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Investigation on antioxidant, angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory activity of Bambara bean protein hydrolysates

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ABSTRACT

Protein isolate was hydrolysed by Alcalase, thermolysin and trypsin. BBPH produced by Alcalase showed highest angiotensin-converting enzyme (ACE) inhibitory properties (IC_{50}: 52 μg/mL). Hydrolysates produced by Alcalase and thermolysin exhibited similar dipeptidyl peptidase-IV (DPP-IV) inhibitory activity (IC_{50}: 1.73 mg/mL), while low inhibitory activity was observed for hydrolysate produced by trypsin. BBPH also showed protective effect against oxidative stress with significant 2,2-diphenyl-1-picrylhydrazyl radical scavenging and ferrous chelating activity. Bioactive peptides of BBPH produced by thermolysin showed better resistance to simulated gastrointestinal digestion (SGID), while the DPP-IV and ACE inhibitory properties were significantly reduced. Molecular weight distribution showed significant reduction in peptides of the molecular weight range 200–400 Da in BBPH produced by Alcalase, after SGID. LC-ESI-TOF-MS and *in silico* analysis showed the presence of potential peptides with both ACE and DPP-IV inhibitory properties in BBPH produced by thermolysin.

1. Introduction

Grain legumes are major foodstuffs in most countries. They are the cheapest source of supplementary proteins in diets in developing countries. Legumes also contain slow release carbohydrates and micronutrients (Tharanathan & Mahadevamma, 2003). Daily consumption of grain legumes reduces the risk factors for cardiovascular disease such as hyper-triglyceridemia, diabetes, and hypertension (Arnoldi, Zanoni, Lammi, & Boschin, 2015). Bambara bean (*Vigna subterranea*) is the third most important legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) in Africa, and known to be drought-tolerant. Studies on Bambara bean protein concentrate showed high protein digestibility and essential amino acid index (Mune Mune, Minka, Mbome, & Etoa, 2011). Bambara bean proteins are usually rich in aspartic acid and glutamic acid, and contain high levels of leucine, arginine, and lysine. Moreover, major proteins had molecular weight between 35 and 115 kDa, with the particularity to not contain subunits linked by disulphide bond (Adebowale, Schwarzenbolz, & Henle, 2011). Bambara bean protein hydrolysates also presented adequate functional properties for use in food formulations (Mune Mune, 2015).

WHO (2013) estimated that the number of people suffering from cardiovascular diseases (CVD) and type-2 diabetes (T2D) is increasing worldwide. Utilization of usual drugs in the management of these diseases showed many side effects (Acharya, Sturrock, Riordan, & Ehlers, 2003). In this regard, functional foods have emerged as an alternative to chemotherapy in the prevention and management of human diseases and for maintaining optimum health state. Moreover, food protein hydrolysates and peptides possess multiple bioactive properties that could find application in functional foods (Udenigwe & Aluko, 2012). Bioactive properties of peptides produced from plant proteins include antioxidant activity (Vaštag, Popović, Popović, Peričin-Starčević, & Krimer-Malešević, 2013), cholesterol-lowering ability (Arnoldi et al., 2015), blood pressure-lowering (ACE inhibitory) effect (Rudolph, Lunow, Kaiser, & Henle, 2017) and anti-hyperglycemic (DPPIV inhibitory) effect (Lacroix & Li-Chan, 2012). Limited enzymatic hydrolysis *in vitro* is widely used to release bioactive peptides from parent proteins, and some of these peptides have been recommended as a component of functional foods or as new drugs for prevention of CVD and T2D (Udenigwe & Aluko, 2012). Rudolph et al. (2017) and Lunow, Kaiser, Brückner, Gotsch, and Henle (2013) produced bioactive peptides from enzymatic hydrolysis of food and plant proteins using Alcalase, thermolysin, trypsin and chymotrypsin. Therefore, grain legumes can be considered promising sources of protein for production of bioactive peptides due to their low cost, low allergenicity and adequate nutritional and functional values.

Some important risk factors for developing CVD are raised blood

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pressure, vascular oxidative stress and associated endothelial dysfunction. Angiotensin converting enzyme (ACE, EC. 3.4.15.1) is a key target for treatment of hypertension, and inhibition of ACE causes a reduction of blood pressure by increasing the level of angiotensin II and decreasing the level of bradykinin, a vasodilatory peptide. Dipeptidyl peptidase IV (EC. 3.4.14.3, DPP-IV) is an important enzyme for the prevention and treatment of T2D. Inhibition of DPP-IV, a serine protease, enhances insulin secretion by modulating the biological activity of the insulintropic hormone, glucagon-like peptide-1 (GLP-1). Hatanaka et al. (2012) reported that inhibitors of DPP-IV have been used as drugs in type-2 diabetes. Moreover, it was estimated that diabetes and hypertension were closely linked. In addition, many pathological conditions such as diabetes, cardiovascular disease, cancer and other chronic and inflammatory diseases were closely related to oxidative damage. It has been demonstrated that natural antioxidants, and more recently plant protein hydrolysates, can be effective for prevention and treatment of oxidative stress-related diseases (Morisco et al., 2004).

