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Original article

Invitro α-amylase Inhibitory Activity and Antioxidant Profile of Carica Papaya Seed Protein Hydrolysate

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Abstract

Carica papaya, a plant belonging to the *Caricaceae* family produces pawpaw fruit; a very useful plant commonly found in most of the tropical African countries, with Nigeria being the third largest producer worldwide. Protein hydrolysates from Pawpaw (*Carica papaya*) seeds were examined for *in-vitro* α-amylase inhibitory and antioxidant activities. Pawpaw seeds proteins were isolated and subsequently hydrolyzed by trypsin and pepsin. The degree of breakdown by trypsin (40.97 ± 0.18%) was more ($p < 0.05$) than pepsin (33.60 ± 0.23%). The peptide yield of tryptic hydrolysis (7.35 ± 0.17%) was significantly higher than that of peptic hydrolysis (5.13 ± 0.04%). All the hydrolysates, including the standards, show α-amylase inhibitory activities and antioxidant activities in a concentration-increasing manner. Trypsin hydrolysate showed the highest α-amylase inhibition (63.64 ± 1.55%). 50% effective concentration (EC50) for Pepsin hydrolysate (0.61 ± 0.02 mg/ml) was lower than trypsin hydrolysate (0.66 ± 0.01 mg/ml). The trypsin hydrolysate displayed the higher ferric reducing antioxidant power (FRAP) and H2O2-Scavenging activities (5.8mg/ml and 22.54 ± 0.12% respectively) while pepsin hydrolysate showed the best DPPH-scavenging activity (75.99%). The results, therefore, suggest that *C. papaya* seeds protein hydrolysates may serve as a food source with curative properties like antioxidant capacity and inhibitory effect against α-amylase.

Keywords: *Carica Papaya*, hydrolysate, antioxidant, α-amylase, inhibitory-activity.

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INTRODUCTION

Pawpaw is the edible part of the *Carica Papaya* plant that belongs to the *Cariceae* family. It is a plant with an inestimable worth that is grown all over tropical Africa with Nigeria being the third-largest producer globally, (FAO 2016). Recently, a lot of attention has been given by researchers and health caregivers on the functions and positive effects of the fruit, leaves and latex of *C. papaya* with little or no emphasis on the seeds. The *Papaya* seeds account for up 16% of the total fresh fruit weight and each seed has an endosperm and sarcotesta (Pungasari *et al.,* 2005). Several studies by researchers revealed that extracts from every part of the plant have a positive effect and have been used against various diseases (Mello *et al.,* 2008).

Diabetes mellitus, a worldwide spread disease, is rampant in most countries and it has multiple biochemical impairments, (Jyothi *et al.,* 2013). The case of diabetes mellitus is in the increase at a very high rate in the world annually. From the statistics of the world health organization, there are about 346 million persons having diabetes mellitus. Ninety percentage (90%) amongst them are suffering from Type 2 Diabetes Mellitus (T2DM) (Jyothi *et al.,* 2013). T2DM is predominantly because of the inefficiency of the body to utilize insulin. The most complex form of diabetes mellitus is postprandial hyperglycemia, which involves a decrease in insulin secretion after meal and increase in glucagon secretion ultimately leading to an increase in postprandial plasma glucose. Carbohydrate hydrolyzing enzymes such as pancreatic alpha-amylase can be inhibited resulting in retarded absorption of glucose. This reduces the risk of postprandial diabetes (Arise *et al.,* 2019).

Alpha-amylase (E.C 3.2.1.1) is an extracellular endoenzyme that catalyses the hydrolysis of α -D-(1, 4)-glycosidic linkages in starch, glycogen and oligosaccharides in a random manner, liberating reducing groups such as glucose and maltose. Most of the drugs used in managing diabetes can trigger the uptake of insulin, discharge insulin from the pancreas, inhibition of α-amylase and α-glucosidase (Baynes, 2015). The primary worries in taking these drugs are the consequences and the drug resistance after lengthy treatment. To curtail these concerns and to figure out inhibitors of alpha-amylases from natural bases is now a major area of research by a scientist, (Jyothi *et al.,* 2013). The hunt for less harmful and acceptable anti-diabetic agents is also stressed by the world health organization.

