RESEARCH ARTICLE

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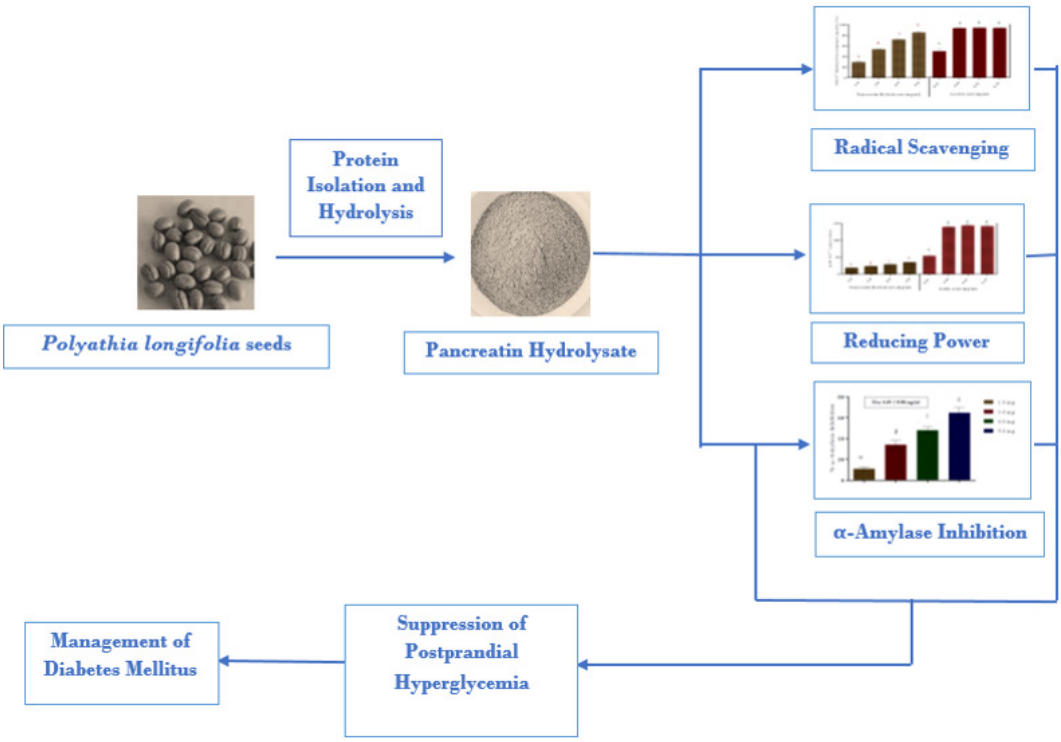


## Highlights

- Pancreatin hydrolysate derived from *Polyalthia longifolia* seed protein has considerable α-amylase inhibitory property.
- Pancreatin hydrolysate demonstrated significant free radical scavenging and reducing potentials.
- Pancreatin hydrolysate can be used to manage postprandial hyperglycemia associated with diabetes mellitus via the inhibition of α-amylase activity and attenuation of free radical production.

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# *In vitro* α-amylase inhibitory and antioxidant properties of pancreatin-derived *Polyalthia longifolia* seed protein hydrolysate

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**Abstract:** Inhibition of α-amylase and free radical scavenging activities are two basic approaches utilized in the management of postprandial hyperglycemia. In recent times, researchers have explored the α-amylase inhibitory and free radical scavenging potentials of plant-derived peptides to circumvent the side effects of synthetic drugs. In this study, proteins from *Polyalthia longifolia (P. longifolia)* were isolated using alkaline solubilisation-acid precipitation method and the proteins were enzymatically hydrolyzed with pancreatin. The hydrolysate derived was investigated for α-amylase inhibitory property, using starch as the substrate; and antioxidant property, using 2, 20-azinobis-3-ethylbenzothiazoline-6-sulphonate cation radical ($ABTS^{+}$) scavenging and ferric ion reducing antioxidant power (FRAP) assays. The hydrolysate demonstrated a concentration-dependent α-amylase inhibitory potential ($IC_{50}$ 6.49 ± 0.08 mg/ml) exhibiting 64.40 ± 5.58% α-amylase inhibition at the highest concentration considered (9 mg/ml). Kinetics analyses revealed that the hydrolysate exhibited uncompetitive inhibition pattern on α-amylase at 9 mg/ml. The hydrolysate exhibited considerable radical scavenging activity (85.11 ± 2.05%) and reducing ability (35.44 ± 0.13 mM $Fe^{2+}$ equivalent) at 9 mg/ml. In conclusion, the study suggests that the hydrolysate possesses the ability to manage postprandial hyperglycemia associated with diabetes mellitus via inhibition of α-amylase activity and attenuation of free radical production.

***Keywords*:** α-amylase; inhibitory properties; antioxidant; *P. longifolia*; hydrolysate.

## INTRODUCTION

Postprandial hyperglycemia and oxidative stress are two inseparable disorders associated with diabetes mellitus. The acute glucose fluctuations during the postprandial period exhibit a more specific triggering effect on oxidative stress than chronic sustained hyperglycemia, primarily caused by defect in nutrient-related first phase insulin response (Parkin and Brooks, 2002; Sies *et al*., 2005; Shihabudeen *et al*., 2011). Persistently elevated postprandial glucose results in glucose auto-oxidation and protein glycosylation leading to increased production of free radicals and oxidative stress (Lipinski, 2001; Kayama *et al*., 2015).

