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DIPEPTIDYL PEPTIDASE IV INHIBITORY ACTIVITY OF PROTEIN HYDROLYZATES FROM *AMARANTHUS HYPOCHONDRIACUS* L. GRAIN AND THEIR INFLUENCE ON POSTPRANDIAL GLYCEMIA IN STREPTOZOTOCIN-INDUCED DIABETIC MICE.

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Abstract

Background: Type 2 diabetes is a chronic metabolic disorder. Recently, dipeptidyl peptidase IV (DPP-IV) inhibitors that protect incretin hormones from being cleaved by DPP-IV have been used as drugs to control glycemia. This study examined the potential hypoglycemic effect of amaranth grain storage protein hydrolyzates to control postprandial glycemia in streptozotocin (STZ)-induced diabetic mice as a model system of diabetes, and their inhibition mode on the enzyme.

Material and Methods: Amaranth grain proteins were isolated and hydrolyzed and fractionated by gel filtration. The DPP-IV inhibitory activity of hydrolyzates as well as their kinetic parameters were assessed. Selected hydrolyzates (300 mg/kg body weight) were administered in a single administration-study (SAS) or in the same concentration during a four-week chronic daily dosing study (FWCDDS) in order to observe the effect on postprandial glycemia of diabetic mice.

Results: Albumin 1, Globulin and Glutelin hydrolyzates (GluH) competitively inhibited DPP-IV *in vitro* (Ki= 0.11-5.61 mg/mL). GluH called Glu.III (IC₅₀= 0.12±0.01 mg/mL) considerably inhibited DPP-IV activity. GluH identified as GluH24 improved glucose tolerance significantly (p<0.05), with remarkable increments in plasma insulin in SAS and FWCDDS (1.25 and 2.25 mg/mL, respectively). This effect could be compared to the one obtained from the mice group that was administered Sitagliptin (580 mg/kg body weight) as positive control (p<0.05).

Conclusion: Amaranth Glutelin hydrolyzates yielded the highest enzyme inhibitory activity reported not only *in vitro*, but also in the STZ-induced diabetic mice in order to control postprandial glycemia.

Key words: Amaranth protein hydrolyzate, DPP-IV inhibitory activity, diabetes

Introduction

Amaranth grain is a traditional crop of Mexico and other countries. It has a great amount of genetic diversity, phenotypic plasticity and it is extremely adaptable to adverse growing conditions: it resists heat and drought and faces no major disease problems. Amaranth can be easily grown in agriculturally marginal lands. It has also been considered a nutritive food because its protein is rich in lysine, which is a limiting amino acid in other crops, and has other nutrients necessary for a well-balanced diet (Rastogi and Shukla, 2013). This food has bio-functional properties (biological activity) which provide medicinal benefits such as the following: it decreases plasma cholesterol levels, stimulates the immune system, exerts an antitumor activity and improves conditions of hypertension and anemia. In addition, it has also been reported as having anti-allergic and antioxidant activities (Caselato-Sousa and Amaya-Farfán, 2012). Some studies have revealed that amaranth's influence on blood glucose level. A previous report claims that the consumption of either the grain or oil may protect against insulin deficiency, but another study claims that the high glycemic index of the starch is a liability to diabetic patients (Kim et al., 2006). What is more, the effect of proteins and their hydrolyzates in regulating glycemia in humans is still largely unknown. This should be taken into account when considering amaranth as a food aimed at people with type 2 diabetes (T2D). It is estimated that by 2030 the number of people affected with diabetes will have reached 366 million (Wild et al., 2004). Thus, this paper focuses on the *in vivo* inhibitory activity of hydrolyzates of amaranth grain storage proteins against dipeptidyl peptidase IV (DPP-IV; EC 3.4.14.5). DPP-IV is a serine protease that modulates the biological activity of circulating peptide hormones by specifically cleaving 2N-terminal amino acids: Xaa-Pro and Xaa-Ala (Bjelke et al., 2006). Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are gut-derived peptides, so-called incretin hormones, that stimulate insulin secretion from the islet β -cells in a glucose-dependent manner. Incretins are responsible for releasing roughly 70% of postprandial insulin. GIP and GLP-1 are secreted within minutes of nutrient ingestion, with plasma levels rapidly falling back to basal values after termination of feeding (Drucker, 2006). GIP and GLP-1 are metabolized extremely rapidly by the DPP-IV. The enhancement of insulin secretion by gut-derived factors such as GIP and GLP-1 is called the "incretin effect". Recently, DPP-IV inhibitors that protect active GLP-1 from being cleaved by DPP-IV have been used as drugs to control postprandial glycemia in T2D (Richter et al., 2008). Thus, complementary strategies to treat this illness currently include the use of protease-resistant GLP-1 analogs used as injectable agents, and the use of orally administered inhibitors of DPP-IV activity (Drab, 2010).

