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Antimicrobial properties and antioxidant activities of pigeon pea seed protein hydrolysates

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In recent times, the biological activities of enzymatic digests of plant and animal proteins have been investigated and have been shown to exhibit multidirectional effects against microorganisms involved in the pathophysiology of a number of diseases. The present study evaluated the antimicrobial and antioxidant effects of pigeon pea protein hydrolysates. Proteins were hydrolyzed using the enzymes pepsin, trypsin and papain. The resulting hydrolysates were evaluated for antimicrobial activities (using selected bacterial strains) and antioxidant effects using DPPH radicals and ferric ions. All hydrolysates displayed varying antimicrobial activities, with papain hydrolysates showing the broadest specificity against four bacterial strains (*Staphylococcus aureus, Actinobacter baumanni, Escherichia coli* and *Salmonella* species). Antioxidant assays indicated that hydrolyates derived from tryptic digestion showed the highest DPPH radical scavenging activity and ferric reducing antioxidant power. These results suggest that the subjection of pigeon pea proteins to enzymatic digestion could yield peptides that can be harnessed to formulate products which could serve as novel alternatives to current therapies in the treatment of infectious diseases.

Key words: Pigeon pea, protein, hydrolysates, pepsin, trypsin, papain, antimicrobial, antioxidant.

INTRODUCTION

Infectious diseases are a significant burden on public health and economic stability of societies all over the world. They have for centuries been among the leading causes of death and disability and present challenges to health (Nii-Trebi, 2017). They are generally caused by microorganisms, and inflict damage on organs and/or systems whenever they gain entry into host organisms (NIH, 2007). Cellular destruction, tissue necrosis and eventual death of the host organisms mainly results from the growth and metabolic process of infectious agents or within body fluids, with the production and release of toxins or enzymes that interfere with the normal functions of organs and or systems (NIH, 2007). The generation of free radicals occurs in a continuous fashion as long as the cell lives (Pham-Huy et al., 2008). They are produced from the oxidation of fuel molecules through in vivo cellular respiration, other metabolic processes, and as a result of exposure to certain drugs, environmental toxicants and pollutants (Majhenič et al., 2007). These reactive metabolites play important biological functions in

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cells in a controlled environment. They are continuously controlled by endogenous antioxidant enzymes or transition metals. Thus, the balance is created between pro-oxidants and antioxidants. The impairment of antioxidant status, either by exogenous or endogenous sources, may disturb the cellular redox balance and the pathological conditions would be the main characteristics and forms oxidative stress in cells or tissues (Noori, 2012). Free radicals are implicated in the pathophysiology of a number of microbial diseases (Handa et al., 2011; Olusola et al., 2020). Some microbial pathogens elicit over-the-top immune responses, while others actively respire, grow and divide in healthy cells. Both processes, cause oxidative stress, lead to cell lysis and eventual tissue necrosis. Certain conventional antibiotics used in the chemotherapy of infectious diseases can potentiate drug-induced oxidative stress, which contribute to tissue injury and death (Olusola et al., 2020). In addition, they are also expensive to procure and place financial strain on patients and their relatives. Also, strains of drug-resistant microbes are on the increase and as such, limit the efficacy of many orthodox antibiotics, and places operational burden on health care workers. Many occurrences of multidrug-resistant bacteria are commonly reported in African countries (Belmonte et al., 2010). Hence there is the need to continually source for safer and cost-effective alternatives from natural sources.

