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Research Article

Prediction and Evaluation of Bioactive Properties of Cowpea Protein Hydrolysates

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Cowpea protein hydrolysates were prepared using thermolysin, alcalase, and trypsin and analysed for bioactive properties, and then, the release of bioactive peptides was investigated *in silico*. It was found that the degree of hydrolysis reached 48% after 24 h hydrolysis with alcalase. The hydrolysate prepared using alcalase showed higher ACE inhibitory (62%) and DPPH scavenging activity (19%). SDS-PAGE analysis revealed that vignin was the major protein in cowpea protein isolate. *In silico* analysis indicated the presence of potential bioactive peptides with potent bioactivity in the primary structure of proteins. The 3D structure of proteins was built, upon which bioactive peptides were mapped using their location in the primary structure. The secondary structure and solvent accessible surface around each bioactive peptide were then calculated. On this basis, the higher degree of hydrolysis and bioactive properties of cowpea protein hydrolysate prepared by alcalase were explained, and structural factors influencing the release of bioactive peptides were investigated.

1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is a valuable crop widely cultivated in the world, and increasingly in Africa, due to its important nutritional properties and the particularity to resist to heat and drought. Production and consumption of cowpea are far beyond that of common dry beans in Africa, where cowpea is the second most important legume after groundnut (*Arachis hypogea*) [1, 2]. Cowpea seeds are rich in protein (22–30%, dwb) with all essential amino acids and low in fat content although they contain poly-unsaturated fatty acids [3]. Their proteins present high functional properties, particularly solubility and emulsion capacity. Cowpea seeds also contain essential minerals and vitamins. Cowpea protein is then an important ingredient in food and non-food formulations. Recently, cowpea consumption has been linked to the prevention of cardiovascular disease (CVD), type-2 diabetes (T2D), and cancer. These properties were related to bioactive peptides encrypted in the protein sequences and polyphenols [4, 5]. Bioactive peptides are usually released through the action of enzymes or microorganisms or by the gastrointestinal proteases. Efficient release of these bioactive peptides from cowpea protein could enhance the application of cowpea hydrolysates in food formulations and then contribute to reducing the high prevalence of CVD and T2D in developing countries.

Generally, enzymatic hydrolysis of plant proteins produced hydrolysates which exhibited a high potential for inhibiting key enzymes for CVD and T2D, as well as cancer cell proliferation and antioxidant properties [6]. Segura Campos et al. [7] hydrolyzed cowpea protein extract with alcalase, flavourzyme, and pepsin-pancreatin and obtained degrees of hydrolysis ranging from 38 to 59%, IC_{50} for angiotensin-converting enzyme inhibitory activity ranging from 1.4 mg/mL to 2.6 mg/mL, and hydrolysates also showed significant antioxidant activity. Lower values for ACE inhibitory activity were obtained by Rudolph et al. [8] when hydrolyzing plant proteins with chymotrypsin and thermolysin. Furthermore, our previous study on the hydrolysis of Bambara bean protein with alcalase, thermolysin, and trypsin produced hydrolysates with high ACE inhibitory

activity and significant antioxidant properties [9]. Many computer-aided approaches for evaluating the release of bioactive peptides from known proteins sequences have been developed recently. Generally, the primary sequence of proteins is used for simulating hydrolysis using known proteases, and the peptides released are compared with those in databank of already characterized peptides with defined activity. These techniques are widely used actually and the profile of bioactive peptides predicted *in silico* is usually observed during *in vitro* experiments. However, the conformation of legume proteins impacts the interaction with proteases and the release of bioactive peptides [10]. Therefore, modulating the biological activity of protein hydrolysates through the control of protein structure will contribute to improving their nutraceutical potential.

Studies on hydrolysis of β-lactoglobulin by trypsin clearly showed that the conformation of this protein influenced the hydrolysis rate and release of individual peptides. Production of bioactive peptides could be improved by denaturation of protein, although the hydrolysis rate was decreased [11]. Furthermore, it was demonstrated in plant proteins that increase in β-sheet secondary structure increased the stability and resistance to gastrointestinal proteases, while α-helix and turns were more accessible to enzymes. In this study, we evaluated the antioxidant and angiotensin-converting enzyme (ACE) inhibitory activity of cowpea protein hydrolysates *in vitro* and compared the results with those obtained by an *in silico* approach. The aim of this study was to analyse the bioactive properties of cowpea protein hydrolysates prepared by alcalase, thermolysin, and trypsin and to investigate the release of bioactive peptides by *in silico* methods. At the end of this work, a structural basis for understanding the release of antioxidant and ACE inhibitory peptides by enzymatic hydrolysis of cowpea protein isolate will be provided.

2. Materials and Methods

2.1. Materials. Cowpea seeds were purchased at the local market of Maroua (Far North region, Cameroon). Alcalase (from *Bacillus licheniformis*, ≥2.4 U/g protein), thermolysin (from *Geobacillus stearothermophilus*, 0.03–0.17 U/g protein), trypsin (from porcine pancreas, 1.5 U/g protein), and ACE (from rabbit lung) were obtained from Sigma Aldrich Chemie (Steinheim, Germany). Hippuryl-L-histidyl-leucin (HHL) was purchased from Bachem AG (Bubendorf, Switzerland). All chemicals were of the highest purity available.

2.2. Preparation of Cowpea Flour. The seeds were cleaned and ground into fine flour (mesh size 200 μm), and then, the flour was defatted using hexane at a liquid-to-solid ratio of 1 : 5 (w/v), according to Maguire et al. [12]. The defatted flour was dried at 45°C in an oven and stored at 4°C in the refrigerator.

2.3. Preparation of Cowpea Protein Isolate (CPI). Protein isolation was done as described by Mune and Sogi [13], with slight modifications. Protein was extracted at pH 9 and then washed twice with distilled water (the pH was adjusted to 4.5 using 1M HCl) for 15 min under stirring. Protein was recovered by centrifugation at 8,000 ×g for 20 min and 4°C. Afterwards, proteins were resuspended in distilled water containing about 5% (w/v) total solids, and the pH was adjusted to 7.0 under stirring. The protein suspensions were then freeze-dried. Moisture and protein content of the protein isolate were determined following AOAC [14] methods, 950.46 and 928.08, respectively.