Antioxidants are substances required in minute quantities for the prevention or retardation of the formation and accumulation of free radicals. Exposure to radiation, smoking, some physiological processes and some of the ways through which free radicals are produced leading to degenerative diseases (Udenigwe and Aluko, 2012). When the body cannot bear the excessive physiological free

radical production, various functions of the immune cells can be impaired by the action of the free radicals worsening different oxidative stress-related diseases like cancer, cardiovascular diseases, etc. (Amit and Priyadarsini, 2011). Due to the less complicated structures of peptide antioxidants to their parent proteins, they possess high stability in the event of heat and exposure to proteases. This makes them non-antigenic and seldom shows enhanced nutritional and functional properties, as well as the, possess antioxidant activity (Xie *et al.,* 2008).

Protein hydrolysates have been defined as mixtures of polypeptides, oligopeptides and amino acids that are from protein sources (Schaafsma *et al*., 2009). They are gotten by the hydrolysis (enzymatic, acid or alkali and heat hydrolysis) of complete protein and are frequently used as a protein supplement in commercial products and are acknowledged effective sources of bioactive peptides. Enzymatic hydrolysis works without destructing amino acid and by avoiding the extreme temperatures and pH levels (Celus *et al*., 2007). A variety of peptides that act against thrombosis, hypertension, microbes, cancer, free radicals, and have immune-modulatory property have been established (Sanchez and Vazquez, 2017). Taking protein hydrolysates with these peptides is potentially supportive in controlling various ailments (Li-Chan, 2015; Dhaval *et al.,* 2016). Therefore, this study tested the *in-vitro* α-amylase inhibitory activity as well as the antioxidant property of *Carica papaya* seed protein hydrolysate.

MATERIALS and METHODS

Materials

Carica Papaya seeds were acquired at Tudun Wada Market in Gombe, Gombe State, Nigeria, and were authenticated at the Herbarium of the Department of Botany, Gombe State University, where a voucher number 58 deposited. The seeds were shade dried and pulverized in a blender. Analytical grade chemicals and reagents were used devoid of any additional refinement. α-amylase, trypsin (from bovine pancreas), pepsin (from porcine gastric mucosa), dinitrosalicylic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Acarbose and Starch were products of Sigma-Aldrich (USA), Trizma-base, n-hexane, trichloroacetic acid (TCA) products of BDH Chemical Limited (Poole, England).

Methods

Preparation of defatted Carica Papaya Seeds Powder

The defatted powder of *Carica Papaya* seed was obtained as described by Sze-Tao snd Sathe, (2004) with slight modification. *Carica Papaya* seeds were ground in a blender. For 1 hour, the powdered seeds were extracted in n-hexane (with a weight to volume ratio of 1:10) by constantly mixing in a beaker and excess solvent filtered. This was done two times. The flakes devoid of fat were opened on a tray and left under a fume cupboard for six hours to dry and to get rid of any remaining solvent. The dried powder was poured in sample bottles and kept at -10^{o}C

Isolation of Defatted Carica Papaya Seeds Protein

Protein constituent of the defatted meal was isolated by alkali solubilization and acid precipitation method according to Alashi *et al*. (2014). Defatted *Carica Papaya* flakes were poured (1:10) in 0.1 M NaOH at a pH of 12.0, mixed for 1 hour and spun for 10 minutes at 18°C and 3000 rpm in a refrigerated centrifuge. This was repeated three times and the supernatants kept. One molar hydrochloric acid solution was added to the supernatant until the pH was 4.0, it was then spun and precipitate obtained washed with distilled water. In adjusting the pH to 7.0, 1M sodium hydroxide was added in drops. The precipitate was freeze-dried and the *C. Papaya* Seed Protein Isolates (PSPI) was refrigerated until needed for enzymatic hydrolysis.

