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Alpha-Amylase – Inhibitory Properties and *in vitro* Antioxidant Potentials of Cowpea Seed Protein Hydrolysates

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Cowpea seed extracts have been investigated for its antioxidant potentials, enzyme-inhibitory activities, antifungal and nematocidal properties among others. Also, protein hydrolysates of cowpea seeds have been evaluated for its antioxidant and antihypertensive potentials *in vitro*. However, there has been relative paucity of information on the α-amylase inhibitory activities of cowpea seed protein hydrolysates. Consequently, this study evaluated the α-amylase – inhibitory activities and antioxidant potentials of protein hydrolysates derived from cowpea seed proteins. Cowpea seeds were defatted using n-hexane and proteins were extracted from the resulting seed meal. The proteinases namely pepsin and trypsin were used for protein hydrolysis and the resulting hydrolysates were investigated for antioxidant properties (using hydrogen peroxide and ferric ions) and α-amylase inhibitory activity. Antioxidant assays indicated that hydrolysates derived from trypsin digestion showed better ferric reducing power even as both hydrolysates demonstrated similar hydrogen peroxide scavenging capacities. α-amylase inhibition studies showed that peptic hydrolysates had better inhibitory activity (IC_{50} = 0.127±0.012 mg/ml). Kinetic data indicated mixed mode of inhibition for both hydrolysates, with peptic hydrolysates showing higher binding affinity (k_i = 0.089 mg/ml). It is suggested that proteins from cowpea might encode certain peptides with potent biofunctionalities beyond their nutritional benefits and as such could be further processed to develop novel anti-diabetic agents and food additives.

Introduction

Diabetes mellitus (DM) is an endocrine system disease, occurring as a result of disturbances in insulin production and function [1]. Elevated plasma glucose level is the main diagnostic symptom which is a direct consequence of impairments in the regulation of metabolism of fuel molecules. These cause a myriad of metabolic derangements resulting in oxidative stress, ketoacidiosis, advanced glycated end-products (AGEs), ultimately leading to multiple organ damage when the disease enters its later stages [2]. Current therapeutic strategies are aimed at regulating blood glucose levels by lifestyle changes, infusion of exogenous insulin or modulating the activities of key enzymes involved either directly or indirectly in glucose metabolism. Important enzymes controlling glucose metabolism such as α-amylase, α-glucosidase and dipeptidyl peptidase-4 have been key pharmacologic targets for many hypoglycemic drugs [2, 3]. However, most conventional chemotherapeutic strategies are rather expensive to procure and thus places an economic strain on patients and their relatives. Also, many of these drugs are not without their untoward side effects, causing eventual damage to vital organs such as liver and kidney [4, 5].

Oxidative stress reflects an imbalance between the systemic production of reactive oxygen species and the body's capacity to detoxify the free radicals or to repair the consequent damage. These reactive intermediates usually alter the normal redox state of cells and can cause rather harmful effects by damaging cellular components such as lipids, DNA and proteins [6]. In humans, oxidative stress is thought to be involved in the pathogenesis of several diseases such as cancer [7], Parkinson's disease, Alzheimer's disease [8, 9], myocardial infarction [10, 11], fragile X syndrome [12], sickle cell disease [13], among others.

Diabetes mellitus has been determined to be a cause and effect of oxidative stress. The production of free radicals results in impairment of tissue function and their eventual damage in the long term [14] and as such there is a growing need to identify newer, more effective, cost effective and considerably safer antioxidant agents and inhibitors of enzymes involved in diabetes mellitus from a number of natural materials [4, 5]. These include peptides and protein hydrolysates from a number of plant and animal sources in recent times [2, 3].

Cowpea (*Vigna unguiculata L.*) is a tropical legume native to both African and Indian continents. It is commonly consumed as a staple food, serving as a rich source of protein in the diets of humans and animals [15]. It is non-toxic and free of anti-metabolites [16]. Protein content of cowpea seeds is within the range of 20-34% [17] and predominant proteins present in cowpea seeds are globulins, glutelins and prolamins, of which the globulins (α, β, and γ-vignins) are the most abundant [16, 18]. Amino acid analysis showed that cowpea seeds are particularly rich in aspartic and glutamic acids, lysine, leucine, arginine and isoleucine but limiting in cysteine and tryptophan [19, 20]. Cowpea seed extracts have been investigated for its antioxidant potentials [21], enzyme-inhibitory activities [22], antifungal and nematicidal properties [23] among others. Its rich protein content makes it an excellent source of biologically active protein hydrolysates and peptides [15]. Protein hydrolysates of Cowpea seeds have also been evaluated for its antioxidant and antihypertensive effects *in vitro* [24, 25]. There has been relative paucity of information on the α-amylase inhibitory activities of cowpea seed protein hydrolysates, and this study is aimed at evaluation of these hydrolysates for these properties with a view to provide cost-effective additives to food products.