Recently, some amaranth components have been isolated and treated by biotechnological procedures and there have been several reports about the assessment of their bio-functionality. From one of these procedures, bioactive peptides have been obtained: these are not only specific, but also small protein fragments that are actually inactive within the sequence of their parent protein (Korhonen and Philanto, 2003). Therefore, enzymatic

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hydrolyzates of amaranth protein have been thought of as a source of these bio-peptides. Several bioactive peptides obtained from amaranth proteins exhibit bio-functionality including antitumoral effects by lunasin (Silva-Sánchez et al., 2008), inhibition of angiotensin I-converting enzyme (Tiengo and Netto, 2009; Tovar-Pérez et al., 2009; Vecchi and Añón, 2009; Barba de la Rosa et al., 2010; Luna-Suarez et al., 2010; Fritz et al., 2011) and antioxidant (Tironi and Añón, 2010; Orsini et al., 2011). Nevertheless, little attention has been given to the possibility that dietary proteins may also contain, within their sequences, fragments corresponding to DPP-IV inhibitory peptides, and thus might be used as natural sources of DPP-IV inhibitors in the management of T2D. Several peptides with DPP-IV inhibitory activity have been identified through an *in silico* analysis from amaranth grain storage protein hydrolyzates and studies were carried out to predict their binding modes at the molecular level (Velarde-Salcedo et al., 2013). Similarly, inhibition of DPP-IV activity has also been found, *in silico*, in 34 food proteins. Caseins from cow milk and collagens from beef and salmon appeared to be the richest potential sources of DPP-IV inhibitors. GA, GP and PG were the most frequently occurring sequences (Lacroix and Lee-Chan, 2012a).

To the best of our knowledge there is no report on *in vivo* assessment of DPP-IV inhibitory activity of amaranth grain storage protein hydrolyzates. Therefore, in this study the potential hypoglycemic effect of amaranth grain storage protein hydrolyzates to control postprandial glycemia in streptozotocin (STZ)-induced diabetic mice as a model system of diabetes, and their inhibition mode on the enzyme were examined.

Materials and methods

Sample preparation

Amaranthus hypochondriacus grain cultivar Revancha obtained from INIFAP-Campus Montecillo, México was used in this research. The whole grain was milled using a Udy mill (Udy Corporation Fort Collins, Co, USA) until flour was obtained; then it was defatted with acetone (5 mL/g). After drying at room temperature, the defatted flour was sieved through a 60-mesh screen and stored at 5°C until further analysis. The defatted flour proximate analysis was carried out according to AOAC (2000) standard procedures. The content of components was (g/100 g of defatted flour): water (11.1±0.08), ashes (3.15±0.12), crude protein (%N X 5.87= 15.43±0.18), ether extract (0.18±0.01), crude fiber (4.91±0.06) and carbohydrates calculated by difference (65.23±0.08). All experiments throughout this study were performed in triplicate.

Extraction and hydrolysis by alcalase of amaranth grain storage proteins

Albumin 1 (Alb1) and globulin (Glo) were extracted from defatted flour according to the method described by Tovar-Pérez et al. (2009). Alb1 was separated from Glo by dialysis of the supernatant for 24 h against distilled water. The residue of defatted flour was treated with 70 % ethanol to discard prolamins. Then glutelins (Glu) were extracted following the method reported by Barba de la Rosa (2010), using 0.1M Tris buffer at pH 8.0, in a residue/buffer ratio 1:10. Protein content was assessed by the Kjeldahl method (AOAC, 2010). Protein extracts were lyophilized and stored at 5°C until alcalase hydrolysis.

