ANTIOXIDANT ACTIVITY OF COWPEA PROTEIN ISOLATE HYDROLYZED BY PEPSIN

M. Mune Mune[a]*, D. Saxena[b] and S. Minka[c]

[a]The Higher Institute of Sahel, The University of Maroua, P.O. Box 46, Maroua. Cameroon
[b]Sant Longowal Institute of Engineering and Technology (SLIET), Longowal. India
[c]Department of Biochemistry, University of Yaounde I, PO Box 812, Yaounde. Cameroon

Antioxidant activity was studied for hydrolysates obtained from cowpea protein isolate by enzymatic hydrolysis using pepsin. Effects of 3 hydrolysis parameters: pH, temperature, and time were analysed using response surface methodology. A second-order polynomial model was used for predicting antioxidant activity of the hydrolysates. This model was adequate to fit experimental data and explained more than 85% of the variation. Interaction between pH and temperature was the major effect affecting antioxidant activity. Optimum antioxidant activity was observed after hydrolysis for 176 min, at pH 2.15, and temperature 38.2 °C. Under these conditions, the predicted antioxidant activity (22.69%) was in agreement with the experimental one. Degree of hydrolysis of the hydrolysate under optimum conditions was 25.70%, and the electrophoretic profile revealed the cleavage of several protein bands present in the isolate.

Keywords: cowpea, hydrolysate, pepsin, optimization, antioxidant activity

There is a continuous search of better and more effective antioxidants for use in food and non-food industries. These substances play a major role in the prevention of lipid oxidation, which causes the formation of free radicals. The free radicals produced are involved in the deterioration of food products, and also associated to many pathological conditions. The use of artificial antioxidants is restricted or prohibited in some countries, because of their toxicity (Ito et al., 1986). Attention is then focused on natural antioxidants, and recently on plant protein hydrolysates (Cumby et al., 2008).

Principally all proteins may be considered as the precursors of antioxidant peptides (Dziuba et al., 2004), which are inactive in the protein sequence and may reveal biological activity after being released by proteolytic enzymes. However, to produce hydrolysates with desirable properties, it is necessary to undertake studies to find the right proteolytic enzyme for a protein substrate, as well as to select factors with major effects on the proteolysis. Tropical legumes can be considered as a source of cheap, accessible, and safe proteins for a large-scale production of antioxidative peptides. In this regards, cowpea (*Vigna unguiculata* L. Walp) is widely produced in the world, with a total annual global production over 3 million tons (CGIAR, 1997). This legume is a good source of protein (18–35%) rich in histidine, leucine, and aromatic amino acids (Mwasaru et al., 1999). Peptides containing these amino acids have been shown to possess strong antioxidant activity (Pihlanto, 2006).

Depending on the enzyme used, hydrolysis of proteins depends on the combination of factors such as time, temperature, pH, ionic strength, and enzyme to substrate ratio. Then, it is necessary to strictly regulate the process, to include the possibility of determining when to

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* To whom correspondence should be addressed.
Phone: (+237) 99309667; e-mail: alainmune@yahoo.fr

stop if the wanted peptides are produced, in order not to lose them. In this regards, response surface methodology (RSM) is a useful technique that has been successfully applied for the optimization of the enzymatic hydrolysis process using different substrates and proteases, with the finality of producing antioxidant peptides. The aim of this study was to study the effects of pH, temperature, and time on DPPH scavenging activity of cowpea protein hydrolysates produced by pepsin, and to find the optimal conditions for hydrolysis.

1. Materials and methods

1.1. Preparation of cowpea flour

Cowpea flour was prepared as described by MUNE MUNE and co-workers (2008), then defatted by the Soxhlet apparatus for 12 h and stored at about 4 °C. The flour contained 4.44% moisture and 24.99% protein, determined according to A.O.A.C. (1990) methods.