Materials and Methods

Materials

Collection of Cowpea Seeds

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

Chemicals and Reagents

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

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

Equipment

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

Methods

Isolation of Cowpea Seed Proteins

The seeds were dried, pulverized and stored in an air-tight container at 4°C. These were defatted using n-hexane as according to the method described by Wani *et al.* [26]. The meal was extracted four times with n-hexane (60–80°C) using a meal/solvent ratio of 1:10 (w/v), after which it was dried at 40°C in a vacuum oven and ground again to obtain a fine powder, termed defatted seed meal, which was stored at -20°C. The protein component of the defatted meal was extracted using the method described by Alashi *et al.* [27]. Defatted cowpea seed meal was suspended in 0.5 M NaOH pH 12.0 at a ratio of 1:10, and stirred for one hour to facilitate solubilization in alkali. The slurry was then centrifuged at 18°C and 3000 g for 10 min. Two additional extractions of the residue from the centrifugation process was carried out with the same volume of 0.1 M

NaOH and the supernatants were pooled. The pH of the supernatant was adjusted to pH 4.0 to facilitate acid-induced protein precipitation using 3 M HCl solution; the precipitate formed was recovered by centrifugation. The precipitate was washed with distilled water, adjusted to pH 7.0 using 0.1 M NaOH, freeze-dried and the protein isolate stored at -20°C until required for further analysis.

Preparation of Cowpea Seed Protein Hydrolysates

The protein isolate was hydrolysed using the method described by Udenigwe *et al.* [28] with slight modifications. The conditions for hydrolysis were specified for each enzyme in order to ensure optimal activity. Hydrolysis was carried out using each of pepsin (pH 2.2, 37°C) and trypsin (pH 8.0, 37°C) The protein isolate (5% w/v, based on the protein content of the isolate) will be dissolved in the appropriate buffer (glycine buffer, pH 2.2 for pepsin and phosphate buffer, pH 8.0 for trypsin). The enzyme was added to the slurry at an enzyme-substrate ratio (E:S) of 2:100. Digestion was performed at the specified conditions for 8 hours with continuous stirring. The enzyme was inactivated by boiling in water bath (95–100°C) for 15 min and undigested proteins were precipitated by adjusting the pH to 4.0 with 2M HCl/2M NaOH followed by centrifugation at 7000 g for 30 minutes. The supernatant containing target peptides were then collected. Protein content of samples was determined using Biuret assay method reported by Arise *et al*. [29] with bovine serum albumin (BSA) as standard.

Determination of Degree of Hydrolysis

Degree of hydrolysis (DH) was determined by calculating the percentage of soluble protein in 10% trichloroacetic acid (TCA) in relation to total protein content of the protein isolate according to the method reported by Arise *et al.* [29]. 1 ml of protein hydrolysate was added to 1 ml of 20% TCA to produce 10% TCA soluble material. The mixtures were left to stand for 30 minutes for precipitation, followed by centrifugation at 4000 g for 20 min. The supernatants were analyzed for protein content using Biuret method reported by Arise *et al*. [29] with bovine serum albumin (BSA) as standard. The degree of hydrolysis (DH) was computed as shown below:

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

Determination of Peptide Yield

The percentage peptide yield was determined using the method described by Girgih *et al.* [32]. The peptide yields (%) were calculated as the ratio of peptide content of lyophilized hydrolysates to the protein content of unhydrolysed protein isolate.

Determination of α-amylase Inhibition

An α-amylase-inhibitory assay was carried out according to the method described by Arise *et al.* [2] with slight modifications. Briefly, 250 µL of hydrolysate (0.2 to 1.0 mg mL–1) was placed in test tubes and 250 µL of 20 mM sodium phosphate buffer (pH 6.9, with 6 mM NaCl) containing α-amylase solution (0.5 mg/mL) added. The content of each tube was pre-incubated at 25°C for 10 min, after which 250 µL of 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9, with 6 mM NaCl) was added at timed intervals. The reaction mixtures were incubated at 25°C for 10 min. The reaction was terminated by adding 250 µL of 1% dinitrosalicylic acid (DNS) colour reagent and further incubated in boiling water for 5 min and cooled to room temperature. The content of each test tube was diluted with 5.0 mL distilled water and the absorbance measured at 540 nm. A control was prepared using the same procedure except that the hydrolysate was replaced with distilled water. The α-amylase-inhibitory activity was determined as shown:

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

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

Determination of Kinetic Parameters of α-amylase Inhibition

The kinetic study of α-amylase inhibition was conducted according to the modified method described by Ali *et al.*[30]. 250

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

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

A double reciprocal plot (1/V versus 1/[S]), where V is reaction velocity and [S] is substrate concentration was plotted. The mode of inhibition and the kinetic parameters (Km, K´m, V'max, V´max, CE and CE´) of α-amylase inhibition by hydrolysates was determined by analysis of the double reciprocal plot. The inhibition constant (Ki) was determined as the intercept on the x-axis from the secondary plot of the slopes of the Lineweaver-Burk plots against inhibitor concentrations.