Alcalase hydrolysis of proteins was carried out following the method reported by Tovar-Pérez et al. (2009) with some modifications: 0.5 M phosphate buffer (pH 7.4) was added to a 5 mg/mL of protein solution. The solution was incubated for 5 min at 50°C; then 2.4 UA/mL of alcalase solution in 0.5 M phosphate buffer was added to each test tube to reach a final ratio E/S=0.8 UA/g protein. The reaction, at the appropriate time between 1 to 48h, was placed in a boiling water bath for 10 min to inactivate the enzyme. Alb1, Glo and Glu hydrolyzates (Alb1H, GloH and GluH, respectively) were obtained at different times (n≤48h) of treatment (Alb1Hn; GlobHn and GluHn, respectively).

Measurement of degree of hydrolysis

The degree of hydrolysis (DH) was conducted according to the method reported by Condés et al. (2009). Free amino groups, released by alcalase hydrolysis, were assessed by their reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS). L-leucine was used as a standard. The DH was calculated using the equation reported by the same authors.

Size-exclusion chromatography

Molecular weight characterization of amaranth grain storage protein hydrolyzates was carried out by gel filtration chromatography using a molecular exclusion column. A Sephadex G-15 column (1.4 x 29 cm; Pharmacia, Uppsala, Sweden) and a Pharmacia LKB FPLC System (Uppsala, Sweden) were used. 500 µL of hydrolyzed proteins (15 mg/mL) dissolved in 32.5 mM K₂HPO₄-2.6 mM KH₂PO₄, pH 7.5, which contained 0.4 M NaCl and 20 mM 2-mercaptoethanol (to reduce disulfide bonds of small peptides in order to avoid peptide aggregation) were injected and eluted with the same buffer at 0.3 mL/min. Absorbance at 214 nm was monitored and 0.5 mL fractions were collected. An ultra-low range molecular weight marker (Sigma-Aldrich, St. Louis, Mo., USA), containing triose phosphate isomerase 26.6 kDa; mioglobin 17 kDa; α-lactalbumin 14.2 kDa; aprotinine 6.5 kDa; insulin 3.5 kDa; bradykinin 1.06 kDa, was used. Peptidic fractions (Alb1: A.I, -II and -III; Glo: G.I, -II, -III and -IV, and Glu: Glu.I, -II and -III) were collected until it was possible to obtain a sufficient amount to assess the IC₅₀ value (see below). The collected peptide fractions were lyophilized and stored at -20 °C until analysis.

Determination of DPP-IV inhibitory activity

DPP-IV from porcine kidney (Sigma-Aldrich; St. Louis, MO, USA) was used to assess the enzymatic activity. The activity was performed by measuring the increase in absorbance at 405 nm using Gly-Pro-p-nitroanilide (Gly-Pro-pNA; Sigma-Aldrich; St. Louis, MO, USA) as DPP-IV substrate. For this purpose an ELx808 absorbance microplate reader (Bio Tek µ QUANT; Bio Tek Instruments, Inc., Winooski, VT, USA.) for 96 well ELISA plates was utilized. The lyophilized hydrolyzates were dissolved in 100 mM Tris buffer (pH 8.0; buffer A) at various concentrations. The hydrolyzate solution (25 µL) was added with 25 µL of 1.59 mM Gly-Pro-pNA, in 100 mM Tris buffer, pH 8.0 (0.1 mM final concentration). The mixture was incubated at 37°C for 20 min, followed by adding 50 µL of DPP-IV, diluted with the same Tris buffer to 4.0 mU/mL (Kojima et al., 1980). The reaction mixture was incubated at 37°C for up to 60 min, and the reaction was stopped every 15 min by adding 100 µL of 1 M sodium

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acetate buffer (pH 4.0). Recorded data were plotted versus time, and the DPP-IV activity was quantified. The % DPP-IV inhibition was defined as the percentage of DPP-IV activity inhibited by a given concentration of hydrolyzate.

