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Review Article

Glycine max Seed Protein Hydrolysates: Antiglycation Potentials and Inhibition of Carbohydrate-hydrolyzing Enzymes

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ABSTRACT

The present study explored the inhibitory effects of hydrolysates of *Glycine max* seed protein on α-amylase, α-glucosidase, and protein glycation. Proteins were extracted from *Glycine max* seeds via alkaline solubilization followed by isoelectric point precipitation. The proteins were then hydrolyzed using trypsin, chymotrypsin, and a combination of both enzymes in equal proportions. The resulting hydrolysates were evaluated for bioactivities against α-amylase, α-glucosidase, and albumin glycation. The results revealed that all hydrolysates inhibited albumin glycation (above 70% inhibition), with tryptic hydrolysates having the best activity. All hydrolysates displayed varying inhibitory effects on α-amylase and α-glucosidase, with hydrolysates obtained from dual-enzyme proteolysis eliciting better activities (77.06 ±0.73% and 71.489 ±3.489% against α-amylase and α-glucosidase, respectively) than hydrolysates from single enzyme digestion. Kinetic data indicated that chymotrypsin digests inhibited α-amylase via an un-competitive mechanism while tryptic hydrolysates and dual-enzyme digests exhibited mixed mode of inhibition, with tryptic hydrolysates having the lowest binding affinity (k_I = 0.508 mg/mL). For α-glucosidase inhibition, an uncompetitive subtype of mixed inhibition was observed for tryptic digests, whereas mixed-enzyme derived hydrolysates inhibited α-glucosidase non-competitively. Mixed inhibition was obtained for chymotrypsin hydrolysates, and they had the lowest binding affinity for α-glucosidase (k_I = 0.250 mg/mL). The study concludes that the enzymatic digestion of proteins from soybean seed yielded bioactive peptide products that may possess antidiabetic capacities, via inhibition of carbohydrate-degrading enzymes and slowing down protein glycation. It is suggested in further studies that these hydrolysates are fractionated and sequenced to identify actual peptides responsible for these effects.

Keywords: α-amylase, α-glucosidase; Antiglycation; Soybean; Proteolysis; Hydrolysates

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INTRODUCTION

The compounds obtained from the hydrolysis of the peptide bonds in proteins, which yields peptides with various length and free amino acids, are known as protein hydrolysates. (Xia and Severin, 2006). Food-based bioactive peptides have been continuously popular due to their therapeutic abilities. Of particular importance are peptides with multifunctional abilities that can be used in the prevention and treatment of diet-related diseases

such as diabetes (Zambrowicz *et al.* 2014). The potential therapeutic benefits and other health-promoting advantages of bioactive peptides and protein hydrolysate preparations generated from microbial fermentation of plant and animal proteins or enzymatic hydrolysis of such proteins have been studied (Lopez-Barrios *et al.,* 2014, Ulagesan *et al.,* 2018). Peptide products have shown bioactivities against hypertension (Yamada *et al.,* 2013; Majumder and Wu 2015), cancer (Vileghe *et al.,* 2010), oxidative stress (Olusola *et al.,* 2018), pathogenic microorganisms (Ulagesan *et al.,* 2018, Ekun *et al.,* 2024[a]), and diabetes mellitus (Olusola and Ekun 2019[a], Famuwagun *et al.,* 2020), in various in vitro assays and/or animal models.

Soybeans (Glycine max) originated from Asia where they have had a long history of use as a food crop and economic commodity (Shurtleff and Aoyagi, 2010). *Glycine max* contains 35–40% protein on a dry-weight basis, of which, 90% is comprised of two storage globulins, 11S glycinin and 7S β-conglycinin (O'Keefe *et al.,* 2015). These proteins contain all essential amino acids, which makes soy products almost equivalent to animal sources in protein quality but with less saturated fat and no cholesterol. *Glycine max L* also contains the biologically active protein components hemagglutinin, trypsin inhibitors, α-amylase, and lipoxygenases (O'Keefe *et al.,* 2015). *Glycine max* L is not only a high-quality protein but it is now thought to play preventive and therapeutic roles for several diseases. Dietary proteins from soybeans have been shown to offer health benefits in vivo and/or in vitro either as intact proteins or in partially digested forms, also called bioactive peptides. (Agyei, 2015).

Diabetes mellitus is a metabolic condition that results from an absolute or relative insulin insufficiency (Rhoades and Bell, 2013). It is currently the fourth most common cause of illness worldwide (IDF 2020). Chronic hyperglycemia linked to abnormalities in the control of protein, lipid, and carbohydrate metabolism its defining feature (Olusola and Ekun 2019). Poorly controlled diabetes mellitus causes the appearance of advanced glycated end products (AGEs), which occur when glucose reacts non-enzymatically with blood proteins such as hemoglobin and albumin (Ramasamy *et al.,* 2005). These adducts are reactive because they activate the receptor for advanced glycated end-products (RAGE). The consequent downstream signaling in affected cells results in the activation of pro-inflammatory reactions, which in turn generate free radicals which are deleterious to cellular and tissue function, especially the retina and kidney (Singh *et al.,* 2014). Apart from the activity of glycated adducts, metabolites such as ketones cause continued organ damage as the disease progresses (Arise *et al.,* 2016).

Conventional treatment strategies have been geared towards keeping of blood glucose levels within normal ranges through a combination of lifestyle changes and pharmacologic interventions. The carbohydrases (α-amylase and α-glucosidase) and incretin-degrading enzymes such as dipeptidyl peptidase IV) have been identified as important pharmacologic targets for numerous hypoglycemic medications (Arise *et al.,* 2019). Consequently, these drugs are utilized in the treatment of diabetes mellitus along with dietary modifications. However, due to a number of these medications' side effects (such flatulence, diarrhoea, and stomach pain) as well as their high cost of acquisition, particularly in developing nations, interest has shifted to natural alternatives like peptide products made from the proteins found in plant seeds and leaves (Adisakwattana *et al.,* 2012; Yu *et al.,* 2012).

