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Production and *in vitro* studies of Bitter Gourd (*Momodica charantia*) seed protein hydrolysates with antidiabetic activity

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Abstract

Diabetes mellitus is a multifactorial chronic disease that affects the human population and it is the third most common cause of death worldwide. *Momordica charantia* is commonly known as Bitter melon, Bitter guard and used as a food and natural medicine. The scientific name, Momordica means "to bite," in Latin which is refers to the jagged edges of the leaves. Including fruits, all parts of the plant, contains a bitter compound, momordicinso and very bitter in taste. It has long been used as a traditional medicine for some ailments. Its high protein content (35.18%) makes it a promising candidate as a source of bioactive protein hydrolysates. In this study, the potential of enzymatically hydrolyzed Bitter guard seed protein to generate functional bioactive peptides was evaluated. Bitter guard seed protein concentrate was hydrolyzed using three different proteolytic enzymes (Alcalase, Bromelain, and Papain) at their respective optimum pH and temperature. The choice of enzyme affected the bioactivities to a certain degree as Bitter guard seed were shown to possess high antidiabetic activity. Bromelain Bitter guard seed protein hydrolysate showed the highest DPP-IV inhibitory activity with 79.27% inhibition, α-Glucosidase inhibitory activity with 72.15% inhibition and α-Amylase inhibitory activity with 59.98% compared to other enzymatic hydrolysates, and therefore was chosen for further investigation of its antidiabetic activity.

Keywords: Bitter gourd (*Momordica charantia*); Bitter gourd seed protein hydrolysate; Antidiabetic activity

1. Introduction

Diabetes mellitus is a disorder that affects the body's ability to make or use insulin. Insulin is a hormone produced in the pancreas that helps transport glucose (blood sugar) from the bloodstream into the cells so they can break it down and use it for fuel. People cannot live without insulin (A. D. A. 2007). Diabetes results in abnormal levels of glucose in the bloodstream. This can cause severe short-term and long-term consequences ranging from brain damage to amputations and heart disease (A. D. A. 2007).

Metabolic forms of diabetes *include; Type 2 diabetes:* Also known as insulin dependent diabetes mellitus (IDDM), this accounts for 90 - 95% of diabetic cases, according to the U.S. National Institutes of Health (NIH). Some of these patients have had prediabetes that went uncontrolled. Once considered a disease of middle and old age, type 2 is also becoming more common in youths as the incidence of childhood obesity grows, Autoimmune*:* The body's immune system can mistakenly destroy the insulin-producing beta cells of the pancreas. The causes of autoimmune diabetes are poorly understood, but genetics and family history play a role, and viruses or other environmental factors are believed to figure in. Autoimmune forms of diabetes include: Type 1 diabetes formerly known as juvenile diabetes, this form generally develops in children and young adults. This variation of type 1 can occur later in life. Individuals with autoimmune diabetes who overeat, are sedentary, gain weight or have certain genes can, like people with metabolic forms of diabetes, develop insulin resistance. This state is known as double diabetes. Diabetes can also result from another disease, such as pancreatitis, or even from a medical treatment, including pancreatectomy (surgical removal of the pancreas) or

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certain medications. This is known as secondary diabetes. In addition, there are uncommon inherited disorders that cause diabetes, such as maturity-onset diabetes of the young and Wolfram syndrome. Most cases of diabetes last the rest of a person's life. However, gestational diabetes generally ends when the pregnancy does, and some cases of secondary diabetes are also temporary (Cefalu *et al*., 2007). Gestational diabetes; Develops in 2 percent to 5 percent of all pregnancies but usually disappears when a pregnancy is over. Hormonal changes contribute to this condition which can develop in any previously nondiabetic woman during pregnancy, especially those who are overweight. The root causes of diabetes are complex. Most cases begin with one of two processes which include unhealthy lifestyle factors such as overeating, physical inactivity and obesity can impair the body's ability to use insulin. This is called insulin resistance. Uncontrollable risk factors which include genetics, family history and age can also be involved.

Diabetes Mellitus (DM) is a name among the first described disease and is one of the most widespread chronic endocrine disorders all over the globe (Akbarzadeh *et al*., 2007). As per the survey of 2014, it has been estimated that there are 387 million of people carrier of this sweet killer disease among which 90% of the carriers are associated with Type 2 Diabetes Mellitus (T2DM). From 2012 to 2014 only diabetes is assessed to conclude in 1.5 to 4.9 million deaths each year. The number of people with diabetes is expected to rise to 439 million by 2030 among which 87 million will be from India (Bachchawat *et al*., 2011). The global economic cost of diabetes in 2014 was estimated to be $ 610 billion USD. It is recognized as a global epidemic by the World Health Organization. It is a well-known and clear fact that managing diabetes is a complex and challenging task; therefore, over the period of last few decades, development of various anti-diabetic drugs has shown an emerging tool to tackle this over growing disease in combination with proper eating habits and physical exercises.

