Multifunctional bioactive peptides from germinated soy (*Glycin* max) and voandzou (*Vigna* subterranea) beans: *in vitro* anti-diabetic potential through α-amylase α-glucosidase inhibition, and antioxidant ability by DPPH reducing

ABSTRACT

Legumes, protein-rich foods containing various peptides, offer the possibility of modulating both sugar absorption and free radical production, in order to prevent diabetes and oxidative stress diseases. This study aimed to reveal the antioxidant and anti-diabetic potential of peptides from germinated soy and voandzou beans by checking the efficacy of these beans peptides generated by germination and even after post-meal digestion. Thus, beans crude proteins were extracted and digested with pepsin and pancreatic enzymes. Digested proteins were fractioned by dialysis. Each type of proteins ability to inhibit α-amylase and α-glucosidase activity and to scavenge DPPH free radical, were tested. Total protein content determined by spectrophotometry at 241 nm and extraction yield evaluated showed no significant difference ($p < 0.05$) with an average of 88% (soy) and 86% (voandzou) and 64.43% (soy) and 60.30% (voandzou), respectively; as opposed to the soluble protein content assessed in the supernatant of protein isolates. Simulated digestion of isolate protein generated peptide extracts showed a better inhibitory capacity of α-amylase and α-glucosidase with the digested voandzou protein isolate D2 exhibiting optimum inhibition (72.39%) for α-glucosidase near to acarbose (75.99%) and the digested soy protein isolate D2 displaying the optimum inhibition (89.25%) for α-amylase better than acarbose (75.99%). Moreover, it is the external fractions of size inferior to 10 kDa which are endowed with this excellent capacity of inhibition of these enzymes involved in the regulation of postprandial glucose. Finally, the high DPPH free radical scavenging capacity of all germinated protein isolate between 64.72 (D0) and 72.27% (D2) for soy and 73.45 (D0) and 81.43 % (D4) for voandzou is practically maintained after digestion and dialysis. Germinated soy and voandzou seeds thus have shown promising potential for being used as nutraceutical or functional ingredients for treatment and prevention of diabetes and diseases related to oxidative stress.

Keywords: germination, soy/voandzou peptides, enzyme inhibition, DPPH inhibition, diabetes, oxidative stress

1. INTRODUCTION

Metabolic diseases including type 2 diabetes mellitus (T2DM) and cardiovascular diseases represent a public health problem nowadays [1]. Indeed, diabetes is undergoing a very

significant expansion. According to the latest [2] estimates, 463 million people worldwide have diabetes and this could reach 578 million by 2030. In addition to these alarming data, it is necessary to take into account people who do not know they have diabetes because the development of the pathology is silent and insidious. As for cardiovascular diseases, they are responsible for the largest number of deaths with 17.9 million per year [1]. Africa is not exempt from this trend. Cardiovascular disease and type 2 diabetes mellitus, once rare, are now rapidly increasing in developing countries. Epidemiological forecasts estimate that the prevalence of diabetes will have increased by 98% in sub-Saharan Africa by 2030 [3]. As a result, the need to reduce these disease rates is emerging as one of the major challenges for development.

Type 2 diabetes is characterized by chronic hyperglycemia leading to insulin resistance or insufficient insulin production for various reasons. This disease is generally linked to age, sedentary lifestyle, and one of the factors not indexed but often a source of complication is that related to the reactive oxygen species. However, there are drug treatments for type 2 diabetes including metformin (glucophage) which improves insulin sensitivity and inhibits endogenous glucose production, sulfonylureas which stimulate and/or potentiate endogenous insulin secretion, acarbose which inhibits the action of α-amylase and α-glucosidase in postprandial hyperglycemia. These drugs used in diabetes management, although effective, are not accessible due to their high cost and are known to trigger clinical symptoms and various side effects such as nausea, vomiting, weight gain, increased cardiovascular risk [4].
Faced with these problems, several approaches of research are being explored to provide a sustainable solution. One of them related to the exploration of the functional aspect of food through the research of the bioactive molecules they contain. Indeed, food is the essential means by which the human body obtains biologically active substances. This multitude of substances, beyond constituting the basic nutrients, exerts a significant impact on human health [5]. In recent years, numerous researches carried out in this field [6; 5] have shown the close relationship between food and health. Diet plays a central role in establishing the balance between health and disease, beyond satisfying the satiety of the human body. Foods contain biologically active molecules that impact positively the biological functions of the body. These molecules of lipid, carbohydrate, phenolic and especially protein nature contribute positively to health beyond their basic nutritional functionality [5].