Determination of Hydrogen Peroxide Scavenging Activity

The ability of the hydrolysate to scavenge hydrogen peroxide was determined according to the method reported by Keser *et al.* [31] with slight modifications. Hydrogen peroxide solution (4 mM) was prepared in 0.2 M phosphate buffer (pH 7.4). Varying concentrations of the hydrolysates (0.2 – 0.8 mg/mL) in distilled water was added to 0.6 ml of 4 mM hydrogen peroxide solution. Absorbance value of test samples (As) was read at 230 nm after 10 minutes against a blank solution containing the phosphate buffer without hydrogen peroxide. Absorbance of hydrogen peroxide (Ac) was taken as the control. Ascorbic acid was used as a standard antioxidant. The percentage of scavenging effect was calculated by comparing the absorbance values of the control and test samples using:

% Scavenging Capacity (% Scavenged [H_2O_2]) = [(AC– AS)/AC] x 100

IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression plot.

Determination of Ferric Reducing Antioxidant Property (FRAP)

The reducing power of the hydrolysates was measured according to a slightly modified method described by Zhang *et al.* [33]. An aliquot of 1 ml of different hydrolysate concentrations (0.2 – 0.8 mg/ml), (0.2 M PBS, pH 6.6) was mixed with 1 ml of 1% potassium ferric cyanide solution. The mixture was incubated at 50°C for 30 minutes followed by the addition of 1 ml 10% (w/v) TCA. 1 ml of the incubation mixture was added with 1 ml of distilled water and 0.2 ml of 0.1% (w/v) ferric chloride in test tubes. After a 10 min reaction time, the absorbance of resulting solution was read at 700 nm. Higher absorbance indicated stronger reducing power. Ascorbic acid was used as the reference antioxidant. An aqueous solution of known Fe (II) concentrations ($FeSO_4 \cdot 7H_2O$; 2.0, 1.0, 0.5, 0.25 and 0.125 mM) was used for calibration. Results were expressed as mM Fe^{2+}/mg hydrolysate. All tests were performed in triplicate.

The EC_{50} of hydrolysates was determined from the graph of A700 against concentration.

Statistical Analysis

Results were expressed as mean of replicates ± standard error of mean. The data were statistically analyzed using One Way Analysis of Variance (ANOVA) and Duncan's multiple range tests. Differences was considered statistically significant at $p<0.05$ GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel software version 2013.

Results

Protein Yield of Isolate, Peptide Yield and Degree of Hydrolysis

The protein yield of isolation, peptide yield and degree of hydrolysis are presented in Table 1. The yield of isolation of cowpea seed proteins was 24.00%. Peptide yield of hydrolysates obtained by pepsin and trypsin treatment were

85.536±1.776% and 14.742±0.020% respectively, while the degree of hydrolysis of hydrolysates obtained from peptic and tryptic digestion were found to be 26.940±2.966% and 9.000±0.631% respectively. Peptide yield and degree of hydrolysis of peptic hydrolysates were significantly higher ($p<0.05$) than those of tryptic hydrolysates.

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

Parameter/Enzyme	Yield of Isolation (%)	Peptide Yield (%)	Degree of Hydrolysis (%)
Protein Isolate	24.00	-	-
Pepsin	-	85.536 ± 1.776[a]	26.940±2.966[a]
Trypsin	-	14.742±0.020[b]	9.000±0.631 [b]

Alpha-amylase Inhibitory Activity

The α-amylase inhibitory activities of cowpea seed protein hydrolysates are illustrated in Figure 1. Both hydrolysates demonstrated a concentration-dependent inhibition of α-amylase. Also, both hydrolysates showed percentage inhibitory activities above 50% at concentrations of 0.20 mg/ml to 1.0 mg/ml. Peptic hydrolysates showed significantly higher ($p<0.05$) inhibitory activity at 0.2 mg/ml and 0.4 mg/ml and 1.0 mg/ml, while tryptic hydrolysates displayed a significantly higher ($p<0.05$) inhibitory activity at a concentration of 1.0 mg/ml.

The 50% α-amylase inhibitory concentrations (IC_{50}) of cowpea seed protein hydrolysates are shown in Figure 2. The IC_{50} of peptic hydrolysates (0.127±0.012mg/ml) was significantly lower ($p<0.05$) than that of tryptic hydrolysates (0.223±0.009 mg/ml).