Peptides are polymers or two or more amino acids linked by amide bonds. They are usually considered to be chains of less than 100 amino acids in length (Voet and Voet, 2011). Peptides and protein hydrolysates from plant and animal sources have continuously gained attention due to their multifunctional abilities ranging from uses as food additives to their utilization in the treatment of disease conditions (Arise et al*.,* 2016b). Studies have demonstrated the abilities of these peptides to inhibit key enzymes and scavenge free radicals involved in the pathophysiology of conditions such as microbial infections and oxidative stress (Ulagesan et al*.,* 2018). One plant that has been studied for its numerous bioactivities is pigeon pea. *Cajanus cajan,* commonly called pigeon pea, is a legume belonging to the species of the genus *Cajanus.* It is economically and nutritionally important and is a major source of protein for the poor communities of many tropical region of the world such as Africa (Sharma et al., 2011). The leaf has been reported to process antimicrobial activity (Okigbo and Omodamiro, 2017) while the seeds serve as source of food and protein for man and animals (Sharma et al., 2011) In recent times, protein hydrolysates obtained from *C. cajan* have been demonstrated to possess antihypertensive activities in spontaneously hypertensive rats (Olagunju et al*.,* 2018). However, there is paucity of information regarding the antimicrobial activities of protein digests of pigeon pea seeds; hence this study was aimed at evaluating the antimicrobial and antioxidant properties of hydrolysate obtained from pigeon pea seed proteins.

MATERIALS

Pigeon pea (*C. cajan*) seeds were purchased from Ogbese market, Ogbese, Ondo State, Nigeria. They were identified and voucher samples deposited at the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba-Akoko. Bacterial isolates were obtained from the stock of Microbiology Laboratory of Adekunle Ajasin University, Akungba Akoko. Pepsin (from porcine gastric mucosa), Papain (from *Carica papaya*), Trypsin (from bovine pancreas), were products of Kem-Light laboratories, Mumbai, India. 1,1 diphenyl 1-2 picrylhydrazyl (DPPH), ascorbic acid, trichloroacetic acid (TCA), potassium ferricyanide, ferric chloride. These were products of sigma-Aldrich laboratories, Co-Artrim, United Kingdom. All chemicals and reagents used were of analytical grade.

METHODS

Isolation of pigeon pea seed proteins

The pigeon pea seeds were dried, pulverized and stored in an air-tight vessel at 10°C. They were defatted using n-hexane as according to a slightly modified method previously described by Olusola and Ekun (2019). The seed meal was cold-extracted twice with n-hexane using a meal/solvent ratio of 1:10. The meal was then dried at 45°C in a vacuum oven and ground again to obtain a fine powder, called the defatted seed meal which was then kept at -20°C.

The protein component of the defatted meal was extracted using the method described by Alashi et al. *(*2014). Defatted watermelon seed meal was suspended in 0.5 M NaOH pH 12.0 at a ratio of 1:10, and stirred for 1 h to facilitate alkaline solubilization. This was then centrifuged at 18°C and 3000 g for 10 min. Two more extractions of the residue from the centrifugation process were performed with the same volume of 0.5 M NaOH and the supernatants were pooled together. The pH of the supernatant was carefully adjusted to pH 4.0 to achieve acid-induced protein precipitation using 0.5 M 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 0.1 M NaOH, freeze-dried and the protein isolate was stored at -20°C until required for further analysis.

Preparation of pigeon pea seed protein hydrolysates

The protein isolate was hydrolyzed using the methods described by Onuh et al. (2015) with some modifications. The conditions for hydrolysis were optimized for each enzyme to facilitate efficient proteolytic activity. Hydrolysis was performed using each of pepsin (pH 2.2, 37ºC), trypsin (pH 8.0, 37ºC) and papain (pH 8.0, 60ºC). The protein isolate (5 % w/v, based on the protein content of the isolate) was dissolved in the appropriate buffer. The enzyme was added to the slurry at an enzyme-substrate ratio (E:S) of 2:100. Digestion was performed at the specified conditions for 6 h with continuous stirring. The enzyme was inactivated by boiling in water bath for 15 min and undigested proteins was precipitated by adjusting the pH to 4.0 with 3 M HCl/3M NaOH followed by centrifugation at 7000 g for 30 min. The supernatant containing target peptides was then collected. Protein content of samples was determined using biuret assay method with bovine serum albumin

(BSA) as standard.