Most of the protein hydrolysates and peptide fractions obtained from plant and animal sources in the literature have been generated by single enzyme proteolysis. However, the use of enzyme combinations to generate bioactive hydrolysates and peptides have been relatively underexplored, when compared to single enzymes. Hence this study aims to explore the antidiabetic potentials of soybean protein hydrolysates obtained using dual-enzyme digestion (with trypsin/chymotrypsin), and comparing them with hydrolysates obtained from proteolysis with trypsin and chymotrypsin used as single enzymes.

MATERIALS AND METHODS

Collection of *Glycine max* Seeds

Glycine max L Seeds were purchased from a local store in Owo, Ondo State, Nigeria, and were identified, authenticated and voucher samples were deposited at the herbarium of the Department of Plant and Science, Adekunle Ajasin University, Akungba Akoko, Ondo State, Nigeria.

Chemicals and Reagents

The proteolytic enzymes used in this study were trypsin (from bovine pancreas), and chymotrypsin (from bovine pancreas). The carbohydrate-hydrolyzing enzymes used were α-amylase (from Saccharomyces cerevisiae) and α-glucosidase (human). Trypsin and α-amylase were procured from Kem Light Laboratories, Mumbai, India; whereas chymotrypsin and α-glucosidase were products of Sigma-Aldrich Laboratories, United Kingdom. Other reagents used in this study were of analytical grade.

Isolation of *Glycine max. L* Seed Proteins

The seeds were dried, pulverized, and kept in a cold and dry container. They were defatted using n-

hexane as described by Olusola *et al*.,(2018). The meal was extracted twice with n-hexane using two hundred grams (200 g) of seed meal suspended in 1000 ml of n-hexane, (meal/solvent ratio of 1:5 w/v). The meal was then air dried and a fine powder was obtained, termed defatted seed meal, which was stored at -10oC. The protein component of the defatted meal was extracted using the method reported by Ekun *et al*. (2022). Defatted soybean seed meal was suspended twice in 0.2 M NaOH pH 12.0 at a ratio of 1:10, and stirred for one hour for the purpose of alkaline solubilization, using a glass rod. This was centrifuged at 18°C and 3000 g for 10 min. One additional extraction of the residue from the centrifugation process was performed with the same volume of 0.2 M NaOH and the supernatants were decanted. The pH of the supernatant was adjusted to 4.2 to facilitate acid-induced protein precipitation using 0.5 M HCl solution; the precipitate formed was recovered by centrifugation. The precipitate was washed with distilled water, adjusted to pH 7.0 using 0.1 M NaOH, freeze-dried, and the protein isolate was then stored at -10°C until required for further analysis.

Preparation of *Glycine max. L Seed* Protein Hydrolysates

The protein isolate was hydrolyzed using the methods described by Ekun *et al.,* (2022) with slight modifications. The conditions for hydrolysis were optimized for each enzyme for maximized activity. Hydrolysis was performed using each of trypsin and chymotrypsin, and a combination of both enzymes at pH 8.0 and 37ºC. The protein isolate (5% w/v, based on the protein content of the isolate) was dissolved in the phosphate buffer at conditions earler specified. The enzymes was added to each suspension at an enzyme-substrate ratio (E:S) of 1:50. Digestion was performed at the specified conditions for nine (9) hours. The enzyme was then inactivated by boiling in hot water (90ºC) for 20 mins. Undigested proteins were precipitated by adjusting the pH to 5.1 with 2 M HCl/2 M NaOH followed by centrifugation at 3000 g for 30 min. The supernatant containing the peptides was then collected. The protein content of samples was determined using the biuret assay method with bovine serum albumin (BSA) as standard.

Inhibition of Albumin Glycation

This was investigated by estimating the degree of non-enzymatic albumin glycation according to the method described by Venu, *et al.* (2016). Glucose solution (2%), 0.06% albumin and 0.02% Gentamycin solutions were prepared in phosphate buffer 0.1 M, pH 7.4. One milliliter (1 ml) each of above solution was mixed. 0.2 mg/mL - 1.0 mg/mL of hydrolysate was added to above mixture. Gallic acid was used as standard. The mixture was kept in dark at room temperature for incubation for 72 hours. At 520 nm, albumin glycation was measured with a spectrophotometer and % inhibition was calculated thus:

Percentage albumin glycation = Abs (sample) - Abs (control)/ Abs (sample) x 100%

Determination of α-amylase inhibitory activity

An alpha amylase-inhibitory assay was performed according to the method reported by Oboh *et al*., (2011). Briefly, 125μL of hydrolysate (0.5 to 2.0 mg mL^{-1}) was placed in test tubes and 125μL of 20 mM sodium phosphate buffer (pH 6.9, with 6mM NaCl) containing alpha amylase solution (0.5 mg/ml) added. The content of each tube was pre-incubated at 25℃ for 10min, after which 125μL of 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9, with 6 mM NaCl) was added at intervals. The reaction mixtures were incubated at 25℃ for 10min. The reaction was terminated by adding 250μL of dinitrosalicylic acid (DNS) color reagent and further incubated in boiling water for 5 min and cooled at room temperature. The content of each test tube was diluted with 2.5 ml distilled water and the absorbance measured at 540 nm. A control was prepared using the same procedure except that the hydrolysate was replaced with distilled water. The alpha amylase-inhibitory activity was evaluated by as the ratio of change in absorbance ($A_{control}$ – A_{sample}) to the absorbance of control, expressed in percentage.