2.4. SDS-PAGE Electrophoresis. The molecular weight profile of the protein isolate was established using SDS-PAGE according to the method of Adebowale et al. [15].

2.5. Enzymatic Hydrolysis. Enzymatic hydrolysis of CPI was performed as described by Lunow et al. [16] with slight modifications. Protein solutions at 5% (w/v) in double-distilled water were incubated in the presence of alcalase (enzyme/substrate ratio, ESR, of 4%, pH 7), trypsin (ESR of 1%, pH 7) at 55°C, and thermolysin (ESR of 1%, pH 8) at 70°C. The selected enzyme doses were found efficient for the release of bioactive peptides from plant proteins [8]. The reaction proceeded for 24 h in an autoclaved closed-top vessel, and the pH was adjusted every 30 min using NaOH 0.5 M. The enzymatic reaction was stopped by heat inactivation at 95°C for 5 min. The mixture was then centrifuged at 8,000 ×g for 10 min at 4°C, and the supernatant was freeze-dried and used for further analysis.

2.6. Degree of Hydrolysis. The degree of hydrolysis was determined as a percentage of free amino groups following reaction with TNBS [17]. The total number of amino groups was determined in a sample by complete hydrolysis using 6 N HCl at 120°C for 24 h. Isoleucine (0.2–3.0 mM) was used as a standard.

2.7. Bioactive Properties Evaluation. The ACE inhibitory activity of cowpea protein hydrolysates (CPH) was measured via RP-HPLC and UV detection, using the substrate HHL as described by Lunow et al. [16].

The scavenging effect of CPH on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was measured according to the method of Zhu et al. [18].

2.8. BIOPEP Analysis. The FASTA sequence of each identified protein was retrieved from the GenBank database at http://www.ncbi.nlm.nih.gov/sites/entrez. The cleavage sites of the enzymes within the protein sequences listed in Table 1 were predicted using Peptide Cutter (http://web.expasy.org/peptidecutter/) [19]. The profiles of potential ACE and antioxidant peptides generated using thermolysin, alcalase, and trypsin were predicted by BIOPEP (https://biochemia.uwm.edu.pl/biopep-uwm/). Following *in silico* digestion, BIOPEP was also used to compare the generated peptides with previously described bioactive peptides in the database.

Table 1: Proteins identified in cowpea protein isolate and their GenBank identity.

N°	Protein	Abbreviation	Source	Mass (Da)	Length (amino acid)	GenBank id
1	8S globulin beta isoform precursor (*Vigna radiata*)	8SG	*Vigna radiata*	51.782	453	ABG02262.1
2	7S globulin-3	7SG3	*Vigna angularis*	49.759	433	2EAA_A
3	Vicilin protein	VIC	*Vigna unguiculata*	49.685	433	CAP19902.1

Furthermore, the quantitative parameters of proteolysis were calculated, including the theoretical degree of hydrolysis (DHt), the frequency of the release of peptides with ACE inhibitory and antioxidant activity by selected enzymes (AE), and the potential biological activity of the protein (B, in mM^{-1}). The calculation of these parameters was done using the following equation:

$$DHt = \frac{dx100}{D}, \quad (1)$$

where d is the number of hydrolyzed peptide bonds and D is total number of peptide bonds in a protein chain.

$$AE = \frac{d}{N}, \quad (2)$$

where d is the number of peptides with specific bioactivity in a protein sequence that can be released by the enzyme, and N is the number of amino acid residues of protein.

$$BE = \frac{\left(\sum_{i=1}^{k} (ai/EC50i)\right)}{N}, \quad (3)$$

where ai is the number of repetitions of ith bioactive fragment, EC50 is the concentration of half-maximum activity of ith protein fragment, mM, k is the number of different fragments with a given activity.

2.9. Molecular Modelling of Cowpea Isolate Proteins and Evaluation of Secondary Structure. Three-dimensional models of the three major proteins presented in the SDS-PAGE analysis of cowpea protein isolate were predicted using the I-TASSER algorithm using their amino acid primary sequence [20]; [21].

The Gromacs 6.2.1 (Groningen University, Netherlands) package [22] was further used to optimize and equilibrate the resulting 8SG, 7SG3, and VIC models. The topologies of the models were defined with OPLS force fields. A weak coupling Berendsen thermorestat was used to prepare the proteins by tempering the solvated protein models at temperature of 55°C and 70°C, in agreement with the hydrolysis conditions. The ionization state of amino acid residues was fixed manually and interactively in Gromacs according to the hydrolysis pH used for alcalase, trypsin, and thermolysin. Afterwards, the system was equilibrated at constant temperature using a HP EliteBook Workstation (8570w, core i7, 7-cores, 2.4 GHz, and 16 GB RAM). The particle mesh method was used for calculating the electrostatic interactions using a Coulomb cutoff of 1.7 nm, and the Lennard–Jones potential and a switching function was employed for the van der Waals interaction. Dedicated *in silico* tools specific to Gromacs, VMD [23] and PDBsum [24], were finally used for characterizing the protein models equilibrated at different temperatures and pH.

2.10. Statistical Analysis. All experiments were replicated at least three times. Mean values with standard deviations were reported. Means were compared by one-way ANOVA, and Tukey post hoc was applied to check the significant differences among means. The computer software used in this study was SPSS (version 10.1, 2000, SPSS Inc., USA).

