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EFFECT OF HYDROLYSIS TIME AND PEPTIDE CONTENT ON DPPH* RADICAL SCAVENGING ACTIVITY OF BROMELAIN- AND PAPAIN-GENERATED HYDROLYSATES FROM DEFATTED COCOA BEANS (*Theobroma cacao* L.) POWDER

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ABSTRACT
Enzyme generated protein hydrolysates have received considerable attention due to their numerous health effects. Among these, the anti-oxidative hydrolysates with DPPH* radical scavenging activity can play a significant role in the prevention of oxidative stress and the resulting chronic disease conditions such as cancer. In the present study, defatted cocoa beans (DCB) powder was dialyzed and then hydrolyzed for 8 h to produce DCB protein hydrolysates using two different proteases (bromelain and papain) at an optimum pH (6.8), temperature (40^{0}C) and enzyme/substrate ratio (1:50). The resulting DCB protein hydrolysates were evaluated for peptide content (PC) and DPPH* radical scavenging activity. The highest DPPH* radical scavenging activity of 78.07% and 53.04% were obtained at a peptide content of 4.85 mg/dL and 3.13 mg/dL for the bromelain- and papain-generated DCB protein hydrolysates at 6 h hydrolysis time, respectively. The effect of hydrolysis time and peptide content on the DPPH* radical scavenging activity of the DCB protein hydrolysates was then studied. The result obtained indicated positive correlation between the DPPH* radical scavenging activity of both bromelain- and papain-generated DCB protein hydrolysates to their peptide content based on Pearson's correlation analysis with R^2 values of 0.946 and 0.772, respectively.
Key word: Defatted cocoa beans, proteases, DPPH* radical scavenging activity, peptide content, hydrolysis time

INTRODUCTION

The generation of free radicals including reactive nitrogen species (RNS) and reactive oxygen species (ROS) can be triggered by external factors such as cigarette smoking, drugs/toxins, ozone, insecticides, heavy metal ions, allergens, ionizing radiations and UV radiations (Sharifi-Rad *et al.,* 2020). The process can also be endogenously mediated by non-enzymatic reactions or via strictly regulated enzymes that are involved in the cellular generation of redox-reactive species (Dorward *et al.,* 2015; Antunes dos Santos *et al.,* 2018; Mahajan *et al.,* 2018; Oke *et al.,* 2019; Sharifi-Rad *et al.,* 2020). Overproduction/long term exposure to free radicals promotes oxidative stress by exerting deleterious effects on the lipids, proteins, and DNA component of living cells leading to chronic degenerative diseases (Biswas *et al.,* 2017; Sharifi-Rad *et al.,* 2020). As previously reported, these chronic/noncommunicable (NCC) diseases including stroke, heart disease, diabetes, pulmonary disease, cancer and neurological diseases accounted for more than 70% of all global deaths (WHO, 2020).

The oxidative stress related damages can be prevented by endogenous antioxidants including the enzymes; catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and some non-enzymatic antioxidants (e.g. thiols antioxidants and coenzyme Q10) which the cell can synthesize from smaller building blocks (Da Pozzo *et al.,* 2018; Sharifi-Rad *et al.,* 2020). Nonetheless, when the endogenous antioxidant defence mechanism become overwhelmed by the excess free radical generation, the use of synthetic antioxidants/drugs becomes necessary to manage the prevailing chronic conditions. These drugs have been reported to exert their pharmacological effect via different mechanisms such as donating an electron to the free radical and converting it to a harmless product, reducing the energy of the free radical, suppressing the radical formation or breaking the chain propagation and by repairing the damage to reconstitute membranes (Sen and Chakraborty, 2011; Merinal *et al.,* 2012; Sridevi *et al.,* 2018).

However, long term use of such synthetic antioxidants/drugs e.g. alkylating agent,

anthracyclines, camptothecins, platinum coordination complexes and podophyllin derivatives may cause several unpleasant side effects including nausea, vomiting and diarrhea. Persistent vomiting may cause dehydration, loss of electrolyte balance, metabolic alkalosis, weight loss and weakness e.t.c. (Joensuu, 2008; Singh *et al.,* 2018). Hence, there is need for safe exogenous antioxidants from natural food sources as alternative to the synthetic antioxidants/drugs to supplements the endogenous antioxidants in the management of the free radical induced oxidative stress and its associated chronic disorders (Sridevi *et al.,* 2018; Lourenço *et al.,* 2019).