In Nigeria, the current prevalence of Diabetes Mellitus among adults aged 20–69 years is reported to be 1.7% (I.D.F. 2017). It is widely perceived that prevalence figures reported by the IDF grossly under-report the true burden of DM in Nigeria, given that they are derived through the extrapolation of data from other countries. Various researchers have reported prevalences ranging from 2% to 12% across the country in recent years (Nyenwe *et al*., 2003). The last time a nationwide population estimate of DM was undertaken in Nigeria was during the 1992 Nigerian National Non-communicable Diseases (NCD) survey, where DM was said to occur in 2.2% of the population. There has been no nationwide health (diabetes) survey in Nigeria since then. However, it is important to determine the actual burden of DM in Nigeria to facilitate appropriate health resource allocation, advocacy, and planning. Thus, in the work reported in the present paper, we aimed to determine the prevalence of and risk factors for DM in Nigeria using a systematic review and meta-analysis. Treatment of diabetes mellitus typically includes diet control, exercise, home blood glucose testing, and in some cases, oral medication and/or insulin. Approximately 40 percent of people with type 2 diabetes require insulin injections.

Momordica charantia is commonly known as Bitter melon, Bitter guard and used as a food and natural medicine. The scientific name, Momordica means "to bite," in Latin which is refers to the jagged edges of the leaves. Including fruits, all parts of the plant, contains a bitter compound, momordicinso and very bitter in taste. The plant grows in tropical regions such as India, China, America Malaya, Bangladesh, tropical Africa, Thailand, Middle East. *Momordica charantia* contains a different biologically active phytochemicals, which includes proteins, triterpens, saponins, flavonoids, steroids, alkaloids, and acids. The plant is beneficial for its anti-tumorous, anti-fungal, anti-parasitic, anti-cancer, antiviral, anti-fertility, anti-bacterial and hypoglycaemic properties due to the presence of numerous phytochemicals. In traditional medication, fruits and leaves are used to cure several diseases like: gout, rheumatism, colic, worms, illness of liver and spleen. Momordica contains alkaloids and peptides which resemble like insulin and charantin, a collection of steroidal sapogenins due to which it has hypoglycaemic property.

The use of synthetic antidiabetic such as Insulin, Metformin, and Glibanclamide has serious limitations due to high cost and potential sight effect on human health. Therefore, alternative medications are necessary for the production of antidiabetic compounds from natural sources without side effects on human health (Jin *et al*., 2017).

Protein hydrolysates and biopeptides have various biological activities such as antidiabetic, antioxidant, antibacterial, and antihypertensive, potential depending on amino acid composition, sequencing, hydrophobicity, and chain length (Nasri, 2017). In recent years, various food protein hydrolysates have been reported to be antidiabetic and antioxidant potential in barley, Palmaria palmata, egg yolk protein, pinto beans, and cumin seeds (Siow & Gan, 2016).

However, there are some inadequate information's about bitter gourd seeds regarding the production of bioactive peptides hydrolysate and impact of the bioactive peptides as antidiabetic compound. Therefore, there is need to produce major principally active protein(s) in *Momodica charantia* especially those of seeds which normally are being discarded and evaluate their antidiabetic properties which may be considered cheap, reliable, and with low side effects. This study

aimed to produce *Momodica charantia* seed protein hydrolysates using different enzymes (Alcalase, Papain, and Bromalain), with *in vitro* antidiabetic activity which may be considered cheap, reliable, and with low side effects.

2. Material and methods

2.1. Collection and identification of Plant material

Fresh fruit of Bitter Gourd (*Momodica charantia*) was collected from their natural habitat, within Jos. And was identified by a botanist in Department of Plant Biology, Bayero University Kano, Nigeria with herbarium accession number BUKHAN 0562.

2.2. Sample preparation

Seed of *Momodica charantia* was separated from the fruits, they were washed under running tap water, and freezed dry using freeze drier. The dried seeds were pulverized to a fine powder using laboratory blander. The powdered sample of *Momodica charantia* seed was then kept in air-tight polythene bag at -20 °C.

2.3. Proximate analysis

Determination of proximate composition will be carried out in accordance with A.O.A.C. methods described by (Hussain *et al*., 2009) with slight modifications. Proximate composition of a substance constitutes the different classes of nutrients present in the samples such as carbohydrates, protein, fat, crude fiber, ash and moisture as well as caloric value calculated from values of carbohydrate, fat and protein.

2.4. Preparation of defatted bitter gourd seed powder

Defatting of bitter gourd seed powder was carried out using method reported by (Zaharuddin *et al*., 2020). With slight modifications.

- Powdered Bitter gourd seed was passed through a sieve (0.5 mm size).
- The sieved Bitter gourd seed powder was defatted by mixing with petroleum ether at 1:3 (w/w) ratio and stirred continuously for 30 min at room temperature (28 °C).
- The solvent was decanted, and extraction was repeated for three (3) more times to achieved maximum defatting.
- The defatted Bitter gourd seed powder was dried overnight under a fume hood.

2.5. Protein extraction of bitter gourd seed

Protein extraction was carried out using method reported by (Zaharuddin *et al*., 2020) with slight modifications.

2.5.1. Procedure

- Defatted Bitter gourd seed powder was mixed with distilled water at a 1:30 ratio (w/v) and the pH was adjusted to 9 using 4 M NaOH.
- The mixture was agitated for 1 hr at 80 rpm and 50 °C, followed by centrifugation at 10,000 rpm for 15 min at 4 °C.
- The supernatant was collected, and the pH was adjusted to 4.5 using 0.1 M HCl.
- Protein precipitate was collected after centrifugation at 10,000 rpm for 15 min at 4 °C (No 2 above), washed with water, and it was freeze dried to obtain Bitter gourd seed protein concentrate.