In the mouth, salivary α-amylase initiates the hydrolysis of starch; and in the small intestine, pancreatic α-amylase completes its conversion into disaccharides and short oligosaccharides (Arise *et al*., 2016). Inhibition of α-amylase is an approach utilized in the management of postprandial hyperglycemia associated with diabetes mellitus. The therapeutic effects of α-amylase inhibitors are elicited by delaying the breakdown of polysaccharides in the mouth and small intestine; thereby, reducing the postprandial blood glucose excursion in diabetic patients. Unfortunately, many commercially available α-amylase inhibitors have been reported to be associated with gastrointestinal side effects such as abdominal pain, flatulence, and diarrhea in diabetic patients (Hanefeld *et al*., 1998). Therefore, the discovery of safer α-amylase inhibitors is of great essence. In order to broaden the management options of diabetes mellitus, it will be interesting to have α-amylase inhibitors with the ability to attenuate the production of free radicals that accompanies postprandial hyperglycemia.

In the light of the above, research efforts have led to the discovery of protein hydrolysates with multifunctional abilities such as α-amylase inhibition and attenuation of free radical generation (Olusola *et al*., 2018; Arise *et al*., 2016; Arise *et al*., 2019; James *et al.,* 2020a, b). These hydrolysates are crude preparations of bioactive peptides that differ in chain length, hydrophobicity, net charge, and activity; and can be separated using known separation techniques (Aluko *et al*., 2018). Nonetheless, peptide separation does not always guarantee increased activity when compared to the original hydrolysate (Aluko *et al*., 2018). Indeed, protein hydrolysates are sometimes more effective than separated peptide fractions (Girgih *et al*., 2011). Interestingly, bioactive peptides contained in hydrolysates hold certain advantages over synthetic drugs. They have the advantage of specificity (limited negative side effects), potency (therapeutic efficiency), and low toxicity such that they can be administered even at high doses (Ishida *et al*., 2011; Thayer, 2011). Therefore, the desire for safer alternatives to commercially available pharmacological agents can be satisfied by bioactive peptides.

One plant that has been implicated in the management of diabetes mellitus is *Polyalthia longifolia*

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*(P. longifolia)* (*Annonaceae*) (Nair *et* al., 2007; Sivashanmugam and Chatterjee, 2013; Anigboro *et al*., 2018). Indeed, this plant has great therapeutic applications in traditional medicine and exerts fascinating antipyretic, anticancer, antimicrobial, anti-inflammatory, hypotensive, antiulcerogenic and antioxidant effects (Katkar *et al*., 2010). Despite the avalanche of biological activities known with the plant parts, very little is credited to the plant seed. Indeed, the plant seed can be termed grossly under-researched and underutilized for biological activities. The moderate protein content of the plant seed (12.40±0.25%) makes it a likely candidate for the isolation of bioactive peptides; and the α-amylase inhibitory and antioxidant properties that have been observed with other parts of the plant makes for a reasonable research effort to be geared towards the plant seed (Sivashanmugam and Chatterjee, 2013; Ajayi and Ifedi, 2016). Therefore, the present study was conducted to investigate the *in vitro* antioxidant and α-amylase inhibitory properties of the hydrolysate obtained from pancreatin digestion of *P. longifolia* seed protein.

## MATERIALS

*P. longifolia* seed was obtained from Tanke area, Ilorin, Kwara State, Nigeria and was authenticated at the Department of Plant Biology Herbarium, University of Ilorin with voucher number: UILH/10/004/892/2021. Analytically graded chemicals and reagents were used without further purification. Pancreatin, α-amylase from *Penicillium fellutanum*, bovine serum albumin, maltose, dinitrosalicylic acid and starch were products of Loba-Chemie Biotech., India and Sigma-Aldrich Chemicals, USA.

## METHODS

### Isolation of *P. longifolia* seed protein

The *P. longifolia* seeds were dried and pulverized before being kept in an air-tight container at 4ºC. The powder was defatted using n-hexane as was previously described by Wani *et al.* (2011). The defatted meal was then de-solventized at room temperature and ground again to obtain a fine powder, termed defatted seed meal, which was then refrigerated until later use. The protein component of the defatted meal was extracted using the method described by Alashi *et al.* (2014) with slight modifications. Defatted *P. longifolia* seed meal was suspended in 0.1 mol $dm^{-3}$ NaOH pH 12.0 at a meal to solvent ratio of 1:10 and stirred for 1 hour to facilitate alkaline solubilisation. This was then centrifuged at 18 °C and 3000 rpm for 10 min. Two additional extractions of the residue from the centrifugation process were carried out with the same volume of 0.1 mol $dm^{-3}$ NaOH and the supernatants were pooled. The pH of the supernatant was adjusted to pH 4.0 to facilitate acid-induced protein precipitation using 1 mol $dm^{-3}$ HCl solution; the precipitate formed was recovered by centrifugation as described above. The precipitate was washed with distilled water, adjusted to pH 7.0 using 1 mol $dm^{-3}$ NaOH, freeze-dried and the protein isolate was refrigerated until required for further analysis. Following isolation, the protein yield was determined as described by Arise *et al.* (2015) using the formula below:

$$\text{Protein Yield \%} = \frac{\text{Mass of protein isolate (g)}}{\text{Mass of defatted } P.\ longifolia \text{ seed meal (g)}} \times 100\ (\%)$$