3. Results and Discussion

3.1. Characterization of Cowpea Protein Isolate. Protein content of CPI was 94.93% (in dwb) and found in the range of those reported for isolates prepared from other seeds such as Bambara bean [13]. The SDS-PAGE pattern of CPI under reducing conditions showed three main bands with molecular masses, respectively, of 58 kDa, 52 kDa, and 47 kDa (Figure 1). Previous studies on cowpea seeds showed that albumin and globulin were the two major water extractable proteins and represented more than 96% of the whole seed proteins. The major globulin in cowpea seeds was vignin with three components termed α-vignin with a sedimentation coefficient of 16.5 S, β-vignin with a sedimentation coefficient of 13 S, and a minor component termed γ-vignin. Moreover, vignin presented a particular thick 60 kDa band in SDS-PAGE analysis [25]. In this regard, the band at 58 kDa in Figure 1 probably represents vignin. The amino acid sequence of the main globulin in cowpea seeds was determined using genetic techniques and identified as GenBank id. CAP19902.1 [26]. On the other hand, Zhang and Li [27] identified two major water-soluble proteins in cowpea seeds with molecular masses of 57 and 51 kDa. These authors made molecular analyses of the two proteins and found high similarity with those found in the NCBInr database, identifying them as 8S globulin beta isoform precursor (*Vigna radiata*) with a molecular mass of 52 kDa (GenBank id. ABG02262.1) and 7S globulin-3 (*Vigna angularis*) with a molecular mass of 50 kDa (GenBank id. 2EAA_A). From the above, the bands with molecular masses 47 and 52 kDa in Figure 1 were assigned as 7S globulin-3 (*Vigna angularis*) and 8S globulin beta isoform precursor (*Vigna radiata*), respectively. Therefore, the amino acid sequences of the three proteins with GenBank identities CAP19902.1, ABG02262.1 and 2EAA_A were retrieved from the database and used for *in silico* analysis and molecular dynamics studies (Table 1).

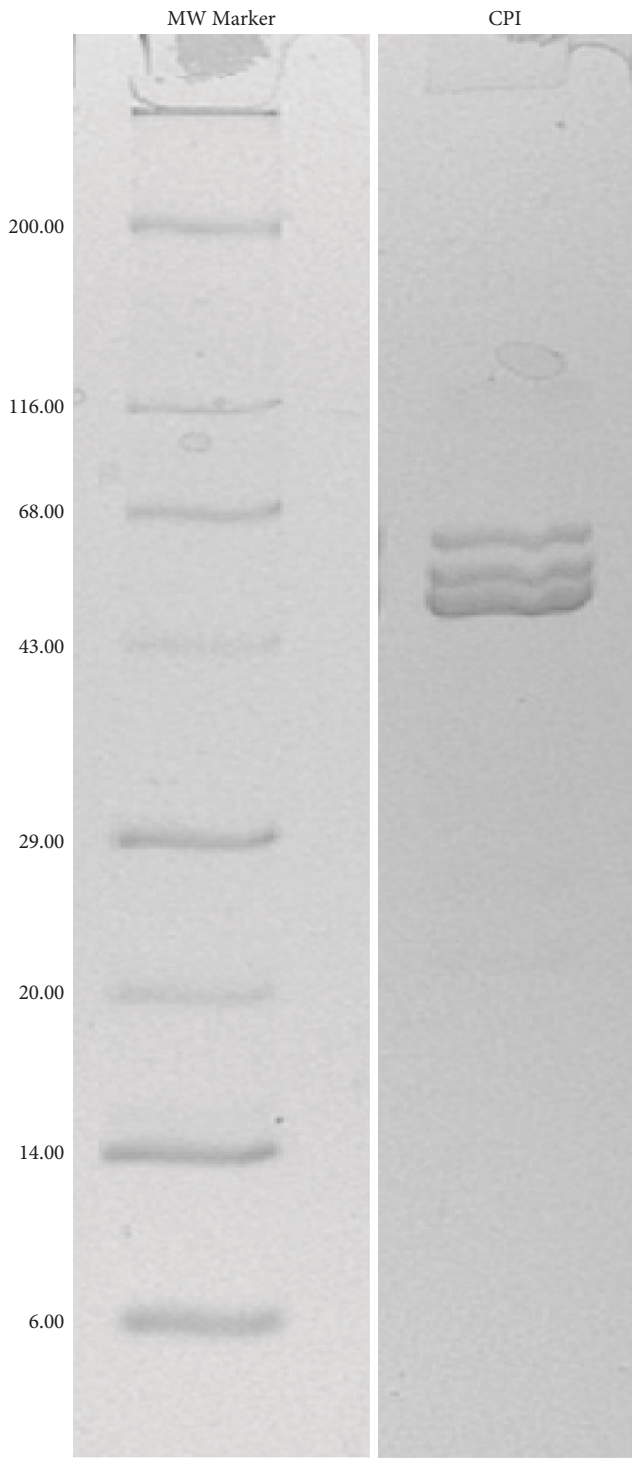


Figure 1: SDS-PAGE analysis of cowpea protein isolate (CPI).

3.2. In Vitro Bioactive Properties of Protein Hydrolysates. Alcalase, thermolysin, and trypsin were used to hydrolyze cowpea proteins with the aim to produce bioactive peptides with antioxidant and ACE inhibitory properties, as these enzymes had been used with similar proteins and good results were obtained [9]. Degree of hydrolysis (DH) was used to monitor the hydrolysis process, and results are presented in Figure 2(a). Generally, it was observed that alcalase produced higher DH for CPI compared to thermolysin and trypsin at all hydrolysis times. Similar results were obtained for hydrolysis of Bambara bean proteins [9]. The typical hyperbolic trend was observed for hydrolysis of CPI with thermolysin and trypsin, with high hydrolysis rate during the first 2 h, then steady state from 6 to 24 h. The maximum DH was 20% and 14% for CPI hydrolyzed by thermolysin and trypsin, respectively. A different trend was observed for CPI hydrolyzed by alcalase, with a high rate of DH during the first 2 h, and finally reached 48% after 24 h hydrolysis. Similarly, DH for CPI hydrolyzed by Alcalase was reported by Segura Campos et al. [7]. Alcalase and thermolysin are particularly indicated for the production of peptides with ACE inhibitory activity and antioxidant properties because alcalase is known to hydrolyze most peptide bonds with a preference for those containing aromatic amino acid residues [28], while thermolysin is an endopeptidase which recognizes amino groups on residues displaying a bulky and hydrophobic side chain, in the order Ile > Leu > Val > Phe [29]. Moreover, ACE prefers competitive inhibitors containing amino acid residues with aromatic or branched lateral chains [30], and the antioxidant properties of peptides are enhanced by hydrophobic amino acid residues [31].