2.6. Determination of enzymatic activity of Alcalase, Bromelain and Papain

The enzymatic activity of Alcalase, Bromelain and Papain were determined according to the casein digestion unit (CDU) method using Hammersten grade casein (0.6%) as substrate with slide modification.

2.7. Protein hydrolysis of bitter gourd seed

Bitter gourd seed protein hydrolysis was carried out with three different enzymes (Alcalase, Papain, and Bromalain) using method reported by (Zaharuddin *et al.,* 2020). With slight modification.

2.7.1. Hydrolysis

- 1 g of Bitter gourd protein Concentrate was mixed with 25 ml of buffer for each enzyme [i.e at a ratio of 1:25 (w/v)] for Papain (50 mM phosphate buffer, pH 6.5, 55 °C), Alcalase (50 mM phosphate buffer, pH 7.5, 55 °C), and Bromelain (50 mM acetate buffer, pH 5.0, 55 °C).
- 0.02g of each enzyme (Alcalase, Papain, and Bromelain) were added in each case [i.e using an enzyme:substrate ratio of 1:50 (w/w)] and hydrolysis was conducted for 8 hrs (12:00PM – 8:00PM).
- 1 ml of each sample were taken initially (0hr), 30 min and every hour thereafter in each case and put in the correspondent labelled 1.5 ml centrifuge tube base on type of enzyme and time, the labelled 1.5 ml centrifuge tube containing the bitter gourd seed protein hydrolysate (BGSPH) was immersed in boiling water (100 °C) for 10 min to inactivate the enzyme in each case.
- The reaction mixture was then cooled and centrifuged at 10,000 rpm for 25 min using refrigerated centrifuge, and it was filtered through 0.22 µm syringe filter to remove any insoluble, residual material.
- The supernatant containing the Bitter gourd seed protein hydrolysate (BGSPH) was collected, and it was used to test for antidiabetic activity (*In vitro* studies).

2.8. Determination of degree of hydrolysis (DH)

The degree of hydrolysis (DH) refers to the percentage of free amino terminal groups cleaved from proteins during hydrolysis and was determined using OPA (o-phthaldialdehyde) according to the method reported by (Awad *et al*., 2017) with minor modifications.

2.8.1. Procedure

- On the 96 well plates, 36 well were labelled appropriately and 300 uL (0.3 mL) of OPA working reagent was added to all the label 36 wells of the 96 well plate.
- 20 uL of Better Gourd Seed Protein Hydrolysates (BGSPH) in each case (For the different time) was added to corresponded label 30 wells of the 96 well plate except the wells label Blank, S10, S8, S6, S4, and S2.
- 20 uL of distilled water was added to the well label Blank.
- 20 uL of different concentration of L-serine 10, 8, 6, 4, and 2 mg/mL was added to the well label S10, S8, S6, S4, and S2 respectively.
- The 96 well plate was incubated for 2 min at room temperature.
- The absorbance was taken at 340 nm using microplate reader.
- The degree of hydrolysis was calculated using the formula below from the standard calibration curve constructed for L-serine:

$$DH = h/htot \times 100\%$$

Where h = is the number of equivalents of peptide bonds hydrolyzed = (A × b)/m. b = is the y intercept from equation of the L-serine standard calibration curve and m = is slope of the L-serine standard calibration curve. h_{tot} = is the total amount of peptide bonds (8.0).

2.9. *In vitro* measurement of enzymatic inhibitory activity

2.9.1. α-Glucosidase inhibition assay

Inhibition of α-glucosidase (rat intestinal) by Bitter gourd seed protein hydrolysates and fractions was measured using the method reported by (Connolly *et al.,* 2014) with slight modifications.

- 100 uL of Bitter gourd seed protein hydrolysates solution (20 mg/ml) were mixed with 200 µl of α-glucosidase (rat intestinal) and will be incubate at 37°C for 10 min.
- After preincubation (No 2 above), 5 mM PNPG (4-nitrophenyl α-d-glucopyranoside) solution (100 µl) was added and incubate at 37°C for 30 min and the absorbance of the solution was measured in every 2 min at 405 nm using microplate reader.
- Phosphate buffer was used as a control. The IC50 value of acarbose was used as the positive control.
- The following equation below was used to calculate percentage inhibition of α-glucosidase activity:

$$\text{Inhibition of } \alpha - \text{glucosidase } (\%) = [Ac - As/Ac] \times 100$$

Where As and Ac represent the slope of curve for absorbance of samples and control, respectively.

2.9.2. α-Amylase inhibition assay

α-Amylase inhibition was determined according to the method reported by (Alu'datt *et al.,* 2012) with slight modifications.

