. 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 Boyer (2012) and Tounkara *et al*., (2014) with some modifications. 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 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.

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 Olusola *et al*. (2018). One ml of protein hydrolysate was added to 1 ml of 20% TCA to produce 10% TCA soluble material. The mixtures was left to stand for 30 minutes to allow for precipitation, followed by centrifugation at 4000 g for 20 minutes. The supernatants were then analyzed for protein content using Biuret assay method with bovine serum albumin (BSA) as standard. The degree of hydrolysis (DH) was then calculated as the ratio of soluble peptide in 10% trichloroacetic acid (in milligrams) to the total protein content of isolate (in milligrams), expressed in percentage.

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.

Determination of α-amylase Inhibition
An α-amylase-inhibitory assay was performed according to the method reported by Oboh *et al*. (2011). Briefly, 125 µL of hydrolysate (0.2 to 1.0 mg mL^{-1}) was placed in test tubes and 125 µL of 20 mM sodium phosphate buffer (pH 6.9, with 6mM NaCl) containing α-amylase solution (0.5 mg mL^{-1}) added. The content of each tube was pre-incubated at 25°C for 10 min, after which 125 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 were incubated at 25°C for 10 min. The reaction was terminated by adding 250 µL 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 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:

$$\%\ Inhibition = (Acontrol - Asample) / Acontrol \times 100$$

The concentration of hydrolysate resulting in 50% inhibition of enzyme activity (IC_{50}) was determined from a plot of percentage inhibition against 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 method described by Olusola and Ekun (2019[a]). 125 µL of the hydrolysate was pre-incubated with 125 µL of α-amylase solution for 10 min at 25°C in a set of tubes. In another set of tubes, 250 µL of phosphate buffer (pH 6.9) was also pre-incubated with 125 µL of α-amylase solution. Starch solution (125 µL) of increasing concentrations (1.0 to 8.0 mg mL^{-1}) were added to both sets of reaction mixtures to initiate the reaction. The mixture were then incubated for 10 min at 25 °C, and then boiled for 5 min after the addition of 250 µL 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:

$$Specific\ Activity\ (\mu mol\ mg\ protein - 1)\ min - 1) = Maltose\ released\ /\ Incubation\ time \times ME.$$

where:
maltose concentration is in µmol mL^{-1};
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 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 and Bonner, 2007), by plotting a graph of inverse of initial

velocities on the x-axis against inhibitor concentrations on the x-axis, at 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. 100μL of α glucosidase (1.0 U/mL) was pre-incubated with 50μL of the different concentrations of the hydrolysates for 10 min. Then 50μL 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 2mL of 0.1 M Na_2CO_3 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 percentage of the blank control. Percentage inhibition was calculated as:

$$\%\, Inhibition = (Acontrol - Asample) / Acontrol \times 100$$

Determination of Kinetic Parameters of α-Glucosidase Inhibition

The kinetic parameters of α-glucosidase by the hydrolysates was determined according to the modified method described by Ali *et al*. (2006). Briefly, 50 μL of the (5 mg mL^{-1}) hydrolysate was pre-incubated with 100 μL 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 50μL of phosphate buffer (pH 6.9). 50 μL of pNPG at increasing concentrations (0.5–2.0 mg mL^{-1}) 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 μL of Na_2CO_3 solution was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically at 405nm using a paranitrophenol 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 and Bonner, 2007), by plotting a graph of inverse of initial velocities on the x-axis against inhibitor concentrations on the x-axis, at fixed concentration of substrate.

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

Peptide Yield and Degree of Hydrolysis

The enzyme chymotrypsin was used to hydrolyze the protein isolate and the degree of hydrolysis was found to be 51.180 ± 2.461 %. The peptide yields of the hydrolysate and its fractions are displayed in Table 1.

Table 1. Peptide Yield of *M. oleifera* seed proteins hydrolyzed by chymotrypsin and fractionated using gel filtration chromatography.

