

Bioactivity Evaluation of Peptic-Tryptic Hydrolysates Derived from Cowpea (*Vigna Unguiculata*) B-Vignin: Antioxidant, A-Amylase Inhibitory, and Antimicrobial Activities

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Abstract

*Cowpea (Vigna unguiculata) is a leguminous plant of major economic and nutritional importance across the developing world, and a rich, low-cost source of protein-derived bioactive peptides. This study aimed to generate peptides from cowpea protein isolate (β -vignin) by sequential pepsin-trypsin hydrolysis and to screen the resulting hydrolysate for antioxidant, α -amylase, and antimicrobial activities. Cowpea protein isolate was extracted by isoelectric precipitation and subjected to sequential peptic-tryptic hydrolysis. The peptic-tryptic hydrolysate obtained was evaluated for DPPH free radical scavenging activity, α -amylase inhibitory activity, and antimicrobial activity by agar well diffusion against *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Candida sp*. DPPH scavenging activity of the hydrolysate (13.25–17.05% across 50–400 $\mu\text{g/mL}$) was significantly lower ($p < 0.05$) than that of the ascorbic acid standard (85.92–87.23%). In contrast, the hydrolysate showed dose-dependent α -amylase inhibition, rising from 11.40% at 200 $\mu\text{g/mL}$ to 67.63% at 500 $\mu\text{g/mL}$. No antibacterial or antifungal activity was observed against any of the test organisms at concentrations of 250–1000 $\mu\text{g/mL}$, whereas the standard drugs cefuroxime and fluconazole produced clear inhibition zones. These findings indicate that peptic-tryptic hydrolysis of cowpea (β -vignin) protein yields peptide hydrolysates with weak antioxidant and no detectable antimicrobial activity, but appreciable α -amylase inhibitory potential. These findings support further investigation of cowpea-derived peptides as potential sources of α -amylase inhibitors relevant to the modulation of postprandial carbohydrate digestion.*

Keywords: Cowpea; β -vignin; bioactive peptides; enzymatic hydrolysis; α -amylase inhibition; antioxidant activity; functional food.

Introduction

In recent years, the value of food protein has increased considerably because of growing recognition of their potential to serve as sources of physiologically active peptides (Chakrabarti et al., 2018). Bioactive peptides are protein-derived food fragments that have a beneficial impact on health when ingested by humans and show various possible physiological roles in the body (Kaur et al., 2019). During digestion in the gastrointestinal tract, peptides are released by enzymatic proteolysis. Peptides can also be released in vitro by protein hydrolysis using food-grade proteases and during food processing (cooking, ripening and fermentation) (Abdel-Hamid et al., 2017).

Studies have shown that the processing of bioactive peptides is mainly through enzymatic hydrolysis of food proteins or fermentation. (Lee & Hur, 2017). The production of bioactive peptides by hydrolysis provides a controlled approach for generating bioactive peptides because hydrolysis conditions, including enzyme specificity, pH, temperature, hydrolysis time, and enzyme-to-substrate ratio, can be systematically regulated. A major factor to consider is the enzyme to substrate ratio in order to obtain a high degree of hydrolysis (Sangsawad et al., 2017; Zhang et al., 2016). The sequence of a particular peptide and its bioactivity depend on the type of enzyme used for hydrolysis (Mojica & de Mejía, 2016). Following hydrolysis, the melange is purified and evaluated for bioactivity and finally identified (Xu et al., 2019). The peptides are then purified using one-dimensional or two-dimensional separation techniques to quickly desalt and then separated based on their sizes (Daliri et al., 2017). Peptides with low molecular weight less than 3 kDa have shown high effectiveness as antioxidants and antihypertensive than peptides with high molecular weight, therefore, enzymes that produce low molecular weight peptides would be valuable in large production of antihypertensive and antioxidant peptides (Ketnawa et al., 2018; Wattanasiritham et al., 2016). Diseases including cardiovascular diseases, hypertension, type 2 diabetes, cancer, etc. have become prevalent globally with millions dying annually due to complications from them. Although synthetic drugs have been shown to be quite effective in the treatment of these conditions, safety of these compounds remains a concern (Singh et al., 2021). In addition, repeated use of any drug has its own negative effects that can lead to health degeneration and in turn raise the cost of health care. As a result of this, there is a need for holistic therapies through safe lifestyle choices and early interventions for disease prevention (Girgih et al. 2014). Bioactive peptides from many dietary protein sources including cowpea have shown to be beneficial to health by functioning as antimicrobial (Zanutto-Elgui et al., 2019), anticancer (Karami et al.), anti-inflammatory (Hao et al., 2019), antioxidant (Tonolo et al., 2019), etc. These characteristics are the reasons they are tagged as biologically-active peptides.