- 100 µl of Bitter gourd seed protein hydrolysate (10 mg/ml) and 100 µl of α-amylase solution (0.5 U/ml) will be incubate at 37°C for 5 min.
- After the preincubation (No 1 above), 100 µl of 0.5% (w/v) starch solution was added to the solution of 1 above. The reaction mixture was incubated for 20 min at 37°C.
- The reaction mixture was heated at 100°C for 10 min, the mixture was allowed to cool down to room temperature and centrifuged for 2 min at 16060 g to separate the undigested starch.
- 20 uL of supernatant were mixed with 1 ml of PAHBAH (4-hydroxybenzhydrazide) and heat to 70°C for 10 min.
- Finally, the solution was allowed to cool at room temperature and absorbance will be measure at 410 nm using microplate reader.
- IC50 value of acarbose will be use as the positive control.

The following equation was used to determine percentage inhibition of α-amylase activity:

$$\text{Inhibition of } \alpha - \text{amylase } (\%) = [1-(As-Ab)/Ac] \times 100$$

Where As, Ab, and Ac represent the absorbance of sample, blank (phosphate buffer, enzyme, sample), and control (starch, buffer, enzyme), respectively.

2.9.3. Dipeptidyl peptidase-IV (DPP-IV) inhibition assay

DPP-IV inhibition was determined following the procedure reported by (Nongonierma and FitzGerald, 2013) with slight modifications.

- 25 µl of Bitter gourd seed protein hydrolysates solution (5 mg/ml) were mixed by 25 µl of Gly-Pro-pNA, as substrate (0.2 mM) and the mixture was incubated for 10 min at 37°C.
- DPP-IV (final concentration 0.0025 units/ml) was added to start the reaction for 1 hr at 37°C and the absorbance of p-nitroaniline release during the reaction was read at 405 nm using microplate reader
- The IC50 value of Diprotin A was used as positive control.
- The inhibitory activity of sample on DPP-IV was calculated by the following equation:

$$\text{Inhibition of DPP–IV } (\%) = [(Ac-Acb)-(As-Asb)/(Ac-Acb)] \times 100$$

Where As, Asb, Ac, and Acb represent the Absorbance of sample, Absorbance of sample blank (sample, buffer, substrate), Absorbance of control (enzyme, substrate, buffer), and Absorbance of control blank (buffer, substrate), respectively.

3. Results

Table 1 Proximate composition, protein concentrate yield and protein content of bitter gourd seed powder

Parameter	Value (g/100 g dry basis)
Ash	8.10±0.10
Moisture	9.80±0.11
Crude Fat	16.09±0.18
Crude Fiber	17.11±0.09
Carbohydrate	13.90±0.21
Crude Protein	36.12±0.98
Protein Content in Concentrate	60.15±0.70
Protein concentrate yield	35.18%

Values represent the mean from two replicates ± SEM

Table 1 shows the proximate composition of Bitter Gourd Seed. The results show that the seed contained crude protein (36.12±0.98), crude fiber (17.11±0.09), crude fat (16.09±0.18), Carbohydrate (13.90±0.21), Moisture (9.80±0.11), Ash (8.10±0.10), Protein content in concentrate (60.15±0.70), and Protein concentrate yield (35.18%).

Table 2 Enzyme Activity Assay for Alcalase, Bromelain and Papain

Enzyme	Enzyme Activity (U/mL)
Alcalase	4.72
Bromelain	5.14
Papain	4.22

The activity of Alcalase, Bromelain and Papain were studied using casein as substrate. The activity of Bromelain enzyme was found to be 5.14 U/mL of enzyme while that of Alcalase and Papain were found to be 4.72 and 4.22 U/mL of enzyme respectively as calculated from the standard graph for L-tyrosine.

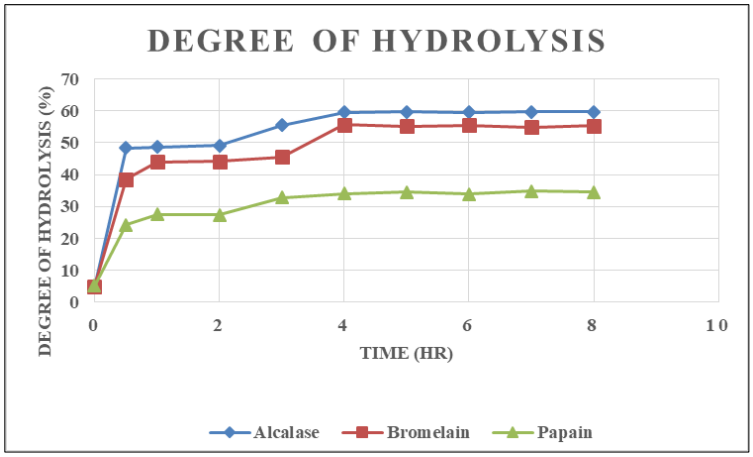

Figure 1 Degree of Hydrolysis (DH) of better gourd seed protein hydrolysate using Alcalase, Bromelain and Papain at different time

The Figure 1 above show the degree of hydrolysis of Bitter Gourd Seed protein isolate. Analysis of the extent of protein hydrolysis of the seed protein by Alcalase, Bromelain and Papain revealed 59.72% after 8 hr of hydrolysis, 55.65% after 4 hr of hydrolysis and 34.76% after 7 hr of hydrolysis respectively.