Determination of degree of hydrolysis

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

Determination of the peptide yield

The percentage peptide yield was determined using the method described by Girgih et al. (2011). It was computed as follows:

Peptide content of lyophilized hydrolysate / Protein content of unhydrolyzed protein isolate × 100

Antimicrobial screening

The antimicrobial screening of the hydrolysates against the bacterial isolates were carried out using the agar well diffusion method (Osuntokun and Olajubu, 2017). The isolates were sub-cultured from stored nutrient agar slants into nutrient agar broth and incubated for 24 h at 37ºC. A stock concentration of 100 mg/ml was constituted by dissolving 1 g each of the hydrolysates in 2.5 ml of Dimethyl sulfoxide (DMSO) and diluted with 7.5 ml of sterile distilled water making 10 ml mixture (ratio 1:3). A 12.5 mg/ml concentration of the extracts was then prepared using dilution formula. Aliquot of 1ml of standardized (0.5 MacFarland turbidity standard) broth culture was mixed with 19 ml of the agar in a sterile universal bottle and poured into sterile Petri dish. The agar plate was left to solidify and wells were bored on the agar plates using 6 mm cork borer. Fifty microliters (50 μL) of the 12.5 mg/ml concentration of the extracts was pipetted into each well, allowed to settled and incubated at 37ºC for 24 h. The diameter zones of inhibition were measured and recorded in millimeter and the results interpreted according to the Clinical Laboratory Standard Institute 2016 guidelines. Levofloxacin (0.125 mg/ml) and Dimethyl sulfoxide (DMSO) were used as positive and negative controls respectively.

Determination of DPPH radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the hydrolysates was measured by using assay method described by Arise et al. (2016a). 1 mL each enzyme hydrolysate at different concentrations (0.2 - 1.0 mg/ml) was added to 1 mL 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken vigorously and incubated in the dark and at room temperature for 30 min. The absorbance was read at 517 nm. Ethanol (95 %) was used as a blank. The control solution consisted of 0.1 mL of 95% ethanol and 2.9 mL of DPPH solution. Analyses were carried out in triplicates. Percentage inhibition of DDPH radical was calculated:

$$\% \text{ DPPH inhibition} = (Abs_{control} - Abs_{sample}) \times 100 / (Abs_{control})$$

Determination of ferric reducing antioxidant property (FRAP)

The ferric reducing power of the hydrolysates was measured according to the method reported by Olusola et al. (2018). An aliquot of 1 ml of different concentrations (0.1 – 0.8 mg/ml) of the hydrolysates (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 min 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 implied 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, 0.125, and 0.063 mM) was used for calibration. Results were expressed as mM Fe^{2+}/mg hydrolysate. All the tests were performed in triplicate.

Statistical analysis

Results were expressed as means ± standard error of mean of triplicate determinations. 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

Yields of pigeon pea protein isolate, hydrolysates and degree of hydrolysis

The percentage protein yield of pigeon pea protein isolate was 36.18%. Three different hydrolytic enzymes; pepsin, trypsin and papain were used to digest the protein isolates, and the percentage peptide yield of the resulting hydrolysates are presented in Table 1. The percentage peptide content of isolate was 65.43 ± 1.43%. Peptic hydrolysates had significantly higher ($p<0.05$) peptide yield when compared to the other two hydrolysates. The degree of hydrolysis of pigeon pea protein hydrolysates is presented in Figure 1. The degrees of hydrolysis were 29.48±1.79%, 8.96±0.72 and 10.82±1.08% for peptic, tryptic and papain hydrolysates respectively. Results are expressed as means± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different from one another.

Antimicrobial activity

The antimicrobial activities of pigeon pea protein hydrolysates are illustrated in Table 2. The hydrolysates and the control were screened against seven bacterial strains. All hydrolysates demonstrated significantly lower ($p<0.05$) antimicrobial activities when compared to the control (Levofloxacin). Among the protein hydrolysates, peptide digests obtained from papain digestion showed the highest ($p<0.05$) activity against *Staphylococcus*

Table 1. Peptide yield of pigeon pea protein hydrolysates.