Peptides from cumin seed have been found to possess antidiabetic functions which they exert by suppressing the activity of α -Amylase. They were observed to act by cutting down starch absorption from the gastrointestinal tract through inhibition of cleavage of large starch moieties into simpler permeable ones, in this way confining assimilation of starch (Siow & Gan, 2016). Peptides from rice bran and cumin seed have cholesterol reducing effects. Cholesterol build-up in the coronary artery may reduce blood flow and thus, supply of oxygen to the heart and incite illness of the cardiovascular system (Singh et al., 2014). These cholesterol lowering peptides act by diminishing cholesterol dissolvability in lipid vectors, and limiting the uptake of cholesterol in a few monolayer cells. Bioactive peptides confer particular cytotoxicity of harmful tissues through different means. A specific peptide HVLSRAPR obtained from *S. platensis* hydrolysates was found to have strong inhibitory effect against HT-29 cancerous cell division however, it showed small limitation against standard liver cells (González-Montoya et al., 2018).

Cowpea (*Vigna unguiculata*) is a leguminous plant originated in the African continent with big economic and societal importance in the developing world. Cowpea is a food that is of great importance to millions of people, especially in less developed tropical countries, and is the main source of protein diets for a large part of the world's population. (Baptista et al., 2017). Amino acid analysis showed that cowpea seeds are made up of high levels of aspartic and glutamic acids, leucine, lysine, arginine as well as isoleucine but limiting in cysteine and tryptophan (Ilesanmi & Gungula, 2016). Cowpea seed extracts have been investigated for its antioxidant (Sombié, 2018), antibacterial (Osman, 2021), hypoglycemic activities. Its rich protein content makes it an excellent source of biologically active protein hydrolysates and peptides (Jayathilake, 2018).

Various studies on cowpea protein have shown that they have peptides with biological activities. In a study by Venancio et al. (2003), cowpea protein isolate was detailed to have a similar molecular weight and amino acid sequence as bovine insulin, and thought to be a possible functional food with antioxidant and anti-diabetic properties. In vitro research on the impact of cowpea peptides on lipid metabolism showed that cowpea peptides may function as antioxidants and inhibit the enzyme required for biosynthesis of cholesterol, and its micellar solubility (Marques et al., 2015). This study therefore aims to synthesize peptides from cowpea protein by enzymatic hydrolysis and to screen the peptides for antioxidant, anti-inflammatory, and α -amylase inhibitory bioactivities.

Materials and Methods

Sample Collection

Vigna unguiculata (cowpea) var. *Ifebrown* was obtained and identified from the Institute of Agricultural Research and Training (IAR&T), Ibadan, Oyo State.

Preparation of Cowpea Flour

The cowpea seeds were sorted to remove extraneous material such as stones, sand, chaff, metals, unhealthy seeds, and insect infested seeds. Cowpeas seeds were thoroughly rinsed in distilled water, soaked for 5mins at room temperature (25°C) and dehulled manually. The dehulled seeds were oven dried at 40°C for 36 hours. The seeds were milled to flour and sieved through a mesh nylon filter. The cowpea flour was defatted by soaking in hexane (1:5w/v) for 18 hrs with intermittent shaking. The hexane was decanted and the mixture filtered using Whatman No. 1 filter paper. The defatted flour was allowed to air-dry in a fume hood until residual solvent had evaporated, after which it was pulverized and stored in an airtight container until protein extraction.