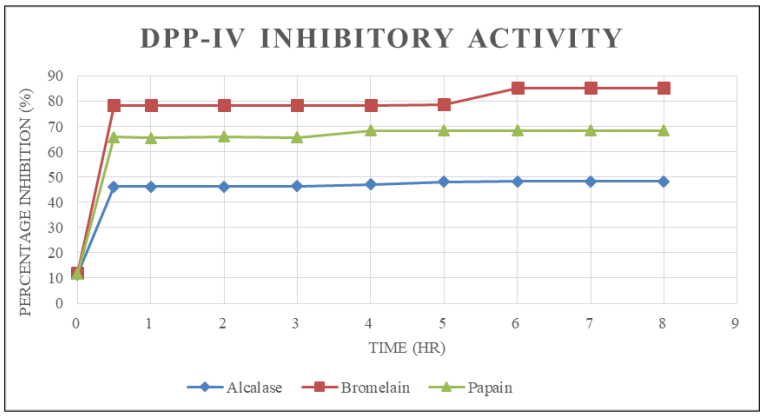

Figure 2 Dipeptidyl peptidase-IV (DPP-IV) inhibitory activity of Bitter Gourd Seed Protein Hydrolysates (BGSPH) using Alcalase, Bromelain and Papain

Dipeptidyl peptidase-IV (DPP-IV) inhibitory activity from the three enzyme (Alcalase, Bromelain and Papain) hydrolysates ranged from 46.20 to 85.15%, as shown in Figure 2 above. Bromelain better gourd seed protein hydrolysate showed the highest DPP-IV inhibitory activity at 85.15% after 6 hr hydrolysis, followed by Papain better gourd seed protein hydrolysate at 68.34% after 4 hr hydrolysis, while Alcalase better gourd seed protein hydrolysate at 48.24% after 8 hr hydrolysis showed the lowest DPP-IV inhibitory activity.

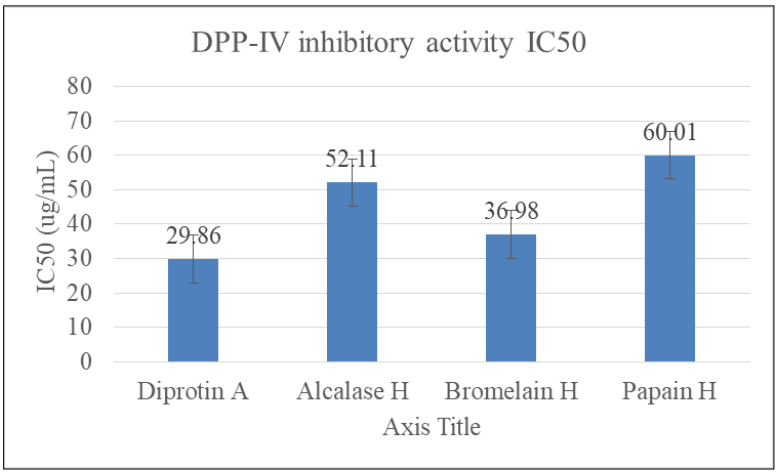

Figure 3 50% inhibition concentration (IC50) values of Diprotin A and Bitter gourd seed protein hydrolysate (BGSPH) on Dipeptidyl peptidase-IV (DPP-IV) inhibitory activity using Alcalase, Bromelain and Papain

Figure 3 shows the 50% inhibition concentrations (IC_{50}) of Alcalase, Bromelain, Papain bitter gourd seed protein hydrolysate and diprotin A that inhibited 50% Dipeptidyl peptidase-IV (DPP-IV). The IC_{50} value of diprotin A is 29.86 ug/mL, followed by Bromelain bitter gourd seed protein hydrolysate with 36.98 ug/mL while Alcalase and Papain bitter gourd seed protein hydrolysate are 51.11 and 60.01 ug/mL respectively.

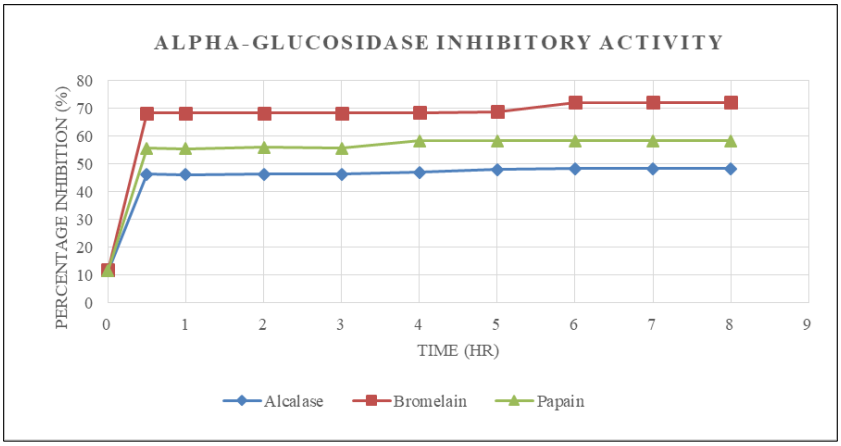

Figure 4 α-Glucosidase inhibitory activity of better gourd seed protein hydrolysate using Alcalase, Bromelain and Papain

In Figure 4, α-Glucosidase inhibitory activity showed relatively high values from the generated better gourd seed protein hydrolysate ranged from 46.21–72.15%, with Bromelain better gourd seed protein hydrolysate exhibiting the highest value at 72.15% after 6 hr hydrolysis, followed by Papain better gourd seed protein hydrolysate at 58.34% after 4 hr hydrolysis, while Alcalase better gourd seed protein hydrolysate showed the lowest chelating metal ion activity at 48.24% after 8 hr hydrolysis.