Evaluation of kinetic parameters of α-amylase inhibition

The method described by Olusola *et al.* (2018) was employed. Aliquot volume (125 µL) of the hydrolysate was pre-incubated with 125 µL of α-amylase solution for 10 min at 25 °C in a set of tubes. In another set of tubes, 250 µL of phosphate buffer (pH 6.9) was also pre-incubated with 125 µL of α-amylase solution. Starch solution (125 µL) of increasing concentrations (1.0 to 8.0 mg mL–1) were added to both sets of reaction mixtures to initiate the reaction. The mixture were then incubated for 10 min at 25 °C, and then boiled for 5 min after the addition of 250 µL of dinitrosalicylic acid (DNS) reagent to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically from a maltose standard curve and converted to reaction velocities. A Lineweaver-Burk plot (1/V versus 1/[S]), where V is reaction velocity and [S] is substrate concentration was plotted. The mode of inhibition and the kinetic parameters (Km, K′m, V′max, V′max, CE and CE′) of α-amylase inhibition by hydrolysates were determined by analysis of the double reciprocal plot. The inhibition constant (K_i) was determined using a secondary plot known as

the Dixon plot (Palmer and Bonner, 2007), by plotting a graph of inverse of initial velocities on the y-axis against inhibitor concentrations on the x-axis, at fixed concentration of substrate.

Determination of α-Glucosidase Inhibition

The effect of the hydrolysates on α-glucosidase activity were determined according to the method described by Olusola and Ekun, (2019[b]) with slight modifications, using α-glucosidase from Saccharomyces cerevisiae. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, and pH 6.9. 200μL of α-glucosidase (1.0 U/mL) was pre-incubated with 100μL of the different concentrations of the hydrolysates for 10 min. Then 50μL of 3.0 mM (pNPG) as a substrate dissolved in 20 mM phosphate buffer (pH 6.9) will be added to start the reaction. The reaction mixture was incubated at 37^{0}C for 20 min and stopped by adding 2 mL of 0.1 M Na_2CO_3 solution. The α-glucosidase activity was determined by measuring the yellow-colored para-nitrophenol released from pNPG at 405 nm. The results were expressed as percentage of the blank control. Percentage inhibition were calculated as:

% Inhibition = (A control– A sample) / A control × 100

Evaluation of Kinetics of α-Glucosidase Inhibition

The mode of inhibition of α-glucosidase by soybean protein hydrolysates determined using the procedure reported by Ekun *et al.,* (2023). Aliquot volume (50 μL) of each hydrolysate was added to 100μL of the glucosidase solution and incubated for 10 minutes at 25^{o}C in a set of test tubes. Another set of test-tubes were pre-incubated with the enzyme and 50 μL of phosphate buffer, pH 6.9. The reaction in both set of tubes were started with the addition of 50 μL of p-nitrophenyl glucopyranose (p-NPG) at graduated concentrations (0.5-2.5 mg/mL). After a further 10-minute incubation of both sets of tubes, the reaction was stopped by the addition of 500 μL of 0.1 M sodium trioxocarbonate (iv) solution (Na_2CO_3). The amount of p-nitrophenol (and glucose) released was determined by spectrophotometric method at 405nm, using a paranitrophenol standard graph and the values were converted to reaction velocties. A double reciprocal plot (1/V on the y-axis versus 1/[pNPG] on the x-axis) to determine the mode of inhibition and kinetic parameters was plotted. The inhibition constant (K_i) was determined using the Dixon plot (Palmer and Bonner, 2007), by plotting a graph of inverse of initial velocities on the y-axis against inhibitor concentrations on the x-axis, at fixed concentration of substrate.

Data Analysis

Results were expressed as mean of triplicate observations ± standard error of mean. The data were statistically analyzed using One Way Analysis of Variance (ANOVA) and Duncan's multiple range tests. Differences were considered statistically significant at $p<0.05$ using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Inhibition of Albumin Glycation

The effect of Soybean seed protein on albumin glycation is illustrated in Figure 1. All digests exhibited significantly lower antiglycation activities when compared to gallic acid (control). In addition they inhibited albumin glycation in a concentration-dependent manner. Among the digests, tryptic hydrolysates had significantly higher ($p<0.05$) inhibitory activity than the others at lower concentrations. However, there is no significant difference in activities among all hydrolysates at higher concentrations, with all hydrolysates having above 70% inhibition at final concentration of 2.5 mg/mL.

α-Amylase inhibitory Activity

The percentage α-amylase inhibitory activity of Glycine max (Soybean) seed protein hydrolysates are illustrated in Figure 2. All hydrolysates demonstrated inhibitory activities above 50% at a final concentration of 2.5mg/ml. Among the hydrolysates, those obtained using a combination of trypsin and chymotrypsin had significantly higher activity at all study concentrations, achieving a maximal inhibitory extent of 77.06 ±0.73% at a concentration of 0.5 mg/mL. Also, chymotrypsin hydrolysates displayed significantly higher inhibitory activities than tryptic hydrolysates at three of the five study concentrations, achieving an activity value of 59.70± 1.33% at 2.5 mg/mL as against 52.83± 2.03% recorded by tryptic digests.