One enzymatic unit was defined as the quantity of enzyme which releases 1 μ mol of *p*-nitroaniline per min under the assay conditions. The kinetic parameters were evaluated using 0.05 – 2 mM Gly-Pro-pNA in the assay mixture. K_m of Gly-Pro-pNA was estimated as 0.25 ± 0.01 mM. The IC_{50} value was considered as the concentration of sample needed to inhibit DPP-IV by 50%. IC_{50} values were determined plotting the DPP-IV inhibition rate as a function of the log of hydrolyzate concentration (mg/mL). The peptide Diprotin A and the antidiabetic drug Sitagliptin (STG), which inhibit DPP-IV activity, were used as controls in order to compare the protein hydrolyzates IC_{50} values.

Induction of diabetes and animal treatments

Male mice strain CD1 of 4-6 weeks old (Charles River) with 35-40 g body weight were supplied by Universidad Autónoma Metropolitana, Campus Iztapalapa (UAM-I). The animals were housed in polypropylene cages containing sterile sawdust as bedding. The animals were maintained under a controlled temperature (23 ± 2 °C), humidity (40-70%) and a 12 h light-dark cycle. Mice were fed on a diet of commercial mouse chow (Harlan® Rodent diet); water and food were provided *ad libitum*. The animals were randomly divided into 5 groups ($n = 5$): diabetic mice (vehicle; citrate buffer only) as negative control; STG as positive control; Alb1H48, GloH48 and GluH24, which were chosen because of their higher DPP-IV inhibitory activity. The handling of the laboratory animals was performed in agreement with the statutes of the CICUAL (Institutional Committee for the Care and Use of the Animals) based in the international and national rules established in the "Official Mexican Rule" for the care and use of the laboratory animals" [NOM-062-ZOO-1999]. Moderate diabetes was induced by two intraperitoneal injections of streptozotocin (40 mg/kg body weight) freshly dissolved in a citrate buffer (100 mM, pH 4.5), in non-fasted mice on two consecutive days. This method induced partial destruction of β -cells pancreatic (Ventura-Sobrevilla et al., 2011). Control mice were injected with the vehicle. Blood glucose concentration was determined using an Accu-Chek® system (Roche). Mice were considered to be diabetic when non-fasted blood glucose level was ≥ 200 mg/dL. Two protocols were used for administration of amaranth protein hydrolyzates: a single administration study (SAS) of protein hydrolyzates or STG, or four-week chronic daily dosing study (FWCDDS) of protein hydrolyzates or STG.

For the SAS, blood glucose concentration was assessed at the beginning of this study for all groups of diabetic mice. Afterwards, Alb1H48, GloH48 or GluH24 (300 mg/kg) or vehicle or STG (580 mg/kg) for the positive control group was orally administered. After their administration, blood glucose was further assessed after 30 min in order to observe any effect. Oral glucose tolerance test (OGTT) was performed by oral administration of glucose (2 g/kg). Blood glucose level was assessed at 0.5, 1, 1.5 and 2 h in order to obtain a curve of glucose tolerance. Blood insulin and glucagon (0.5 mL of blood collected from caudal vein) were also measured, after 10 min of the glucose load, using an ELISA ALPCO™ kit (80-INSMS-E01 and E10 48-GLUHU-E01, respectively). In the FWCDDS, mice were orally administered once a day with the same hydrolyzates as in the SAS (300 mg/kg) or STG (580 mg/kg) or vehicle, everyday for four weeks. Once the hydrolyzate administration to mice was over, the blood glucose level as well as blood insulin and glucagon were measured, similarly to the SAS.

Statistical analysis

One-way ANOVA was used to determine treatment effects and these were subsequently compared among groups with Tukey's range test. Values were reported as mean \pm SD, and were considered to be significant at $p < 0.05$. Stargraphics Plus v. 5.1 (Statistical Graphics, Manugistics, MD, USA) was used for this purpose.