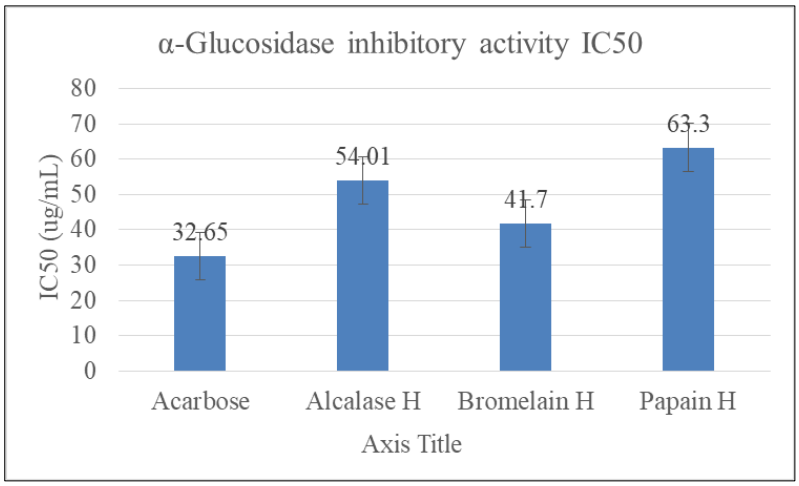

Figure 5 50% inhibition concentration (IC50) values of Acarbose and Bitter gourd seed protein hydrolysate (BGSPH) on α-Glucosidase inhibitory activity using Alcalase, Bromelain and Papain

Figure 5 shows the 50% inhibition concentrations (IC_{50}) of Alcalase, Bromelain, Papain bitter gourd seed protein hydrolysate and acarbose that inhibited 50% α-Glucosidase. The IC_{50} value of acarbose is 32.65 ug/mL, followed by Bromelain bitter gourd seed protein hydrolysate with 41.70 ug/mL while Alcalase and Papain bitter gourd seed protein hydrolysate are 54.01 and 63.30 ug/mL respectively.

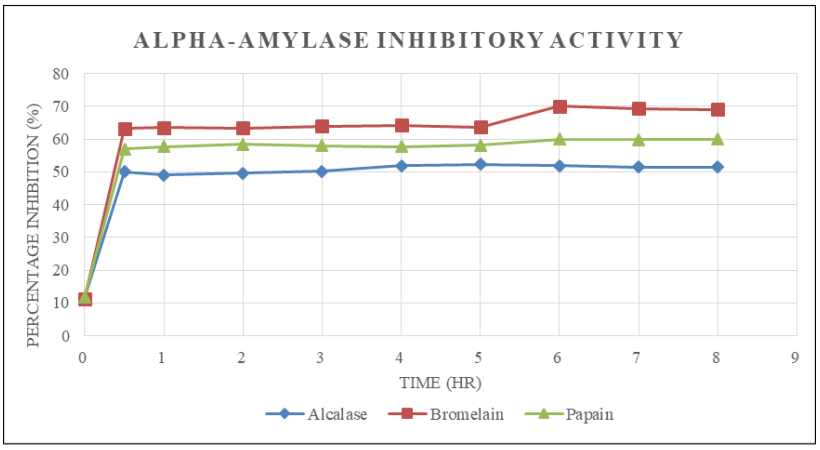

Figure 6 α-Amylase inhibitory activity of better gourd seed protein hydrolysate using Alcalase, Bromelain and Papain

In Figure 6, α-Amylase inhibitory activity showed relatively high values from the generated better gourd seed protein hydrolysate ranged from 49.96–69.97%, with Bromelain better gourd seed protein hydrolysate exhibiting the highest value at 69.97% after 6 hr hydrolysis, followed by Papain better gourd seed protein hydrolysate at 59.98% after 8 hr hydrolysis, while Alcalase better gourd seed protein hydrolysate showed the lowest chelating metal ion activity at 51.87% after 6 hr hydrolysis.