Extraction of Cowpea β -vignin

The extraction and of cowpea seed protein was carried out according to the method of Frota et al. (2018). The defatted cowpea flour obtained was stirred in a low ionic strength buffer (50 mM Tris-HCl and 200 mM NaCl, pH 8.5), 1:10 (w/v) for 2 hours at room temperature to solubilize the protein. This slurry was filtered through muslin cloth, and the residue was discarded. The filtrate was centrifuged at 10,000 x g for 20 minutes; the supernatant was removed and the pellet discarded. The pH of the supernatant was adjusted to 4.5 (vicilin isoelectric point) to facilitate acid-induced protein precipitation using 1M HCl. The mixture was refrigerated for 12 hours at 4°C for the protein to precipitate. After this, the suspension was centrifuged at 10,000 x g for 20 minutes. The precipitated protein fraction was collected as the cowpea protein isolate, lyophilized, and stored at -20°C until further analysis.

Enzymatic Hydrolysis Using Pepsin and Trypsin

The protein isolate was hydrolysed using the method described by Udenigwe et al. (2009) 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 solution) was dissolved in phosphate buffer (pH 2.2 for pepsin). The enzyme was added to the slurry at an enzyme-to-substrate (E:S) ratio of 2:100 (w/w). Digestion was performed at the specified conditions for 4 hours with continuous stirring, pepsin was inactivated by heating the reaction mixture at 95-100°C for 15 min. After cooling to room temperature, the pH was adjusted to 8.0 using 2 M NaOH, and trypsin was added at an E:S ratio of 2:100 (w/w). Hydrolysis was continued

for 4 h at 37°C under continuous stirring. Trypsin was subsequently inactivated by heating at 95–100°C for 15 min. Trypsin was added to the slurry at an E:S ratio of 2:100 and incubated for another 4 hrs with continuous stirring. The enzyme was inactivated by boiling in water bath (95–100°C) for 15 min.

Filtration and Partial Purification of Cowpea Hydrolysate

The hydrolysate was filtered through a sterile 0.22 µm nylon membrane syringe filter to remove particulate matter and obtain a clarified sample for subsequent assays

The sample was loaded into the syringe and the syringe attached securely to the filter by a twisting motion. The syringe plunger was then pressed gradually to push the sample through the filter.

Determination of Protein Concentration of the Hydrolysate

Protein concentration of the hydrolysate was determined using a modified Biuret method with bovine serum albumin (BSA) as the standard.

DPPH Radical Scavenging activity (2, 2-diphenyl-1-picrylhydrazyl)

DPPH has been extensively used to determine the free radical scavenging activity of samples due to its relative stability. The DPPH activity was measured using the method of (Wang et al., 2012). Ascorbic acid served as a standard.

Different concentrations of the hydrolysate (50 – 400 µg/mL) were added to 0.5 mL of distilled water (DH₂O) and 1mL of 0.2 mM DPPH in 95% methanol in various test tubes and incubated for 30 mins at room temperature in the dark. This group was treated as the experimental group. The control sample was prepared by adding 1mL of DH₂O and 1mL of DPPH in a test tube. In addition, 95% methanol substituted for DPPH solution in the blank sample. The absorbance was read using a UV-Vis spectrophotometer (BOSCH, Germany) at 517nm. The antioxidant activity of the sample was evaluated by the half inhibition concentration (IC₅₀). DPPH radical scavenging activity was calculated with the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{1 - A_i - A_j}{A_0} \right) \times 100$$

where A_i is the sample absorbance, A₀ and A_j are the absorbance of control group and the blank, respectively.

α-Amylase Inhibitory Activity of Cowpea β-vignin Hydrolysate

The α-amylase-inhibitory assay was measured to determine the hypoglycemic activity of the partially purified isolate according to the method described by Xiao et al. (2006) with slight modifications.

Varying concentrations of the hydrolysate (200 - 500 µg/mL) were pipetted into test tubes containing 0.2 - 1mL of 20 mM sodium phosphate buffer (pH 6.9, with 6 mM NaCl) and 1.5ml α-amylase solution. The content of each tube was incubated in a water bath (Lemfield Medical, England) at 37°C for 10 min, after which 0.5mL of 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9, with 6 mM NaCl) was added and incubated for another 15mins. 0.06mL of 1 M HCl was added to stop the enzymatic reaction, followed by the addition of 0.3mL of iodine reagent (5 mM I₂ and 5 mM KI). The absorbance was read at 620 nm after noting the color change. The control reaction representing 100% enzyme activity did not contain any plant extract. To eliminate

the absorbance produced by plant extract, appropriate extract controls without the enzyme were also included. In the starch-iodine assay, the intensity of the blue colour reflects the amount of residual starch, with greater colour intensity indicating reduced starch hydrolysis.

$$\% \text{ Inhibition} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

The IC₅₀ value was estimated from a dose response curve of an inhibitor versus the enzyme activity.