In silico analysis has been useful in the prediction of release of bioactive peptides from various food proteins, and this technique is complementary to experimental procedures. Chang and Alli (2012) predicted the release of 34 and 35 peptides having ACE inhibitory potential from chickpea proteins using ficain and proteinase K, respectively. Lacroix and Li-Chan (2012) also demonstrated that plant and animal proteins contained fragments with potential DPP-IV inhibitory activity which could be released through enzymatic hydrolysis. The aim of this work was to evaluate the bioactive properties of Bambara bean protein hydrolysates produced by Alcalase, thermolysin and trypsin, and study effect of simulated gastrointestinal digestion on these properties. Furthermore, the release of bioactive peptides was analyzed by *in silico* and molecular analysis.

2. Materials and methods

2.1. Materials

Bambara bean 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), α-chymotrypsin (from bovine pancreas, ≥0.04 U/g protein), pepsin (from porcine gastric mucosa, 3200–4500 U/mg protein), Prolidase (106 U/mg protein), aminopeptidase M (28 U/mg protein), ACE (from rabbit lung), 2,4,6-trinitrobenzenesulphonic acid (TNBS) were obtained from Sigma Aldrich Chemie (Steinheim, Germany). Hippuryl-L-histidyl-Leucin (HHL) was purchased from Bachem AG (Bubendorf, Switzerland). Pronase E (4000 PU/mg protein) was purchased from Merck (Darmstadt, Germany). DPP-IV Drug Discovery Kit containing H-Gly-Pro-p-nitroaniline and DPPIV enzyme was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). All chemicals were of the highest purity available.

2.2. Preparation of Bambara bean flour

The seeds were cleaned and ground into fine flour (mesh size 200 μm), and defatted using a hexane/isopropanol mixture at 3/2 ratio and according to Maguire, O'sullivan, Galvin, O'connor, and O'brien (2004). The defatted flour was air-dried at ambient temperature and stored at −4 °C.

2.3. Preparation of Bambara bean protein isolate (BBPI)

Protein isolation was done as described by Mune Mune (2015) with slight modifications. The extracted proteins were washed twice at pH 4.5 for 15 min and recovered by centrifugation at 8000*g* for 20 min and 4 °C. Proteins were re-suspended in double distilled water maintaining about 10% (m/v) total solid, and the pH adjusted to 7.0 and freeze-dried. Moisture and protein contents of the protein isolate were determined following AOAC (1990) methods, and were respectively 7.20 and 90.26%.

2.4. Preparation of Bambara bean protein hydrolysates (BBPH)

Enzymatic hydrolysis of BBPI was performed as described by Lunow et al. (2013) 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 (Rudolph et al., 2017). The reaction proceeded for 24 h in an autoclaved closed-top vessel, and the pH was adjusted each 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 8000*g* for 10 min and 4 °C, and the supernatant was freeze-dried and used for further analysis.

2.5. Simulated gastro intestinal digestion

The hydrolysis of BBPH by gastrointestinal proteases was performed by the method for simulated gastrointestinal digestion described by Vastag et al. (2013). BBPH at 20 mg/mL in HCl 0.01 M was initially treated with pepsin at the ESR of 0.04 mg/mg, at pH 2.10, 37 °C for 30 min. pH was adjusted to 7.5 and the solutions of α-chymotrypsin (ESR of 0.02 mg/mg) and trypsin (ESR of 0.08 mg/mg) were added. The hydrolysis by pancreatic enzymes was performed at pH 7.5 and 37 °C for 90 min. At the end of hydrolysis by pepsin and pancreatic enzymes, the reaction mixture was immediately heated (100 °C, 5 min) and the mixture was centrifuged at 8000*g* for 10 min). The collected supernatants were freeze-dried and used for further analysis.

2.6. Amino acid analysis

Amino acid analysis was performed as described by Hellwig and Henle (2013) and Hellwig and et al. (2014) with hydrolysis of proteins by the following enzymatic procedure. Briefly, proteins were successively hydrolyzed by pepsin, pronase E, aminopeptidase and prolidase. Then, the mixtures were incubated for further 24 h at 37 °C and then lyophilized. Enzymatic hydrolysates of proteins were reconstituted in 1 mL of 5 mM heptafluorobutyric acid, diluted if necessary and membrane filtered (0.45 μm).

Measurement of aminoacids was performed with the amino acid analyzer S 433 (Sykam, Fürstenfeldbruck, Germany) using a PEEK column filled with the cation exchange resin LCA K07/Li (150 × 4.6 mm, 7 μm) as described by Hellwig and Henle (2013). Tyrosine was used as an internal standard for the evaluation of amino acid chromatograms.

2.7. Degree of hydrolysis

The degree of hydrolysis was determined as percentage of free amino groups following reaction with TNBS (Adler-Nissen, 1979). 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 standard.

2.8. Determination of bioactive properties

The samples hydrolyzed for 24 h were used for evaluating the bioactivities of BBPH.

2.8.1. Antioxidant properties

The scavenging effect of BBPHs on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was measured according to the method of Zhu,