Protein Yield of Isolate

Following isolation, the protein yield was calculated using the following expression as used by Arise *et al.*, (2015).

$$ProtienYield(\%) = \frac{Massofproteinisolate\ (g)}{MassofDefattedCarica\ papayaseedmeal\ (g)} X\ 100\%$$

Protein Content of Isolate

The percentage protein content of isolate was obtained as a percentage ratio of peptide content of isolate (actual/experimental concentration) to the concentration of lyophilized isolate on which protein determination assay was run (theoretical concentration), (Alashi *et al*., 2014).

Preparation of Carica Papaya Seed Protein Hydrolysates

Methods used by Udenigwe *et al*. (2009) was employed to hydrolyze the protein isolate. Hydrolysis was carried out at body temperature by each of Pepsin (pH 2.2) and Trypsin (pH 8.0,). 5g of PSPI was solubilized in 100ml of phosphate buffer at alkaline pH for trypsin hydrolysate and glycine buffer at acidic pH for pepsin hydrolysate and the pepsin and trypsin were added at a ratio of 1:100 (E:S). For 5hrs the reaction proceeded, after which it was immersed into boiling water for 15min. The pH of the solution was set to 4.0 then spun at 4000rpm for 30min. The supernatant was pipetted out, the extent of enzymatic breakdown determined and then lyophilized. The dried hydrolysate termed PSPH was kept in a refrigerator until required for further analysis.

Assay of Peptide Degree of Hydrolysis

The Degree of hydrolysis (DH) was obtained by computing the percentage of protein that dissolves in 10% trichloroacetic acid divided by the total protein content of the isolate as done by Hoyle and Merritt, (1994). 1ml each of protein hydrolysates and 20% TCA was poured into a beaker to yield 10% TCA soluble solution. After 30mins, the mixture was centrifuged at

4000rpm for 25mins. The solution above the sediment was analyzed for protein content by the Biuret method put forward by Gornall *et al.* (1949) using BSA as a reference. The extent of enzyme breakdown was determined by the following expression:

$$\mathrm{DH} = \frac{\text{Dissolved short amino acids in 10\% Trichloroacetic Acid (mg/mL)}}{\text{Overall protein amount of PSPI (mg/mL)}} \mathrm{X}\ 100$$

Assay of DPPH free radical scavenging activity of seed protein

The DPPH radical-scavenging activity of *Carica papaya* seed protein hydrolysate was carried out as reported by Arise *et al.* (2016). 0.05 mM of DPPH was dissolved in absolute ethanol and *Carica papaya* seed hydrolysates were added to distilled water. A portion (0.60 ml) of *Carica papaya* seed hydrolysate (0.2 – 1.0 mg/ml) was mixed with 1.00ml DPPH (0.05mM). The mixture was shaken strenuously and brooded at 25^{o}C for 30 min. Light absorption was measured at 517 nm. Distilled water was measured as blank and 0.60 ml distilled water and 1.50 ml DPPH as control. Percentage of DPPH$^{\cdot}$ inhibition was obtained as follows.

$$\mathrm{DPPH}^{\cdot} - \text{scavenging Activity (\%)} = \frac{\mathrm{Absorbance}_{\mathrm{control}} - \mathrm{Absorbance}_{\mathrm{sample}}}{\mathrm{Absorbance}_{\mathrm{control}}} \times 100\%$$

The 50% Effective Concentrations, EC50 of PSPH and vitamin C were transcluded from a non-linear regression graph of DPPH-scavenging ability against PSPH concentrations with GraphPad Prism version 6.0. Experiments were carried out three times

Hydrogen Peroxide Scavenging Activity

PSPH ability to scavenge hydrogen peroxide was carried out as reported by Arise *et al.* (2016). 4mM H_2O_2 was made and poured into a 0.20M phosphate buffer of pH 7. 1.00mL of PSPH at the concentration range of 0.20 to 1.0 mg/ml in distilled water was mixed with 0.15 ml H_2O_2 solution. The mixture was left to stand for 10mins and the absorbance taken at 230nm against a blank solution that has the phosphate buffer only. The absorbance of H_2O_2 was used as the control, and ascorbate as the reference antioxidant. H_2O_2 scavenging ability was obtained by the expression below.