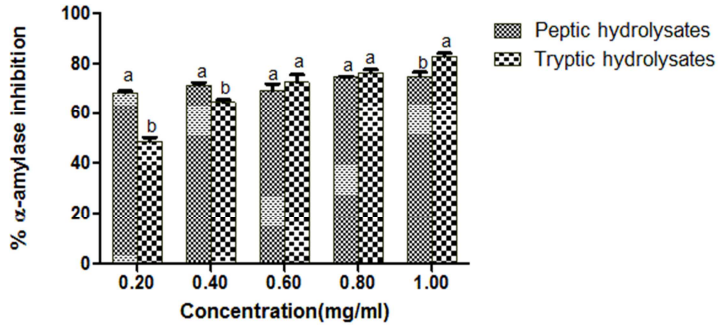


Figure 1. *Percentage α-amylase inhibition by cowpea seed protein hydrolysates. Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Comparison is strictly within the same concentration value. Bars with the same letters do not differ significantly while values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

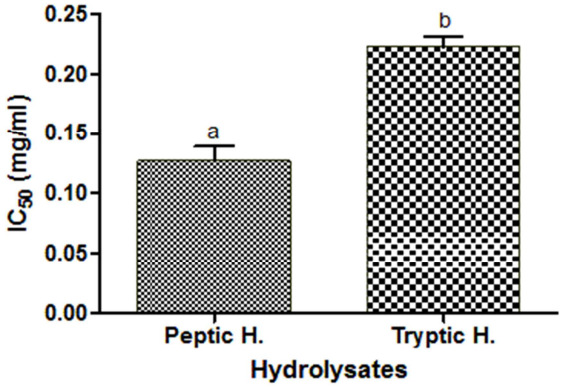


Figure 2. *IC_{50} values of α-amylase inhibition by cowpea seed protein hydrolysates. Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Bars with the same letters do not differ significantly while values with different letters are significantly different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

Kinetics of α-amylase Inhibition

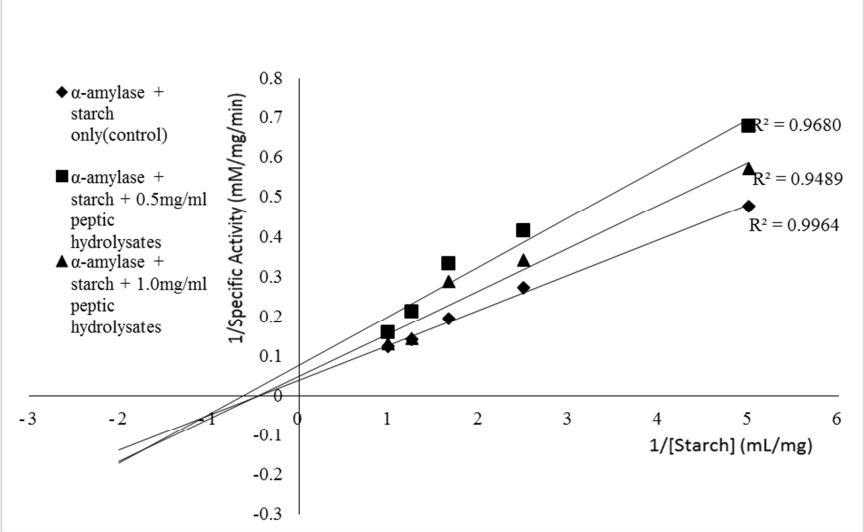
The effects of peptic and tryptic hydrolysates of cowpea seed proteins on the catalytic activity of α-amylase in converting starch to maltose are shown in Figures 3 and 4. Kinetic parameters determined from Lineweaver-Burk plots in the absence and presence of two different concentrations of each of peptic and tryptic hydrolysates are summarized in Table 1. The km of the

enzyme for its substrate was determined to be 2.301mg/ml of starch, while Vmax was 25.974 mmol/mg/min. The presence of increasing concentrations of the hydrolysates appeared to have no effect on the Km of the enzyme, while maximal velocity, Vmax and catalytic efficiency, CE of α-amylase were reduced in the presence of the hydrolysates. Tryptic hydrolysates showed a more reduced Vmax and catalytic efficiency when compared to peptic hydrolysates. The enzyme-inhibitor dissociation constant, ki, of α-amylase inhibition by peptic hydrolysates (0.089 mg/ml) was lower than that obtained for tryptic hydrolysates (0.155 mg/ml). The mode of inhibition of both hydrolysates was the uncompetitive type of mixed inhibition.