1.2. Preparation of cowpea protein concentrate

Cowpea protein concentrate was prepared as described by MUNE MUNE and co-workers (2008) with some modifications. An aliquot (10 g) of defatted cowpea flour was mixed with 100 ml of NaCl solution (0.15 M), the pH was adjusted to 9.9 and the mixture was stirred for 120 minutes at room temperature (max 30 °C). The resultant slurry was then centrifuged at 5000 r.p.m. for 20 min using an Eltek centrifuge (Mumbai, India). The pH of the resultant supernatant was adjusted to 4.5 under stirring, the precipitated proteins were washed once in distilled water and recovered by centrifugation at 5000 r.p.m. for 20 min. The protein was resuspended in water at about 1:1 (w/v), the pH was adjusted to 7.0, then freeze-dried, ground, and passed through a 150 µm mesh sieve. The concentrate contained 86.87% protein determined by the Kjeldahl method (A.O.A.C., 1990).

1.3. Experimental design

A central composite rotatable design (CCRD) with three variables was used to study the response pattern and to determine the optimum combination of the variables. The variables optimized were pH (1.50–2.50), temperature (32–42 °C), and time (30–240 min), each at 5 coded levels -1.682, -1, 0, 1 and 1.682 as shown in Table 1. The CCRD shown in Table 2 was arranged to allow fitting an appropriate regression model using a multiple regression program. Four replicates (treatments 15–18) at the centre of the design were used to allow the estimation of the pure error sum of squares. Experiments were randomized in order to minimise the effects of unexplained variability in the observed responses due to extraneous factors.

Table 1. Experimental values and coded levels of the independent variables for CCRD

Variable	Symbols		Levels				
	Coded	Uncoded	-1.682	-1	0	1	1.682
pH	X_1	x_1	1.50	1.70	2.00	2.30	2.50
Temperature (°C)	X_2	x_2	32	34	37	40	42
Time (min)	X_3	x_3	30	73	135	197	240

Table 2. CCRD Design arrangement and responses in terms of DPPH scavenging activity

Exp.	pH (X_1)	Temp. (X_2)	Time (min) (X_3)	DPPH (%) (Y_1)
1	−1	−1	−1	13.24
2	1	−1	−1	0.15
3	−1	1	−1	11.18
4	1	1	−1	13.25
5	−1	−1	1	22.15
6	1	−1	1	8.90
7	−1	1	1	0.65
8	1	1	1	20.85
9	−1.682	0	0	20.85
10	1.682	0	0	10.42
11	0	−1.682	0	6.84
12	0	1.682	0	5.32
13	0	0	−1.682	11.07
14	0	0	1.682	16.94
15	0	0	0	23.34
16	0	0	0	20.74
17	0	0	0	22.04
18	0	0	0	23.50

*coding is done as follows: $X_1 = (x_1 - 2)/0.3$; $X_2 = (x_2 - 37)/3$; $X_3 = (x_3 - 135)/62$

The variables were coded according to the following equation:

$$X_i = (x_i - \bar{x}_i)/\Delta x_i \tag{1}$$

where X_i is the coded value of an independent variable, x_i is the real value of an independent variable, $\bar{x}_i$ is the real value of an independent variable at the centre point, Δx_i is the step change.

1.4. Enzymatic hydrolysis with pepsin

The proteolysis was performed based on the method described by MUNE MUNE and co-workers (2011). Cowpea protein concentrate was homogenised in distilled water, the pH was adjusted to 1.50–2.50 using 2 M HCl, and the mixture was stirred for 2 h at room temperature (25±2 °C). The mixture was preincubated at 32–42 °C for 30 min, then a pepsin (1:3000, Himedia) solution of 2.3 mg ml⁻¹ in water was added to obtain a final enzyme/substrate ratio (E/S) of 2% (w/w), and a solid/liquid ratio of 1/20 (w/v). The E/S was chosen low for an economical reason and potential industrial application. The reaction was allowed to proceed under constant stirring at 37 °C for 30 to 240 min, and the pH was maintained at 1.5–2.50 by the addition of 1 M NaOH. At appropriate reaction time, the pH of the mixtures was adjusted to 7 with 2.5 M NaOH. The reaction was stopped by heating at 100 °C for 10 min, than

cooled in an ice bath at 4 °C. The mixtures were then centrifuged at 5000 r.p.m. for 15 min, the supernatants freeze-dried and stored at 4 °C.

1.5. Degree of hydrolysis

The DH was defined as the percent ratio of the number of peptide bonds broken to the total number of peptide bonds in the substrate studied and the calculation formula was: DH(%)=alpha-amino nitrogen content/total nitrogen content×100%. The alpha-amino nitrogen was determined by a formol titration method (CHEN et al., 2003) and the total nitrogen content was determined by A.O.A.C. (1990) method.