Results and Discussion

Degree of hydrolysis

Fig. 1a shows hydrolysis of Alb1, Glo and Glu fractions. Alb1 and Glo were more resistant to alcalase hydrolysis than Glu. Alb1 and Glu fractions reached their highest DH at 24 h ($43 \pm 0.51\%$ and $75 \pm 0.51\%$, respectively), but the Glo fraction had its highest DH from 48 h ($59 \pm 0.29\%$). Glu fraction was less resistant to alcalase hydrolysis from the first hour of treatment. Alb1 and Glo fractions showed a similar behavior to alcalase hydrolysis during the 14 h of treatment. Although Alb1 fraction had its maximum DH 24 h earlier than the Glo one, Alb1 hydrolysis was 16% lower than that reached by the Glo fraction. In our laboratory we have observed that hydrolysis of amaranth proteins with alcalase takes a long time in order to reach an extensive hydrolysis ($DH\% > 20$). Glo has been usually more resistant to alcalase hydrolysis. Unlike the previously reported studies, we have found that hydrolysis resistance of proteins with alcalase is surpassed in rather long periods (≥ 24 h). A longer protein hydrolysis yields smaller peptides whose enzymatic inhibitory properties should be studied, such as DPP-IV. High molecular mass (Mr) proteins were hydrolyzed during the first hour of digestion, but peptides of around 22 kDa from Glo and Glu did not change during 48 h of hydrolysis. Barba de la Rosa et al. (2010) stated that Glu hydrolysis with trypsin did not change after 4 h treatment. Limited protein hydrolysis had already been observed by Orsini et al. (2011) and Ventureira et al. (2012), who hydrolyzed an amaranth protein isolate using alcalase for 20 min ($DH\% = 1.7$) and 4 h ($DH\% = 9.5$) and could only achieve a very low partial hydrolysis. Additionally, it has also been reported that globulin-P is associated with protein-bodies that do not allow easy access to hydrolytic enzymes. Konishi et al. (1991) observed that Glo is nowhere as prone to proteolysis as Alb because the former can be found inside protein bodies and this slows down proteolysis during the first 12 h. Condés et al. (2009) found that a polypeptide of 45 kDa from 7S globulin was more resistant to the trypsin action (serine protease) and Vecchi and Añón (2009) reported a great resistance to alcalase hydrolysis of hydrophobic B polypeptides of 11S globulin.

Inhibitory activity of DPP-IV by protein hydrolyzates and their peptide fractions

We have found out that the higher the DH in Alb1 and Glo, the more peptides released with inhibitory activity of DPP-IV (Fig. 1b). The highest DPP-IV inhibition by Alb1 and Glo hydrolyzates was observed at 48 h ($45 \pm 0.2\%$ and $63 \pm 0.5\%$, respectively) and 24 h for Glu ($85 \pm 0.2\%$). It was observed that the Glu hydrolysis released peptides that strongly inhibited DPP-IV activity as early as the first hour of treatment (Fig 1b). Velarde-Salcedo et al. (2013) identified several inhibiting DPP-IV peptides from amaranth hydrolyzates by an *in silico* trypsin digestion analysis, and predicted their binding modes at the molecular level. By LC-ESI/MS/MS and bioinformatics analysis, they identified the amino acid sequences PPP,

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GP, PP, MP, VA, MA, KA, LA, FA, AP, FP, PA, LP, VP, LL, VV, IPA, IPI that displayed an inhibition of DPP-IV activity. Of those peptides, the sequence IPI was identified by these authors as Diprotin A. This is known as an inhibitor of DPP-IV [31-33] (Lacroix and Lee-Chan, 2012b; Rahfeld et al., 1991; Umezawa et al., 1984), and functions as a non-covalent inhibitor that temporarily binds to the catalytic site of the DPP-IV and blocks the degradation of larger polypeptidic chains such as incretins (Wiedeman and Trevillayn, 2003). These di-, tri-, oligopeptides may explain the inhibition of the enzyme during the initial period of hydrolysis.