### Preparation of pancreatin-derived *P. longifolia* seed protein hydrolysate

The protein isolate was hydrolyzed using the methods described by Udenigwe *et al.* (2009) and Onuh *et al.* (2015) with slight modifications. The conditions for hydrolysis were tailored for each enzyme in order to optimize its activity. Hydrolysis was done using pancreatin (pH 8.0, 37 ºC). The protein isolate (5% w/v) was dissolved in phosphate buffer to maintain the pH (6.9–8.0) for maximal hydrolytic condition. The enzyme was added to the slurry at an enzyme-substrate ratio (E: S) of 1:100. Digestions were performed at the specified conditions for 6 hours with continuous stirring before the enzyme was inactivated by immersing the reaction vessel in boiling water (95–100ºC) for 15 min. Undigested proteins were precipitated by adjusting the pH to 4.0 with 2 mol $dm^{-3}$ HCl/2 mol $dm^{-3}$ NaOH followed by centrifugation at 4000 rpm for 30 min. The supernatant containing target peptides was then collected, analyzed for degree of hydrolysis and percentage peptide yield. The supernatant was then refrigerated until further analysis. Protein content of samples was determined using the 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 peptide 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. Five hundred microliters (500 µL) of protein hydrolysate were added to 500 µL of 20% TCA to produce 10% TCA soluble material. The mixtures were left to stand for 30 min to allow precipitation, followed by centrifugation (3500 rpm for 15 min). The supernatant was analyzed for protein content using biuret assay method of Gornall *et al*. (1949) with bovine serum albumin (BSA) as standard. The experiment was performed in triplicates. The degree of hydrolysis (DH) was computed as shown below:

$$\text{Degree Hydrolysis (\%)} = \frac{\text{Soluble peptide in 10\% TCA (mg mL}^{-1}\text{)}}{\text{Total protein content of isolate (mg mL}^{-1}\text{)}} \times 100\ (\%)$$

### Determination of peptide yield

The percentage peptide yield was determined using the method described by Girgih *et al*. (2011). The experiment was performed in triplicates. The peptide yield (%) of the hydrolysate was calculated as the ratio of peptide weight of each hydrolysate to the protein weight of protein isolate as shown:

$$\text{Peptide Yield \%} = \frac{\text{Peptide weight of each hydrolysate (mg mL}^{-1})}{\text{Protein weight of lyophilized isolate (mg mL}^{-1})} \times 100$$

**Determination of α-amylase inhibition**

Alpha-amylase inhibitory assay was carried out according to the method of Bernfield (1951) as reported by Oboh *et al*. (2011). One hundred and twenty-five microliters (125 µL) of hydrolysate (1.0 – 9.0 mg/mL) was placed in test tubes and 125 µL of 20 mmol $dm^{-3}$ sodium phosphate buffer (pH 6.9, with 6 mmol $dm^{-3}$ NaCl) containing α-amylase solution (0.5 mg/mL) (optimum pH for α-amylase from *Penicillium fellutanum* is 6.5 – 7.0) was 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 mmol $dm^{-3}$ sodium phosphate buffer (pH 6.9, with 6 mmol $dm^{-3}$ 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 prepared using the same procedure except that the hydrolysate was replaced with distilled water. The experiment was performed in triplicates. The α-amylase-inhibitory activity was calculated as shown below:

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

The concentration of hydrolysate resulting in 50% inhibition of α-amylase activity ($IC_{50}$) was estimated by the plot of concentrations (x-axis) against percentage inhibition (y-axis) (linear regression) in triplicates using Microsoft Excel Version 2016. From the line of best fit, the intercept (C) and the gradient (M) were obtained.

$$\text{50\% Inhibitory Concentration } (IC_{50}) = \frac{\text{50 - Intercept}}{\text{Gradient}}$$

**Determination of kinetic parameters of α-amylase inhibition**

The kinetic study of α-amylase inhibition by the hydrolysate was conducted according to the modified method of Ali *et al*. (2006). One hundred and twenty-five microliters (125 µL) of the hydrolysate were pre-incubated with 125 µL of α-amylase solution for 10 mins at 25 ºC in one set of tubes. In a second set of tubes, 250 µL of phosphate buffer (pH 6.9) was pre-incubated with 125 µL of α-amylase solution. Starch solution (125 µL) of increasing concentrations (1.0 – 8.0 mg/mL) was added to both sets of reaction mixtures to initiate the reaction. The mixture was then incubated for 10 minutes at 25 ºC, and then boiled for 5 minutes after the addition of 250 µL of DNS reagent to stop reaction. The amount of reducing sugars released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities as shown below.

$$\text{Specific Activity (µmol/ /min)} = \frac{\text{[Maltose released]}}{\text{Incubation Time} \times M_E}$$

ME = Amount (in mg) of enzyme in the reaction mixture

Incubation time: 10 minutes

A double reciprocal plot of 1/ [Starch] (x-axis) against 1/ [Specific Activity] (y-axis) was plotted. The mode of inhibition and the kinetic parameters (*Km* and *Vmax*) of α-amylase inhibition by the hydrolysates were determined by analysis of the double reciprocal plot using Microsoft Excel Version 2016.

*Calculations*

*Vmax* (Maximum Velocity) and *Km* (Michaelis constant) were derived from the double reciprocal plot of 1/ [Starch] (x-axis) against 1/ [Specific Activity] (y-axis) using Microsoft Excel Version 2016. From the line of best fit, the intercept (C) and the gradient (M) were obtained for the presence and absence of hydrolysate.

$$\text{Maximum velocity } (Vmax) = \frac{1}{\text{Intercept}}$$

$$\text{Michaelis Constant } (Km) = Vmax \text{ x Gradient}$$

**2, 20-azinobis-3-ethylbenzothiazoline-6-sulphonate cation radical scavenging capacity**

The effect of the hydrolysate on 2, 20-azinobis-3-ethylbenzothiazoline-6-sulphonate Cation ($ABTS^{+}$) radical was determined by the ABTS cation decolourization assay described by Re *et al*. (1999). ABTS cation radical was produced by the reaction between 7 mmol $dm^{-3}$ ABTS in water and 2.45 mmol $dm^{-3}$ potassium persulfate (1:1), stored in the dark at room temperature for 12-16 h before use. ABTS cation solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. After the addition of 5 µL of the respective concentrations (1 to 9 mg/mL) of the hydrolysate to 4 mL of diluted ABTS cation solution, the absorbance was measured at 30 min after the initial mixing. An appropriate solvent blank was run in each assay. The experiment was performed in triplicates. The capacity of the hydrolysate to scavenge the ABTS cation radical was calculated using the equation below:

$$\text{Percentage Scavenging Activity} = \frac{\text{Absorbance}_{blank} - \text{Absorbance}_{sample}}{\text{Absorbance}_{blank}} \times 100\ (\%)$$