In due course, scientific evidences have shown considerably higher bioavailability and efficacy of protease-generated antioxidative hydrolysates from different food proteins as natural exogenous antioxidants to reduce the risk of chronic diseases such as cancer without manifesting life threatening side effects (Lobo *et al.,* 2010; Sridevi *et al.,* 2018; Sharifi-Rad *et al.,* 2020). Among these, alcalase-generated *Pleurotus geesteranus* hydrolysate exerted its cytoprotective effects against H_2O_2-induced oxidative stress in PC12 cells by stimulating the activities of the antioxidant enzymes and decreasing the intra cellular levels of the ROS (Liao *et al.,* 2020). Similarly, the effect of ultrafiltered chia protein hydrolysates fraction (molecular weight < 3-10 kDa) on the suppression of oxidative stress and increased survival of *Saccharomyces cerevisiae* (exposed to hydrogen peroxide) has been reported (Coelho *et al.,* 2019).

The cocoa bean used in this study, is one of the leading cash crops with great economic significance that is cultivated in the south-western Nigeria. It has been identified as a rich source of protein (Rawel *et al.,* 2019) and phytochemicals (Cádiz-Gurrea *et al.,* 2020) with many health benefits including antioxidant activity, antidiabetic activity, antimicrobial activity and antihypertensive activity (Oracz and Nebesny, 2016; Ludovici *et al.,* 2017; Ramos *et al.,* 2017; Todorovic *et al.,* 2017; Dugo *et al.,* 2018).

To the best of our knowledge, no work has been reported on the radical scavenging activity of DCB protein hydrolysates. Hence, the present study is targeted to hydrolyze defatted cocoa beans (DCB) protein using bromelain and papain to produce protein hydrolysates with antioxidative potential that can be safely used as free radical scavenger against DPPH* and its related radicals in the management of chronic degenerative diseases and other free radicals triggered inflammatory conditions.

MATERIALS AND METHODS

Collection, Identification and Preparation of Plant Material

The cocoa pods (*Theobroma cacao* L.) sample was obtained from Gbongan town in Ayedaade Local Government area, Osun State, Nigeria. The sample was identified in the Department of Plant Biology, Bayero University Kano. The seeds were removed, cleaned and dried, and the recovered cocoa almonds were ground into fine powder and stored at −4°C before further analysis.

Chemicals and Reagents

The proteases (bromelain and papain) and DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical were purchased from Sigma Chemical Co., USA. Linoleic acid, Ferric Chloride, potassium ferricyanide, trichloroacetic acid (TCA), monobasic and dibasic sodium phosphate and methanol were purchased from Merck (Germany). All other chemicals and reagents used were of analytical grade.

Proximate Composition Analysis

The cocoa beans powder (CBP) was analysed for moisture, ash, crude fat and crude protein and total carbohydrates content according to the methods of the Association of analytical communities as previously described (Horwitz and Latimer, 2005).

Preparation of Defatted Cocoa Beans Powder (*Theobroma cacao* L.)

The CBP was defatted according to the method described by Zaharuddin *et al.* (2021) with some modifications. The CBP was mixed with petroleum ether in a 1:3 ratio and agitated for 30 min. The mixture was decanted to remove the solvent layer and the process was repeated two more times for optimal defatting. The defatted sample was then air-dried at room temperature under a fume hood overnight to remove excess solvent.

Preparation of the DCB Protein Hydrolysates Using Bromelain and Papain

The defatted cocoa beans powder (5 g) was dialysed using (MWCO) 8000-14000kDa dialysis membrane tube for 96 h (for 4 h against deionized water at room temperature and then against phosphate buffer at 4°C in dark for 92 h). The dialyzed DCB sample was then preincubated with phosphate buffer (0.1M, pH 6.8) and heated the mixture to 40^0C. Each enzyme was then separately added to the 5 g of the dialysed sample at an enzyme/substrate ratio of 1:50 w/w and thoroughly mixed. The hydrolysis reaction for the proteolytic digestion

of the DBC protein was continued under shaking for 8 h. Afterwards, the reaction mixture was heated in a boiling water bath at 100^0C for 5 min to inactivate the enzyme. The mixture was then cooled and centrifuged at 10,000 rpm for 15 min and supernatant obtained was lyophilized to obtain the DCB protein hydrolysates which was then stored at -20^0C for further analysis.