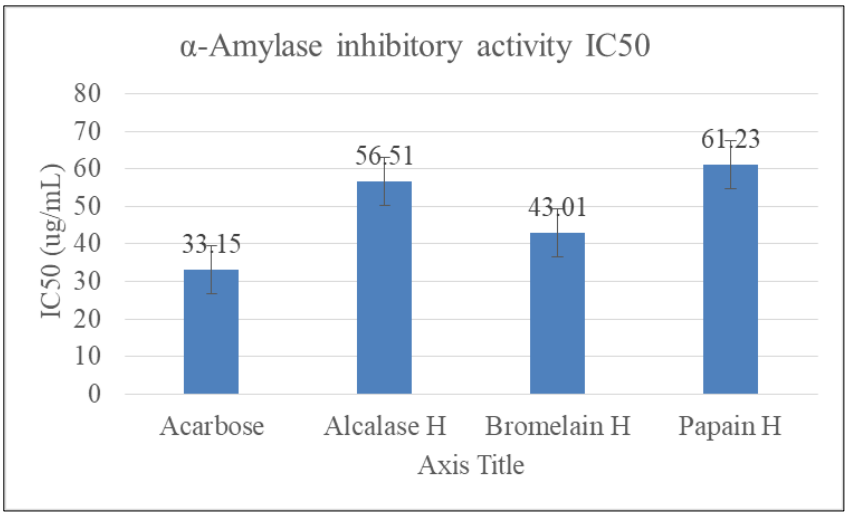

Figure 7 50% inhibition concentration (IC50) values of Acarbose and Bitter gourd seed protein hydrolysate (BGSPH) on α-Amylase inhibitory activity using Alcalase, Bromelain and Papain

Figure 7 shows the 50% inhibition concentrations (IC_{50}) of Alcalase, Bromelain, Papain bitter gourd seed protein hydrolysate and acarbose that inhibited 50% α-Amylase. The IC_{50} value of acarbose is 33.15 ug/mL, followed by Bromelain bitter gourd seed protein hydrolysate with 43.01 ug/mL while Alcalase and Papain bitter gourd seed protein hydrolysate are 56.51 and 61.23 ug/mL respectively.

4. Discussion

Proximate composition aims at estimation and determination of how much of the major food components exist in a given plant or animal component. The proximate composition of Bitter Gourd Seed reported in this study showed that the crude protein content (36.12%), Crude fat (16.09%), Crude fiber (17.11%) and Carbohydrate (13.90%) is high when compared with the previously reported 19.50%, 11.50%, 29.60% and 9.18% respectively for the same seed (Bakare *et al.*, 2010). This may be due to the differences in the source of the seed and probably experimental conditions. However, the percentage crude ash (8.10%) and moisture content (9.80%) obtained in this study is higher than 9.73% and 2.69% earlier reported for the same seed (Bakare *et al.,* 2010). The protein content of the concentrate was 60.15%, while other researchers obtained values of 55.6% (Zaharudden *et al*., 2020), 63.1% and 56.5% (Ijarotimi *et al*., 2018) from seeds of kenaf, *Prosopis cineraria* and *Buchholzia coriacea*, respectively.

The percentage peptide yield gives a measure of the extractable protein in a protein extraction procedure. 35.18% protein yield was obtained in this study during extraction which is higher than 19.5% obtained from *Momodica charantia* seed (Bakare *et al.,* 2010), and 18.91% obtained from *Citrullus lanatus* seed (Arise *et al.,* 2016) as other protein source. This may be because *Citrullus lanatus* seed contains less salt soluble protein fraction (Singh and Matta, 2010). The result obtained in this study is lower than the 52% protein yield obtained from South African bambara groundnut landraces by Arise *et al.,* (2015). This might be attributed to the extraction method employed because the level of protein yield is a function of the extraction method (Boye *et al.,* 2010). Bromelain peptide yield which is the highest is significantly different ($p<0.05$) from that of Alcalase and Papain. This result suggests more bioactive peptides of Bromelain may have been produced during the enzymatic hydrolysis. The higher value for Bromelain could be due to the fact that it is a non-specific enzyme.

One unit of enzyme activity was defined as the amount of enzyme, releasing a product equivalent to 1 µg of tyrosine/min/ml under the standard assay conditions (of 37^0C) and expressed as U/mL or CDU/mL. The casein hydrolysis activity of the protease preparations was examined over the pH range 5.0–7.5, using the commercially available substrate casein (Table 2). The casein hydrolysis activity of Alcalase, Bromelain, and Papain protease preparations appeared to be higher in Bromelian with enzyme activity value of 5.14 U/mL at a pH value of 5.0 and temperature value of 37^0C under the assay conditions, in comparison to the Alcalase and Papain protease with activity value of 4.72 U/mL and 4.22 U/mL at a pH value of 7.5 and 6.5 respectively. However, Alcalase activity is higher than Papain activity but lower than Bromelain enzyme activity.

To ascertain the extent to which peptide bonds have been broken to release short peptides, the proteolysis monitoring parameter known as degree of hydrolysis (DH) is often used (Jrad *et al.,* 2014). The degree of hydrolysis of all bitter gourd seed protein hydrolysate showed initial rapid increase followed by a gradual reduction in rate of hydrolysis, a pattern similar with other plant-based protein hydrolysis such as horse gram and sweet sorghum (Zaharudden *et al*., 2020). Depending on the enzyme used, different levels of degree of hydrolysis were attained among the bitter gourd seed protein hydrolysate. This suggests that different enzymes affect substrates differently, attacking different sites on bitter gourd seed proteins, producing peptides in a range of molecular weights and amino acid composition. Moreover, the rapid increase in degree of hydrolysis during the initial hydrolysis phase also reflects the abundance of cleavage sites available to the enzymes.