In the case of proteins, many studies have shown the effectiveness of bioactive peptides in the prevention and treatment of diabetes and oxidative stress causing cardiovascular diseases. For example, research conducted by [7] and [8] have shown the existence of some oligopeptides with antihypertensive, antioxidant and antidiabetic activities that can help reduce metabolic diseases such as diabetes mellitus, cancer, cardiovascular diseases, etc. Although less effective than chemical molecules, these peptides and hydrolysates (mixture of amino acids, oligopeptides and polypeptides) of dietary origin have the advantage of having fewer side effects based on their natural sources and mechanisms of action [5].
Cereals and especially legumes are an excellent source of peptides because of their high protein content. Among legumes, soy (*Glycine* max) and voandzou bean (*Vigna* subterranea) as well as their press-cakes have high protein contents ranging from 40 to 45% and 14 to 20.74%, respectively that can be exploited in the search for bioactive peptides [9]; [10]. Generally peptides are existing in the food protein supply and can only be released as a result of a hydrolysis reaction. This hydrolysis reaction, which occurs during certain metabolic pathways such as gastrointestinal digestion, fermentation, and germination, can lead to oligopeptides with multiple biological activities ([11]; [12]).

It should be noted, that some studies such as those of [11] conducted on germinated soybeans at 6 days only have identified peptides of interest in the treatment of diabetes.

However, to date, no study has investigated the search for the optimum production of germinated soy and voandzou bean peptides over multiple days. Similarly, the potential of voandzou bean to generate bioactive peptides has been very little explored to date. This work, which is part of the research context of bioactive peptides in the prevention and sustainable treatment of type 2 diabetes mellitus and diseases related to oxidative stress, has the general objective of enhancing the value of germinated soy and voandzou bean by highlighting their anti-diabetic and antioxidant potential.

2. MATERIAL AND METHODS

2.1. Material

The biological material is made up of two legumes seeds, soy (*Glycine* max L.) and voandzou (*Vigna* subterranea) white with red spots, and of the digestive juice of snails (*Achatina* achatina). This material was purchased at the forum market of Adjamé (5° 29′ 17″ north, 4° 01′ 56″ west) and then transported to the laboratory of the Pedagogical and Research Unit (UPR) of Biotechnology of the Laboratory of Biotechnology Agriculture and Valorization of Biological Resources.

The reagents used were α-amylase from aspergillus orizae (EC 3.2.1.1; 30 mg U/mg solid), pancreatin (EC 232-468-9; 8X USP, the mixture of several digestive enzymes produced by exocrine cells of the porcine pancreas) and glucose oxidase activity assay kit (colorimetric) from SIGMA-Aldrich (Madrid, Spain), pepsin (EC 3.4.23.1; 0,7 FIP- U/mg) from porcine gastric from PanReac AppliChem, arcabose (a drug for glycemia modulation) and pure distilled water both puchased in a pharmacy officine, bovine serum albumin (BSA, >98% pure) and a semi-permeable hydrophilic membrane with a cut-off diameter of 10-12 KDa (SIGMA-Aldrich, USA).

2.2. Methods

2.2.1 Preparation of germinated soy and voandzou seeds

Soy and voandzou seeds were germinated for six days following the method described by [13]. Thus, seeds of each legume type (four batches of 100 g) were soaked in 600 mL of distilled water for 2 h at 30 °C. The soaked seeds were then placed in a germination chamber (Seed Processing Holland, The Netherlands) at 30 °C for six days in the dark with a 15 s irrigation cycle every 6 h. On day D0 and every second day until day 6, D6) the seeds of a batch of germinated legumes were oven dried (UN-110 Memmert, Germany) at 40 °C for 24 h, then ground in a Moulinex type mixer and sieved. The resulting flour was then delipidated by suspension in petroleum ether in a ratio of 1/2 (W/V) under magnetic stirring for 12 h at room temperature (28 °C). After decantation, the supernatant containing the suspended lipids was removed and the pellet was recovered and dried in an oven at 45°C for 24 h to constitute the press-cake (delipidated flour).

2.2.2 Preparation and quantification of protein isolate from germinated soy and voandzou seeds

Protein isolates from soybean and voandzou at different germination times were prepared from the previously obtained delipidated flour by alkaline extraction (pH 8.0) and isoelectric precipitation (pH 4.3) [14]. A mass of 2.5 g of the different soybean and voandzou press-cakes were suspended in 25 mL of distilled water (1/10 W/V) and the pH of the mixture was adjusted to 8.0 with a 0.1 M NaOH solution at 35°C. Then, the suspension was put under magnetic stirring at 35°C for 1 h for protein solubilization. Finally, the solubilized proteins

were recovered in the S1 supernatant after centrifugation of the mixture at 5000 rpm for 15 min at 4 °C (SIGMA 3-30K, Germany). The pellet recovered and processed again as before twice to recover successively the S2 and S3 supernatants. The solubilized proteins from the different supernatants S1, S2 and S3 were mixed for isoelectric precipitation. After adjusting the pH of the previously obtained supernatants (S1, S2 and S3) to 4.3 with a 2 M HCl solution under constant magnetic stirring, the mixture was centrifuged at 10,000 rpm for 20 min at 4 °C (SIGMA 3-30K, Germany). The pellet containing the precipitated proteins was recovered for soluble sugar extraction

Soluble sugars were extracted from protein isolates by ethanolic extraction (80%) following the optimized method of [15]. To 5g of protein isolates were added 25 mL of ethanol (80%), the mixture was put under magnetic stirring for 15 min then centrifuged at 3000 rpm at 4°C for 3 min. The pellet separated from the supernatant was suspended in ethanol (80%) and the procedure was repeated twice successively (time after which no trace of sugar was detected in the discarded supernatant using the phenol sulphuric acid reagent method [16]. The pellet was then recovered and dried at 45°C in the oven for 24 h. The resulting pellet corresponding to the pure protein isolate is suspended in distilled water to constitute a 5% (w/v) prepared protein isolate.