1.6. 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

The scavenging effects of cowpea protein hydrolysates (CPH) on the DPPH free radical were measured basically as described by SHIMADA and co-workers (1992). Briefly, a 1.5 ml aliquot of CPH solution (4 mg ml^{-1} in 0.1 M phosphate buffer pH 7) was added to 1.5 ml of 0.05 mM DPPH in 95% ethanol. The mixture was vortexed for 10 s and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm using the Hach DR 5000 spectrophotometer. The scavenging effect was expressed as:

DPPH radical scavenging activity (%)=
(blank absorbance–sample absorbance)×100/blank absorbance

where the DPPH blank is the value of 1.5 ml of 0.1 M phosphate buffer pH 7 mixed with 1.5 ml of ethanol containing 0.05 mM DPPH. All experiments were carried out in triplicate.

1.7. Polyacrylamide gel electrophoresis

SDS-PAGE is performed by the method of LAEMMLI (1970). The gel system consisted of a 10% polyacrylamide-resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). The lengths of the resolving and stacking gels are 12 and 3 cm, respectively, with a gel thickness of 1.5 mm. Molecular weight markers (30–150 kDa) were purchased from Gencript Corp (NJ, USA).

1.8. Statistical analysis

A second-order polynomial equation was used to fit experimental data given in Table 2. The model proposed for the response is given as follows:

$$Y_i=a_0+a_1X_1+a_2X_2+a_3X_3+a_{11}X_1^2+a_{22}X_2^2+a_{33}X_3^2+a_{12}X_1X_2+a_{13}X_1X_3+a_{23}X_2X_3 \qquad (2)$$

where $Y_{i(1-2)}$ is predicted response for DH and solubility of cowpea protein hydrolysates. The a_0 value is that of the fitted response at the centre point of the design, a_1, a_2, and a_3 are linear terms, a_{12}, a_{13}, and a_{23} are interaction effects, a_{11}, a_{22}, and a_{33} are squared effects. Optimization of the fitted polynomial was carried out by the partial derivative method. The optimum condition was verified by conducting experiments under that condition. Experimental and predicted responses were compared. The fitted polynomial equation was expressed as surface and contour plots. The computer software used for this study was STATISTICA (version 5.5, 2002; Statsoft Inc., USA).

2. Results and discussion

2.1. Fitting the models

Antioxidant activity (AA) was measured as DPPH scavenging activity and used as the response during hydrolysis of cowpea protein isolate by pepsin under different pH, temperature, and time conditions. Multiple regression equations were generated relating the predicted response to coded levels of variables. The regression coefficients were determined by the least square method, the following model explained the experimental data:

$$Y=22.401.58X_1-2.39X_1{}^2-0.08X_2-5.77X_2{}^2+1.80X_3-2.96X_3{}^2+$$
$$+6.07X_1X_2+2.25X_1X_3-2.57X_2X_3 \tag{3}$$

where Y is the predicted DPPH scavenging activity, X_1, X_2, and X_3 are the coded values of pH, temperature, and time.

Analysis of variable was calculated to test model adequacy. The data obtained using ANOVA appropriate to the experimental design are presented in Table 3. The probability of the model was significant at P<0.000. The high value of coefficient of determination (R^2adj=0.852) demonstrated that the model explained 85.2% of the variation in the response. In addition, plot of experimental versus predicted value indicated a good fit ($R{\geq}0.96$; P<0.000). On the other hand, the lack of fit was found to be insignificant (P>0.05). The lack of fit measures the failure of the model to represent data in the experimental domain, at points which are not included in the regression (MYERS & MONTGOMERY, 2002). It can be concluded from these results that the model satisfactorily describes the process.