$$\mathrm{H_2O_2}\text{-scavenging Capacity (\%)} = \frac{\mathrm{Absorbance}_{\mathrm{control}} - \mathrm{Absorbance}_{\mathrm{sample}}}{\mathrm{Absorbance}_{\mathrm{control}}} \times 100$$

The EC50 of PSPH and vitamin C were transcluded from a non-linear regression graph of H_2O_2-scavenging ability against PSPH concentrations with GraphPad Prism version 6.0. The procedure was carried out three times

Ferric Reducing Antioxidant Power

Reducing Fe^{3+} by PSPH was carried out as reported by Chen *et al.* (2009). Briefly, 100µL of hydrolysates with various concentrations were mixed with 0.7 mL of 0.2mol/L sodium phosphate buffer and 2mL of 30 mmol/L potassium ferricyanide mixture and was brooded for 25min at 45^{o}C, then 2mL of 10% trichloroacetic acid solution added. Then, the mixture was spun at 3000rmp for 10min. 1 ml supernatant was finally mixed with 3mL of 1.7 mmol/l of aqueous ferric chloride, and the absorbance was then taken at 700 nm. Reducing power varies directly to the absorbance of the mixture.

α-Amylase Inhibition Assay

The method by Bernfield (1951) was employed in α-Amylase Inhibition determination as reported by Arise *et al.,* (2016). 125µL of various concentration of the hydrolysate (0.2 – 1.0 mg/mL) was added to test tubes and the same volume of 20mM sodium phosphate buffer (pH 6.9, 6mM NaCl) having 0.5mg/ml α-amylase mix was added to the test tube. The content of the various tubes was pre-incubated for 10min at room temperature, followed by adding 125µL of 1% starch mixture in 20mM sodium phosphate buffer at pH 6.9 having 6mM sodium chloride at various times. The mixture was allowed for 10min. at room temperature. The process was completed by dispensing 250 µL of dinitrosalicylic acid as a color reagent and again brooded for 5min in hot water and then left to cool. Absorbance was taken at 540nm after the addition of 2.5ml of distilled water. The same procedure was followed for the control but with distilled water in place of the hydrolysate. The α-amylase inhibitory activity was evaluated using the expression below.

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

The hydrolysate concentration that gives 50% inhibition of α-Amylase activity (IC_{50}) was obtained using a graph of percentage inhibition versus the various hydrolysate concentrations with GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA, USA).

Statistical Analysis

All values are presented as mean ± standard deviation (SD), and Analysis of variance and Tukey's multiple range tests were applied with the use of GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA, USA). Values were said to be significant at $p < 0.05$.

RESULTS and DISCUSSION

The proteolysis checking feature called the degree of hydrolysis (DH) is seldom applied to obtain the level of peptide bonds cleavage (Jrad, 2014). There is a greater DH ($p < 0.05$) by tryptic digestion when compared to peptic digestion indicating that trypsin hydrolyzes *Carica papaya* seed protein to a greater extent (Table 1). This means that the hydrolysate might consist of more positively charged amino

acids to which trypsin is specific (Naik, 2012). The DH values could be indicative of the length of the peptide formed because higher values mean shorter chain length of peptides, while lower values indicate a longer peptide chain (Malomo *et al.,* 2015). Pepsin with a lower DH (33.60%) suggests that the hydrolysate may contain longer peptide chains and trypsin with a higher DH (40.97%) may have bioactive peptides with shorter chain length (Malomo *et al.,* 2015).

The extent of breakdown by pepsin resulting from this research is higher than the one obtained for the protein of hemp and neem seeds, hydrolyzed with the same enzyme (Malomo *et al.,* 2015, Arise *et al.,* 2019). Arise *et al.,* (2016), also obtained a lower value of tryptic hydrolysis (26.26 ± 0.27%) for watermelon seeds than the one obtained in the present study (40.97 ± 0.18%). Similarly, 21.79 ± 0.77% reported earlier for the hydrolysis of whey by trypsin had the highest value (Kamau *et al.,* 2010). Australian canola and hemp seeds were reported to have lower values of DH than the one reported in this study when pepsin was used for the hydrolysis (Girgih *et al.,* 2011; Alashi *et al.,*2014).