The total protein content (in the whole extract) and the soluble protein content measured in the supernatant of protein isolate were evaluated using the Folin reagent method of (Singleton et al 1999) [17]. This method is based on the ability of the phosphomolybdate molecules contained in the Folin-Ciocalteu yellow staining reagent to form complexes with the aromatic amino acids present in the solution to give a blue coloration. The optical density was measured at 660 nm with a solution containing all the reagents except the proteins as a control. Bovine serum albumin (BSA) solution was used as a standard.

Similarly, the extraction yield was calculated as the ratio between the amount of delipidated press-cake and the mass of protein isolate obtained.

2.2.3 Simulation of gastrointestinal digestion of germinated soy and voandzou seeds protein isolate

Soy and voandzou bean protein isolate were sequentially digested with porcine pepsin and pancreatin as previously described [18]. Hence, 1 mL of a 160 mg/ml pepsin solution (EC 3.4.23.1) prepared in 0.1 M HCl were mixed with 25 mL (4%, v/v Enzyme/Protein isolate) of the different legume seed and the pH of the medium adjusted to 2 with a 1 M HCl solution. The reaction medium was homogenized by vortexing and incubated at 37 °C for 1 h. Then, 1 mL of pancreatin solution was added, and the pH was adjusted to 7.5 with 1 N NaOH. The solution was incubated at 37 °C for 2 h. The reaction was stopped by immersing the samples in a boiling bath for 10 min. After cooling to room temperature, the mixture was centrifuged at 16,000 rpm for 10 min and the resulting supernatant (containing hydrolysates) were recovered and stored at -18 °C for further analysis.

2.2.4 Fractionnement of hydrolysate from germinated soy and voandzou seeds protein isolate

Protein isolates ppreviously obtained were fractioned by dialysis through a cellophane membrane (10 kDa) in a ratio of extract to pure distilled water (1/10, v/v) for 2 h. Both inside and outside contents of dialyse bag were concentrated using speed vac and polyethylene glycol (PEG), respectively and stored at -20 °C for further assay.

2.2.5 Essays for α-amylase inactivation by germinated soja and voandzou seeds protein isolate

α-amylase inhibition potential of the different studied legume seeds extracts was evaluated and compared to arcabose as a positive control. Different reaction media of 200 µL made of 30 µL of protein extract (5% W/V), or positive control (5% W/V, acarbose), or negative control (water in 0.02 M sodium phosphate buffer pH 6.9), 50 µL of α-amylase (1.5 IU/mL) (prepared in 0.02 M sodium phosphate buffer pH 6.9), and 10 µL of phosphate buffer (0.02 M sodium phosphate buffer pH 6.9). After pre-incubation at 37 °C in a water bath for 5 min, the reaction is initiated by addition of 110 µL of corn starch solution (prepared at 1%, W/V in 0.02 M phosphate buffer pH 6.9) and incubation at 37 °C in a water bath for 10 min. A volume of 200 µL of 3,5-Dinitrosalicilic acid acid solution was added to develop the reaction and the whole set was put in a boiling bath for 5 min for staining development. Then, 3.6 mL of distilled water was added, and the absorbance measured at 540 nm with a spectrophotometer (Pioway, China V5000) against a blank (all the reagents in phosphate buffer, except starch). The results were expressed as percentage of α-amylase inhibition which is calculated according to the following formula:

$$\text{Inhibition of } \alpha\text{-amylase (\%)} = \frac{A_{SA} - A_{PEA}}{A_{SA}} X100$$

A_{SA} : Absorbance of the standard assay (reaction medium with enzyme (50 µL) and substrate (110 µL of corn starch solution 1%, w/v in 0.02 M phosphate buffer pH 6.9) in 40 µL of phosphate buffer (0.02 M sodium phosphate buffer pH 6.9) ;

A_{PEA}: Absorbance of the Protein Extract Assay (reaction medium with Enzyme (50 µL)+substrate (110 µL of corn starch 1%) + protein Extract (30 µL) ;

2.2.6 Essay for α-glucosidase inactivation by germinated soy and voandzou seeds protein isolate

Inhibition of α-glucosidase activity by the different protein extracts is demonstrated by assessing the amount of glucose released by the enzyme from a natural substrate (sucrose), following the method described by [19] with slight modification.