Kinetics α-Amylase Inhibition

The double reciprocal plots of α-amylase inhibition by Soybean seed protein hydrolysates are illustrated by double-reciprocal plots in Figures 3-5 and kinetic parameters are summarized in Table 1. In the absence of the hydrolysates as inhibitors, the Michaelis constant (k_m) and maximum velocity (V_{max}) were 0.142 mg/mL and 0.987 mM/mg/min respectively. Tryptic hydrolysates and digests obtained from dual-enzyme digestion inhibited α-amylase via a mixed mechanism, whereas an uncompetitive mode of α-amylase inhibition was observed in the presence of chymotrypsin hydrolysates. Also, the enzyme-inhibition dissociation constant, Ki, obtained for trypsin hydrolysates (0.508 mg/mL) was lower than those of the other protein digests.

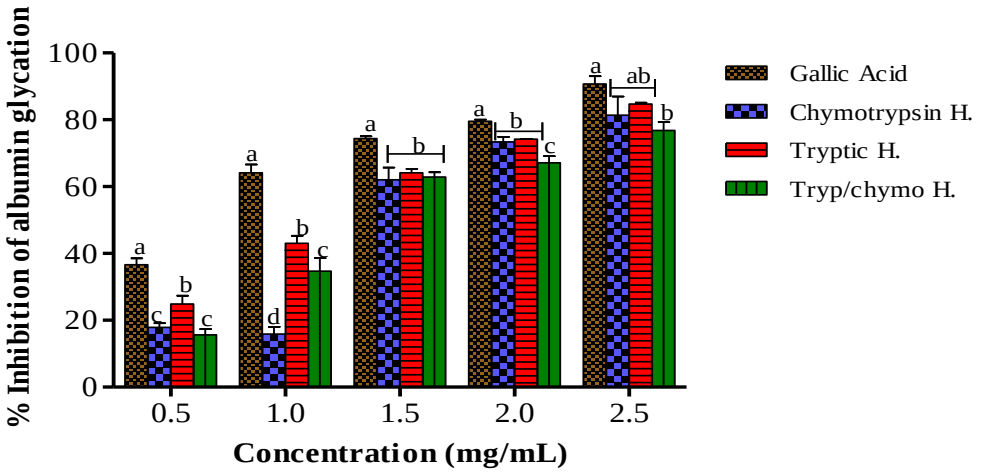

Figure 1. Antiglycation Properties of *Glycine max* seed Protein Hydrolysates
Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Comparison is made strictly among samples with the same concentration. Bars carrying different letters are significantly different ($p<0.05$) from one another. Bars carrying the same letter are not significantly different ($p>0.05$).

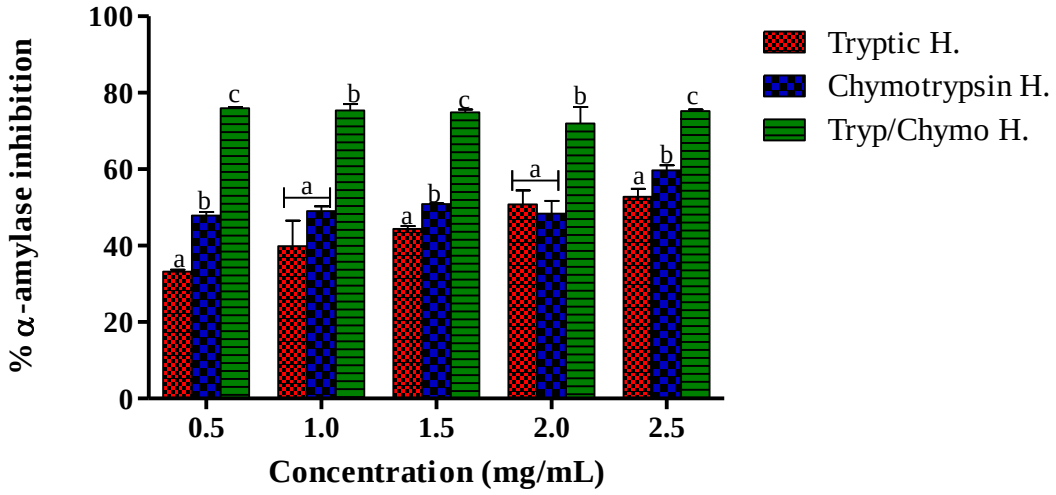

Figure 2. α-Amylase Inhibitory Activities of *Glycine max* seed Protein Hydrolysates
Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Comparison is made strictly among samples with the same concentration. Bars carrying different letters are significantly different ($p<0.05$) from one another. Bars carrying the same letter are not significantly different ($p>0.05$).

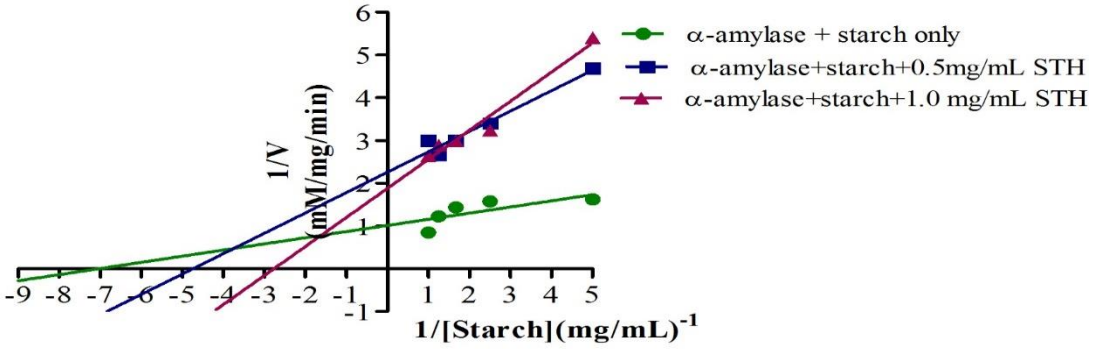

Figure 3. Kinetics of α-Amylase inhibition in the Presence of Soybean protein hydrolysates obtained from Trypsin proteolysis.
STH: Soybean Trypsin hydrolysate