Hydrolysate	Peptide yield (%)
Peptic hydrolysates	89.03 ± 3.10[a]
Tryptic hydrolysates	25.72 ± 1.79[c]
Papain hydrolysates	75.38± 4.13[b]

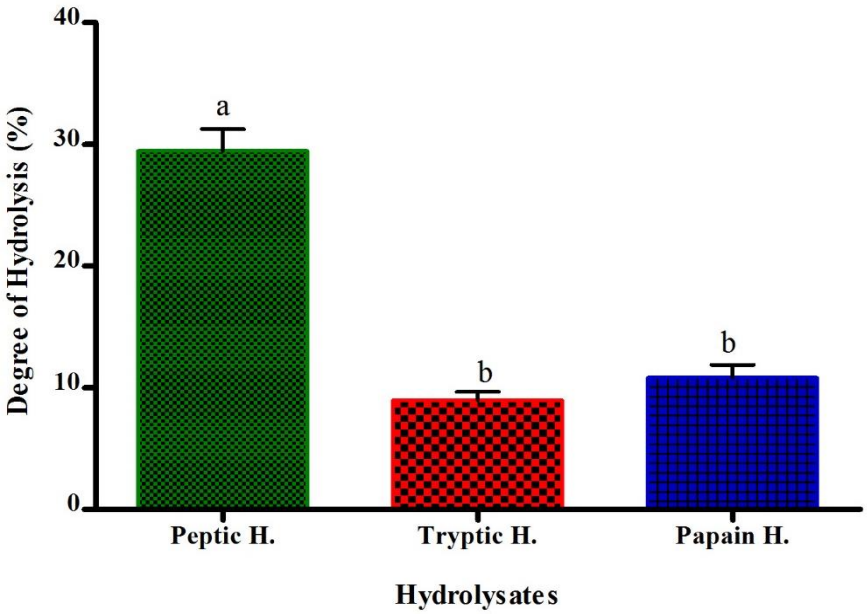

Figure 1. Degree of hydrolysis of pigeon pea protein hydrolysates. Bars are expressed as means± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different from one another.

aureus when compared to peptic and tryptic hydrolysates. Papain hydrolysates also showed activities against *Acinetobacter baumanni, Escherichia coli* and *Salmonella* species. Peptic hydrolysates showed higher ($p<0.05$) activity against *S. aureus* than tryptic hydrolysates, and also displayed antibacterial effects when screened against *Enterobacter cloacae* and *Vibrio* species. Tryptic digests exhibited the lowest ($p<0.05$) activity against *S. aureus*, but showed antibacterial effects against *Klebsiella pneumonia.*

DPPH radical scavenging activity

Figure 2 displays the DPPH radical scavenging activity of ascorbate (control) and pigeon pea protein hydrolysates, at a concentration range of 1.0 to 2.0 mg/ml. The hydrolysates had significantly ($p<0.05$) lower scavenging activities when compared to ascorbate at all study concentrations. Peptic hydrolysates showed a concentration-dependent decrease in DPPH scavenging activity, such that it attained 22.27±1.07% at 0.5 mg/ml and 9.99±1.82% at 2.0 mg/ml. Tryptic and papain hydrolysates, on the other hand demonstrated a concentration-dependent increase in DPPH scavenging activity, reaching a maximal scavenging activities of 75.52±1.69 and 26.04±1.08%, respectively at a concentration of 2.0 mg/ml. Tryptic hydrolysates displayed the highest ($p<0.05$) DPPH radical scavenging activity when compared to those of peptic and papain hydrolysates.

Ferric reducing antioxidant properties (FRAP)

The Ferric reducing antioxidant properties of ascorbic acid (control) and the hydrolysates are displayed in Figure 3. All hydrolysates had significantly ($p<0.05$) reduced antioxidant activities at different concentrations when compared to ascorbic acid. Peptic, tryptic and papain hydrolysates achieved FRAP values of 5.59±0.20, 9.32±0.49 and 5.85±0.21 mM Fe(II) respectively.