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

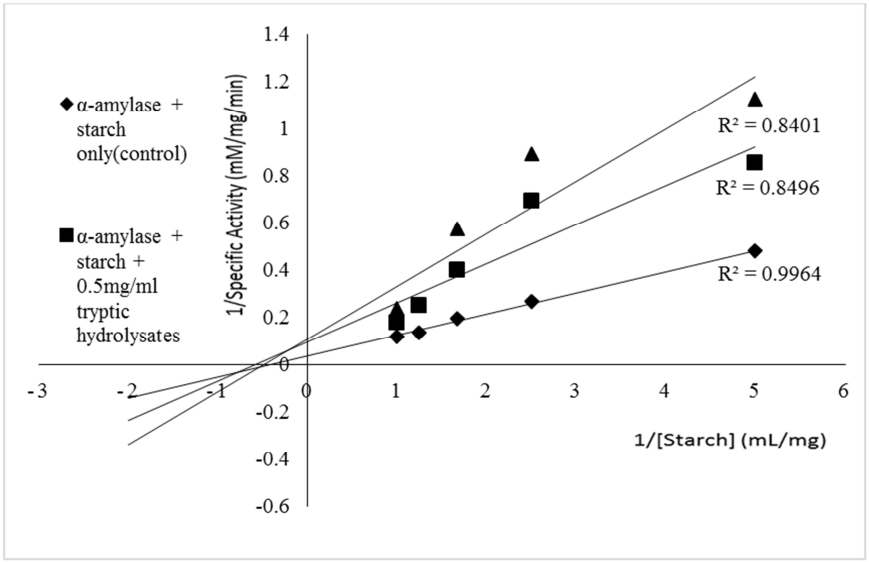
Kinetic Parameter	No inhibitor	Peptic Hydrolysates (mg/ml)		Tryptic hydrolysates (mg/ml)	
		0.5	1.0	0.5	1.0
K_m or K'_m (mg/ml)	2.301	2.216	1.639	1.694	2.032
V_{max} or V'_{max} (mM/mg/min)	25.974	20.576	13.210	10.225	9.124
CE (mmol/ml/min)	11.288	9.285	8.060	6.036	4.490
K_i (mg/ml)	-	0.089		0.155	

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



R^2 – Coefficient of determination

Figure 3. *Lineweaver-Burk plot of α-amylase inhibition by cowpea seed protein hydrolysates derived from peptic proteolysis.*



R^2 – Coefficient of determination

Figure 4. *Lineweaver-Burk plot of α-amylase inhibition by cowpea seed protein hydrolysates derived from tryptic proteolysis*

Ferric Reducing Antioxidant Power

The ferric reducing antioxidant properties of ascorbate (control) and cowpea seed protein hydrolysates are illustrated in Figure 5. All samples displayed a concentration-dependent increase in ferric reducing power, except for tryptic hydrolysates the showed a reduction at 0.6 mg/ml. All hydrolysates had significantly ($p<0.05$) reduced antioxidant activities at different concentrations when compared to ascorbate. However, tryptic hydrolysates had significantly ($p<0.05$) higher antioxidant activities than peptic hydrolysates at concentrations of 0.2 mg/ml, 0.4 mg/ml and 0.8 mg/ml.

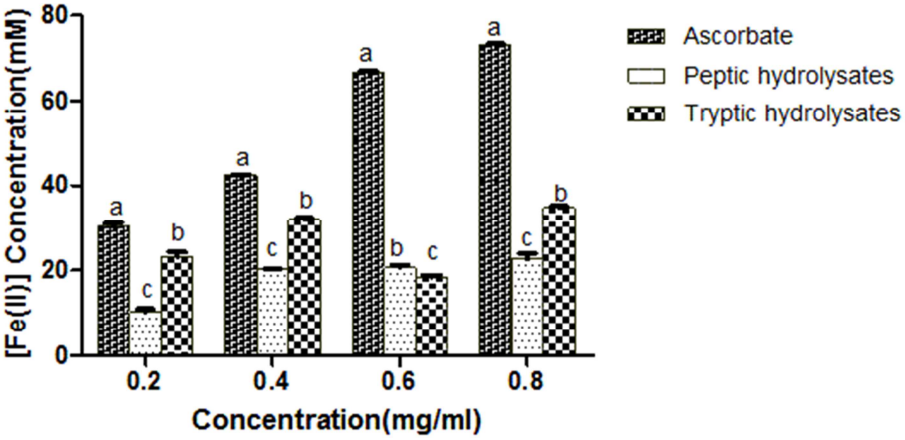


Figure 5. *Ferric reducing antioxidant properties of cowpea seed protein hydrolysates.*

Bars are expressed as means ± standard error of mean (SEM) of triplicate determinations (n=3). Comparison is strictly within the same concentration value. Bars with the same letters do not differ significantly while values with different letters are significantly different from one another at $p<0.05$.

Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging activities of ascorbate and cowpea seed protein hydrolysates are presented in Figure 6. All samples showed a concentration dependent increase in scavenging H_2O_2. Both hydrolysates demonstrated a significantly lower ($p<0.05$) H_2O_2 scavenging activities when compared to control. There was no significant difference in H_2O_2 scavenging activities of both hydrolysates. Figure 7 shows EC_{50} values of cowpea seed protein hydrolysates in scavenging hydrogen peroxide, as compared to ascorbate (control). Peptic hydrolysates scavenged H_2O_2 to a 50% inhibition at a concentration of 0.6020 ±0.331 mg/ml, while tryptic hydrolysates had 50% scavenging activities at a concentration of 0.672±0.231 mg/ml. Tryptic hydrolysates had IC_{50} values comparable to peptic hydrolysates.