The ACE inhibitory activity of cowpea protein hydrolysates (at the concentration 100 μg/mL) and their DPPH scavenging activity (at the concentration 5 mg/mL) are presented in Figure 2(b). ACE inhibitory was significantly higher ($p < 0.05$) for CPI hydrolyzed by alcalase (62%, IC_{50} 54.34 μg/mL), compared to that hydrolyzed by thermolysin (54%, IC_{50} 77.31 μg/mL) and trypsin (19%, $IC_{50} > 1000$ μg/mL). Results with alcalase were higher than those reported by Segura Campos et al. [7], but in the same range as the cowpea protein hydrolysate ultrafiltrated fraction with MW cutoff sizes 1–3 kDa. Higher ACE inhibitory activity was also observed for Bambara bean protein hydrolysates prepared by alcalase and thermolysin, compared to those prepared by trypsin [9]. Hydrophobic and aromatic residues containing-peptides probably played an important role in the high ACE inhibitory properties of cowpea protein hydrolysates [8]. In another hand, antioxidant activity of CPI hydrolyzed by alcalase, thermolysin, and trypsin followed the same trend as ACE inhibitory activity. Significantly higher ($p < 0.05$) DPPH scavenging activity was obtained by hydrolysis with alcalase, and CPI hydrolyzed with trypsin showed lower DPPH scavenging activity. A different trend for DPPH scavenging activity was observed for Bambara bean protein isolate hydrolyzed by the same enzymes [9]. Therefore, besides looking at the specificity of proteases in hydrolyzing peptide bonds in the protein substrate, it could also be important for the efficient release of bioactive peptides to investigate peptide bond accessibility and the structural characteristics of the polypeptides.

3.3. In Silico Analysis of the Bioactive Potential of CPH Using BIOPEP. The analysis of the release of bioactive peptides by enzymatic hydrolysis of protein sequences using BIOPEP is actually one of the most commonly used procedures to

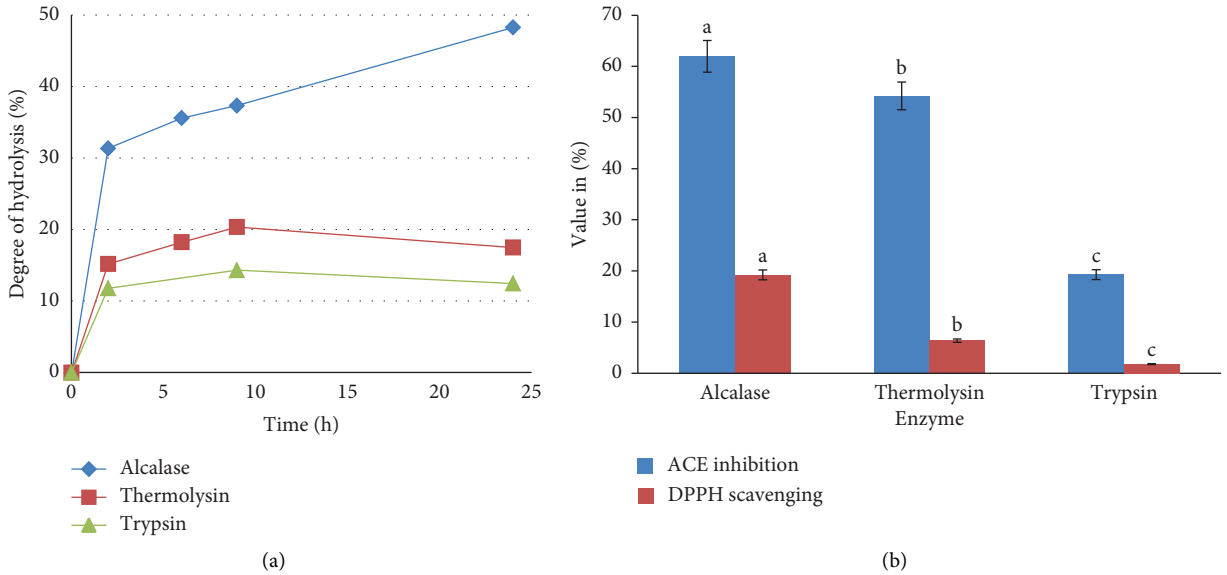


Figure 2: Degree of hydrolysis (a) and ACE inhibitory activity and DPPH scavenging activity (b) of CPI hydrolyzed with thermolysin, alcalase, and trypsin.

evaluate the bioactive potential of proteins by *in silico* methods. Table 2 shows the results obtained for the proteins 7SG3, 8SG, and VIC, the major proteins identified in the CPI, which are hydrolyzed by alcalase, thermolysin, and trypsin. The sequence and location of antioxidant and ACE inhibitory peptides produced from each polypeptide were provided, and then, the theoretical degree of hydrolysis (DHt), the average frequency of bioactive peptides (AE), and the potential biological activity (BE) were calculated. It appeared that CPI major proteins were good sources of bioactive peptides which could be efficiently released through the action of Alcalase (DHt 31.01–32.18%) and thermolysin (DHt 34.26–36.28%). CPI was less efficiently hydrolyzed by trypsin, with DHt ranging from 11 to 12%.

Hydrolysis of CPI major proteins with Alcalase produced six known peptides (AE ranging from 0.0044–0.0046), while hydrolysis with thermolysin produced 5 peptides (AE: 0.0022–0.0046) with antioxidant activity. The two peptides KAI and PHY were more frequently released by Alcalase, while VKL was less found. The peptide LPHH was more frequently released by the action of thermolysin. In addition, LHE and LHR were also found. No antioxidant peptide was found during the hydrolysis of CPI major proteins by trypsin.

The number of peptides exhibiting ACE inhibitory activity and released during hydrolysis of CPI major proteins by thermolysin was higher (AE: 0.03–0.0375) compared to hydrolysis by Alcalase (AE: 0.0162–0.0208) and trypsin (AE: 0.0023–0.0044). In contrast, the potential biological activity of bioactive peptides released by Alcalase was higher (BE: 0.00060–0.00066) than those released by thermolysin (BE: 0.00010–0.00025) and trypsin (BE < 0.00010). The main ACE inhibitory peptides produced from cowpea major proteins following hydrolysis by Alcalase were VF, GI, NF, DF, IL, AIP, AF, and GS (Table 2). Most of these peptides were also found during fermentation of soybean protein by *Aspergillus oryzae* [32] and from other plant protein hydrolysates (VF, IL, GI, and GS) [33]. However, it should be noticed that hydrolysis of CPI major proteins with thermolysin produced bioactive peptides containing aromatic amino acids and proline (FP, YP, IP, and FG). Generally, these peptides produced potent ACE inhibitory activity *in vivo*.