Table 2. Antimicrobial activity of pigeon pea protein hydrolysates.

Microorganism	Diameter of zone of inhibition (mm)			
	Control (Levofloxacin)	Peptic hydrolysates	Tryptic hydrolysates	Papain hydrolysates
Staphylococcus aureus	28.067±0.191[a]	15.540±0.216[c]	14.769±0.119[d]	19.530±0.205[b]
Enterobacter cloacae	22.200±0.125[a]	13.600±0.368[b]	0.0	0.0
Klebsiella pneumonia	30.233±0.232[a]	0.0	15.960±0.245[b]	0.0
Acinetobacter baumanni	30.533±0.921[a]	0.0	0.0	22.611±0.354[b]
Escherichia coli	24.167±0.223[a]	0.0	0.0	16.170±0.330[b]
Vibrio spp.	25.867±0.196[a]	16.941±0.098[b]	0.0	0.0
Salmonella spp.	24.823±0.756[a]	0.0	0.0	17.745±0.272[b]

Results are expressed as means± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values bearing different superscripts are significantly different from one another.

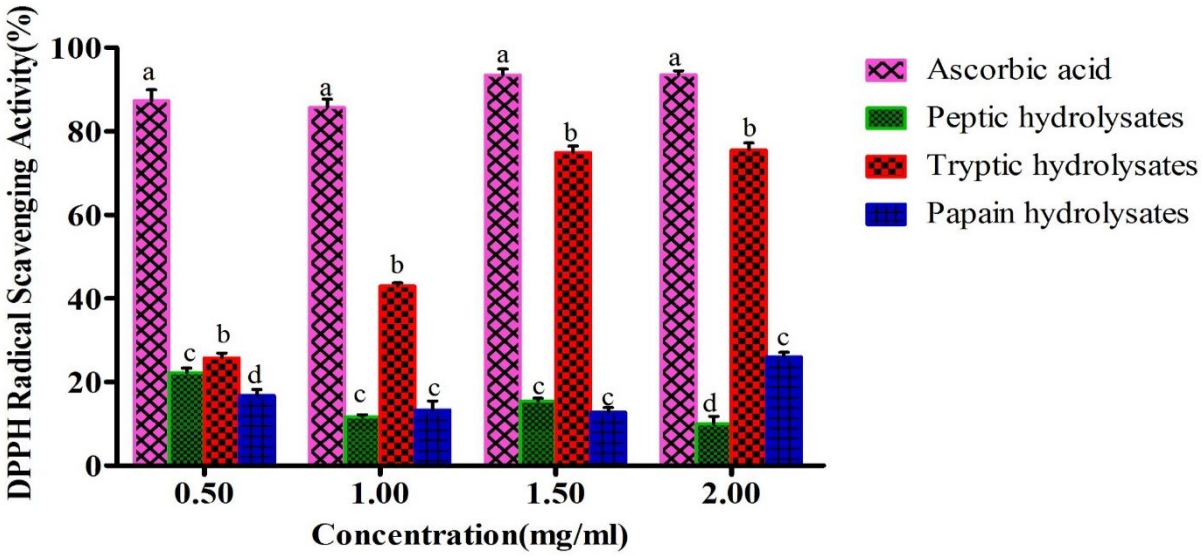

Figure 2. DPPH radical scavenging activities of pigeon pea protein hydrolysates. Bars are expressed as means± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different from one another.

At all concentrations, there was no significant difference in the activities of peptic and papain hydrolysates. However, tryptic hydrolysates had significantly ($p<0.05$) higher antioxidant activities than the other two hydrolysates at all study concentrations.