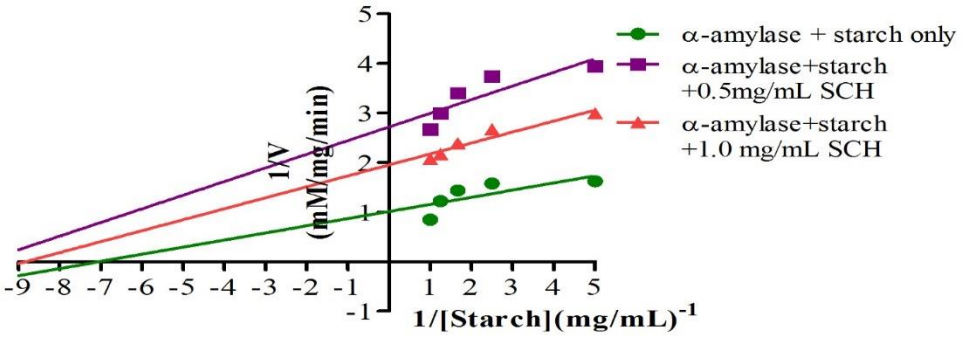

Figure 4. Kinetics of α-Amylase inhibition in the Presence of Soybean protein hydrolysates obtained from chymotrypsin proteolysis.
SCH: Soybean chymotrypsin hydrolysate

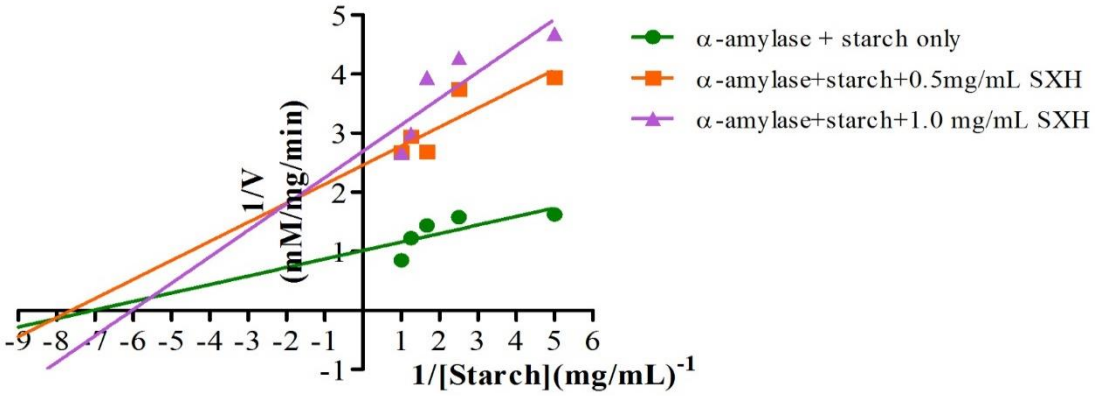

Figure 5. Kinetics of α-Amylase inhibition in the Presence of Soybean Protein Hydrolysates Obtained via the Combined Actions of Trypsin and Chymotrypsin
SXH: Soybean Trypsin/chymotrypsin hydrolysate

Table 1: Kinetic indices of amylase-catalyzed starch hydrolysis to maltose in the presence and absence of soybean seed protein hydrolysates

Kinetic parameters	No inhibitor	Tryptic hydrolysates (mg/mL)		Chymotrypsin hydrolysates (mg/mL)		Tryptic/ Chymotrypsin hydrolysates (mg/mL)	
		0.5	1.0	0.5	1.0	0.5	1.0
K_M or K'_M (mg/mL)	0.142	0.211	0.361	0.101	0.113	0.131	0.166
V_{max} or V'_{max} (mM/mg/min)	0.987	0.443	0.532	0.368	0.512	0.407	0.371
CE (mmol/ml/min)	6.951	2.100	1.474	3.644	4.531	3.107	2.235
K_I (mg/mL)	-	0.508		0.522		0.849	

K_M /K'_M:Michaelis constant in the absence/presence of hydrolysates; V_{max} or V'_{max} : Maximal velocity in the absence/presence of hydrolysates; CE:Catalytic efficiency of the enzyme K_I: Enzyme-inhibitor dissociation constant

α-Glucosidase inhibitory Activity

The effects of *Glycine max* seed protein hydrolysates on **α**-glucosidase in hydrolyzing the artificial substrate paranitrophenyl glucopyranose to glucose and paranitrophenol is illustrated in Figure 6. The hydrolysates obtained from the combined actions of trypsin and chymotrypsin exhibited significantly ($p>0.05$) higher inhibitory activities than hydrolysates obtained from single enzyme hydrolysis at all study concentrations, achieving a maximal inhibition of 71.49±3.49% at a final hydrolysate concentration of 2.5mL. Tryptic hydrolysates demonstrated significantly better activities than chymotrypsin hydrolysates at four of the five study concentrations, attaining a maximum inhibitory extent of 28.79±1.52% at 2.0 mg/mL.

Kinetics of α-Glucosidase Inhibition

Kinetic analysis of α-glucosidase activity in the presence and absence of inhibitory hydrolysates obtained from *Glycine max* seed protein is

displayed in Figures 7-9 and summarized in Table 2. In the absence of inhibitory protein digests, the Km of α-glucosidase for its substrate and Vmax of the reaction were 0.364 mg/mL and 909.091 mM/mg/min respectively. In the presence of tryptic digests, an uncompetitive subtype of mixed inhibition was observed, and a non-competitive inhibition mechanism was obtained for the mixed-enzyme derived hydrolysates. A mixed mode of inhibition was obtained for chymotrypsin hydrolysates of *Glycine max* seed protein.