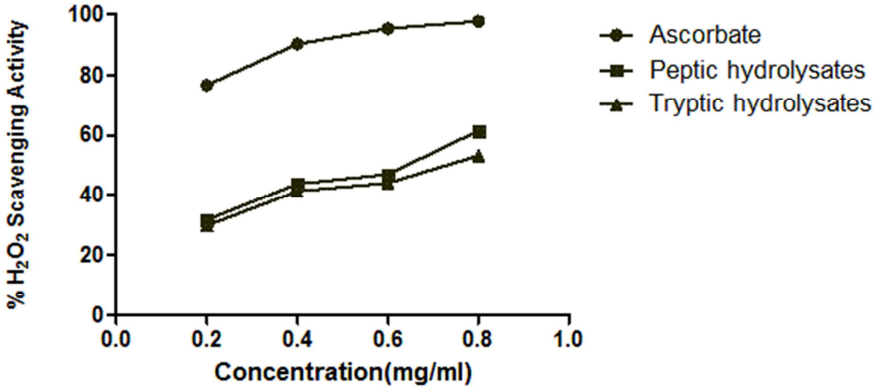


Figure 6. *Hydrogen peroxide scavenging activities of cowpea seed protein hydrolysates.*

Each point on the graph is expressed as mean± standard error of mean (SEM) of triplicate determinations (n=3).

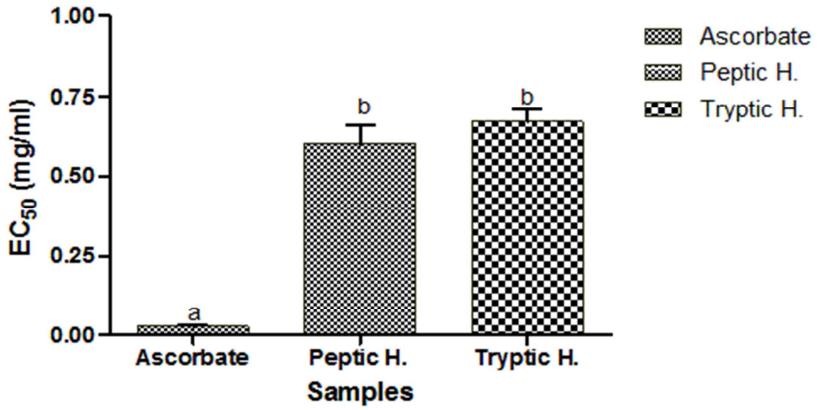


Figure 7. *EC_{50} Values of cowpea seed protein hydrolysates in scavenging hydrogen peroxide.*

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

Discussion

Protein Yield of Isolate, Peptide Yield and Degree of Hydrolysis

The percentage protein yield of 24.0% obtained for cowpea seed protein isolate was within the range of 20-34% reported by Ofuya and Akhidue [17], for cowpea seed proteins, and 22-30% for *Arachis hypogea* seed proteins [34] respectively. This is an indication that alkaline extraction and isoelectric precipitation has proved to be more efficient than other methods of protein extraction [35]. Cowpea seed proteins comprise mainly globulins, prolamins and glutelins [16, 18] all of which are insoluble in HCl and so are easily precipitated, thereby minimizing protein loss from isolation. Peptide yield is an important parameter that is commonly used in determining the efficiency of the entire process of hydrolysis [27]. Peptic hydrolysates had a higher peptide yield of 85.54% and this is higher than 68.90% and 55.0% obtained by Arise *et al.* [2] and Alashi *et al.* [27] for watermelon seed protein hydrolysates and canola seed meal protein hydrolysates respectively. The relatively high yield obtained indicate that most of the proteins in cowpea seeds have regions susceptible to enzymatic hydrolysis by pepsin and could be digested into peptides, which would be largely beneficial for industrial purposes. Also, cowpea seeds have been reported to be especially rich in hydrophobic amino acids [19, 20], and pepsin prefers to cleave amino acids at the C- terminal ends of these hydrophobic residues [36]. This may further explain the reason why tryptic hydrolysates have a higher peptide yield when compared to tryptic hydrolysates. Tryptic hydrolysates have a yield of 14.74%, which is lower than 41.38% obtained for tryptic digests of watermelon seed protein hydrolysates. Trypsin is highly specificity for lysine and arginine residues [36] which may lead to the cleavage of a lower number of peptides, and this may account for the relatively low peptide yield obtained.