Determination of Peptide Content

The peptide content was determined as TCA-soluble peptide content as previously described (Liao *et al.,* 2020) with little modification. A 200 µL each of bromelain- and papain-generated DCB protein hydrolysates were separately mixed with 200 µL of 20% (w/v) TCA solution and allowed to stand for 30 min. The precipitate formed was centrifuged at 7800 x g for 12 min and the TCA-soluble peptide content of the resulting supernatant was determined using BCA protein assay kit method as previously reported (Auwal *et al.,* 2018a; Auwal *et al.,* 2018b) with bovine serum albumin (BSA) as standard. The TCA-soluble peptide yield was calculated as follows:

TCA - soluble peptide (%) = X/Y x 100%

where X: peptide content of the supernatant and Y: total protein content of the sample before enzymatic hydrolysis.

Determination of DPPH* Radical Scavenging Activity

The DPPH* radical scavenging activity of the bromelain- and papain-generated DCB protein hydrolysates was determined according to the method described by Auwal *et al.* (2017). A 100 µL solution of 0.15 mM DPPH prepared in methanol was added to 100 µL of the hydrolysates prepared at a concentration of 10 mg/mL and incubated at room temperature for 30 min. The absorbance was read at 517 nm and the DPPH* radical scavenging activity was calculated as follows;

$$\text{DPPH} * \text{ radical scavenging activity } (\%) = \left(1 - \frac{\text{As} - \text{A}b}{\text{Ac}}\right) \times 100$$

Where As is Absorbance of the sample at 517 nm, Ab is Absorbance of the blank at 517 nm and Ac is Absorbance of the control at 517 nm.

RESULTS AND DISCUSSION

Proximate Composition Analysis

The proximate composition of the cocoa beans is shown in Table 1. The moisture, ash, fat, protein and total carbohydrate contents were 4.41%, 4.32%, 38.83%, 23.33% and 10.28%, respectively. The relatively high protein content is an indication that, the cocoa beans can be a potential source of bioactive protein hydrolysates for different applications in functional foods and nutraceutical industries.

Table 1: proximate composition of cocoa beans

Parameters	Composition (%)
Crude protein	23.33±0.47
Carbohydrates	10.28±0.11
Crude fibre	10.16±0.02
Crude fat	38.83±0.22
Ash content	4.32±0.04
Moisture	4.41±0.60

Values are expressed as mean ± standard deviation

Effect of Hydrolysis Time and Peptide Content on DPPH* Radical Scavenging Activity

As previously reported, the antioxidant capacity of various reducing agents has been studied by assessing their DPPH* radical scavenging ability (Shahi *et al.,* 2020). In the present study, the DCB protein was hydrolyzed for 8 h using bromelain and papain. The proteases acted differently to hydrolyze the peptide bonds in the protein and produce hydrolysates of varying peptides composition and DPPH* radical scavenging activity at different hydrolysis time. An increase in DPPH* radical scavenging activity of the hydrolysates was observed with increase in hydrolysis time up to 6 h, where both bromelain- and papain-generated DCB protein hydrolysates showed their maximum DPPH* radical scavenging activity of 78.07% and 53.04%, respectively (Figure 1).