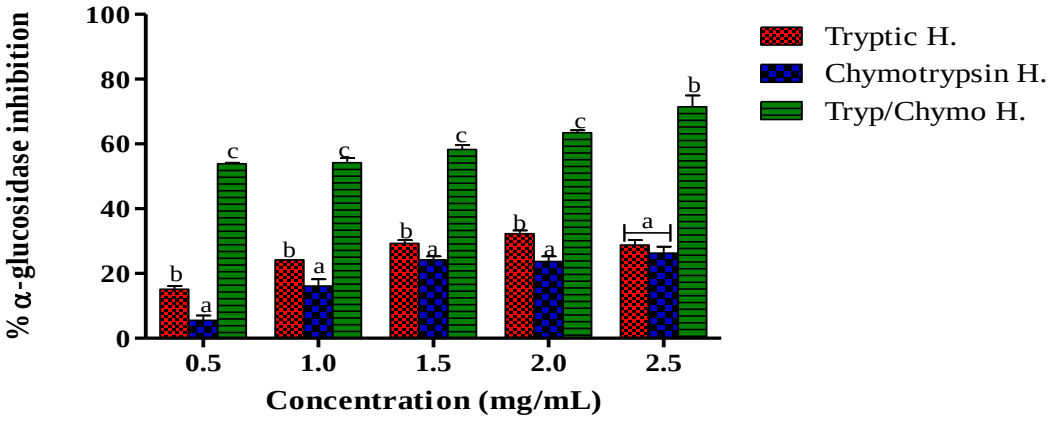

Figure 6. α-Glucosidase Inhibitory Activities of *Glycine max* seed Protein Hydrolysates
Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Comparison is made strictly among samples with the same concentration. Bars carrying different letters are significantly different ($p<0.05$) from one another. Bars carrying the same letter are not significantly different ($p>0.05$).

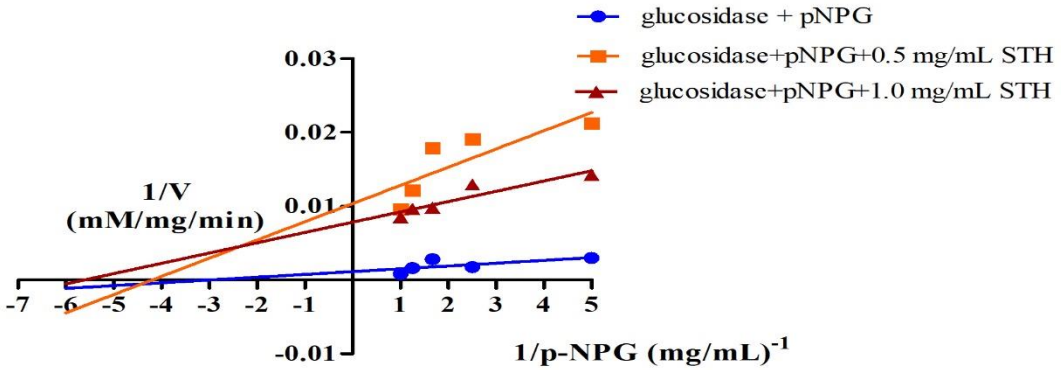

Figure 7. Kinetics of α-Glucosidase inhibition in the Presence of Soybean protein hydrolysates obtained from Trypsin proteolysis.
STH: Soybean Trypsin Hydrolysate

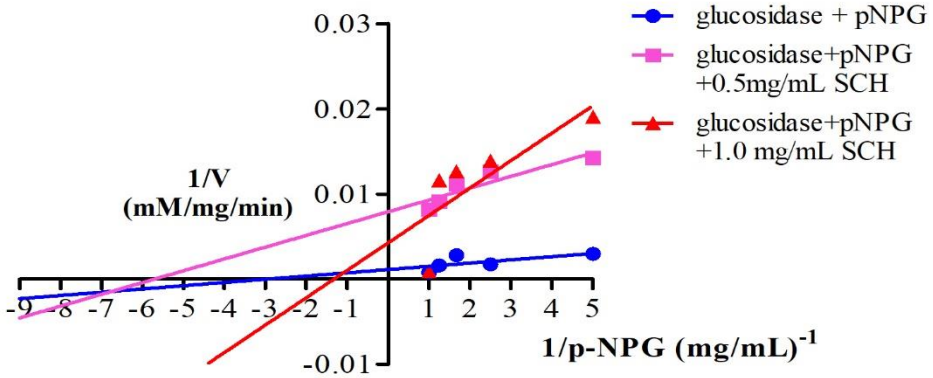

Figure 8. Kinetics of α-Glucosidase inhibition in the Presence of Soybean protein hydrolysates obtained from chymotrypsin proteolysis.
SCH: Soybean Chymotrypsin Hydrolysate