Sample	Peptide Yield
Unfractionated Hydrolysate	87.571 ± 2.342^{a}
Fraction 1 (<1 kD)	12.791 ± 0.535^{c}
Fraction 2 (1-3 kD)	13.548 ± 1.070^{bc}
Fraction 3 (3-5 kD)	13.656 ± 0.076^{b}

α-Amylase Inhibitory Activity

The percentage α-amylase inhibitory activity of the hydrolysate and its fractions in relation to the control, acarbose, was illustrated in Figure 1 and their IC_{50} values were displayed in Figure 2. This result showed that, at lower concentrations, the hydrolysate fractions demonstrated α-amylase inhibitory effects comparable to the control. However, the unfractionated chymotrypsin hydrolysate demonstrated a concentration-dependent reduction in activity, attaining a value of 15.487 ± 1.125 % at a final concentration of 1.0 mg mL^{-1}, which is significantly lower ($p<0.05$) than those of the hydrolysate fractions. Chymotrypsin hydrolysate fractions displayed percentage inhibitions above 50% at all study concentrations, such that the fractions F1, F2 and F3 reached 61.859 ± 2.490 %, 76.440 ± 1.220% and 67.195 ± 0.528 % inhibition respectively at a final concentration of 1.00 mg mL^{-1}. Among the chymotrypsin hydrolysate fractions, fraction F2, had the highest ($p<0.05$) inhibitory activity. The hydrolysate and its peptide fractions F1 – F3, inhibited α-amylase to a 50% extent at concentrations of 1.312 ± 0.267 mg mL^{-1}, 0.172 ± 0.005 mg mL^{-1}, 0.892 ± 0.038 mg mL^{-1} and 0.526 ± 0.093 mg mL^{-1} respectively.

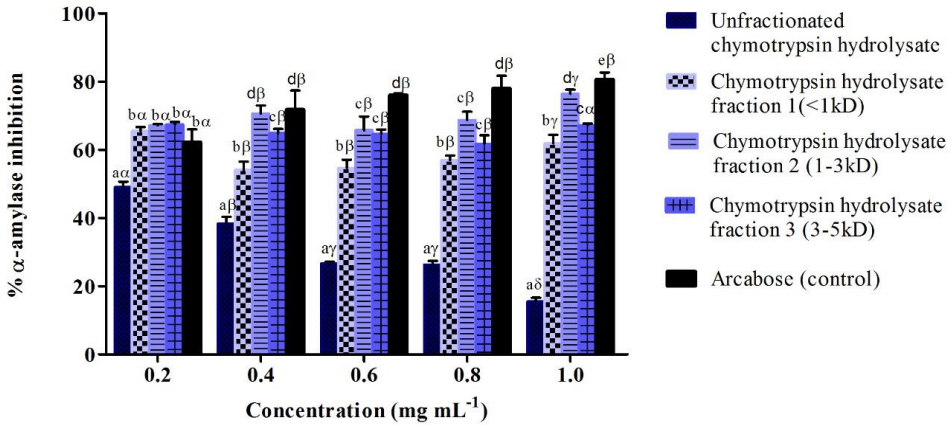

Figure 1. Percentage α-amylase Inhibition by *Moringa oleifera* Seed Protein Hydrolysates and their Fractions Obtained by Chymotrypsin Digestion.

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 ($p<0.05$).

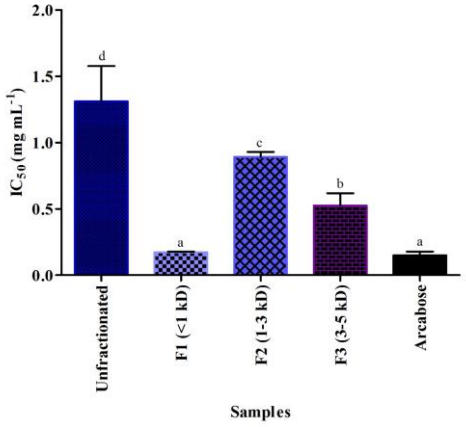

Figure 2. Values of 50% Inhibition of α-amylase Activity by Chymotrypsin-derived *M. oleifera* Seed Protein Hydrolysate and its Fractions.

Bars are expressed as means ± standard error of means of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Kinetics of α-amylase Inhibition

The effect of a selected peptide fraction (fraction 2) on the catalytic activity of α-amylase in converting starch to maltose was presented in Figure 3. Kinetic parameters determined from Lineweaver-Burk plots in the absence and presence of two different concentrations of the hydrolysate fraction were summarized in Table 2. In the absence of the hydrolysate fractions, the Michaelis constant, K_m, of α-amylase for its substrate was found to be 0.552 mg $mL^{-1}$of starch while maximal velocity, V_{max} was 3.890 mM mg^{-1} min^{-1}. 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 peptide fractions. The peptide fraction demonstrated competitive inhibition at both 0.5 mg mL^{-1} and 1.0 mg mL^{-1}.