The degree of hydrolysis (DH) gives an estimate of the number of cleaved peptide bonds in a protein hydrolysate, thus affecting the molecular sizes and amino acid compositions of the peptides and thereby altering the biological activities of the peptides formed during hydrolysis. Therefore, the DH is a vital parameter in determining the functional properties of protein hydrolysates [37]. The degree of peptic hydrolysis obtained in this study (26.94%, enzyme:substrate ratio of 2:100) was higher than 19.38±0.86% (E:S of 1:100) obtained for watermelon seed protein hydrolysates [2] and 8% (E:S of 4:100) for hemp seed peptic protein hydrolysates [38]. This is probably due to the nature of the seed proteins, enzyme-substrate ratio and the conditions of hydrolysis. The degree of tryptic hydrolysis (9.00±0.63%, E:S 2:100) was lower than 26.26 ±0.27% obtained for tryptic hydrolysates of watermelon seed protein hydrolysates [2]. This result is likely due to the enzyme-substrate ratio for tryptic proteolysis as a reduced E:S ratio appears to increase the degree of hydrolysis.

Alpha-amylase Inhibitory Activity and Kinetics of Inhibition

There is relative paucity of information on α-amylase inhibition by peptides and protein hydrolysates when compared to that of plant extracts. In this study, both hydrolysates demonstrated a dose-dependent increase in percentage inhibition, such that tryptic hydrolysates had an overall stronger inhibitory effect (82.86%) than peptic hydrolysates (74.91%) at a final concentration of 1.0 mg/ml. However, peptic hydrolysates inhibited the enzyme to a 50% extent at a much lower concentration

(0.127±0.012 mg/ml) than tryptic hydrolysates (0.223±0.009 mg/ml). The 82.86% inhibition of α-amylase by tryptic hydrolysates is comparable to 82.97% inhibition of the same enzyme obtained for tryptic hydrolysates of *Citrullus lanatus* seed proteins [2], although that was obtained at a concentration of 2.0mg/ml. Also, the extent of inhibition by peptic hydrolysates in this study (74.91%) is slightly higher than 70.19% reported by Arise *et al.* [2] for peptic hydrolysates of *Citrullus lanatus* seed proteins. This indicate that legume seed proteins could release bioactive peptides with potent α-amylase-inhibitory potentials when digested with pepsin and trypsin, simulating gastrointestinal proteolysis and this may have beneficial effects in the management of diabetes mellitus. Cowpea seed proteins are especially rich in aspartic and glutamic acids, lysine, leucine, arginine and isoleucine [19, 20]. Yu *et al.* [39] and Garza *et al.* [40] had reported that phenylalanine, leucine, proline and glycine residues are required for the inhibition of α-amylase. In the same vein, Arise *et al.* [2] also suggested that α-amylase binds to peptides containing cationic residues such as Lys and branched chain residues such as Phe, Tyr and Trp. This could, in part, explain the reason why both hydrolysates have a high α-amylase-inhibitory activity, since trypsin cleaves after lysine and arginine, while pepsin is known to cleave at C-terminals of hydrophobic amino acid residues.

The kinetic parameters determined from the double-reciprocal plots were summarized in Table 2; suggesting that the km of α-amylase in the absence of inhibitory hydrolysates is 2.301 mg/ml of starch which is higher than 1.3 mg/ml [41] and 1.4 mg/ml [42] for α-amylases obtained from *Ganoderma tsugae* and *Aspergillus oryzae* respectively, but lower than 6.639mg/ml reported by Arise *et al.* [2] for *Bacillus licheniformis* α-amylase. The mode of inhibition of both hydrolysates was the uncompetitive subtype of mixed inhibition. These were exemplified by the kinetic data determined at varied concentrations. This indicates that the peptides that made up the hydrolysates could bind α-amylase in both its free and starch bound forms, but having higher affinity for the enzyme in its starch-bound form than in its free form. That means the hydrolysates might bind to other sites distinct from the catalytic sites, resulting in progressive reduction in activity as concentrations increase. The kinetic data in Table 2 also showed a concentration-dependent reduction in Vmax and CE of α-amylase for both hydrolysates. Ki values imply that peptic hydrolysates had higher binding affinity for α-amylase than tryptic hydrolysates. The ki of 0.089 mg/ml obtained for peptic hydrolysates was relatively higher than 0.042 mg/ml reported for *Citrullus lanatus* seed protein hydrolysates [2], while the binding constant of 0.155mg/ml determined for tryptic hydrolysates was lower than 0.449 mg/ml reported by Arise *et al.* [2] for *Citrullus lanatus* seed protein hydrolysates. This lends credence to the position of Yu *et al.* [39] that specific amino acid residues on peptides are required for α-amylase binding and inhibition.