**Ferric ion reducing antioxidant power**

The reducing capacity of the hydrolysate on ferric ion was determined according to the method described by Benzie and Strain (1996). The ferric ion reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mmol $dm^{-3}$

acetate buffer, 10 mL TPTZ in 40 mmol $dm^{-3}$ HCl and 20 mmol $dm^{-3}$ $FeCl_3.6H_2O$ in the proportion of 10:1:1 at 37 °C. Freshly prepared working FRAP reagent was pipetted (3.995 mL) and mixed with 5 µL of the respective concentrations (1 to 9 mg/mL) of the hydrolysate. An intense blue color complex was formed when ferric tripyridyl triazine ($Fe^{3+}$ TPTZ) complex is reduced to ferrous ($Fe^{2+}$) form and the absorbance at 593 nm was recorded against a reagent blank after 30 min incubation at 37 °C. The calibration curve was prepared by plotting the absorbance at 593 nm versus different concentrations of $FeSO_4$. The experiment was performed in triplicates. The concentrations of $FeSO_4$ produced by the standard (gallic acid) and hydrolysates were then extrapolated from the calibration curve and expressed in mM $Fe^{2+}$ equivalent.

### Statistical analysis

Data were expressed as mean of three replicates ± standard deviation (SD). The data were subjected to analysis of variance and Turkey's multiple range tests using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

## RESULTS

### Protein yield of sample and pancreatin hydrolytic parameters

The percentage protein yield, degree of hydrolysis and peptide yield are shown in Table 1. Following isolation, the percentage protein yield was 2.23%. The percentage degree of hydrolysis and peptide yield obtained from pancreatin hydrolytic process were 8.77 ± 0.08% and 54.97 ± 0.36% respectively.

### Percentage α-amylase-inhibitory effect of pancreatin-derived *P. longifolia* seed protein hydrolysate

The percentage α-amylase inhibition and 50% α-amylase inhibitory concentration ($IC_{50}$) of *P. longifolia* pancreatin-derived seed protein hydrolysate are shown in Figure 1. The α-amylase inhibition of *P. longifolia* pancreatin-derived seed protein hydrolysate was significantly different ($p< 0.05$) at all the concentrations considered. The highest inhibitory activity was observed at 9.0 mg/ml with 64.40 ± 5.58%; while the least was observed at 1.0 mg/mL with 10.90 ± 2.14%. The inhibitory activities of other concentrations of the hydrolysate (3.0 mg/mL and 6 mg/mL) were 34.06 ± 4.03% and 47.83 ± 3.36% respectively. From this analysis, it is crystal clear that the hydrolysate exhibited a concentration-dependent inhibition of α-amylase. The 50% α-amylase inhibitory concentration ($IC_{50}$) was observed to be 6.49 ± 0.08 mg/mL and is shown as an in-text in Figure 1.

### Kinetics of α-amylase inhibition by pancreatin-derived *P. longifolia* seed protein hydrolysate

The effects of *P. longifolia* Pancreatin-derived Seed Protein Hydrolysate on the kinetic parameters of α-amylase catalyzed degradation of starch to maltose is illustrated in figure 2. Table 2 highlights the kinetic parameters derived from figure 2. Uninhibited α-amylase activity resulted in a higher $V_{max}$ and $K_m$ (72.46 µmol/mg/min and 34.68 mg/mL respectively), than the $V_{max}$ and $K_m$ (19.38 µmol/mg/min and 9.66 mg/mL respectively) of the *P. longifolia* pancreatin-derived seed protein hydrolysate inhibited α-amylase activity at 9 mg/mL.

**Table 1:** Protein yield and pancreatin hydrolytic parameters.

| | Pancreatin Hydrolytic Parameters | |
|---|---|---|
| **Protein Yield of Isolate (%)** | **Degree of Hydrolysis (%)** | **Peptide Yield (%)** |
| 2.23 | 8.77 ± 0.08 | 54.97 ± 0.36 |

*Values represent mean of triplicate determinations ± standard deviation (SD).*

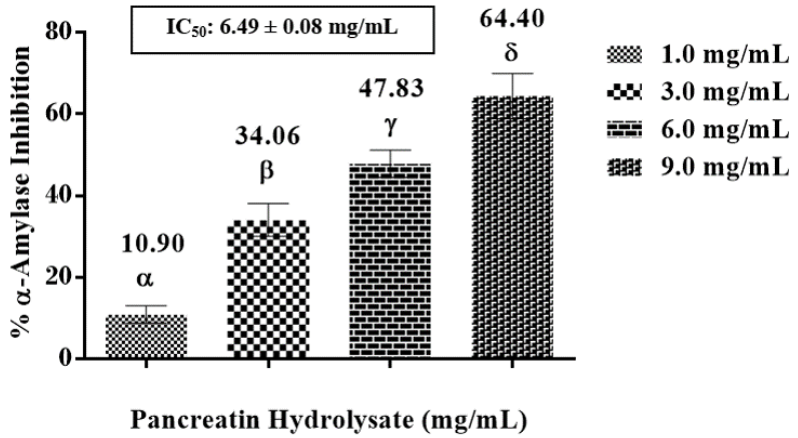


**Figure 1:** α-Amylase Inhibitory Activity of Pancreatin-derived *Polyalthia longifolia* Seed Protein Hydrolysate.

*Each bar represents mean of triplicate determinations ± SD. Bars at the different concentrations with different Greek letters (αβγδ)* are sign*ificantly different at p < 0.05.*