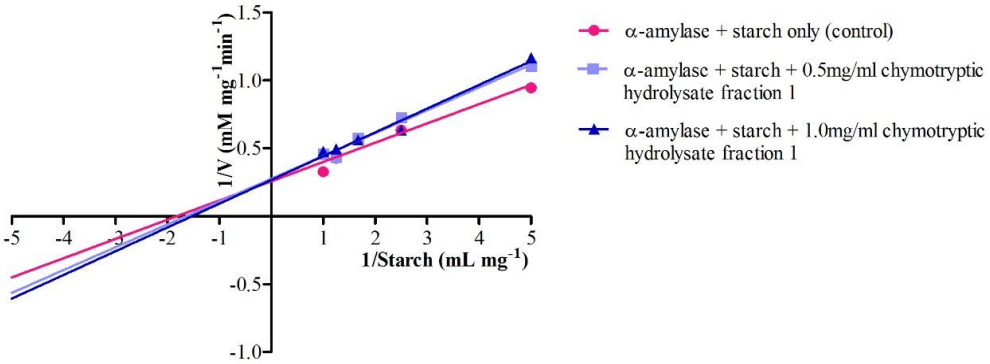

Figure 3. Lineweaver-Burk plot of α-Amylase Inhibition by *Moringa oleifera* Seed Protein Hydrolysate Fraction 2 (1-3 kD) Obtained from Chymotrypsin Hydrolysis

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

Kinetic parameters	No inhibitor	Chymotrypsin hydrolysate fraction 2 (mg mL^{-1})	
		0.5	1.0
K_m or K'_m (mg mL^{-1})	0.552	0.610	0.659
V_{max} or V'_{max} (mM mg^{-1} min^{-1})	3.890	3.632	3.750
CE (mmol mL^{-1} min^{-1})	7.053	5.953	5.717
K_i (mg/ml)	-	0.735	

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

α-Glucosidase Inhibitory Activity

The inhibitory activities of the *M. oleifera* seed protein hydrolysate and its fractions on α-glucosidase – catalyzed hydrolysis of p-nitrophenyl glucopyranoside at varying concentrations in comparison to acarbose (control) are presented in Figure 4. Their IC_{50} values were also depicted in Figure 5. *M. oleifera* seed protein hydrolysate and its fractions, as digested by chymotrypsin, also displayed increased inhibition of α-glucosidase with increasing concentration (Figure 4). However, these were significantly lower ($p<0.05$) when compared to control at all study concentrations. The unfractionated hydrolysate and its gel-filtration fractions F1, F2 and F3 attained maximal inhibitory activities of 38.723 ± 1.508 %, 73.077 ± 1.110 %, 83.474 ± 2.691% and 85.282 ± 1.295% respectively at a final concentration of 1.0 mg/ml. Chymotryptic hydrolysate fraction F2 had significantly higher ($p<0.05$) activity than other fractions at 0.4 mg mL^{-1}, 0.6 mg mL^{-1} and 0.8 mg mL^{-1}, but there was no significant ($p<0.05$) difference in its activity when compared to fraction F3 at 1.0 mg mL^{-1}. In addition, chymotrypsin hydrolysate and its fractions F1, F2 and F3 inhibited α-glucosidase to a 50% extent at concentrations of 0.509 ± 0.025 mg mL^{-1}, 0.651 ± 0.025 mg mL^{-1}, 0.463 ± 0.008 mg mL^{-1}, and 0.696 ± 0.051 mg mL^{-1} respectively. These IC_{50} values were higher ($p<0.05$) than those of acarbose. Chymotrypsin hydrolysate fraction 2 had the lowest ($p<0.05$) IC_{50} value among the hydrolysates and fractions derived from chymotrypsin proteolysis.