The peptide yield reported in this study for peptic breakdown (5.13%) was significantly less than earlier stated for *Azadirachta indica* seed (35.61%), (Arise *et al.,* 2019b). From Table 1, the decreased peptide yield for peptic hydrolysis, when compared to that of trypsin (7.35%), suggests that during hydrolysis, more biologically active peptides may have been formed by trypsin. The more excellent result for trypsin might be because it can breakdown esters and amides of amino acids and also very specific for amino acids that do not readily dissolve in aqueous solution (Naik, 2012). The peptide yield shown by peptic and tryptic breakdown of PSPH was to a greater extent lower than that reported for *Luffa cylindrica* seed peptic and tryptic breakdown with a yield of 16.93% and 34.04% respectively, (Arise, *et al.,* 2019a).

Table 1: Protein isolate yield, content and degree of hydrolysis of C. papaya seed

Parameter and Enzymes		**DH (%)**	**Peptide Yield (%)**
Yield of Isolate (%)	5.13	—	—
Protein content of isolate (%)	55.76 ± 0.27	—	—
Pepsin	—	33.60 ± 0.23[a]	5.13 ± 0.04[a]
Trypsin	—	40.97 ± 0.18[b]	7.35 ± 0.17[b]

Values represent the mean of triplicate determinations ± standard error of the mean (SEM). Results with varying alphabets in the same column are significantly different at $p < 0.05$

Alpha-Amylase inhibitory activity

Alpha-amylase is among the enzymes that participate in the breakdown of dietary starch, discharging oligosaccharides that can again be hydrolyzed to glucose, and quickly used by the body (Gropper and Smith, 2013). Therefore, inhibition of α-amylase is among the promising ways for the treatment of diabetes. Based on the result of this study (Figure 1), tryptic and peptic hydrolysates displayed an appreciable α-amylase inhibitory activity. They showed inhibitory activity which increases

as the concentration increased. Acarbose (72.53%), which is the standard displayed the best α-amylase inhibition at all concentrations, followed by trypsin hydrolysate (63.64%), while the activity of peptic hydrolysate (53.69%) was the lowest at 1.0mg/ml (Figure 1).

The nature of the cleavage product of the protein is probably responsible for the inhibition of α-amylase by tryptic hydrolysate (Naik, 2012). It can be predicted that α-amylase fancies binding to amino acid residues having a positive charge and branched-chain amino acids, e.g Tyrosine, Phenylalanine, Tryptophan, and Lysine. (Arise *et al.*, 2019a)

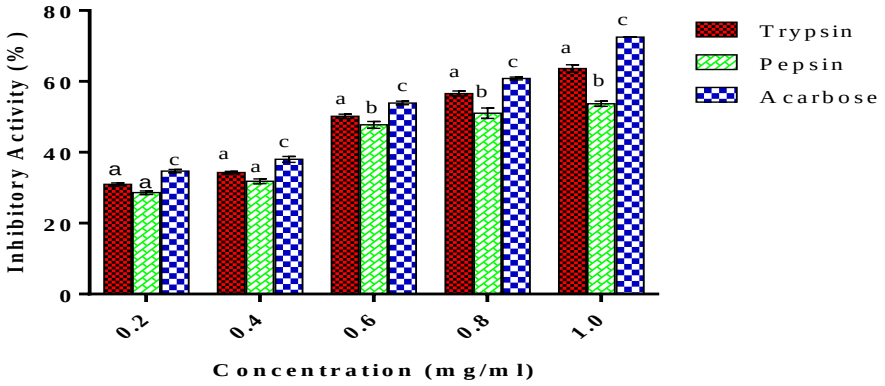

Figure 1: Alpha-Amylase Inhibitory Activities of *Carica papaya* Seeds Protein Hydrolysates

Individual bars represent the average of triple determinations ± SEM. Bars with equal concentration but varying alphabets are significantly different at p <0.05.