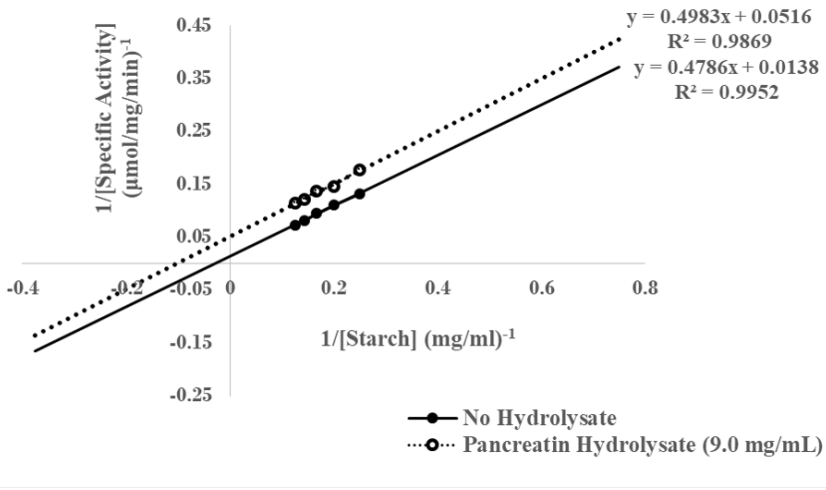


**Figure 2:** Lineweaver-Burk Plot of α-Amylase Inhibition by Pancreatin-derived *Polyalthia longifolia* Seed Protein Hydrolysate.

**Table 2:** Kinetic Parameters of α-Amylase-Catalyzed Degradation of Starch in the Absence (Control) and Presence of Pancreatin-derived *Polyalthia longifolia* Seed Protein Hydrolysate.

| Catalytic Parameter | Control (No Hydrolysate) | Pancreatin Hydrolysate (9.0 mg/mL) |
|---|---|---|
| $V_{max}$ (µmol/mg/min) | 72.46 | 19.38 |
| $K_m$ (mg/mL) | 34.68 | 9.66 |

$K_m$: Michaelis Constant in the presence and absence of hydrolysate; $V_{max}$: Maximum reaction rate in the presence and absence of hydrolysate.

**$ABTS^+$ radical scavenging capacity of pancreatin-derived *P. longifolia* seed protein hydrolysate**

The $ABTS^+$ radical scavenging capacities of ascorbate and *P. longifolia* pancreatin-derived seed protein hydrolysate are shown in figure 3. The $ABTS^+$ radical scavenging capacity of ascorbate was higher ($p < 0.05$) than the hydrolysate at the concentrations considered (0.01 to 0.10 mg/mL and 1.0 to 9.0 mg/mL respectively). The highest $ABTS^+$ radical scavenging capacity of ascorbate was observed at 0.10 mg/mL with 94.47 ± 0.13%, while the least was observed at 0.01 mg/mL with 49.65 ± 4.77%. The $ABTS^+$ radical scavenging capacities of other concentrations of ascorbate, 0.02 mg/mL, and 0.05 mg/mL, were observed to be 93.21 ± 0.35% and 93.64 ± 0.20% respectively. Clearly, from the analysis of the $ABTS^+$ radical scavenging capacity of ascorbate above, there was no significant difference ($p < 0.05$) between 0.02 mg/mL to 0.10 mg/mL, whereas, there was a significant difference ($p < 0.05$) between 0.01mg/mL and other concentrations considered. Therefore, the $ABTS^+$ radical scavenging capacity of ascorbate was not concentration dependent, it flattened out from 0.02 mg/mL to 0.10 mg/mL. In the case of the hydrolysate, the highest $ABTS^+$ radical scavenging capacity was observed at 9.0 mg/mL with 85.11 ± 2.05%, while the least was observed at 1.0 mg/mL with 29.36 ± 0.98%. The $ABTS^+$ radical scavenging capacities of other concentrations of the hydrolysate, 3.0 mg/mL and 6.0 mg/mL, were observed to be 53.35 ± 1.36% and 71.95 ± 1.17% respectively. From the analysis of the $ABTS^+$ radical scavenging capacity of the hydrolysate, it is evident that there was a concentration dependent radical scavenging capacity exhibited by the hydrolysate.

**Ferric ion reducing antioxidant power of pancreatin-derived *P. longifolia* seed protein hydrolysate**

The ferric ion-reducing abilities of gallic acid and *P. longifolia* pancreatin-derived seed protein hydrolysate are shown in figure 4. The ferric ion reducing antioxidant power of gallic acid was higher ($p < 0.05$) than that of the hydrolysate at the concentrations considered (0.01 to 0.10 mg/mL and 1.0 to 9.0 mg/mL respectively). The highest ferric ion reducing antioxidant power of gallic acid was observed at 0.10 mg/mL with 141.43 ± 3.16 mmol $dm^{-3}$ $Fe^{2+}$ equivalent, while the least was observed at 0.01 mg/mL with 54.11 ± 5.20 mmol $dm^{-3}$ $Fe^{2+}$ equivalent. The ferric ion reducing antioxidant power of other concentrations of gallic acid, 0.02 mg/mL, and 0.05 mg/mL, were observed to be 139.62 ± 2.12 mmol $dm^{-3}$ $Fe^{2+}$ equivalent and 143.48 ± 1.55 m mol $dm^{-3}$ $Fe^{2+}$ equivalent respectively. Clearly, in like manner to the $ABTS^+$ radical scavenging capacity of ascorbate, there was no significant difference ($p < 0.05$) between 0.02 mg/mL to 0.10 mg/mL of the ferric ion reducing antioxidant power of gallic acid, whereas there was a significant difference ($p < 0.05$) between 0.01 mg/mL and other concentrations considered of the ferric ion reducing antioxidant power of gallic acid. In the case of the hydrolysate, the highest ferric ion reducing antioxidant power was observed at 9.0 mg/mL with 35.44 ± 0.13 mmol $dm^{-3}$ $Fe^{2+}$ equivalent, while the least