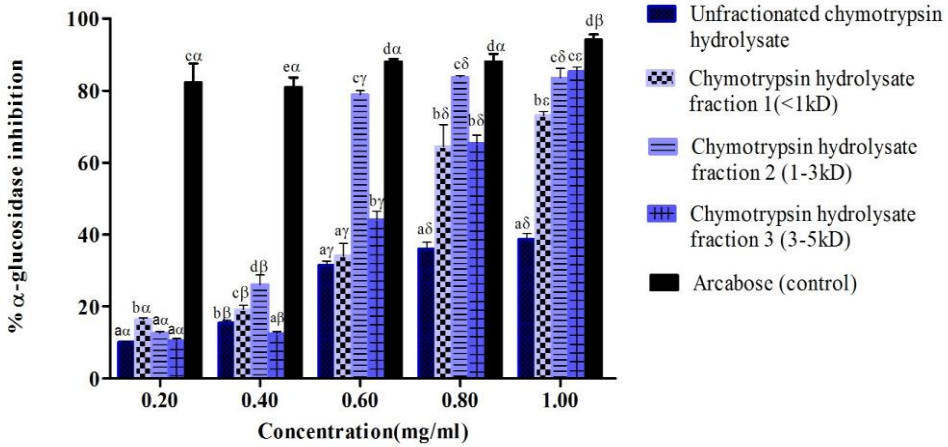

Figure 4. Percentage α-Glucosidase Inhibition by *Moringa oleifera* Seed Protein Hydrolysates and their Fractions Obtained by Chymotrypsin Digestion.

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 ($p<0.05$).

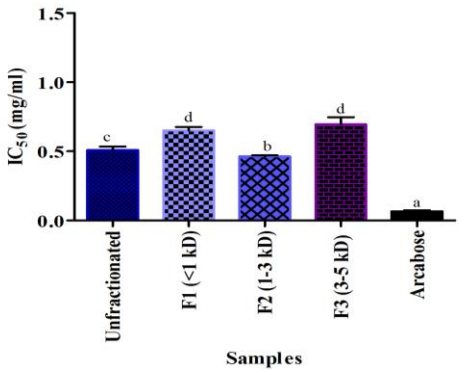

Figure 5. IC_{50} Values of α-Glucosidase Activity by Chymotrypsin-derived *M. oleifera* Seed Protein Hydrolysate and its Fractions.

Bars are expressed as means ± standard error of means of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Kinetics of α-Glucosidase Inhibition

The effects of a selected *M. oleifera* seed protein hydrolysate fraction (fraction 2) on the kinetics of α glucosidase–catalyzed hydrolysis of p-nitrophenyl glucopyranoside, p-NPG, to p-nitrophenol were illustrated in Figure 6, while the kinetic parameters from the resulting Line-weaver Burk plot were summarized in Table 3. In the absence of inhibitory hydrolysates, the Michaelis constant, K_m of α- glucosidase for its substrate was determined to be 0.297 mg mL^{-1} p-NPG, while maximum velocity, V_{max}, was 270.27 mM mg^{-1} min^{-1}. All hydrolysate fractions caused decreases in the V_{max} and catalytic efficiency, CE of the enzyme.

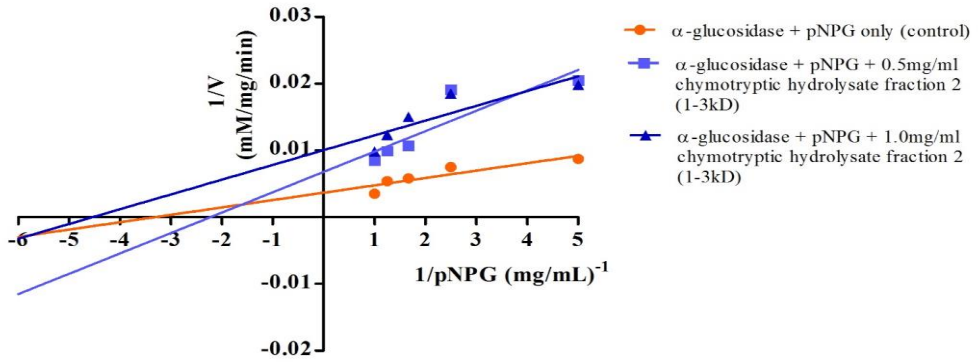

Figure 6. Lineweaver-Burk Plot of α-Glucosidase Inhibition by *Moringa oleifera* Seed Protein Hydrolysate Fraction 2 (1-3 kD) Obtained from Chymotrypsin Digestion.