Ferric Reducing Antioxidant Power

Potassium ferricyanide is commonly used to determine the reducing power of plant extracts as well as hydrolysates. Higher reducing power, that is the production of ferrous ions from ferric ions, denotes stronger antioxidant activity of the extract [43]. This property evaluates the ability of the hydrolysate to serve as proton donors in redox reactions [2]. The hydrolysates generally exhibited low ferric reducing properties when compared with ascorbic acid and correlates with previous studies by Razali *et al.* [43] and Arise *et al.* [2] which revealed a similar trend of results. This may be as a result of the relatively low amount of sulfur-containing aminoacyl residues in the hydrolysates, which would have otherwise contributed positively to antioxidative activity by donating protons to ferric ions in the reaction medium [19, 44]. However, both hydrolysates demonstrated better reducing power than *Citrullus lanatus* seed protein hydrolysates [2]. This could be due to increased hydrolysis time during the preparation of the protein hydrolysates. In this study, trypsin hydrolysates did show better ferric reducing properties when compared with peptic hydrolysates. This may be, in part, as a result of trypsin's specificity to cleave at C-terminals of lysine and arginine residues, yielding peptides capable of donating protons that can reduce ferric ions *in-vitro*. Hydrolysates obtained from peptic digestion also showed better ferric reducing properties, and this was higher than the results obtained for cobia skin gelatin hydrolysates [43]. Factors such as hydrolysis time, degree of hydrolysis, and choice of enzyme are known to affect the functional properties of peptides. In addition, there is evidence indicating that the C-terminal aminoacyl residue of a peptide is crucial to its antioxidant activity [45]. Udenigwe and Aluko [44] had demonstrated that sulphur containing as well as acidic aminoacyl residues are positive contributors to ferric reducing properties of peptides. Ilesanmi and Gungula [20] reported that cowpea seeds are rich in acidic amino acids, and pepsin being less specific, cleaves after hydrophobic and to a lesser extent, acidic amino acids [36, 46], releasing peptides that could act as proton donors to reduce ferric ions *in vitro*.

Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide is a pro-oxidant species that is produced by normal redox and enzymatic reactions occurring in living cells and tissues, such as during the oxidation of purines and hemoglobin [29, 47]. Despite being a non-radical, it is quite unstable and indirectly injurious to cellular structures such as cell membranes, DNA and proteins because it can generate the more dangerous hydroxyl radicals and singlet oxygen both of which are already known to be cytotoxic [48]. Thus,

hydrogen peroxide scavenging assay is another important parameter in evaluating the antioxidant potentials of plant extracts, protein hydrolysates and peptides. The results indicate that both hydrolysates showed concentration-dependent H_2O_2 scavenging activities but these were lower when compared to ascorbic acid. This is in contrast to what was obtained by Arise *et al.* [29] for *C. lanatus* seed protein hydrolysates, which had H_2O_2 scavenging activities higher than ascorbic acid. Peptic hydrolysates demonstrated slightly higher scavenging property than tryptic hydrolysates, and this is most likely due to the nature of peptides released by the enzymes. Previously, Udenigwe and Aluko [44] had suggested that hydrophobic amino acyl residues and acidic amino acids in peptides have positive effects on H_2O_2 – scavenging properties of peptides while on the other hand, positively charged residues show negative effects ability of a peptide to scavenge hydrogen peroxide. Pepsin cleaves at C-terminal residues of hydrophobic amino acids, and to a lesser extent acidic amino acids while trypsin digests protein sequences at C-terminals of arginine and lysine residues [36, 46]. In addition, Li and Li [45] demonstrated that the C-terminal aminoacyl residue of a peptide is the most important domain for the antioxidant activity of a peptide. This implies that there were more hydrophobic and acidic amino acid residues on the C-terminal domains of peptic hydrolysates hence the observed slightly better H_2O_2 – scavenging activity than tryptic hydrolysates. However, their EC_{50} values (0.602 ±0.331 mg/ml for peptic hydrolysates and 0.672±0.231 mg/ml for tryptic hydrolysates) showed that they scavenged H_2O_2 to a 50% exent at nearly the same concentrations. These were comparatively higher than 0.373±0.012 mg/ml recorded for peptic hydrolysates, but lower than 0.945±0.039 mg/ml obtained for tryptic digests, both derived from *Citrullus lanatus* seed proteins [29].

Conclusion

The results from this study showed that both hydrolysates exhibited alpha-amylase inhibitory activity, with peptic hydrolysates demonstrating better inhibitory effects. The hydrolysates also exhibited significant antioxidant properties via different mechanisms, such that tryptic hydrolysates had better overall free radical scavenging activities. These results therefore suggest that both hydrolysates could be promising candidates for development of peptide products which could serve potential therapeutic purposes in the treatment of diabetes mellitus and as dietary supplements antioxidants. Thus, cowpea seed proteins are a good source of peptide products which possess multidirectional functionalities beyond their nutritional value. ■

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