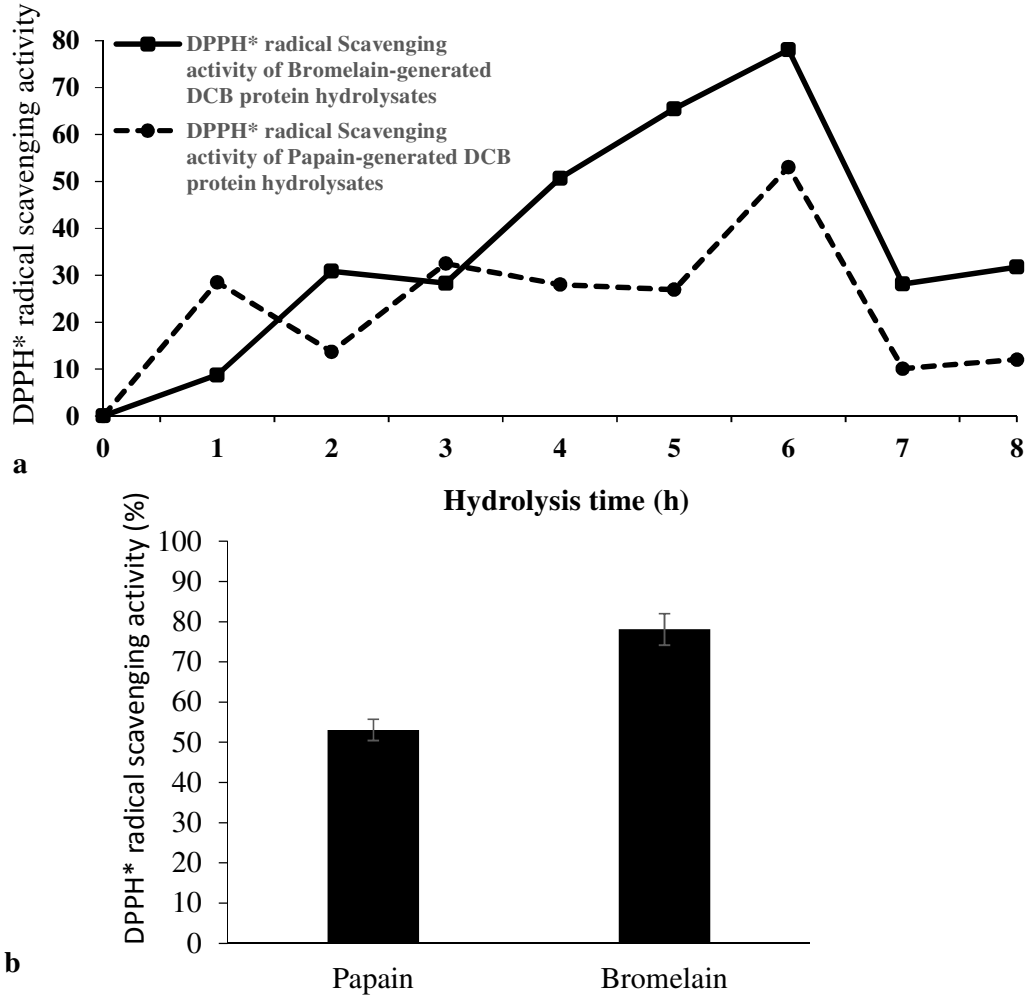

Figure 1: DPPH*radical scavenging activity of bromelain and papain generated hydrolysates (a) Effect of hydrolysis time on DPPH* radical scavenging activity of bromelain and papain generated hydrolysates and (b) maximum DPPH* radical scavenging activity of bromelain- and papain-generated hydrolysates.

Similarly, higher DPPH* radical scavenging activity was obtained at higher hydrolysis time for palm kernel cake protein generated hydrolysates (Zarei *et al.,* 2012). Such observation was attributed to the generation of low molecular weight peptides containing alkaline amino acids (arginine, lysine and histidine) which might have donated electron to neutralize the DDPH* radical and terminated its propagation (Auwal *et al.,* 2017; Sonklin *et al.,* 2021).

However, beyond 6 h, a decrease in DPPH* radical scavenging activity was observed with increase in hydrolysis time. In a related finding, the decrease in DPPH* radical scavenging activity with increase hydrolysis time, has been related to the hydrolysis of the protein into hydrophilic peptides/amino acids (You *et al.,* 2009; Shahi *et al.,* 2020) and increased polarization which retard the interaction of amino acids to the DPPH* radical (Zhao *et al.,* 2010).