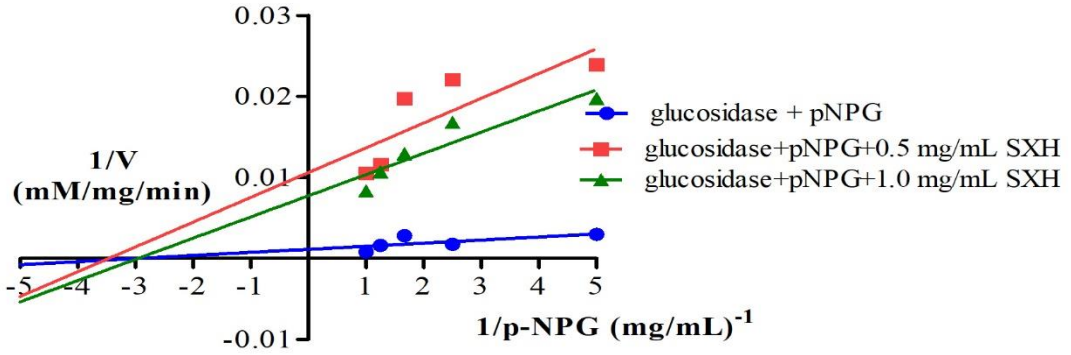

Figure 9. Kinetics of α-Glucosidase inhibition in the Presence of Soybean protein hydrolysates obtained from the Combined Actions of Trypsin and Chymotrypsin
SXH: Soybean Trypsin/chymotrypsin Hydrolysate

Table 2: Kinetic parameters α-Glucosidase-catalyzed hydrolysis of paranitrophenyl glucopyranose to p-nitrophenol in the presence and absence of soybean seed protein hydrolysates

Kinetic parameters	No inhibitor	Tryptic hydrolysates (mg/mL)		Chymotrypsin hydrolysates (mg/mL)		Tryptic/ Chymotrypsin hydrolysates (mg/mL)	
		0.5	1.0	0.5	1.0	0.5	1.0
K_M or K'_M (mg/mL)	0.364	0.24	0.18	0.18	0.74	0.29	0.34
V_{max} or V'_{max} (mM/mg/min)	909.091	96.15	128.21	126.58	232.56	94.34	129.87
CE (mmol/ml/min)	2497.503	400.64	712.25	715.15	312.58	323.08	384.23
K_I (mg/mL)	-	0.33		0.250		0.37	

K_M /K'_M:Michaelis constant in the absence/presence of hydrolysates; V_{max} or V'_{max} : Maximal velocity in the absence/presence of hydrolysates; CE: Catalytic efficiency of the enzyme K_I: Enzyme-inhibitor dissociation constant

DISCUSSION

The appearance of advanced glycated end products in the blood is often a direct consequence of poorly managed diabetes mellitus (Singh *et al.,* 2014, Ekun, 2022). Advanced glycated end products, AGEs, are formed when excess glucose combine with proteins such as hemoglobin and albumin in the blood (Ramasamy *et al.,* 2005). These AGEs promote signaling mechanisms involving the receptor for advanced glycated end products (RAGE), with the consequential rise in the production of reactive oxygen species. These pro-oxidants in turn aggravate inflammation, by producing local pro-inflammatory mediators, causing microvascular damage in blood capillaries which serve vital tissues and organs such as the heart and the retina, as the disease progresses (Caturano *et al.*, 2025). In previous studies, protein digests from various plant sources have been demonstrated to possess anti-glycation activities, slowing down the rate of formation of glycated adducts, with potential implications for diabetes mellitus therapy (Olusola and Ekun, 2019[b], Caturano *et al.,* 2025). In the current study, *Glycine max* seed protein hydrolysates demonstrated varying capacities in slowing down the rate of protein glycation. The inhibition activity of tryptic hydrolysates of *Glycine max* seeds was higher than that of those obtained for unfractionated trypsin digests of *M. oleifera* seed protein (Ekun, 2022), which could be as a result of variabilities in peptide sequences from the two sources. However, there is much to be done in the area of identifying peptide sequences responsible for these effects. However, in this study, hydrolysates obtained from single enzyme digestion, appeared to elicit better effects against protein glycation, than those obtained from dual-enzyme proteolysis. This is because single enzyme digestion with either of trypsin and chymotrypsin, cleave proteins at specific residues (trypsin cleaves proteins after lysinyl and argininyl residues, whereas chymotrypsin catalyze peptide cleavage at aromatic aminocyl residues). High specificity leads to less frequence of cleavage occurrence, therby producing longer peptides in the process (Voet *et al.,* 2016). This is in contrast to enzyme mixtures, showing broader cleavage specificity, may have produced too many short-chained peptides, many of which could form aggregates with one another, which may resulted in lowered inhibition of protein glycation, especially at low concentrations.

Ekun (2022) reported that low molecular weight peptide fractions recorded lower inhibitory effects activity when compared to unfractionated protein

hydrolysates. In this case, the use of enzyme mixtures may have produced similar effects as peptide fractionation, yielding peptide digests consisting of low molecular weight peptides.