However, the inhibitory concentration (IC_{50}) result of *Carica papaya* seed protein hydrolysate presented in figure 2 for α-amylase inhibitory activity showed that tryptic and peptic hydrolysates had an IC_{50} of 0.58 ± 0.02mg/ml and 1.05 ± 0.02mg/ml respectively. This indicates that tryptic hydrolysate has a better inhibitory activity for alpha-amylase than peptic hydrolysate because of the lower the IC_{50} the better (Arise *et al.*, 2019a)

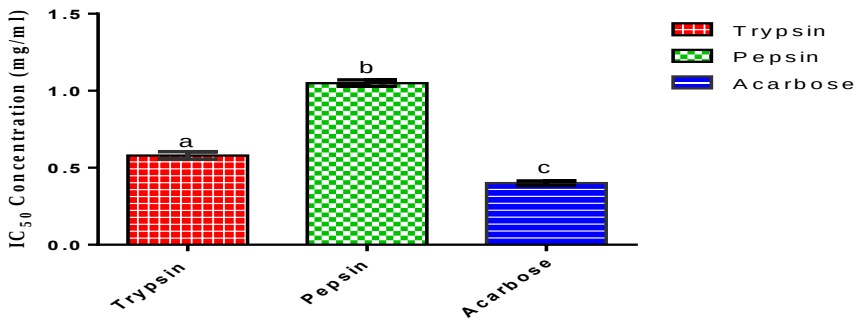

Figure 2: IC_{50} values of Carica papaya seed protein hydrolysates for α-Amylase inhibition

Individual bar denotes the mean of three values of IC_{50} ± SEM. Bars with dissimilar alphabets are different at $p < 0.05$

Antioxidant Profile

The H_2O_2-scavenging action of *Carica papaya* seed protein hydrolysates obtained in Figure 3 revealed an increasing tendency. The tryptic hydrolysate presented the most robust H_2O_2-scavenging action with 22.54% but peptic hydrolysate had a smaller value (20.72%). This could be as a result of the presence of some side chains amino acid that are existing in tryptic hydrolysate, which may perhaps have a scavenging outcome to counter H_2O_2, otherwise not shown by other oxidants. The H_2O_2-scavenging action of the two hydrolysates was significantly dissimilar ($p< 0.05$) from the standard at the several concentrations (Figure 3).

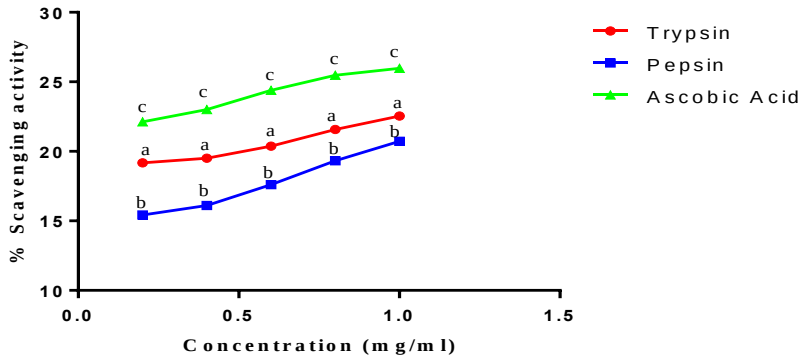

Figure 3: H_2O_2-Scavenging activities of *Carica papaya* seed protein hydrolysates.

The individual dot denotes the average of triple determinations ± SEM. Dots at equal concentration but with varying alphabets are significantly different at $p <0.05$.

The EC50 value governs the concentration of hydrolysates needed to impede 50% of the oxidant, thus commonly used to access antioxidant, antiradical, and reduction competences, (Razali et al., 2015). A small EC50 value is required as a low value indicates high effectiveness. From Figure 4, Tryptic hydrolysate obtains higher EC50 for hydrogen peroxide scavenging action (0.66 ± 0.01 mg/ml) than peptic hydrolysate (0.61 ± 0.02 mg/ml). Ascorbic acid obtains the lowest EC50 value (0.45 ± 0.01 mg/ml).