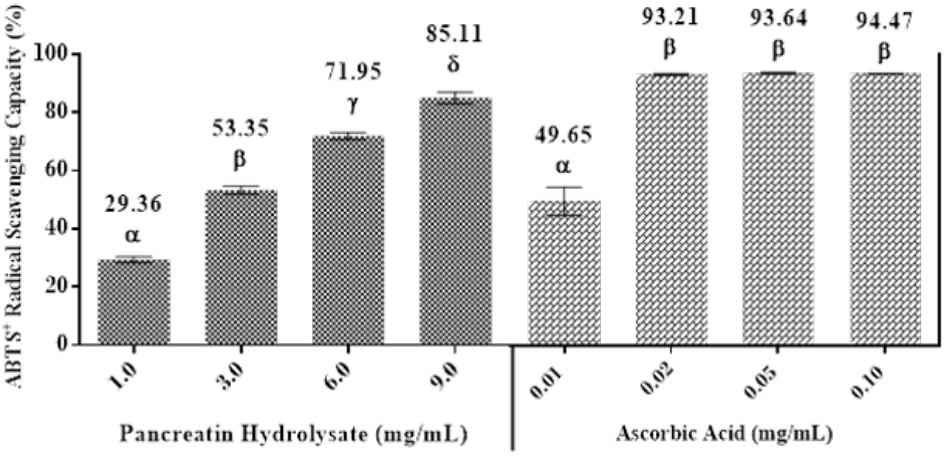


**Figure 3:** ABTS$^+$ Radical-scavenging Activity of Ascorbic Acid and Pancreatin-derived *Polyalthia longifolia* Seed Protein Hydrolysate. *Each bar represents mean of triplicate determinations ± SD. Bars at the different concentrations with different Greek letters (αβγδ)* are significantly different at $p < 0.05$.

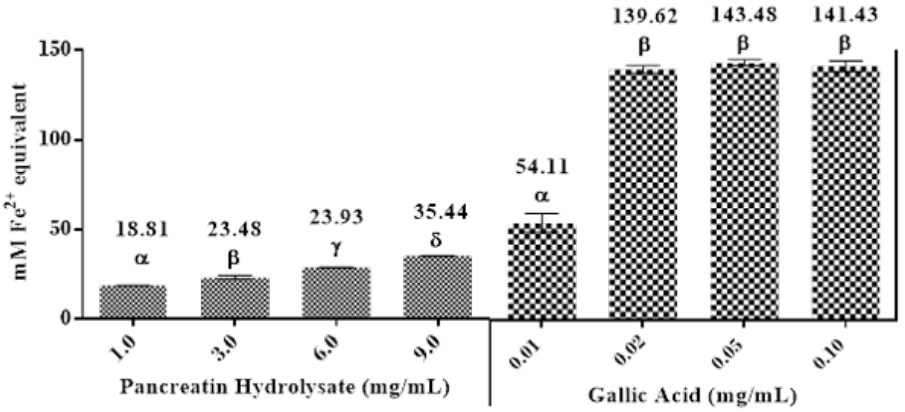


**Figure 4:** Ferric-reducing Antioxidant Power of Gallic Acid and Pancreatin-derived *Polyalthia longifolia* Seed Protein Hydrolysate. *Each bar represents mean of triplicate determinations ± SD. Bars at the different concentrations with different Greek letters (αβγδ)* are significantly different at $p < 0.05$.

was observed at 1.0 mg/mL with 18.81 ± 0.11 mmol dm$^{-3}$ $Fe^{2+}$ equivalent. The ferric ion reducing antioxidant power of other concentrations of the hydrolysate, 3.0 mg/mL and 6.0 mg/mL, were observed to be 23.48 ± 0.93 mmol dm$^{-3}$ $Fe^{2+}$ equivalent and 28.93 ± 0.20 mmol dm$^{-3}$ $Fe^{2+}$ equivalent respectively. Again, as observed with the analysis of the ABTS$^+$ radical scavenging capacity of the hydrolysate, there was a concentration dependent ferric reducing antioxidant power exhibited by the hydrolysate.

## DISCUSSION

### Protein yield of sample and pancreatin hydrolytic parameters

The percentage protein yield accounts for the amount of protein obtained in a protein extraction process. This parameter is typically a function of the extraction method employed (Boyle *et al*., 2010). In this study, the alkaline solubilisation-acid precipitation method was employed; and it extracts only the glutelin fraction of seed storage proteins (Osborne, 1924). The fact that the method extracts only a single fraction might have caused the low protein yield observed (Table 1). In comparison with the percentage protein yield of the seeds of *Psidium guajava* (4.8%), *Carica papaya* (5.13%), *Citrullus lanatus* (18.91%), *Luffa cylindrical* (18.60%) and *Moringa oleifera* (39.53) wherein alkaline solubilisation-acid precipitation method was also employed, a lower percentage protein yield was obtained for *P. longifolia* seed protein (Table 1) (Arise *et al*., 2016; Olusola *et al*., 2018; Arise *et al*., 2019; James *et al*., 2020a,b). This implies the glutelin fraction of *P. longifolia* is lower than that of the seed proteins above.