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

Kinetic parameters	No inhibitor	Chymotrypsin hydrolysate fraction 2 (mg mL^{-1})	
		0.5	1.0
K_m or K'_m (mg mL^{-1})	0.297	0.456	0.220
V_{max} or V'_{max} (mM mg^{-1} min^{-1})	270.270	147.059	100.000
CE (mmol ml^{-1} min^{-1})	910.001	322.498	454.545
K_i (mg mL^{-1})	-	0.136	

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

DISCUSSION

Degree of Hydrolysis and Peptide Yield

The degree of hydrolysis (DH) estimates the amounts of cleaved peptide bonds in a protein hydrolysate. DH can affect the molecular sizes and amino acid compositions of the peptides and consequently influence the biological activities of the peptides formed during hydrolysis (Olusola *et al*., 2018). The degree of chymotrypsin hydrolysis of *M. oleifera* seed proteins was 51.59 ± 3.81 % which was higher than 38.66% gotten for obtained for whey protein hydrolysates at an E/S ratio of 1:100 but slightly lesser than 57.34% obtained for the same whey hydrolysate at an E/S ratio of 2:100 (Galvao *et al*., 2001). Also, it was higher than the DH value of 11.2 ± 1.4% determined for chymotrypsin digests of buffalo casein at an E/S ratio of 1:100 (Shanmugam *et al*. 2015). The observed differences could be due to different protein sources, which invariably leads to varying number of sites susceptible to chymotrypsin hydrolysis, as the enzyme is known to preferentially hydrolyze the C-terminal peptide bonds of aromatic aminoacyl residues (Voet *et al*., 2016). In addition, it appears that an increased enzyme/substrate ratio is required to improve the degree of hydrolysis as regards proteolysis by chymotrypsin. Other factors such as hydrolysis time and pH of the buffer medium could affect enzymatic activity and by extension, the degree of hydrolysis.

Peptide yield measures the amount, in percentage, of peptides generated relative to the whole protein subjected to enzymatic proteolysis; thus representing 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 (Girgih *et al*., 2011). The peptide yield obtained by chymotrypsin digests was higher than the 48.18 ± 0.89% reported for chymotrypsin hydrolysates of yellow field proteins (Awosika and Aluko, 2019). This could be as a result of a relative abundance of

aromatic aminoacyl residues in *M. oleifera* seed proteins (Mune-Mune *et al.*, 2016) which provides more cleavage points for enzymatic hydrolysis. 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 chromatographic sepaprtion 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 hydrolysates and peptide fractions.

α-Amylase Inhibitory Activity and Kinetics

The enzyme α-amylase, a digestive carbohydrase, catalyzes the hydrolysis of complex polysaccharides in the mammalian gut. Thus, the inhibition of α-amylase activity is a key pharmacologic intervention in the management of type 2 diabetes mellitus (Olusola and Ekun, 2019a). In this study the unfractionated hydrolysates showed lower α-amylase inhibitory activities when compared to acarbose, and this is because acarbose is a synthetic inhibitor of α-amylase. In addition, the peptide fractions demonstrated better inhibitory activities than the unfractionated hydrolysate, and this is in consonance with the reports of Awosika and Aluko (2019) that hydrolysate fractionation improves peptide bioactivity, such that more peptides are able to access substrate binding sites on the enzyme. In the same vein, Malomo and Aluko, (2016) reported that unfractionated hydrolysates contained high molecular weight peptides which could act antagonistically to smaller peptides, thus reducing inhibitory activity. Among the peptide fractions, the chymotrypsin hydrolysate fraction F2 (1-3 kD) demonstrated the highest inhibitory activities at a maximum concentration of 1.0 mg mL^{-1}. This could be a direct result of the nature of the peptides released by these enzymes during proteolysis. It is known that chymotrypsin as an endoprotease hydrolyzes peptide linkages from C-terminal residues of phenylalanine, tyrosine and tryptophan. Reports from previous studies stated that phenylalanine, leucine, proline and glycine residues are required for the inhibition of α-amylase (Yu *et al*. 2012, Garza *et al*. 2017). In the same vein, Arise *et al*. (2016) also suggested that α-amylase binds to peptides containing aromatic residues such as Phe, Tyr and Trp. Thus, these peptides obtained from enzymatic proteolysis could contain these specific amino acid residues that locks into sites on the enzyme, inhibiting its activity in the process. The Lineweaver-Burk plot was used to determine the mode of α-amylase inhibition by varying two concentrations of a selected peptide fraction, F2. Also, the kinetic parameters determined from the double-reciprocal plots were summarized in Table 2; suggesting that the Michaelis constant, k_m of α-amylase (from *Saccharomyces cerevisiae*) in the absence of inhibitory hydrolysates is 0.552 mg mL^{-1} of starch, which is lower than 1.4 mg mL^{-1} (Acharya *et al*., 2014) for α-amylases obtained from *Aspergillus oryzae*. The selected peptide fraction in this study exhibited a competitive type of inhibition at both study concentrations. This suggests that these peptides are capable of binding α-amylase in its free form, at the same substrate-binding site for starch, creating a dead-end complex as a result. This is in contrast to the findings of Arise *et al*. (2016) that reported a mixed type of inhibition of α-amylase for peptic, tyrptic and alcalase hydrolysates of *Citrullus lanatus* seed protein hydrolysates, but literature has been scarce for the α-amylase – inhibitory activity of peptides obtained by chymotrypsin hydrolysis. However, The K_i value obtained suggest that these peptides bind α-amylase with lesser affinity when compared to enzyme – substrate binding.