Table 3. ANOVA for the quadratic model

Source	Df	SS	MS	*F*-value	P-value
Model	1	2013.71	2013.71	228.85	0.000
X1	1	34.19	34.19	3.89	0.084
X11	1	72.16	72.16	8.20	0.021
X_2	1	0.08	0.08	0.01	0.925
X_{22}	1	420.60	420.60	47.80	0.000
X_3	1	44.28	44.28	5.03	0.055
X_{33}	1	111.15	111.15	12.63	0.007
X_{12}	1	295.18	295.18	33.55	0.000
X_{13}	1	40.39	40.39	4.59	0.065
X_{23}	1	53.02	53.02	6.03	0.040
Residual	8	70.39	8.80		
Lack of fit	5	65.40	13.08	7.86	0.06
Pure error	3	4.99	1.66		
Total	17	1009.41			

R^2=0.851806; SS: sum of square; Df: degree of freedom; MS: mean square

2.2. Response surface plotting

The response surfaces and contour plots obtained from eq. 3, presenting the effects of 2 variables on AA of CPH where the third one was constant at the central level, are presented in Fig. 1A–C.
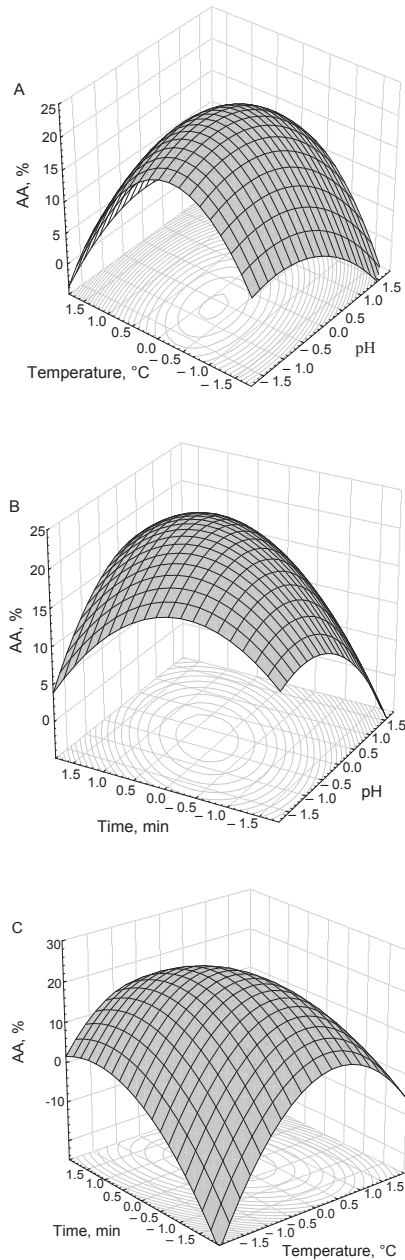

Fig. 1. Response surface and contour plot of the effects of factors on antioxidant activity (AA); A: pH and temperature; B: pH and time; C: temperature and time

2.2.1. Effect of pH and temperature on AA. The AA of CPH is a quadratic function of pH and temperature. The two effects are negative and significant at P<0.05 (Table 3). The AA increased with increasing pH and temperature till the maximum, and then decreased thereafter (Fig. 1A). The maximum value of AA was observed around pH and temperature coded of –1.0 to –0.3 (1.70 to 1.91 uncoded) and –0.5 to 0.0 (35.5 to 37 °C uncoded), respectively. Interaction between pH and temperature was positive and significant at P<0.00. The variation of pH and temperature probably affected the protein structure, leading to exposure to the enzyme of more cleavage sites and exposing the hydrophobic or proton donating residues buried inside the molecule, then contributed to the AA of the hydrolysates (Cheison et al., 2011).

    2.2.2. Effect of pH and time on AA. A curvilinear variation in AA of CPH was observed with increasing pH and time (Fig. 1B). The quadratic effect of time was negative and significant at P<0.05 (Table 3). The maximum AA was observed at coded pH and time around –0.5 to 0.0 (1.85 to 2.0 uncoded) and 0.0 to 0.5 (135 to 166 min uncoded), respectively. And, generally high AA of CPH was observed at combined low or high values of pH and time. Combination of long time and high pH, probably affected AA of CPH by increasing extent of hydrolysis of proteins, and liberation of high antioxidant peptides (Sun et al., 2011).

    2.2.3. Effect of temperature and time on AA. The shape of the response surface indicated that there was significant interaction between temperature and time on AA of CPH (Fig. 1C). The interaction was negative and significant at P<0.05. The AA increased with increasing temperature and time up to the maximum, and then decreased thereafter. The maximum was observed at coded temperature and time around –0.3 to 0.3 (36.1 to 37.9 °C uncoded) and 0.2 to 0.7 (147 to 178 min uncoded), respectively. It could be observed that high or low temperature was not favourable for the production of antioxidant peptides from cowpea protein isolate hydrolyzed by pepsin.