Different reaction media of 200 µL were made in the same proportions as the α-amylase inhibition assay previously mentioned with the difference that 110 µL of sucrose solution prepared at 1% in phosphate buffer (0.02 M, pH 6.9) was used as substrate and the incubation time of the reaction media was 20 min. Then, the reacti5555ns were stopped by immersing the test tubes containing the different media in a boiling bath at 100 °C for 10 min. Afterwards, the released glucose was quantified by the glucose oxidase method using the Amplex Red glucose/glucose oxidase kit [20; 21]. 10 µL of each media were added to 1mL of reagent R consisting of a mixture of glucose oxidase (GOD ≥20000IU/L), phosphate buffer (150 mmol/L), peroxidase (POD ≥1000 IU/L), PAP (4-Amino-antipyrine 0.8 mmol/L), and chromogen (chloro-4-phenol, 2 mM). The absorbance of the extracts was read at 500 nm against a blank (1mL of reagent R and 10 µL of distilled water). Acarbose was used as a positive control and the results expressed as % inhibition were calculated using the following formula:

$$\text{Inhibition of } \alpha\text{-glucosidase (\%)} = \frac{A_{SA} - A_{PEA}}{A_{SA}} X100$$

A_{SA} : Absorbance of the standard assay (reaction medium with enzyme (50 µL) and substrate (110 µL of corn starch solution 1%, w/v in 0.02 M phosphate buffer pH 6.9) in 40 µL of phosphate buffer (0.02 M sodium phosphate buffer pH 6.9) ;

A_{PEA}: Absorbance of the Protein Extract Assay (reaction medium with Enzyme (50 µL)+substrate (110 µL of corn starch 1%) + protein Extract (30 µL) ;

2.2.7 Essays for radical DPPH inactivation by germinated soy and voandzou seeds protein isolate

The capacity of different protein isolates to reduce the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical was evaluated by the method described by [22]. A volume of 500 µL of aqueous protein extract was added to 1,500 µL of DPPH solution contained in a hemolysis tube. The mixture was homogenized and left in the dark for 30 min for staining development. Then the absorbance was read at 517 nm against a blank and the percentage of DPPH radical inhibition was obtained by the following formula:

$$\% \text{ Inhibition (DPPH)} = \frac{DOc-(DOe-DOb)}{DOc} X\ 100$$

Doc: control absorbance (0.5 mL methanol + 1.5 mL DPPH) ;
Doe: extract absorbance (0.5 mL protein extract + 1.5 mL DPPH ;
Dob: blank absorbance (0.5 mL protein extract + 1.5 mL methanol.

2.2.8 Statistical analysis

All assays were performed in triplicate and the statistical analysis of the results was done with STATISTICA 7.1 software. Comparisons between variables were determined by one-factor analysis of variance ANOVA, Duncan's test at the 95% threshold was used to determine significant differences between means.

3. RESULTS AND DISCUSSION

3.1 Soybean and voandzou crude protein extraction yield and content

The results of protein extraction yields, total and soluble protein contents from defatted and without sugar soy and voandzou seeds as a function of germination time are shown on table 1.
Regarding the extraction rate (ratio of the final weight of the protein extract to the initial weight of the press cake) and the total protein content, no significant difference ($p < 5\%$) was observed between the different germination times of soy and voandzou protein isolate which averaged 64.43% for soybean and 60.30% for voandzou. On the other hand, soy protein isolate showed a higher extraction yield than voandzou protein isolate for all germination times studied.

Table 1: Extraction yield, soluble and total protein content of soy and voandzou bean protein isolat at different germination time

Germination time	Soybean	Voandzoubean
Extraction yield (%)		
D0	64.66 ± 0.91^{aA}	60.40 ± 3.63^{aB}
D2	64.17 ± 1.50^{abA}	60.56 ± 3.27^{aB}
D4	64.34 ± 5.70^{aA}	59.69 ± 3.96^{abB}
D6	64.55 ± 7.15^{aA}	60.55 ± 7.15^{aB}
Total protein content (%)		
D0	88.05 ± 2.12^{aA}	85.66 ± 1.40^{aB}
D2	88.21 ± 3.13^{aA}	86.06 ± 4.18^{abB}
D4	88.95 ± 4.91^{aA}	85.83 ± 1.46^{aB}
D6	87.80 ± 2.32^{aA}	85.15 ± 3.98^{aB}
The soluble protein content of the isolate (%)		
D0	73.05 ± 2.56^{bB}	79.55 ± 1.00^{bA}

D2	69.25 ± 1.34^{cA}	67.75 ± 1.04^{dB}
D4	69.05 ± 1.33^{cB}	74.20 ± 1.11^{cA}
D6	77.25 ± 122^{aB}	80.70 ± 2.44^{aA}

Different alphabetical uppercase letters of each group in the same row show significant differences (P < .05) between samples at the same germination time and different alphabetical lower-case letters of each group in the same column show significant differences (P < .05) between samples at the same origin according to Duncan's test. Mean ± S.E.M = Mean values ± Standard error of means of six experiments

D0: no germination; D2, D4, D6: 2, 4 and 6 days of germination, respectively.