α-Glucosidase Inhibitory Activity and Kinetics

The enzyme α-glucosidase occurs mostly as a membrane-bound enzyme on the brush border membranes of the ileum, and digests carbohydrates by hydrolyzing glucose residues from a number of oligosaccharides. Thus, modulating α-glucosidase activity represents another important strategies in the control of blood glucose levels in the management of diabetes mellitus (Qaisar *et al.* 2014). Chymotrypsin hydrolysate fractions displayed a concentration-dependent increase in α-glucosidase inhibitory activities, such that fractions F2 and F3 showed better inhibitory effects. This is consistent with the reports of Awosika and Aluko (2019) who reported that chymotrypsin hydrolysate fractions of yellow field pea protein hydrolysates inhibited α-glucosidase. It therefore follows that the process of fractionation may have helped liberate these bioactive peptides so that they could bind to and inhibit α-glucosidase. In addition, since chymotrypsin cleaves peptides after aromatic residues (Voet *et al.* 2016) and that a tyrosinyl residue is a likely requirement for α-glucosidase inhibitory activity (Ibrahim *et al*., 2018), it is therefore suggested that the presence of a one or more well positioned aromatic side chains may have contributed positively to the inhibition of α-glucosidase activity. The kinetic parameters obtained from the double - reciprocal plots of α-glucosidase inhibition by selected peptide fractions of *M. oleifera* seed proteins in Figure 6 were summarized in Table 3. The Michaelis constant, k_m, of α-glucosidase for p-nitrophenyl glucopyranoside in the absence of inhibitor was determined to be 0.297 mg mL^{-1} p-NPG in this study. This is slightly higher than 0.211 mg mL^{-1} (0.7mM) p-NPG obtained by Awosika and Aluko (2019) but lower than 6.31 mg mL^{-1} reported by Arise *et al.* (2019). V_{max}, in the absence of inhibitory hydrolysates was 270.27mM $mg^{-1}mL^{-1}$. Chymotrypsin hydrolysate fraction F2 demonstrated a mixed mode of inhibition at 0.5 mg mL^{-1} while at 1.0 mg mL^{-1} the inhibition

mechanism shifted to an uncompetitive subtype of mixed inhibition. This is in contrast to what was obtained with the <1 kD fraction of chymotryptic digests of yellowfield pea protein hydrolysate (Awosika and Aluko 2019) as they inhibited α-glucosidase via a non-competitive inhibition mechanism. This suggests that peptide concentration could have significant effects on mechanism of enzyme inhibition and this could be evaluated in further studies. The enzyme-inhibitor binding constant, K_i, revealed that the peptide fraction possessed stronger binding affinity for α-glucosidase than for α-amylase. Ibrahim and others (2018) stated that aminoacyl residues such as tyrosine are needed at terminal ends of a peptide for α-glucosidase inhibitory activity. Chymotrypsin is a protease that could cleave and release peptides with aromatic C-terminal ends (Voet *et al.*, 2016), and this might explain the α-glucosidase inhibitory activities observed by these peptide products.

CONCLUSION

This study concludes that peptide fractions obtained from chymotrypsin digests of *M. oleifera* seed proteins elicited antidiabetic potentials by inhibiting carbohydrate hydrolyzing enzymes – α-amylase and α-glucosidase – by differing mechanisms *in vitro*. In addition, Fraction 2 containing peptide sizes of 1-3 kD recorded the highest activity against the carbohydrases. Further studies such as structural identification, modifications, and chemical synthesis of peptides responsible for the observed bioactivities are being embarked upon, and are currently in progress.

***Conflict of interest*:** The authors declare no conflicts of interest.

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