Antimicrobial Activity of Cowpea β-vignin Hydrolysate

Antimicrobial assay of cowpea hydrolysate was carried out by agar well diffusion method in Mueller Hinton Agar (MHA) plates according to the method of Manandhar et al. (2019).

Before the assay was carried out, the yeast isolate was identified as *Candida* spp., while *Klebsiella pneumoniae* and *Staphylococcus aureus* were identified using Gram staining and the Microbact™ 12A identification system.

Procedure

The test organisms were inoculated in nutrient broth and incubated overnight at 37°C to adjust the turbidity to 0.5 McFarland standards giving a final inoculum of 1.5×10^8 CFU/ml. MHA plate was lawn cultured with standardized microbial culture broth. Cowpea hydrolysate of 50 mg/ml concentration was prepared in Dimethyl Sulfoxide (DMSO). Four wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer (6 mm) and each well filled with 50μl each of hydrolysate, standard drug, undigested protein and phosphate buffer at various concentrations (0.25 – 1mg). Following loading of the wells, the plates were allowed to stand at room temperature for 30 min to permit diffusion and were subsequently incubated at 37°C for 18–24 h. Zones of inhibition were measured in millimetres. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of cowpea extract. The zone of inhibition (ZOI) will be observed and measured in mm. Positive control, (cefuroxin and fluconazole) for bacteria and yeast respectively.

Statistical Analysis

Data were analysed using GraphPad Prism version 6.0. Differences among treatment groups were evaluated using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test to determine the significant difference between samples at $p < 0.05$ level. Results were expressed as mean ± standard error of mean.

Results and Discussion

DPPH radical scavenging activity

The free radical scavenging activity of cowpea hydrolysate and ascorbic acid (standard) at different concentrations (50-400μg/mL) are presented in Figure 4.3. From the result, the DPPH radical-scavenging activity of the cowpea protein hydrolysate increased significantly with increasing concentration. However, the activity of the hydrolysate remained significantly lower ($p < 0.05$) than that of ascorbic acid across the concentrations tested. This indicates that ascorbic acid had a higher free radical scavenging activity than the hydrolysates.

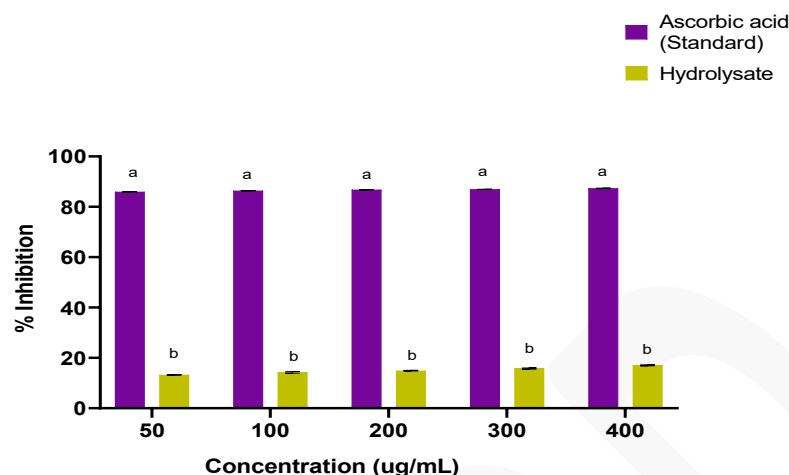


Figure 1: Effects of peptic-tryptic cowpea protein hydrolysate and Ascorbic acid (standard) on DPPH radical scavenging activity

Alpha-amylase Activity of β -vignin hydrolysate

Increasing concentrations of the cowpea protein hydrolysate produced progressively greater inhibition of α -amylase activity, as indicated by increasing residual starch in the starch-iodine assay. This occurred in a dose dependent manner giving a light brown to dark-blue color complex, whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolyzed by α -amylase.