This study revealed that the degree of hydrolysis of Alcalase, Bromelain and Papain were 59.72% after 8 hr of hydrolysis, 55.65% after 4 hr of hydrolysis and 34.76% after 7 hr of hydrolysis respectively. The higher degree of hydrolysis ($p<0.05$) Alcalase by digestion compared to that of Bromelain shows that Alcalase is most effective in hydrolyzing *M. charantia* seed protein. Bromelain digestion also proved effective in the hydrolysis more than Papain but less than Alcalase digestion. Therefore, the higher value of Alcalase digestion show that there could be large number of positively charged residues for which Alcalase is specific to, as well as acidic residues. (Naik, 2012). The results of the degree of hydrolysis obtained in the present study are similar in trend but higher than the degree of hydrolysis obtained in kenaf seed protein, where Bromelain and Alcalase hydrolysate gave >50%, while Papain gave >20% (Zaharuddin *et al.,* 2020). This could be due to the variation in the time taken for the hydrolysis because the degree of hydrolysis is directly proportional to hydrolysis time (Hrckova *et al.,* 2002). The lowest degree of hydrolysis was obtained from Papain hydrolysis (34.76%) is still higher than 15.4% obtained for Flaxseed protein hydrolysate (Hrckova *et al.,* 2002; Karamac *et al.,* 2016).

The extent of hydrolysis can be estimated from degree of hydrolysis values which can be used as an indication of peptide chain length; higher and lower values indicate shorter and longer lengths respectively (Malomo *et al.,* 2015). This therefore suggest that Papain which has the least degree of hydrolysis value (34.76%) may contain longer length of peptides whereas peptides obtained by Alcalase and Bromelain may contain shorter length of bioactive peptide. Peptides produced by Bromelain have diverse biological activities including antioxidant activity.

Antidiabetic activity of better gourd seed protein hydrolysate was evaluated using three different *in vitro* assays, to reflect the different possible antidiabetic mechanisms.

Dipeptidyl peptidase-IV (DPP-IV) inhibitory activity indicates the ability of antidiabetic compound to inhibit the activity of DPP-IV enzyme. DPP-IV inhibitory activity was observed to be relatively high for all better gourd seed protein hydrolysate. Enzymatic hydrolysis improved DPP-IV inhibitory activity, to a certain extent. Bromelain-generated hydrolysates had the highest DPP-IV inhibitory activity at 85.15% after 6 hr hydrolysis. The level of DPP-IV inhibitory activity for all better gourd seed protein remained steady as hydrolysis progressed across the 6 hr hydrolysis period, indicating the release of peptides with inhibitory activities unaffected by proteolysis. Some studies have shown that some proteins are hydrolysable but lack DPP-IV inhibitory activity, while some proteins are resistant towards enzymatic hydrolysis preventing the release of active peptides. This shows the importance of enzyme selection to produce hydrolysates with high DPPIV inhibitory activity. Zaharuddin *et al.,* (2020)

Recent advances in understanding the activity of intestinal enzymes (α-amylase and α-glucosidase both are important in carbohydrate digestion and glucose absorption) had led to the development of newer pharmacological agents. Interestingly, results of our study showed that hydrolysis of bitter gourd seed proteins hydrolysates with bromelain which possess highest activity of 69.97% and 72.15% may reduce the extent of carbohydrate absorption by inhibiting the enzymatic actions of α-amylase and α-glucosidase respectively. α-Amylase and membrane-bound α-glucosidase in the lumen of the small intestine aid the production of glucose from the catabolism of complex starches and oligosaccharides. α-Amylase hydrolyzes complex starches to oligosaccharides, while α-glucosidase hydrolyzes oligosaccharides to glucose and other monosaccharides. Inhibition of these enzymes produces a postprandial antihyperglycemic effect by reducing the rate and extent of glucose absorption from the small intestine with modest reduction of hemoglobin glycosylation in diabetes (Okoli *et al.,* 2011). The potency of postprandial antihyperglycemic and glucose tolerant effects of bromelain bitter gourd seed protein hydrolysate may derive from synergism due to concurrent inhibition of α-amylase and α-glucosidase.

The IC_{50} value determines the concentration of hydrolysates required to inhibit 50% of the enzyme, thus widely used to measure antidiabetic efficiencies (Razali *et al.,* 2015). Low IC_{50} value is desirable as lower values indicate higher effectiveness. The IC_{50} values obtained in this study (53.88±1.55, 40.56±0.90 and 61.51±1.14 ug/ml) for Alcalase, Bromelain and Papain hydrolysates respectively are significantly different ($p>0.05$) from each other and are

comparable to that of the standard antidiabetic (31.22 ± ug/ml). The Bromelain IC_{50} in the present study with IC_{50} value of 40.56 ug/ml is in contrary to that of Kneef seed Alcalase, Bromelain, Flavoenzyme and Papain hydrolysate reported by Zaharuddin *et al*., (2020), water melon seed Alcalase hydrolysate previously reported (Arise *et al.,* 2016

Conclusion

In conclusion, findings from this study showed that hydrolysis of better gourd seed protein with bromelain enzyme owe its high blood glucose lowering properties by inhibition of glucose absorption and enhancement of glucose storage as seen from the results which suggest that bromelain better gourd seed protein hydrolysate efficiently inhibits DPP-IV, α-glucosidase and α- amalyse enzymes *in vitro*. Bromelain better gourd seed protein hydrolysate will be used for further investigation *in vivo*.

Compliance with ethical standards

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