DISCUSSION

Pigeon pea protein isolate

The percentage protein yield of 36.18% obtained for pigeon pea protein isolates was higher than 18.91 and 18.60% obtained for *Citrullus lanatus* and *Moringa oleifera* seed proteins respectively (Arise et al., 2016a; Olusola et al., 2018). It is also found to be higher than 21% in another study on crude pigeon pea proteins (Eltayab et al., 2010) obtained by means of proximate analysis. This may be due to differences in methods used in isolating the proteins, varying techniques of protein determination, as well as the nature and the relative abundance of these proteins in the seeds.

The method of alkaline solubilization followed by acid precipitation has been widely used and considered effective in isolating proteins (Garza et al., 2017). Pigeon pea proteins are abundant in globulins, since they are legumes and they constitute the major portion of the protein isolate.

Peptide yield and degree of hydrolysis

Peptide yield is one of the determinants used to determine the efficiency of the hydrolysis process (Alashi

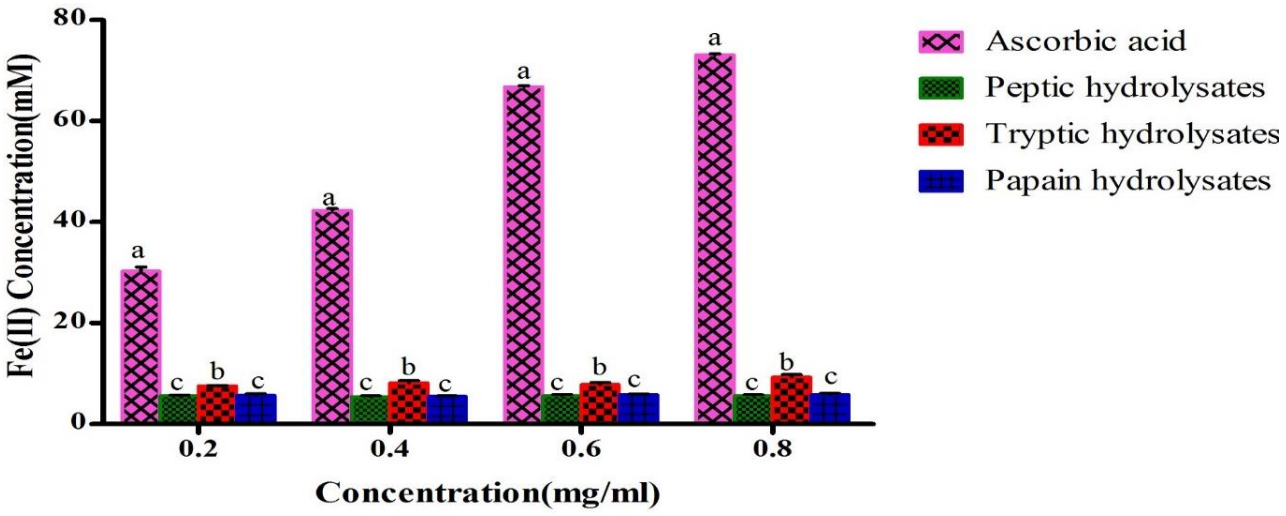

Figure 3. Ferric reducing antioxidant properties of pigeon pea protein hydrolysates. Bars are expressed as means± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

et al., 2014). Peptic hydrolysates had the highest yield of 89.03%, and this is significantly higher than 68.90 and 32.33% obtained by for peptic digests of *C. lanatus* seed protein hydrolysates (Arise et al., 2016b) and *Arachis hypogea* seed protein hydrolysates (Olusola and Ekun, 2018), respectively. The relatively high yield obtained implies that pigeon pea proteins just as other legumes, contain several hydrophobic residues that constitute sites for proteolysis by pepsin (Egbuonu, 2015). Papain hydrolysates with a peptide yield of 75.38%, was higher than that obtained by trypsin hydrolysates. Papain is a relatively non-specific proteolytic enzyme when compared to trypsin, and this has the effect of the former hydrolyzing more peptide linkages, especially those involving basic amino acids such as arginine and lysine residues after bulky residues such as phenylalaine, resulting in increased peptide yield (Olusola et al., 2018).