The two hydrolytic parameters employed, degree of hydrolysis and peptide yield, give the extent to which a protease hydrolyzes a protein extract. In particular, the degree of hydrolysis accounts for the cleaved peptide bonds during proteolysis, while the peptide yield gives the amount of peptides produced after proteolysis. Generally, the degree of hydrolysis is directly related to the peptide yield. One factor that influences the degree of hydrolysis

is the type of enzyme; others include the enzyme/substrate (E/S) ratio, pH value, temperature, and time of hydrolysis (Benitez *et al*., 2018). As regards the type of enzyme, pancreatin, employed in this study, is an enzyme mixture containing endopeptidases (trypsin, chymotrypsin, and elastase) and exopeptidases (carboxypeptidases A and B) (Andriamihaja *et al*., 2013). In comparison with the degree of hydrolysis (15-30%) estimated by formal titration method for pancreatin-derived whey protein concentrate hydrolysate, the value obtained in this study is lower (Table 1) (Silvestre *et al*., 2011). In the same vein, the degree of hydrolysis (35.85%) estimated by the method of soluble protein content in trichloroacetic acid for pancreatin-derived whey protein concentrate hydrolysate by Morais *et al*. (2013) was higher than the value obtained in this study. By inference, it is crystal clear from the study of Silvestre *et al*. (2011) and Morais *et al*. (2013) that the degree of hydrolysis of a protease is influenced by the method of estimation employed. Indeed, Morais *et al*. (2013) went so far as to consider four different methods of estimation and arrived at different degrees of hydrolysis for pancreatin-derived whey protein concentrate hydrolysate. Therefore, it will not be surprising if other studies to determine the degree of hydrolysis of pancreatin-derived *P. longifolia* seed protein hydrolysate are carried out and values different from the ones stated in this study are arrived at. Howbeit, this is only expected for methods of estimation different from the one utilized in this study. Due to the fact that trypsin and chymotrypsin, endopeptidases present in pancreatin, preferentially cleave peptide bonds containing residues of lysine, or arginine and basic, or aromatic amino acids respectively, the higher degrees of hydrolysis obtained by the authors for pancreatin-derived whey protein concentrate hydrolysate assert a higher composition of these amino acids in the seed protein than in *P. longifolia* seed protein (Gupta *et al*., 2002; Hinsberger and Sandhu, 2004; Page and Di Cera, 2008). Furthermore, Malomo *et al*. (2015) asserted that degree of hydrolysis values could be used to predict peptide chain length as higher degree of hydrolysis values specify shorter length peptides while lower values specify longer length peptides. Therefore, the higher degree of hydrolysis values obtained for pancreatin-derived whey protein concentrate hydrolysate shows shorter length peptides were obtained in it than in pancreatin-derived *P. longifolia* seed protein hydrolysate.

### α-amylase inhibitory effect of pancreatin-derived *P. longifolia* seed protein hydrolysate

As stated in the foregoing discussion, inhibition of α-amylase is an approach utilized in the management of postprandial hyperglycemia associated with diabetes mellitus. Consequently, researchers have been most assiduous in their search for novel and effective α-amylase inhibitors. Based on the results of this study, pancreatin-derived *P. longifolia* seed protein hydrolysate exhibited a concentration-dependent inhibitory effect against α-amylase (Figure 1). Two hydrophobic amino acids, leucine, and proline, and one hydrophilic amino acid, arginine, have been implicated in the inhibition of α-amylase (Matsui *et al*., 1999; Yu *et al*., 2012; Ren *et al*., 2016; Zhang *et al*., 2016; Jiang *et al*., 2018; Ramadhan *et al*., 2018; Wang *et al*., 2020). Indeed, Wang *et al*. (2020) asserted unequivocally that these three amino acids in peptides are regarded vital to the inhibition of α-amylase separately or synergistically and should be taken into account in the synthesis of novel bioactive peptides for diabetes management. Therefore, it is hypothesized that the inhibitory activity of pancreatin-derived *P. longifolia* seed protein hydrolysate against α-amylase is due to the abundance of these three amino acids. Apparently, further work on this hydrolysate is needed to lay credence to this hypothesis. Ramasubbu *et al*. (1996) proposed a mechanism of action of peptides in inhibiting α-amylase. He stated that the interaction of the amino acid residues of peptides with the α-amylase active site changes the position of the substrate by displacing it further away from the catalytic domain. However, this proposition cannot be generalized, because the kinetic study on the inhibitory activity of the highest concentration of pancreatin-derived *P. longifolia* seed protein hydrolysate against α-amylase pointed to an uncompetitive mechanism of α-amylase inhibition (Figure 2). One thing that is known about uncompetitive mechanism of enzyme inhibition is that the inhibitor binds only to the complex formed between the enzyme and the substrate and not the free enzyme (Naik, 2012). Therefore, the plausibility of displacing the substrate from the catalytic domain for the peptides to bind to the enzyme does not hold in this study. As known with uncompetitive inhibition mechanism, pancreatin-derived *P. longifolia* seed protein hydrolysate reduced the $V_{max}$ and $K_m$ of α-amylase-catalyzed reaction (Table 2). Although, uncompetitive inhibitory mechanism of action against single substrate reactions like that catalyzed by α-amylase is rare, however, it is encountered to some degrees, even in hydrolysates (Naik, 2012; Olusola *et al*., 2018). The inhibition range (Figure 1) observed in this study is well within bounds, for excessive inhibition of α-amylase is not desirable. Kumar *et al*. (2011) pointed out very clearly that excessive inhibition of α-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon, therefore mild α-amylase inhibition activity is more useful. The 50% inhibitory concentration ($IC_{50}$) for the inhibitory activity observed in this study (Figure 1) was higher than that of most hydrolysates (Olusola *et al*., 2018; Arise *et al*., 2016; James *et al.,* 2020a, b). This implies pancreatin-derived *P. longifolia* seed protein hydrolysate must be taken in higher concentrations than most hydrolysates to achieve a desirable inhibition of α-amylase. Notwithstanding, the promise afforded by this hydrolysate is not diminished, for peptides can be administered at high doses without eliciting grievous side effects (Ishida *et al*., 2011; Thayer, 2011).