The result in Figure 4.4 shows the different degree of alpha amylase inhibitory activity using starch as the substrate. The cowpea protein hydrolysate exhibited concentration-dependent inhibition of α -amylase activity, with inhibition increasing from $11.40 \pm 0.38\%$ at $200 \mu\text{g/mL}$ to $67.63 \pm 0.35\%$ at $500 \mu\text{g/mL}$.

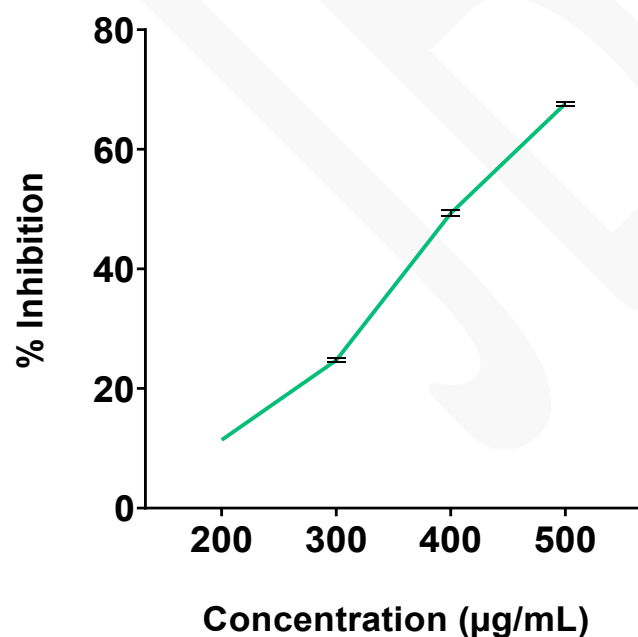


Figure 2: Inhibitory effect of cowpea protein hydrolysate on α -amylase activity

Antimicrobial Activity of β -vignin hydrolysate

As shown in Table 4.1, no detectable antibacterial or antifungal activity was observed for the hydrolysate against the test organisms at the concentrations evaluated. The standard drug cefuroxime showed activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* while fluconazole showed activity against *Candida sp.*

Table 1: Zones of Inhibition (mm) of Treatment Groups Against Microbial Strains at concentrations 0.25-1mg/ml

Treatment Group	Concentration / Load	<i>Klebsiella pneumoniae</i>	<i>Staphylococcus aureus</i>	<i>Candida sp.</i>
Phosphate Buffer	All levels	0	0	0
DMSO Solvent	All levels	0	0	0
Undigested Cowpea Isolate	0.25–1.0 mg	0	0	0
Cowpea Hydrolysate	0.25–1.0 mg	0	0	0
Standard Drug*	0.25 mg	27	31	35
	0.50 mg	29	32	37
	1.00 mg	31	33	39

Cefuroxime was used as standard drug for bacteria (Kleb. & Staph.) while fluconazole was used as the standard drug for yeast (Candida).



Figure 3: Plates showing the inhibitory ability of some test subjects on *Candida albicans* at different concentrations

Standard drug (S.D), filtered cowpea hydrolysate,

Undigested cowpea protein (U.D), phosphate buffered saline (buffer).



Figure 4: Plates showing the inhibitory ability of some test subjects on *Staphylococcus aureus* at different concentrations
Standard drug (S.D), filtered cowpea hydrolysate,
Undigested β -vignin protein (U.D), phosphate buffered saline (buffer).



Figure 5: Plates showing the inhibitory ability of some test subjects on *Klebsiella pneumoniae* at different concentrations

Standard drug (S.D), filtered cowpea hydrolysate,
Undigested β -vignin protein (U.D), phosphate buffered saline (buffer).

Food-derived bioactive peptides have attracted increasing attention because of their potential antioxidant, enzyme-inhibitory, antimicrobial, and other physiological activities. Their biological properties are influenced by peptide sequence, molecular size, hydrophobicity, charge, and the enzymes used to generate them (Koehbach & Craik, 2019; Mitra, et al., 2019).