3.4. Molecular Dynamics Analysis. A tridimensional model was constructed for each CPI major protein in Table 1 from their FASTA sequence using the I-TASSER algorithm. This 3D structure was used to map the encrypted antioxidant and ACE inhibitory peptides detected by BIOPEP analysis in order to find the structural information around these peptides. Models were further equilibrated at hydrolysis conditions for each enzyme (55°C and pH 7 for alcalase and trypsin and 70°C and pH 8 for thermolysin). The structural quality of models was evaluated using the Eval23D tool, and their characteristics are shown in Table 3 and Figures 3(a)–3(f).

3.4.1. Structural Analysis. Results showed that the three models (7SG3, 8SG, and VIC) had a central region rich in strands and beta sheets, which was surrounded by regions containing helix and turn secondary structures (Figure 3). This conformation is characteristic of the cupin superfamily domain. Cupins are the major globulins found in cowpea [34]. Cowpea major proteins were rich in strands (27–30%) compared to helices (10–16%) and also contained β-sheet, β-hairpin, and β-bulge secondary structures. The presence of high content in β-sheet conformation of legume proteins has been related to the resistance to denaturation during gastrointestinal digestion, and β-hairpin conferred additional heat stability [10]. Therefore, heat treatment at 55°C and

Table 2: Bioactive peptides (ACE inhibitory and antioxidant) produced during in silico hydrolysis of cowpea protein isolate major proteins (8SG, 7SG2, and VIC).

Enzyme	Protein	Sequence	Location	Activity	DHt (%)	AE	BE
Thermolysin	7SG3	LPHH	[74–77]	Antioxidant	34.2593	0.0046	0
		LHR	[44–46]				
		FP	[385-386]	ACE inhibitor		0.030	9.579×10^{-5}
		YP	[338-339]	ACE inhibitor			
		IP	[114-115] [138-139] [334-335]	ACE inhibitor			
		VG	[302-303]	ACE inhibitor			
		FG	[353-354]	ACE inhibitor			
		IE	[299-300]	ACE inhibitor			
		VE	[63-64] [204-205]	ACE inhibitor			
		LN	[348-349]	ACE inhibitor			
		LR	[41-42] [131-132]	ACE inhibitor			
Thermolysin	8SG	LPHH	[97–100]	Antioxidant	36.2832	0.0022	0
		FP	[405-406]	ACE inhibitor		0.0375	0.00016
		YP	[358-359]	ACE inhibitor			
		IP	[137-138] [161-162] [354-355]	ACE inhibitor			
		FG	[373-374] [22-23]	ACE inhibitor			
		LG	[10-11]	ACE inhibitor			
		VR	[2-3]	ACE inhibitor			
		AR	[86-87]	ACE inhibitor			
		VE	[86-87] [227-228]	ACE inhibitor			
		LN	[368-369]	ACE inhibitor			
		YE	[273-274]	ACE inhibitor			
		LR	[64-65] [154-155]	ACE inhibitor			
Thermolysin	VIC	LPHH	[93–96]	Antioxidant	35.1852	0.0046	0
		LHE	[268–270]	Antioxidant			
		FP	[403-404]	ACE inhibitor		0.0323	0.00025
		YP	[356-357]	ACE inhibitor			
		VK	[153-154]	ACE inhibitor			
		IP	[352-353]	ACE inhibitor			
		VP	[1-2] [157-158]	ACE inhibitor			
		FG	[18-19] [371-372]	ACE inhibitor			
		LG	[6-7]	ACE inhibitor			
		IE	[318-319]				
		VE	[82-83]	ACE inhibitor			
		LN	[366-367]	ACE inhibitor			
		LR	[60-61] [150-151]	ACE inhibitor			
Alcalase	VIC	VKL	[153–155]	Antioxidant	32.1759	0.0046	0
		KAI	[304–306]	Antioxidant			
		VF	[284-285] [349-350]	ACE inhibitor		0.0162	0.0006
		VP	[157-158]	ACE inhibitor			
		GI	[19-20]	ACE inhibitor			
		NF	[367-368]	ACE inhibitor			
		DF	[195-196]	ACE inhibitor			
		IL	[124–126]	ACE inhibitor			

Table 2: Continued.

Enzyme	Protein	Sequence	Location	Activity	DHt (%)	AE	BE
Alcalase	7SG3	VF	[265-266] [331-332]	ACE inhibitor	31.0185	0.0208	0.00066
		AIP	[137–139]				
		AF	[352-353]				
		GS	[430-431]				
		NF	[349-350]				
		DF	[176-177]				
		IL	[105-106] [428-429]				
		PHY	[280–282]	Antioxidant		0.0046	0
		KAI	[285–287]				
Alcalase	8SG	PHY	[303–305]	Antioxidant	31.8584	0.0044	0
		KAI	[308–310]	Antioxidant			
		VF	[288-289] [351-352]	ACE inhibitor		0.0221	0.00063
		AIP	[160–162]	ACE inhibitor			
		AF	[372-373]	ACE inhibitor			
		GI	[23-24]	ACE inhibitor			
		GS	[450-451]	ACE inhibitor			
		DF	[199-200]	ACE inhibitor			
		IL	[128-129] [448-449]	ACE inhibitor			
Trypsin	8SG	GR	[440-441]	ACE inhibitor	11.5044	0.0044	2.38×10^{-5}
		AR	[4-5]				
Trypsin	VIC		—	—	10.6481	—	—
Trypsin	7SG3	GR	[420-421]	ACE inhibitor	12.0370	0.0023	7.217×10^{-7}

AE is the occurrence frequency of fragments with a given activity in polypeptide chain, $A = a/N$, where a is the number of fragments with a given activity and N is the number of amino acid residues. BE is the potential biological activity of the protein chain, mM^{-1}, $\mathrm{BE} = (\sum_{i=1}^{k} (ai/EC50i))/N$, where, ai is the number of repetitions of ith bioactive fragment; EC50i is the concentration of half-maximum activity of ith protein fragment, mM; and k is the number of different fragments with a given activity. DHt is the theoretical degree of hydrolysis, $\mathrm{DHt} = (d/D) \times 100$, where d is the number of hydrolyzed peptide bonds and D-total number of peptide bonds in a protein chain.