### $ABTS^+$ radical scavenging capacity of pancreatin-derived *P. longifolia* seed protein hydrolysate

The ABTS method is a uniquely rapid means of determining the free radical scavenging ability of bioactive compounds. The $ABTS^+$ radical is produced by the oxidation reaction between ABTS and potassium persulfate (Miller *et al*., 1993; Miller and Rice-Evans, 1994; Miller and Rice-Evans,

1996). The radical produced is stable for several minutes and reduced on exposure to a bioactive agent by electron or $H^{\bullet}$ transfer (Braca *et al*., 2003; Ismail *et al*., 2004; Santoz-Sanchez *et al*., 2019). Lorenzo *et al*. (2018) chronicled quite thoroughly the role of protein hydrolysates and isolated bioactive peptides in scavenging $ABTS^{+}$ radical. In particular, pancreatin-derived protein hydrolysates have shown great effectiveness in this regard (Bayarjargal *et al*., 2014; Karamac *et al*., 2014; Liao *et al*., 2020). In this study, ascorbate yielded considerable $ABTS^{+}$ radical scavenging capacity at lower concentrations than the hydrolysate (Figure 3). This implies ascorbate has higher radical scavenging abilities than the hydrolysate. However, the hydrolysate did exhibit this property to a much encouraging extent at the concentrations considered. Numerous studies have revealed that the difference in the free radical scavenging activity of hydrolysates is partly ascribed to their amino acid composition (Hwang and Winkler-Moser, 2017; Wong *et al*., 2020). In particular, high level of hydrophobic amino acids (including Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu and Phe) has been correlated with radical scavenging capacity through hydrophobic interaction between free radicals and active functional groups on these amino acids; thereby, making free radicals unavailable for interaction with other molecules (Udenigwe and Aluko, 2011). Furthermore, negatively charged amino acids (including Glu and Asp) have been suggested to confer radical scavenging activity to hydrolysates due to the abundance of electrons that can be donated to quench free radicals (He *et al*., 2013). In addition, aromatic amino acids (including Tyr, Trp and Phe) have been suggested to potentiate the radical scavenging ability of hydrolysates by donating protons to stabilize electron deficient radicals while retaining their stability through resonance structures (Sarmadi and Ismail, 2010). Therefore, the radical scavenging capacity of pancreatin-derived *P. longifolia* seed protein hydrolysate may be attributable to the abundance of these classes of amino acids. The values obtained in this study (Figure 3) were similar to that obtained in pancreatin-derived *Pleurotus geesteranus* fruit hydrolysate (>80%) at 0.5 mg/mL but higher than that of pancreatin-derived *Bunium perscium Bioss.* press cake hydrolysate (~60%) at 4 mg/mL (Liao *et al*., 2020; Shahi *et al*., 2020).

**Ferric ion reducing antioxidant power of pancreatin-derived *Polyalthia longifolia* seed protein hydrolysate**

The FRAP method measures the total antioxidant activity of a bioactive agent by its ability to reduce ferric ion ($Fe^{3+}$) to ferrous ion ($Fe^{2+}$) (Santos-Sachez, 2019). Therefore, this method is based on electron transfer rather than hydrogen atom transfer observed in some methods of antioxidant estimation (Prior *et al*., 2005). Several studies have chronicled the ferric ion reducing antioxidant power of hydrolysates (Lorenzo *et al*., 2018). More particularly, the reducing power of pancreatin-derived hydrolysates has had some attestations (Karamac *et al*., 2014; Karamac *et al.,* 2016; Liao *et al*., 2020; Nnamezie, 2021). In this study, gallic acid yielded considerable reducing power at lower concentrations than the hydrolysate (Figure 4). This implies gallic acid has higher reducing abilities than the hydrolysate. Notwithstanding, the hydrolysate exhibited a reducing ability worthy of note (Figure 4). Certain studies have revealed that the amino acid content and peptide size play important roles in conferring ferric ion reducing ability on hydrolysates (Rajapakse *et al*., 2005; Li *et al*., 2008; Ajibola *et al*., 2011; Udenigwe and Aluko, 2011; Vastag *et al*., 2011; Carrasco-Castilla *et al*., 2012) By specific reference, Udenigwe and Aluko (2011) asserted that sulfur-containing and acidic amino acid residues are positive contributors to ferric reducing properties of peptide. Also, smaller size peptides were demonstrated to exhibit better reducing ability than high molecular weight peptides (Li *et al*., 2008; Ajibola *et al*., 2011). Therefore, the reducing power of the hydrolysate here studied can be attributable to the presence of sulfur-containing and acidic amino residues in its peptides' sequences and the abundance of small-sized peptides. The values of $Fe^{2+}$ mM equivalent obtained in this study (Figure 4) were similar to that obtained in pepsin-derived *Moringa oleifera* seed protein hydrolysate at 0.2 to 0.8 mg/ml (Olusola *et al*., 2018).

## CONCLUSION

The results obtained in this study revealed that pancreatin-derived *P. longifolia* seed protein hydrolysate possesses very encouraging alpha-amylase inhibitory properties and antioxidant effects. Therefore, it can be said that the hydrolysate has the ability to manage postprandial hyperglycemia associated with diabetes mellitus via α-amylase inhibition and attenuation of free radical production. Further studies are required to fractionate the hydrolysate, characterize its effects, and identify its constituent bioactive peptides.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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