A study on cowpea seed peptide by Xiong et al., (2013), reported a DPPH scavenging rate of about 70% (at 10mg/ml) which is higher than 17.05% (at 400µg/ml) DPPH scavenging activity of peptic-tryptic cowpea protein hydrolysate recorded in this study; however, their assay was conducted at a much higher concentration than that used in the present study. Therefore, direct comparison of the absolute percentage inhibition should be interpreted cautiously. A study on alcalase hydrolysed soybean (Zhang et al., 2019) showed a higher scavenging activity of 47.4% (at 1.0mg/ml) than that recorded in this study. Another study by Arise et al., (2016) on peptic *Citrullus lanatus* seed protein hydrolysate showed a higher DPPH scavenging activity $56.39 \pm 0.45\%$ (at 2.5 mg/mL) which was also substantially higher than the activity observed for the cowpea hydrolysate in this present study. The relatively low DPPH activity observed may reflect differences in peptide composition, peptide concentration, molecular size distribution, amino acid sequence, hydrolysis conditions, or assay conditions compared with previously reported cowpea hydrolysates and peptides. The inhibition of α -amylase enzyme in the digestive tract of human is being considered to be effective in controlling hyperglycaemia by decreasing the absorption of glucose from starch (Gautam et al., 2013). Since high amylase inhibition results in many harmful side effects in human beings, low level of alpha - amylase inhibitors from natural fruits, vegetables and legume grains are reported to offer a good strategy to control postprandial hyperglycaemia over the synthetic drugs (Abeykoon et al., 2021). The extent of inhibition by peptic-tryptic hydrolysates in this study (67.63%) is slightly lower than 70.19% reported by Arise et al. for peptic hydrolysates of *Citrullus lanatus* seed proteins. Also, the percentage inhibition of alpha amylase of a study on peptic hydrolysates of cowpea seed protein by Ekun and Olusola (2019) showed a higher percentage of 74.91%. These findings suggest that the cowpea hydrolysate contains components capable of inhibiting α -amylase in vitro and therefore warrants further investigation as a potential source of peptides relevant to the modulation of postprandial carbohydrate digestion. Cowpea seeds are especially rich in aspartic and glutamic acids, leucine, arginine, lysine and isoleucine but limiting in cysteine and tryptophan (Ilesanmi & Gungula, 2016). Also, a study by Arise et al. (2016), proposed that α -amylase binds to peptides containing cationic residues such as Lys and branched chain residues such as Phe, Tyr and Trp. The observed inhibitory activity may partly reflect the peptide sequences generated by the combined action of pepsin and trypsin. Trypsin preferentially cleaves peptide bonds on the carboxyl side of lysine and arginine residues, whereas pepsin preferentially cleaves peptide bonds involving hydrophobic or aromatic residues. Such cleavage patterns may generate peptide sequences with physicochemical characteristics favourable for interaction with α -amylase. The standard drugs used exhibited antimicrobial activity against the test organisms at various concentrations, whereas no detectable inhibition was observed for the cowpea hydrolysate under the experimental conditions used. In contrast to the present findings, Abdel-Shafi et al. (2019), detected the antimicrobial activity of 7S and 11S globulin isolated from *Vigna unguiculata* against a number of organisms. They found different degrees of antimicrobial activity against some tested gram positive and gram-negative bacteria including *Klebsiella pneumoniae* and *Staphylococcus aureus*. A peptide derived from a Japanese soybean fermented food showed antibacterial activity against *Streptococcus pneumoniae* and *Bacillus subtilis* group but no activity was recorded for *S. aureus* and *Candida albicans* which is similar to this study (Kitagawa et al., 2017). Ib-M peptides (containing 6-20 amino acid units) have shown high bacterial inhibitory activity against *Escherichia coli* in a study done by Prada-Prada et al. (2020).

Conclusion

Sequential pepsin–trypsin hydrolysis of cowpea (*Vigna unguiculata*) protein generated a hydrolysate with concentration-dependent α -amylase inhibitory activity in vitro. In contrast, the hydrolysate exhibited relatively weak DPPH radical-scavenging activity compared with ascorbic acid and showed no detectable antibacterial or antifungal activity against the tested organisms under the assay conditions. The findings indicate that the hydrolysate may represent a potential source of α -amylase-inhibitory peptides. Further studies involving peptide fractionation, sequence identification, structural characterization, and validation using additional biological models are required to establish the specific peptides responsible for the observed activity.

Ethical Statement

Not applicable.

Availability of Data and Material

All data is published in this research article.

Informed Consent

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

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