Table 3: Structural changes in the predicted main cowpea proteins under different pH and temperature treatment for enzymatic hydrolysis.

	7SG3		8SG		VIC	
	55°C, pH7	70°C, pH8	55°C, pH7	70°C, pH8	55°C, pH7	70°C pH8
Secondary structure descriptors						
No strands	27 (30.49%)	28 (30.72%)	25 (29.09%)	26 (29.53%)	25 (28.06)	25 (27.39%)
No beta sheets	4	6	6	4	6	6
No beta hairpins	6	6	5	6	5	5
No beta bulges	6	4	8	9	5	6
No helices	11 (15.92%)	11 (14.13%)	11 (12.93%)	8 (10.35%)	9 (13.03%)	10 (12.92%)
Helix-helix interactions	4	4	4	4	4	4
No beta turns	73	71	83	91	77	74
No gamma turns	11	13	19	19	10	11
Overall structure descriptors						
Volume (nm^3)	89.309	88.318	93.190	92.839	88.243	86.655
SAS (nm^2)	211.836	210.991	226.506	226.260	208.161	207.840

SAS, solvent accessible surface.

70°C caused little change in the structure of the major cowpea proteins. A minor increase was observed in the number of beta turns of the 8SG model at 70°C compared to 50°C, while the number of helices decreased. Generally, the structure of cowpea proteins remained very stable at 55°C and 70°C, as evidenced by their similar volume and solvent accessible surface, which made their tridimensional models at these temperatures highly similar to those at 25°C, as shown in Figure 3. Therefore, adequate substrate pretreatment should be considered to modulate the release of bioactive peptides from cowpea protein isolate following enzymatic hydrolysis.

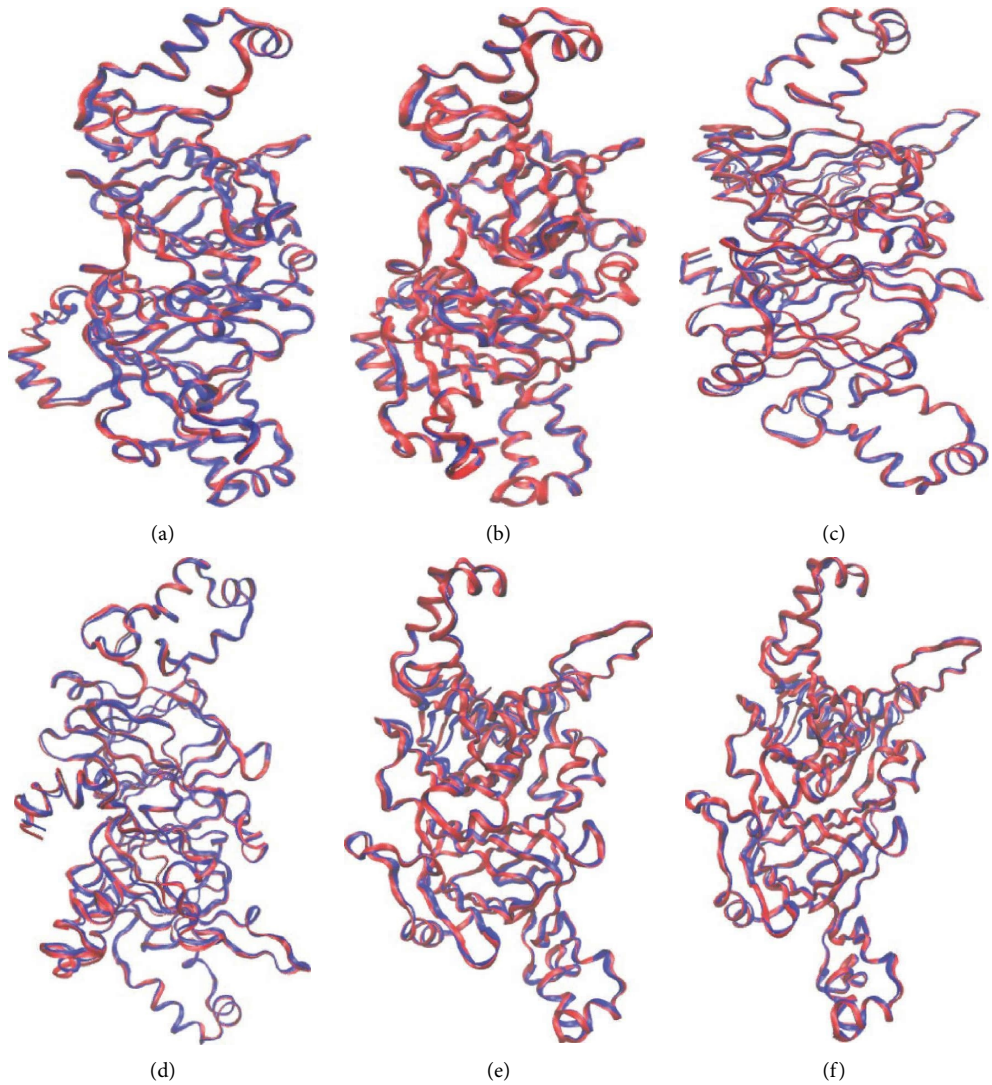


Figure 3: Superposition of the 7S3 (at 55°C and 70°C (a, b)), 8SG (at 55°C and 70°C (c, d)), and VIC (at 55°C and 70°C (e, f)). Models at 25°C (blue) and 55°C or 70°C (red). Images were prepared with VMD software.

3.4.2. Secondary Structure of Encrypted Bioactive Peptides and Solvent Accessible Surface (SAS). The tridimensional model of major proteins found in cowpea protein isolate at different hydrolysis conditions for thermolysin, alcalase, and trypsin were used to calculate SAS for each amino acid. The mean SAS for each peptide (SASM) was calculated on the basis of SAS of each amino acid. The location of encrypted bioactive peptides in relation to their corresponding secondary structure was determined using the BIOPEP and PDBSum tools. The results are shown in Table 4 and the 3D model of proteins presenting potentially accessible peptides is shown in Figure 4.