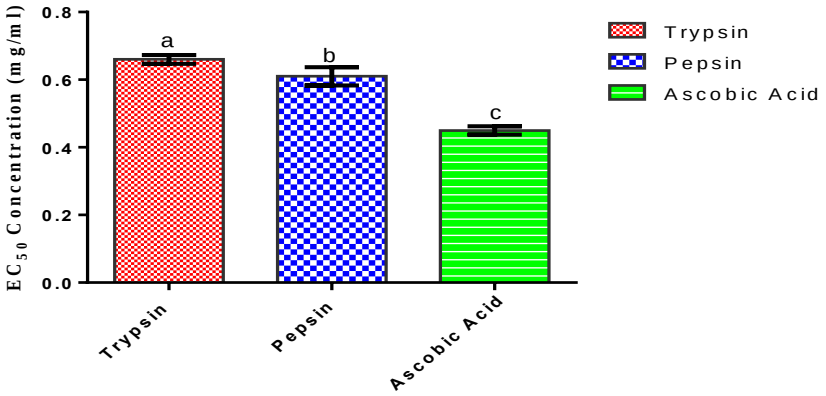

Figure 4: EC_{50} values of Carica papaya seed protein hydrolysates for H_2O_2 activity

Individual bar denotes the mean of three values of EC_{50} ± SEM. Bars with dissimilar alphabets are different at $p < 0.05$

The free radical scavenging action of hydrolysates of *C. papaya* seed protein evaluated by the DPPH scavenging assay showed a concentration-dependent inhibition of DPPH activity (Figure 5). Out of the two hydrolysates, Pepsin showed the greatest inhibition of DPPH action (75.99% at 1.0 mg/ml) and can be comparable to the scavenging action of ascorbic acid (82.53%) while trypsin hydrolysates showed a lower DPPH action (56.58%). At 0.4mg/ml, no significant difference ($p < 0.05$) exist amongst ascorbic acid and tryptic hydrolysate likewise at 0.8mg/ml, between ascorbic acid and peptic hydrolysate. The results of the DPPH-free radical scavenging action (Figure 5) suggest that the pepsin protein hydrolysate of *C. papaya* is more capable of scavenging free radicals and EC_{50} of the hydrolysates and ascorbic acid were gotten as 0.5050, 0.4267 and 0.5494mg/ml for trypsin, pepsin, and ascorbic acid respectively (Figure 6). EC_{50} of ascorbic acid was significantly higher than the peptic hydrolysate (0.4267 ± 0.0143 mg/ml) having the lowest. The lesser the EC_{50} value, the greater the antioxidant action (Arise *et al.,* 2019a). Therefore, peptic CSPH has higher antioxidant activity and can be considered a more effective antioxidant compared to ascorbic acid and trypsin in DPPH assay. The dissimilarities in the radical-scavenging ability found here could be attributed to the different sizes of the peptide and their makeup, the specificity of the hydrolyzing enzymes, the breakdown time, and conditions (Arise *et al.*, 2016)

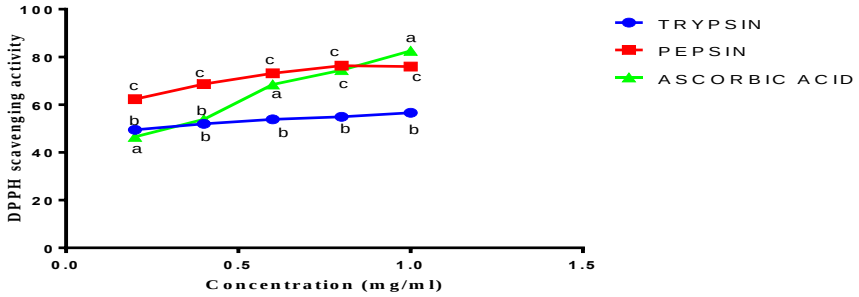

Figure 5: in vitro DPPH radical-scavenging action of C. papaya seed protein hydrolysates

The individual dot denotes the average of triple determinations ± SEM. Dots at equal concentration having varying alphabets are significantly different at p <0.05.