2.3. Optimization

The predicted optimum conditions were found by the partial derivative method. The first derivatives of the eq. 3 were done as follows:

$$-4.78X_1+6.07X_2+2.25X_3-1.58=0$$
$$-11.54X_2+6.07X_1+2.57X_3-0.08=0 \tag{4}$$
$$-5.92X_3+2.25X_1+2.57X_2+1.80=0$$

    The system of linear eq. 4 was solved and the predicted optimum AA corresponding to 22.61% was reached at pH coded of 0.49 (2.15 uncoded), temperature coded of 0.40 (38.2 °C uncoded) and time coded of 0.67 (176 min uncoded).

    The validity of the mathematical model was confirmed by conducting additional experiments under the predicted optimum conditions. The mean of tri replicates determination was 22.69 (±0.92)%, and non significant (P>0.05) difference was found between predicted and experimental values of AA activity of CPH. At these optimum conditions, degree of hydrolysis of hydrolysate was 25.70%.

2.4. Electrophoretic profile

The SDS-PAGE profiles of cowpea protein isolate and the hydrolysate obtained under optimum conditions are shown in Fig. 2. Two major bands of about 65 and 30 kDa were observed on the SDS-PAGE profile of the isolate. These bands probably corresponded to subunits of 7S globulin (vignin) as noted by FREITAS and co-workers (2004). Production of antioxidant peptides is associated with the hydrolysis of vignin, as observed by loss of intensity of bands and appearance of a new band of about 42 kDa. Therefore, small and medium size peptides had a significant effect on AA of cowpea protein hydrolysate.
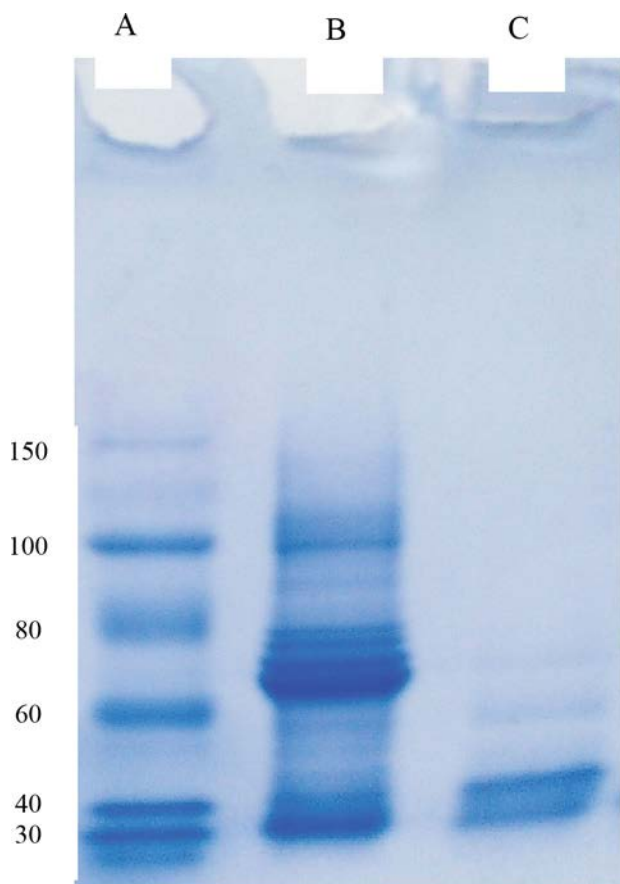

Fig. 2. SDS-PAGE profile of cowpea protein isolate (column B) and hydrolysate (column C). Column A consists of molecular weight markers

3. Conclusions

This study showed that cowpea protein isolate exhibited noticeable antioxidant activity following hydrolysis with pepsin. Adequate combination of hydrolysis parameters, pH, temperature, and time, was important for the production of cowpea protein hydrolysates with

high AA, and interaction between pH and temperature was significant. Optimum conditions were found by response surface methodology, and under these conditions DPPH scavenging activity of CPH was 22.69%. SDS-PAGE analysis revealed the cleavage of several protein bands present in the isolate after hydrolysis. So, cowpea protein hydrolysates could be useful ingredients of functional foods.

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