Moreso, proteins from legumes are abundant in positively charged amino acids (Lopez-Barrios et al., 2014; Egbuonu, 2015), which implies more cleavage sites for the enzyme papain. Tryptic hydrolysates with a yield of 25.72% had the lowest peptide yield in this study. This might be due to the high specificity of trypsin, for positively charged residues lysine and arginine only (Voet and Voet, 2011), which could have limited the yield of pigeon pea protein hydrolysis by trypsin and hence reduced the peptide yield. The degree of hydrolysis determines the number of cleaved peptide linkages in a protein hydrolysate. It has direct effects on the molecular weights and amino acid compositions of the peptide and as a result, influences their eventual functional properties (Jamdar et al., 2010; Arise et al., 2016b; Olusola et al., 2018). The degree of hydrolysis for each of the hydrolysates is presented in Figure 1. Peptic hydrolysates had the highest degree of hydrolysis, with a value of 29.48± 1.79%. This value is significantly higher than 26.933± 0.668% obtained by Olusola et al. (2018) for peptic hydrolysates from *M. oleifera* seed proteins. This might be a direct result of the broad specificity of the enzyme pepsin, with preferences for peptide cleavage at hydrophobic and acidic amino acid residue leading to the generation of several peptides of different sizes (Voet and Voet, 2011). Tryptic hydrolysates had a degree of hydrolysis 8.96± 0.72% which was significantly lower than 10.305± 0.072% obtained for tryptic hydrolysates of *M. oleifera* seed proteins (Olusola et al., 2018), and also lesser than 21.26 ± 0.76% determined for tryptic digests of *Cryptozona bistralis* proteins (Ulagesan et al., 2018). This could be as a result of low enzyme-substrate ratio of the reaction mixture, such that there were limited enzyme active sites avaliable for binding and hydrolysis of the protein molecules.

Antimicrobial activity

Three different protein hydrolysates (peptic, tryptic and papain hydrolysates) were analyzed for their antibacterial activity against seven (7) bacterial strains which include *S. aureus, E. cloacae, K. pneumoniae, A. baumanni, E. coli, Vibrio* spp. and *Salmonella* spp. Levofloxacin was used as control. Peptic hydrolysates had activity against *S. aureus, Vibrio* spp. and *E. cloacae*. Tryptic hydrolysates had activity against *S. aureus* and *K. pneumoniae,* while papain hydrolysates displayed the broadest specivity against 4 bacterial strains (*S. aureus, A. baumanni, E. coli* and *Salmonella* spp). There is a relative paucity of research regarding the antimicrobial properties of protein hydrolysate preparations. The relative broad specificity of hydrolysates obtained from

papain digestion was consistent with the reports of Ulagesan et al. (2018) who had previously stated that hydrolysis obtained from papain digestion had antibacterial activity against *S. aureus* and *Pseudomonas aeruginosa.* Though, in this study, papain hydrolysates, at a concentration of 12.5 mg/ml, had a lower zone of inhibition against *S. aureus* when compared to Ulagesan et al. (2018). This may be due to differences in protein source (which invariably give rise to hydrolysates with varying peptide and amino acid compositions) and enzyme/substrate ratio used in hydrolysis.

Protein hydrolysates and peptides which contain cationic residues in their composition have potential antimicrobial activity. This is because their respectively charged residues interacts with the cell membranes of the bacteria, disrupting membrane intergrity and consequently lysing the cells (Nguyen et al., 2011). This also accounts for the antimicrobial activites of peptic and tryptic hydrolysates. It is therefore suggested that the amino acid sequences is closely related to the antimicrobial activity of the protein hydrolysis (Nguyen et al., 2011; Ulagesan et al., 2018). However, this result contrasts with the reports of Ratnayani et al. (2017) that protein hydrolysates obtained from germinating pigeon pea proteins digested by pancreatin had no antibacterial activity against *S. aureus* and *E. coli*. This could be that the germinating sprouts may have used up essential amino acids and proteins in the seed for growth processes which could have otherwise contributed to their antimicrobial activity. Also, it could be due to the nature of the proteolytic enzyme used in their work, as quite a number of reports (Arise et al., 2016b; Olusola and Ekun, 2019) have demonstrated that choice of hydrolytic enzymes used in protein digestion, as a result of differing amino acid cleavage specificities, can determine the biofunctionalities of peptides and protein hydrolysates.