As shown in Table 4, most of potential antioxidant peptides produced by hydrolysis of cowpea major proteins with thermolysin were located in β-sheet secondary structure, with just LHR present in β-turn-β-hairpin secondary structures and a SASM higher than 0.3 nm^2 (0.620 nm^2). The other peptides had their SASM less than 0.3 nm^2. It was reported by Vecchi and Añon [35] that bioactive peptides were found in exposed zone when SAS was higher than 0.3 nm^2 and in the buried zone when SAS was less than 0.3 nm^2. Exposed peptides are more expected to be released by proteases following enzymatic hydrolysis *in vitro* and then exert their action *in vivo*. It was also observed that most of the potential ACE inhibitory peptides released following hydrolysis by thermolysin were present in β-sheet secondary structure (65%, 11 peptides), while the exposed peptides were mostly located in β-turn and ɤ-turn (23%, 8 peptides). Moreover, 7% peptides (3 peptides) with the most potent ACE inhibitory activity (IC50 < 200 μM) were exposed to the solvent, among them 7% (3 peptides) are located in turn and 2% (1 peptides) in β-sheet. VK was the most potent peptide (IC50 = 13 μM) potentially released by thermolysin hydrolysis, but with SASM of 0.053 nm^2 and located in β-sheet secondary structure.

It is shown in Table 4 that most of the antioxidant peptides (3 peptides) potentially released following hydrolysis by Alcalase were exposed to the solvent

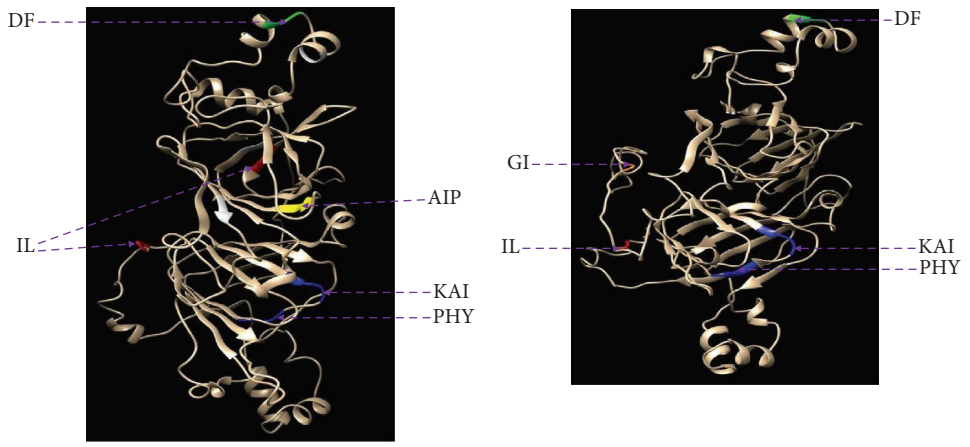
Table 4: Predicted ASA and secondary structure of bioactive peptides produced from hydrolysis of cowpea protein isolate major proteins (8SG, 7SG3, and VIC).

Enzyme	Protein	Sequence (N° of location)	ASA (nm^2)					Secondary structure	IC50 in μM
			SASM	SASaa1	SASaa2	SASaa3	SASaa4		
Thermolysin	7SG3	LPHH	0.258	0.064	0.356	0.034	0.579	Beta sheet	—
		LHR	0.620	0	1.121	0.74		Beta turn; beta hairpin	—
		FP	0.828	0.443	1.212			Gamma turn	315
		YP	0.252	0.253	0.25			Beta turn	720
		IP (1)	0.311	0	0.622			Beta sheet; beta turn	130
		IP (2)	0.231	0.394	0.067			Beta sheet	130
		IP (3)	0.125	0.064	0.186			Beta sheet; beta turn	130
		VG	0.231	0.462	0			Beta sheet	1100
		FG	0.000	0	0			Beta sheet	3700
		IE	0.404	0	0.807			Beta sheet	—
		VE (1)	0.014	0	0.027			Beta sheet	—
		VE (2)	0.166	0.053	0.279			Beta sheet	—
		LN	0.084	0	0.167			Beta sheet	—
		LR (1)	0.188	0.091	0.284			Beta sheet	158
		LR (2)	0.084	0	0.167			Beta sheet	158
Thermolysin	8GS	LPHH	0.211	0.186	0.265	0	0.394	Beta sheet	—
		FP	1,127	0.693	1.56			Gamma turn	315
		YP	0.339	0.257	0.421			Beta turn	720
		IP (1)	0.298	0	0.595			Beta turn	130
		IP (2)	0.222	0.277	0.167			Beta sheet	130
		IP (3)	0.053	0	0.106			Beta turn	130
		FG (1)	0.046	0.091	0			Beta sheet	3700
		FG (2)	0.749	1,431	0.067			Beta turn	3700
		LG	0.610	0.952	0.267			Beta turn	8800
		VR	1.594	0.939	2.249			Beta turn	52.80
		AR	0.000	0	0			Beta sheet	95.50
		VE (1)	0.000	0	0			Beta sheet	—
		VE (2)	0.372	0.08	0.664			Beta sheet	—
		LN	0.114	0.027	0.201			Beta sheet	—
		YE	0.276	0.353	0.198			Beta sheet	630.90
		LR (1)	0.146	0	0.292			Beta sheet	158
		LR (2)	0.154	0	0.307			Beta sheet	158
Thermolysin	VIC	LPHH	0.214	0.208	0.33	0.034	0.284	Beta sheet	—
		LHE	0.100	0.053	0.08	0.167		Beta sheet	—
		FP	1.107	0.708	1.505			Gamma turn	315
		YP	0.241	0.295	0.186			Beta turn	720
		VK	0.053	0	0.106			Beta sheet	13
		IP	0.053	0	0.106			Beta sheet; beta turn	130
		VP (1)	0.000	0	0			Beta turn	420
		VP (2)	0.191	0.314	0.067			Beta sheet; beta turn	420
		FG (1)	0.853	1,639	0.067			Beta turn	3700
		FG (2)	0.000	0	0			Beta sheet	3700
		LG	0.336	0.671	0			Beta turn	8800
		IE	0.143	0	0.285			—	—
		VE	0.000	0	0			Beta sheet	—
		LN	0.064	0.033	0.095			Beta sheet	—
		LR (1)	0.186	0.08	0.292			Beta sheet	158
		LR (2)	0.080	0	0.159			Beta sheet	158
Alcalase	VIC	VKL	0.020	0	0.027	0.033		Beta sheet	—
		KAI	0.338	0.897	0	0.117		Beta turn; beta sheet	—
		VF (1)	0.032	0	0.064			Beta sheet	9.20
		VF (2)	0.000	0	0			Beta sheet	9.20
		VP	0.226	0.352	0.1			Beta sheet	420
		GI	0.050	0.067	0.033			Beta turn	1200
		NF	0.040	0.027	0.053			Beta sheet	46.30
		DF	0.755	0.786	0.724			Helix	363.10
		IL	0.080	0.159	0			Beta sheet	54.95