In addition, the higher free radical scavenging activity due to bromelain, could be related to its broad specificity in cleaving peptide bonds with neighboring C-terminal arginine or lysine which show higher tendency to donate electron and stabilize the free radical. Similarly, bromelain has been reported to hydrolyze peptide bonds in proteins with preference to carbonyl end of alanine, lysine, glycine, and tyrosine, but with higher preference for small molecular substrates

such as Z-Arg-ArgNHMec (Rowan *et al.,* 1990; Yu and Mikiashvili, 2020). Furthermore, the DPPH* radical scavenging activity of both bromelain- and papain-generated hydrolysates was found to correlate positively to their peptide contents with R^2 values of 0.946 and 0.772 based on Pearson's correlation analysis (Fig. 2). Thus, an increase in the DPPH* radical scavenging activity of the protein hydrolysates was observed with increase in peptide content according to a second-order polynomial model. A similar observation which showed an increase in the DPPH* radical scavenging activity of the hydrolysates with increase in hydrolysis time was made based on the Pearson's correlation analysis according to the second order polynomial model (Fig. 2).

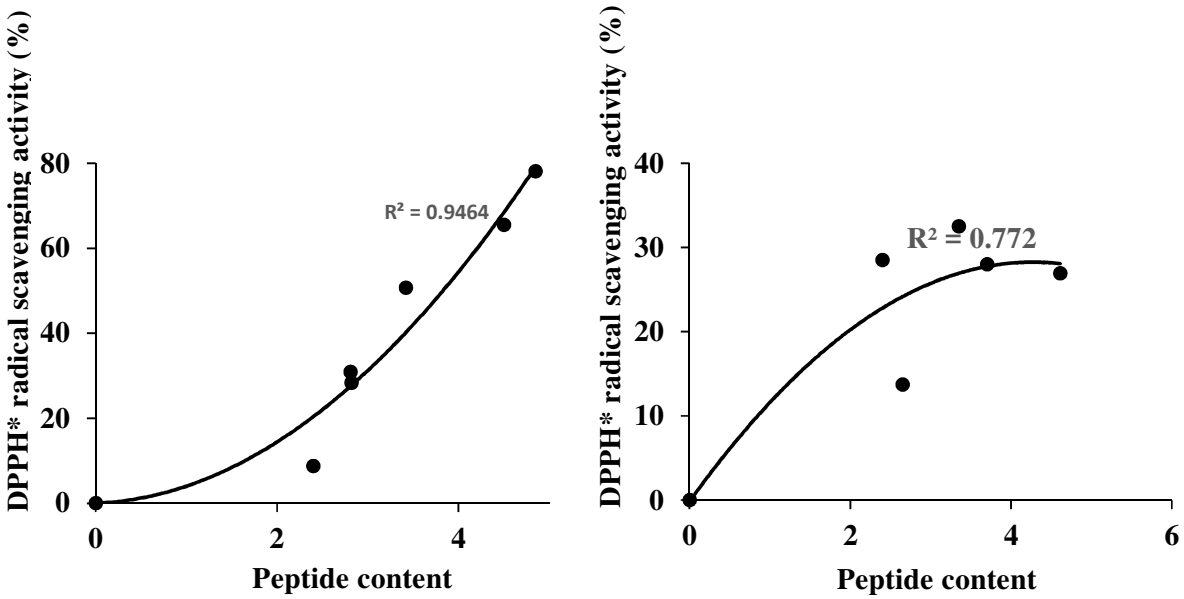

Figure 2: Correlation between peptide content and DPPH* radical scavenging activity of DCB protein hydrolysates: (a) Bromelain; (b) Papain.

CONCLUSION

In the present study, the bromelain- and papain-generated hydrolysates were found to exhibit strong DPPH* radical scavenging activity under the influence of hydrolysis time and peptide content. Bromelain-generated hydrolysates showed the highest DPPH* radical scavenging activity of 78.07% compared to 53.04% revealed by papain-generated hydrolysates. The DPPH* radical scavenging activity of both bromelain- and papain-generated hydrolysates correlated positively to their hydrolysis time and peptide content during the first 6 h of hydrolysis. Thus, protease-mediated hydrolysis of DCB protein can be used to generate hydrolysates containing potent bioactive peptides with DPPH* radical scavenging activity that can serve as potential ingredients in the industrial formulation of functional foods and nutraceuticals for the management of chronic conditions.

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