DPPH radical scavenging activity

The DPPH radical has a single electron and shows maximum absorbance at 517 nm. It is commonly used to determine the scavenging activity of some organic compounds such as phenolics, flavonoids as well as peptides and protein hydrolysates (Sun et al., 2013). As DPPH reacts with a proton-donating substances, the radical is scavenged, leading to a reduction in absorbance (Arise et al., 2016a). The DPPH radical scavenging activities of pigeon pea protein hydrolysates in comparison to ascorbic acid (control) were presented in Figure 2. Tryptic hydrolysates displayed the higest DPPH radical activity at all study concentrations and had a value of 75.52±1.69% at a maximum concentration of 2.0 mg/ml. This is higher than the 47.10±1.33% obtained at a maximum concentration of 2.5 mg/ml for tryptic digests of *C. lanatus* seed proteins (Arise et al., 2016a).

However, in this study, peptic hydrolysates had a maximum scavenging activity of 22.27±1.07% at a concentration of 0.5 mg/ml, which was lower than 56.39±1.07% obtained for peptide fragments of *C. lanatus* derived by pepsin digestion, as reported by Arise et al. (2016a). The observed results may be due to the nature of the aminoacyl residues present in the peptide sequences released during hydrolysis. Li and Li (2013) reported that the nature of the amino acid at the C-terminal region determines antioxidant activity of peptide fragments. Also, trypsin being able to cleave at C-terminal ends of positively charged residues (Voet and Voet, 2011), could release bioactive peptides with net positive charges, which could act as proton donors to neutralize the DPPH radical.

Ferric reducing antioxidant power

Ferric reducing antioxidant power assay is also widely used to determine the ability of naturally occurring substances from plant and animal sources to donate protons to ferric ions *in vitro* (Olusola and Ekun, 2018). The ability of bioactive compounds to reduce Fe^{3+} to Fe^{2+} ions is strongly correlated with their antioxidant properties. The hydrolysates generally exhibited low ferric reducing properties at all concentrations in this study, when compared to ascorbate. This is comparable to a previous study on fractionated cobia skin gelatin hydrolysates by Razali et al. (2015) which revealed a similar trend of results. One reason could be that pigeon pea proteins have low amounts of cysteinyl aminoacyl residues which are normally strong contributors to antioxidant properties of peptides, owing to their thiol-containing side chains (Udenigwe and Aluko, 2011; Lopez-Barrios et al., 2014). Also, in this study, tryptic hydrolysates showed higher ferric reducing activity than peptic and papain hydrolysates. However, peptic and tryptic hydrolysates, in this study had lower ferric reducing properties than those obtained for cowpea seed protein hydrolysates (Olusola and Ekun, 2019). This could be that trypsin, being highly specific for cleaving at C-terminals of lysine and arginine residues released peptides that were able to reduce ferric ions *in-vitro.* In addition, a range of other factors which include plant source, hydrolysis time, enzyme:substrate ratio, degree of hydrolysis, among other conditions also contribute to the antioxidative properties of peptides (Olusola and Ekun, 2019).

Conclusion

The subjection of pigeon pea proteins to enzymatic hydrolysis using selected enzymes yielded hydrolysates which displayed antimicrobial and antioxidant activities. Protein digests obtained from papain hydrolysis displayed

the broadest antibacterial activity while tryptic hydrolysates exhibited the strongest antioxidant effects. Fractionation of these hydrolysates and characterization of the resulting peptides is recommended, which is already underway, so as to further elucidate their effects and also to harness their potential as cost effective and safer alternatives to current antimicrobial agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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