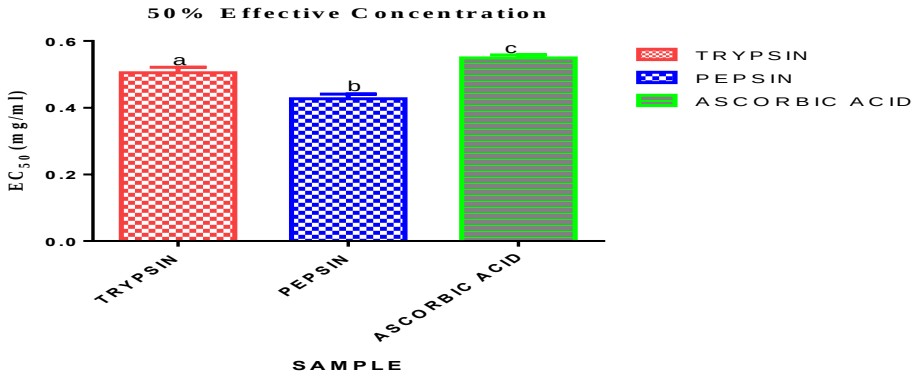

Figure 6: EC_{50} values of *Carica papaya* seed protein hydrolysates for DPPH activity

Individual bar denotes the mean of three values of EC_{50} ± SEM. Bars with dissimilar alphabets are different at p < 0.05

The ferric reducing ability of *C. papaya* seed protein hydrolysis product of pepsin and trypsin was matched with ascorbic acid at varying concentrations (Figure 7). The ferric-reducing action of both the ascorbic acid and that of the pepsin and trypsin hydrolysates was concentration-dependent. The ascorbic acid, which is the standard, shows the highest ferric reducing power (6.4 mg/ml), followed by that of trypsin hydrolysate (5.8mg/ml). However, the pepsin hydrolysate has the lowest ferric reducing power (5.3mg/ml). The greater ferric reducing the action of trypsin hydrolysate may occur as a result of the revelation of a lot of amino acid R-groups during enzymatic breakdown (Matoba, 2002). Thus, vitamin C has considerably higher ($p < 0.05$) reducing capacity at every concentration used in this study.

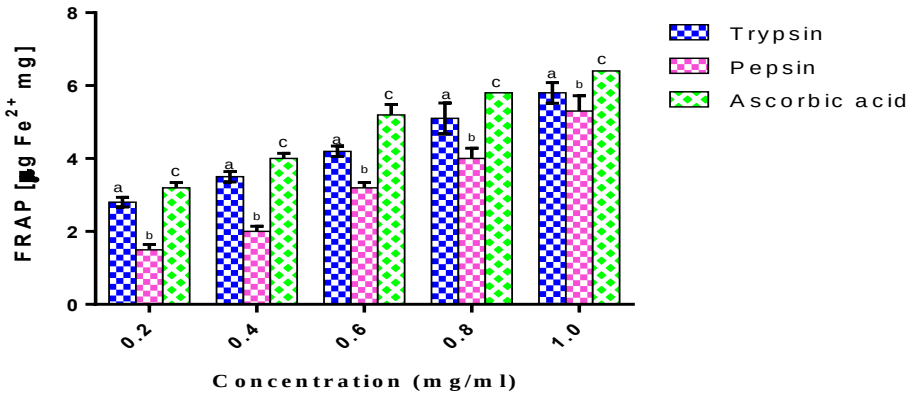

Figure 7: Ferric-reducing antioxidant ability of C. Papaya seed protein hydrolysate

Individual bars denote the average of triple determinations ± SEM. Bars at equal concentration having varying alphabets are significantly different at p <0.05.

Conclusion

The result from this study has shown that *Carica papaya* seed protein product of hydrolysis may be rich in biologically effective peptides with α-amylase and antioxidant inhibitory actions. Consequently, it may as well be exploited further as a probable substitute for synthetic anti-diabetics and antioxidants.

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