Analysis of these results showed, for each type of seed (soybean and voandzou) germinated and non-germinated, a protein extraction level significantly identical and greater than 60% regardless of germination time (Table 1). Thus, these yield values greater than 60% are higher than the yields found in the literature on hemp protein isolates generally between 35 and 50% extraction. This reflects the efficiency of the method (solubilization at alkaline pH and precipitation at pI) used to extract almost all of the protein contained in the studied plant matrix [23]. In addition, this technique, which consisted of precipitating the solubilized proteins at their isoelectric point (pH 4.3) easy to implement, also remains less expensive than the usually used membrane techniques.

The assessment of the total protein content in the crude protein extracts allows the purity of the preparation to be evaluated. The results of the quantification of total protein content in the studied legumes protein isolate revealed an average content of 88% (soybean) and 86% (voandzou bean) significantly identical regardless of the germination time. These high contents recorded in these crude protein isolates are far superior to those obtained (74.10 %) by [24] for quinoa protein isolated at the same pH used in the extraction process. This highlights the purity of our protein extracts which with protein contents between 80 and 95% can be qualified as protein isolates [25; 26].

As concern the soluble protein, their content has varied significantly during the different germination times of soy and voandzou. Indeed, during the first four days of germination, the quantity of soluble protein initially of 73.05 (soybean) and 79.55% (voandzou bean) decreased and was maintained at 69.05 % (soybean) and 74.20 % (voandzou bean). On the other hand, after these first four days of germination (D4) an increase is observed at 77.25 % (soybean) and 80.70 % (voandzou bean). During germination, proteins started to be degraded by endogenous endopeptidases from cotyledons to form oligopeptides. These peptides are then hydrolyzed by exopeptidases to form free amino acids, which are used to synthesize new molecules and tissues [27]. Consequently, this justifies the variation in the amount of soluble protein observed. A similar increase in the amount of protein after D4 of soybean germination was observed by [27; 28].

3.2. Antidiabetic and antioxidant potential of crude proteins from soybean and voandzoubean protein isolate

This study investigated the antidiabetic potential and antioxidant property of the studied protein isolate.

3.2.1 Antidiabetic potential: α -amylase and α-glucosidase inhibitory activity

α-Amylase and α-glucosidase are two key enzymes responsible for postprandial hyperglycemia and the search for peptides capable of inhibiting the action of these enzymes could be an alternative pathway in the management of type 2 diabetes according to [1].

α-amylase (1A) and α-glucosidase (1B) inhibitory activities by germinated soybean and voandzoubean seeds protein isolate are given in Figure 1. The crude protein isolates (D0) and the different germinated protein isolates from both soybean and voandzou bean showed significant ($p < 0.05$) lower inhibitory activity with average inhibition of 19.73% (soybean) and 21.76% (voandzou) for α-amylase and 16.55% (soybean) and 28.47% (voandzoubean) for alpha glucosidase respectively compared to those of acarbose control 75.99 % (α-amylase) and 89.25 % (α-glucosidase).

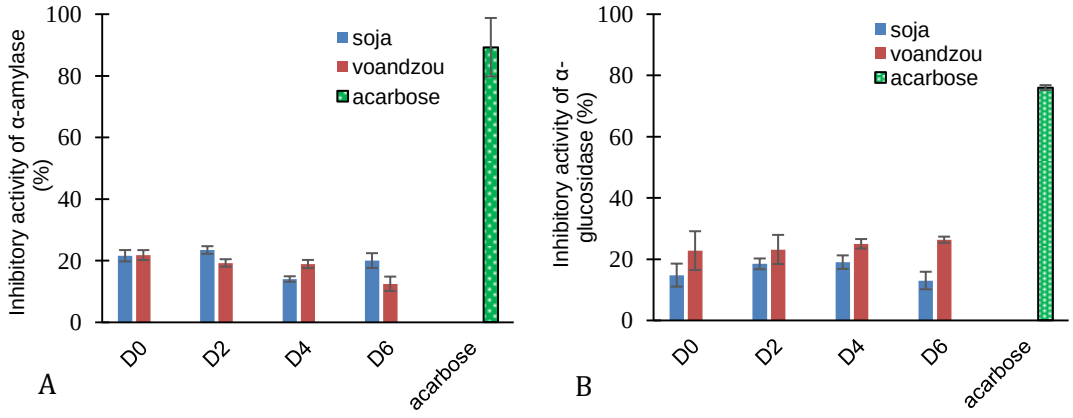

Figure 1: Percentage inhibition of α-amylase (A) and α-glucosidase (B) by soybean and voandzoubean seed protein isolates as a function of germination time