The digestion and absorption of starchy foods require α-amylase activity. It is a hydrolase present in pancreatic secretions and in saliva, as it is involved in glycosidic bond cleavage of complex carbohydrates, especially starch. These polysaccharides are hydrolyzed at the α-(1-4) glycosidic bonds to yield simple sugars such as glucose and maltose (Arise *et al.,* 2016). Studies are now increasingly geared toward the investigation of peptides and peptide products as enzyme inhibitors with positive implications for the treatment of cardiovascular diseases such as hypertension and diabetes mellitus (Arise *et al.,* 2019, Ekun *et al.,* 2022). In the present study, all three hydrolysates inhibited amylase activity in different capacities, with the hydrolysates from the trypsin/chymotrypsin proteolysis achieving better effects than other protein digests. The combination of proteases in producing biologically active protein hydrolysates have been explored in recent times. Garza *et al.*(2017) utilized pepsin-trypsin combination to produce antidiabetic peptide fractions from *Moringa oleifera* seed proteins, which had higher α-amylase inhibitory activity than those obtained from single enzyme hydrolysis. The trypsin/chymotrypsin combination used in this study may have helped to broaden the amino acid specificity, in addition to liberating peptide sequences with requisite aminoacyl residues required to inhibit amylase activity. Chymotrypsin hydrolysates had better inhibitory properties when compared with tryptic hydrolysates in this study, and this was comparable to the α-amylase inhibitory activities obtained for unfractionated chymotrypsin hydrolysates of *M. oleifera* seed protein (above 50% inhibition at 1.0 mg/mL) (Ekun *et al.,* 2022). Olusola *et al.* (2018) had suggested that amino acid residues such as phenylalanine, and tryptophan in addition to lysine and tyrosine were essential aminoacyl residues in peptides which inhibited activity. It is evident that protease action may have liberated these residues in peptides, which in turn accounted for the inhibitory activities observed. The Lineweaver-Burk plots used to establish the modes of α-amylase inhibition indicated that, mixed inhibitory patterns were observed for tryptic hydrolysates. Arise *et al* (2016) and Olusola and Ekun (2019[a]) also observed a mixed pattern of α-amylase inhibition by tryptic digests of *Citrullus lanatus* seed protein and Cowpea seed protein respectively. This suggests that the proteolytic cleavage of proteins by trypsin yielded peptides which inhibit amylase activity in its unbound state and when it has bound substrate, creating dead end enzyme-inhibitor complexes in both cases. Information has been scarce about the kinetics of amylase inhibition by hydrolysates derived from two-protease combinations, but in this study, a mixed mode of inhibition was observed. Chymotrypsin hydrolysates of *Glycine max* seed protein inhibited α-amylase in an uncompetitive manner. However, this was in contrast to the mixed inhibition mechanism exhibited by a <1kD peptide fraction obtained from chymotrypsin hydrolysis of *M. oleifera* seed protein (Ekun *et al.,* 2022). This is likely as a result of peptide size differences in both hydrolysates. The enzyme-inhibitor dissociation constant, Ki, is a measure of the binding affinity of the enzyme for the inhibitor (Palmer and Bonner, 2007). Trypsin hydrolysates had the lowest Ki value of all three hydrolysates, which indicated highest binding affinity. This was higher than the duo of 0.449 mg/mL and 0.148 mg/mL obtained for trypsin digests of *C. lanatus* seed proteins (Arise *et al* 2016) and *Arachis hypogea* seed proteins (Olusola and Ekun, 2018) respectively. This indicates that specific aminoacyl residues such as lysine residues on peptides were required for amylase inhibition (Olusola and Ekun, 2018).

α-Glucosidase is an important enzyme which mediates the cleaving of glucosyl residues from oligosaccharides in the mucosa cells of the small intestine (Voet *et al.,* 2016). Thus, the inhibition of glucosidase activity remains a key intervention strategy in the treatment of diabetes mellitus, as it is a pharmacologic target for glucose-lowering drugs (Qaisar *et al.,* 2014; Ekun *et al.,* 2024[b]). In the present study, hydrolysates obtained by single enzyme proteolysis had lower α-glucosidase inhibition when compared with protein digests obtained from dual-protease action. The maximal inhibitory extent of 28.79% by trypsin digests at a maximum concentration of 2.5mg/ml was lower than 54.54% obtained by Arise *et al*., (2019) for tryptic hydrolysates of *Luffa cylindrica* seed protein hydrolysates. According to Ibrahim *et al.* (2018) structural requirements of peptides for α-glucosidase inhibition suggested that side chains such as basic amino acids, hydroxyl amino acids and proline have to be present in these peptides for optimal inhibitory effects on the enzyme. It therefore follows that the protease combination may have helped cleaved the protein to liberate peptides possessing requisite amino acid residues. Also, tryptic hydrolysates performed better than chymotrypsin hydrolysates in inhibiting α-glucosidase activity. Kinetic data as depicted by the double reciprocal plots (Figures 7-9) and Table 2 showed that in the absence of inhibitors, the

Michaelis constant, Km was 0.364 mg/mL of p-nitrophenyl glucopyranose. This was higher than the duo of 0.297 mg/mL p-NPG (Olusola and Ekun, 2019[b]) and 0.7mM (0.211 mg/mL) (Awosika and Aluko, 2019). The maximal rate of reaction also obtained under this condition was 909.09 mM/mg/min, which was also higher than 270.27 determined by Olusola and Ekun(2019[b]). All three hydrolysates inhibited α-glucosidase via non-competitive mechanism, which suggests that *Glycine max* seed protein hydrolysates are able to bind and inhibit the enzyme in both free and substrate-bound states, altering catalysis in both instances. The enzyme-inhibitor dissociation constant, Ki, was lowest in chymotrypsin hydrolysates This indicated that they had the highest binding affinity for α-glucosidase among all three hydrolysates. However this result was higher than the Ki values obtained for *M. oleifera* seed protein hydrolysates (Olusola and Ekun, 2019[b]) as they inhibited α-glucosidase activity.

CONCLUSION

The results of this study demonstrated that soybean proteins, on enzymatic proteolysis by single enzymes – trypsin and chymotrypsin, and a combination of both enzymes (dual-enzyme digestion) produced hydrolysates capable of slowing down non-enzymatic albumin glycation to varying degrees. In addition, these hydrolysates inhibited α-amylase and α-glucosidase activities via different mechanisms. Single enzyme digestion produced hydrolysates capable of inhibiting protein glycation better than dual-protease derived hydrolysates, whereas protein digests obtained from dual-enzyme proteolysis yielded protein digests with better enzyme-inhibitory activities, than digestion with single enzymes. It is therefore recommended to further explore protease combinations for the production of biologically active protein hydrolysates. In addition, fractionation of the hydrolysates is also suggested, to unravel their sequences for the development of novel antidiabetic peptides.

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