Table 4: Continued.

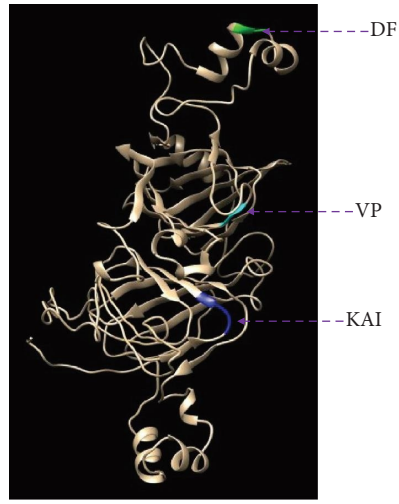
Enzyme	Protein	Sequence (N° of location)	ASA (nm^2)					Secondary structure	IC50 in μM
			SASM	SASaa1	SASaa2	SASaa3	SASaa4		
		VF (1)	0.000	0	0			Beta sheet	9.20
		VF (2)	0.000	0	0			Beta sheet	9.20
		AIP	0.165	0	0.394	0.1		Beta sheet	670
		AF	0.033	0.027	0.038			Beta sheet	190
		GS	0.040	0.08	0			Beta turn	3800
Alcalase	7SG3	NF	0.110	0.193	0.027			Beta sheet	46.30
		DF	0.689	0.771	0.606			Helix	363.10
		IL (1)	0.192	0.383	0			Beta sheet	54.95
		IL (2)	1,045	0.894	1.195			Beta turn	54.95
		PHY	0.367	0.462	0.027	0.613		Beta sheet	—
		KAI	0.380	0.89	0	0.25		Beta turn	—
		PHY	0.304	0.489	0.053	0.369		Beta sheet	—
		KAI	0.383	0.91	0	0.239		Beta turn	—
		VF (1)	0.000	0	0			Beta sheet	9.20
		VF (2)	0.000	0	0			Beta sheet	9.20
		AIP	0.126	0	0.277	0.1		Beta sheet	670
Alcalase	8SG	AF	0.038	0	0.075			Beta sheet	190
		GI	0.318	0.067	0.569			Beta turn	1200
		GS	0.014	0	0.027			Beta turn	3800
		DF	0.763	0.711	0.815			Helix	363.10
		IL (1)	0.102	0.204	0			Beta sheet	54.95
		IL (2)	0.595	0.531	0.659			Beta turn	54.95
Trypsin	8GS	GR	0.754	0.027	1.481			Gamma turn	3200
		AR	1.154	0.609	1.698			Beta turn	95.50
Trypsin	VIC								
Trypsin	7SG3	GR	0.171	0.027	0.314			Beta turn	3200

SASaai, solvent accessible surface of the *i*th amino acid; SASM, mean solvent accessible surface of the peptide; the IC50 values were provided by the BIOPEP database.



(a) (b)

Figure 4: Continued.



(c)

Figure 4: Mapping of bioactive peptides in the 7SG3 protein (a), 8SG protein (b), and VIC protein (c). Images were prepared with Chimera software.

(SASM > 0.3 nm^2) then expected to be released *in vitro*. These peptides are equally distributed in β-sheet and β-turn secondary structures. The only nonexposed peptide with antioxidant activity was VKL, and it was located in β-sheet. ACE inhibitory peptides potentially released by the Alcalase digestion were located in β-sheet (62%, 6 peptides), turns (23%, 3 peptides), and α-helix (12%, 1 peptide). Most of the ACE inhibitory peptides were then located in β-sheets. However, the solvent-accessible peptides were mostly found in β-turns (12%, 2 peptides) and α-helix (12%, 1 peptide, DF). It was noteworthy that the peptide IL with IC50 54.95 μM was potentially released *in vitro* by hydrolysis with Alcalase since its SASM was >0.3 nm^2. Figure 4 shows the location of solvent-accessible bioactive peptides potentially released by Alcalase in the 3D structure of cowpea isolate proteins.

The ACE inhibitory activity of CPI hydrolyzed by trypsin was probably mostly explained by the release of two peptides, GR and AR, which showed SASM of 0.754 and 1.154 nm^2, respectively (Table 4).

4. Conclusion

It has been demonstrated in this study that cowpea protein hydrolysates showed good potential for antioxidant activity and inhibition of ACE. However, to optimize the release of bioactive peptides by enzymatic hydrolysis, the structure of vignin, the major protein found in CPH, should be carefully monitored. In addition, the choice of enzyme for the preparation of CPH was also important. Cowpea protein hydrolysate produced by Alcalase presented higher ACE inhibitory and antioxidant activity *in vitro*. Simulation of the hydrolysis of cowpea protein isolate major proteins by BIOPEP confirmed the higher bioactive potential of the hydrolysate prepared by Alcalase. In addition, the tridimensional structure of the major proteins was constructed and equilibrated at hydrolysis pH and temperature, depending on the enzyme. The equilibrated structure was used to map bioactive peptides and understand the structural factors which could affect the release of bioactive peptides. The results were permitted to be concluded on the potential bioactive composition of cowpea protein hydrolysate.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The author declares that there are no conflicts of interest.

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