The mixture of peptides present on the non-germinated protein isolate (D0) from soybean and voandzoubean exhibited an inhibitory capacity of the two glycosidases on average around 20% for both soybean and voandzou. This finding was also reported by [29] for crude extracts of soybean (21.39%) and common bean (19.52%) proteins on alpha amylase. This ability to inhibit both the two glycosidases, highlighted in crude (non-germinated) protein isolates suggested that these legume beans might be a promising ingredient that could be used in the design of functional foods for glycemic control and prevention of type 2 diabetes mellitus.
Similarly, germination does not improve the inhibition capacity of the protein isolate on the two studied glycosidases since the inhibition averages fluctuated around 20 % with 21.3 and 20.80 % (D2), 16.42 and 22 % (D4) and 16.22 and 19.66 % (D6) for α-amylase and α-glucosidase, respectively. However, although germination did not substantially improve the ability of the protein isolate to inhibit these glycosidases, it did not have a negative effect either since the level of inhibition remains the same.

3.2.2 Antioxidant capacity

Oxidative stress is one of the factors that potentiate the onset of multi-factorial diseases including cardiovascular diseases and type 2 diabetes mellitus [30]. The search for potentially antioxidant peptides (oligopeptides and polypeptides) could help in the development of prevention strategies for these major pathologies [31].
DPPH radical scavenging activity was used to evaluate the antioxidant potential of the germinated protein isolates from soy and voandzou seeds. The antioxidant capacities of germinated protein isolates from soy and voandzoubean are given in Table 2.

Table 2: DPPH radical scavenging inhibition by the crude germinated protein isolates at different germination times (%)

Germination times	**soybean**	**Voandzou bean**	**Vitamin C**
D0	64.72 ± 1.21^{dC}	73.45 ± 1.22^{eB}	
D2	72.27 ± 2.50^{bC}	75.75 ± 2.41^{dB}	
D4	72.08 ± 2.05^{bC}	81.43 ± 3.16^{bB}	
D6	70.58 ± 1.98^{cC}	78.70 ± 2.33^{cB}	
			100 ± 0.00^{aA}

Different alphabetical uppercase letters of each group in the same row show significant differences (P < .05) between samples at the same germination time and different alphabetical lower-case letters of each group in the same column show significant differences (P < .05) between samples at the same origin according to Duncan's test. Mean ± S.E.M = Mean values ± Standard error of means of six experiments

The two protein isolates of soybean and voandzou from ungerminated seeds (D0) initially displayed a good level of DPPH free radical scavenging (67% on average) compared to that of vitamin C (100%) with the protein isolate of voandzou (73.45%) exhibiting a higher DPPH free radical scavenging capacity than soybean (64.72%). As concern germination, this process significantly improved the free radical inhibition capacity since both types of protein isolates, soybean (71.6%) and voandzou (78;62%), showed on average a higher level of DPPH inhibition than the non-germinated seed protein isolates. This high level of antioxidant activity recorded by both soybean and voandzou protein isolate could be explained by their richness in certain amino acids such as hydrophobic amino acids since authors [31] indicated that this kind of amino acids are able to interact on the active site of glycosidases and prevent the fixation of the substrate.

3.3. α-amylase and α-glucosidase inhibition by peptides resulting from enzymatic digestion of germinated soy and voandzou beans crude proteins

3.3.1 Antidiabetic potential of digested crude proteins from germinated soy and voandzou beans

The results of the study on the α-amylase (Figure 2A) and α-glucosidase (Figure 2B) inhibition capacity by the digested extracts from germinated and ungerminated soy and voandzou seeds are given in Figure 2. The analysis of the results showed that the different digested protein isolate, whether germinated or not, have significantly different inhibition capacities on the two types of studied enzymes (p < 5%).

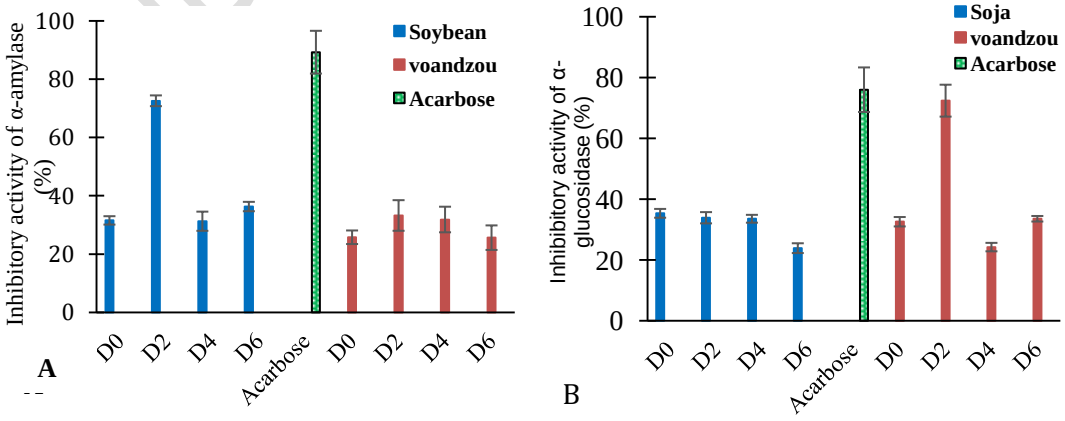

Figure 2: Inhibition of α-amylase (A) and α-glucosidase (B) activity by digested protein extracts of soybean and voandzou as a function of germination time

NB : D0 : ungerminated; D2 : 2nd day of germination; D4 : 4th day of germination ; D6 : 6th day of germination

Indeed, the D2 digested isolate of soy germinated seeds showed the highest inhibition capacity (72.58%) nearly that of the control acarbose (89.25%) for α-amylase (Figure 2A), as well as the D2 digested isolate of voandzou (72.39%) with the highest inhibition capacity comparable to the acarbose control (75.99%) for α-glucosidase (Figure 2B). On the other hand, the inhibition capacity of a digested isolate of other germination times remains relatively weak compared to that of acarbose control since they were between 23.87 to 35.33% (soybean) and 25.63 to 33.24% (voandzou bean) for α-amylase and between 13.93 to 19.15% (soybean) and 24.22 to 33.54% (voandzou) for α-glucosidase. Thus, gastrointestinal digestion generated hydrolysates containing potential bioactive peptides only with D2 germination time for each soy and voandzou beans protein isolate.

According to [32], digestion is the natural site of enzymatic hydrolysis of proteins resulting in a wide variety of bioactive peptides. The specificity of the acid endoprotease named pepsin for hydrophobic amino acids allowed to release of peptides with hydrophobic ends capable of interacting on the active site of the two studied glycosidase enzymes resulting in these inhibitions [33].

A similar inhibition rate compared with acarbose obtained for the D2 digested isolate of soy and voandzou germinated seeds indicates the presence of bioactive peptides that can be directed in the treatment of type 2 diabetes. The same finding has been reported by [11] with the protein extracts of soybean germinated over 6 days. However, they should be combined with other types of antidiabetic drugs [34]. For example, the combined action with biguanides could in addition to improving the sensitivity of insulin target cells (muscle cells, liver cells), promote the regulation of postprandial hyperglycemia. So *in vitro* simulation of gastrointestinal digestion by porcine pepsin in one hour followed by porcine pancreatin in two hours appears to be a good technique for obtaining bioactive peptides, unlike what was previously observed on the crude protein extracts during germination with local endopeptidase.
On the other hand, the levels of inhibition exhibited by the digested protein isolates at other germination times (D0, D4 and D6), even if they are lower than that of acarbose, can be exploited in the prevention of chronic hyperglycemia (pre-diabetes) that can lead to type 2 diabetes mellitus as well as the higher levels of inhibition exhibited by D2 digested protein isolates. Indeed, the amount of sugar in the blood is constantly modulated by the body's activities. Digestion, for example, is responsible for the postprandial hyperglycemia observed after the consumption of mostly starchy foods. Regular exposure of the body to hyperglycemia can in some cases lead to complications in insulin synthesis or utilization [35]. Thus, the integration of soybean and voandzou germinated seeds containing these peptides into the diets of populations could help prevent metabolic diseases. This control of the flow of glucose in the blood could then help to prevent the establishment of chronic hyperglycemia which is responsible for the occurrence of type 2 diabetes mellitus. Therefore, they could be used as nutraceutical foods in powder or capsule form to reduce the massive influx of glucose into the bloodstream and thus avoid exposure of populations to the state of chronic hyperglycemia.

3.3.2- Antidiabetic potential of dialysed peptids from soy and voandzou beans crude proteins digestion

Generally, biopeptides with hypoglycemic effect used in the treatment of diabetes are small peptides [36]. Thus, the identification of peptides that generated the inactivation of α-

amylase and α-glucosidase comparable to that of acarbose, was performed after separation of the peptides according to their size through a dialysis membrane with a 10 kDa cut-off diameter (figure 3A and 3B).

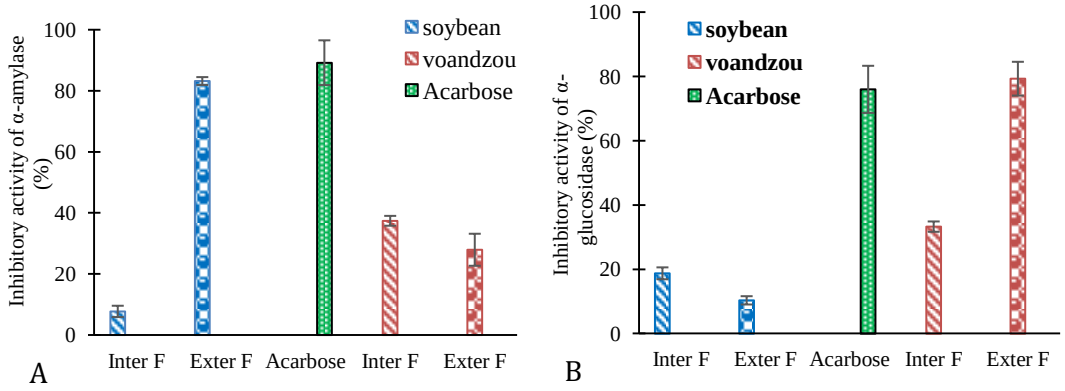

Figure 3: Inhibition of α-amylase (A) and α-glucosidase (B) by the internal and external fractions of dialyzed hydrolysates of soy and voandzou seeds at two days (D2) of germination

***Legend** : Inter F : internal fraction (<10 kDa) ; Exter F : external fraction (≥10 kDa)*

Concerning the hydrolysates from the D2 germinated protein isolate of the two studied legume seeds, the results revealed that the two peptides fraction obtained through the dialyses process displayed significant different inhibition capacity on the studied glycosidases. Thus, the outer peptides fractions, with size <10 kDa exhibited the highest inhibitory level (83.44%) similar to the control acarbose (89.25 %) for α-amylase inhibition. The same trend was recorded with the outer peptide fraction (size<10 kDa) from voandzou bean with a rate of 79.31% close to that of acarbose control (79.99%) for α-glucosidase inhibition. This onset of peptide separation revealed that the high acarbose-like activity of digested D2 protein extracts was due to peptides (oligopeptides and polypeptides) smaller than 10 kDa in size from soybean and voandzou. Similar results were reported in previous studies where peptide fractions of sizes smaller than 5 and 10 kDa from 6-day germinated soybean [11] and quinoa [18] hydrolysates showed the strongest inhibition of α-amylase and α-glucosidase; confirming that low molecular weight peptides are responsible for this inhibition.

3.4- *In vitro* antioxidant potential of digested protein extracts and dialyzed peptides from soy and voandzou beans crude proteins digestion

The inhibition rates of DPPH (2,2-Diphenyl-1-Pycrilhydrazyl) free radical by germinated and non-germinated soybean and voandzou digested protein isolates as a function of germination time and by vitamin C (positive control) are shown in table 3. Statistical analysis revealed a significant difference ($P < .05$) level of free radical inhibition by the two types of digested protein isolates of soybean and voandzou and by vitamin C (positive control).

Table 3: Percentage of DPPH radical inhibitory by digested protein extracts and peptide fractions of soybeans and voandzou germinated at D2

Germination time	Soybean	Voandzou	Vitamin c
Digested protein isolates (%)			
D0	72.26 ± 2.22^{bB}	66.72 ± 1.75^{cC}	
D2	$69.2\pm2.2.01^{cB}$	65.38 ± 1.25^{dC}	
D4	73.50 ± 2.20^{aB}	67.88 ± 1.50^{bC}	

D6	67.88±1.85^{dC}	70.80±0.97^{aB}	100±0.55^{aA}
Peptides fraction from D2 digested protein isolates			
Internal fraction (≥10 kDa)	69.86±1.55^{aB}	62.95±1.21^{bC}	
External fraction (<10 kDa)	61.16±1.57^{bC}	71.64±1.33^{aB}	

Different alphabetical uppercase letters of each group in the same row show significant differences (P < .05) between samples at the same germination time and different alphabetical lower-case letters of each group in the same column show significant differences (P < .05) between samples at the same origin according to Duncan's test. Mean ± S.E.M = Mean values ± Standard error of means of six experiments

On the other hand, enzymatic hydrolysis during germination and hydrolysis during in vitro gastrointestinal digestion by pepsin (1 h) and pancreatin (2 h) did not alter the antioxidant capacity of these two extracts, which always showed DPPH radical inhibition capacities higher than 60% on average. These results were similar to those obtained by [37] with quinoa (*Chenopodium* quinoa) and kiwicha (*Amaranthus* caudatus).

This good level of antioxidant activity recorded by both soybean and voandzou protein extracts could be explained by their richness in certain amino acids such as histidine and hydrophobic amino acids such as tryptophan [31]. Thus, the good level of free radical inhibition (antioxidant potential) by soy and voandzou protein-derived peptides (ungerminated, germinated, and hydrolyzed) could inhibit lipid peroxidation and thus avoid the development of cardiovascular diseases [38 ; 31].

4. CONCLUSION

The germination of soybean and voandzou seeds and enzymatic hydrolysis of their protein isolates generated bioactive peptides with both antioxidant capacity and inhibitory activity of both α-amylase and α-glucosidase.
However, only small (less than 10 kDa) digested peptides from D2-germinated isolates were most involved in modulating sugar absorption compared with the control. Contrary to the antioxidant activity which concerned all peptide fragments as well as protein isolates independently of the germination time. The ingestion of soy and voandzou beans may provide bioactive peptides that can manage and reduce the risk of diabetes and oxidative stress-related diseases and could be suitable to be used as a functional or nutraceutical ingredient.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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