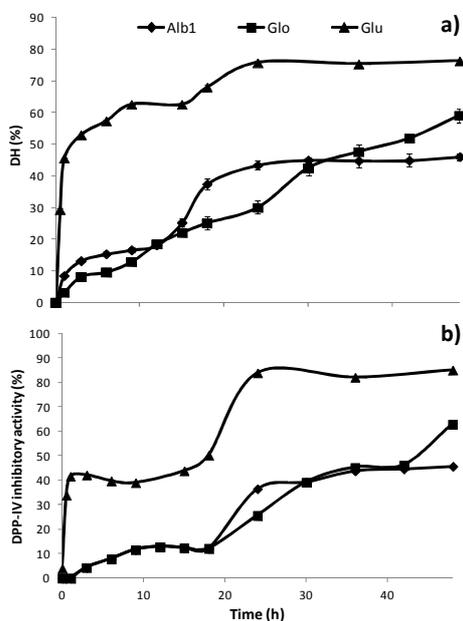


Figure 1: (a) Hydrolysis degree of Albumin 1 (Alb1), Globulin (Glo) and Glutelin (Glu), and (b) Inhibitory activity of DPP-IV by Alb1, Glo and Glu hydrolyzates as a function of reaction time.

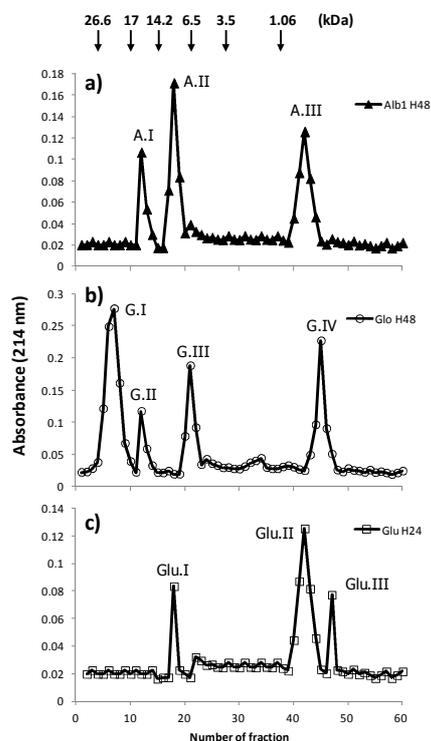


Figure 2: Size-exclusion chromatography elution profile of their respective peptide fractions of Alb1H48, GloH48 and GluH24.

Of the hydrolyzates obtained in this work, Alb1H48, GloH48 and GluH24 showed the maximum DPP-IV inhibitory activity and were partially characterized by size-exclusion chromatography (Sephadex G-15). Alb1H48 consisted of three fractions (Fig. 2a) that were named: A.I (Mr=17 kDa); A.II (Mr=8.1 kDa) and A.III (Mr=0.9 kDa). A.I fraction, still made up of proteins, did not show any DPP-IV inhibitory activity. A.II and A.III fractions did show peptides with DPP-IV inhibitory activity. IC₅₀ values were: 8.34 ± 0.09 and 1.98 ± 0.09 mg/mL, respectively (Table 1). A.III fraction consisted of peptides with about 7-8 amino acids, which is in agreement with Huang et al. (2012) as well as Lacroix and Li-Chan (2012a) and

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Velarde-Salcedo et al. (2013) findings. All of them claimed that di-, tri-peptides and oligopeptides are capable of inhibiting DPP-IV. Peptides larger than 13 residues have also been reported as novel DPP-IV inhibitors (Uchida et al., 2011). GloH48 was made up of four different fractions (Fig. 2b), named: G.I (Mr=24 kDa); G.II (Mr=14.9 kDa), G.III (Mr=6.3 kDa) and G.IV (Mr=0.65 kDa). Similarly, G.I and G.II contained mainly low Mr proteins and did not show any DPP-IV inhibitory activity. Furthermore, G.III and G.IV fractions showed inhibitory activity of the enzyme; IC₅₀ values were 5.6 ± 0.1 and 0.25 ± 0.04 mg/mL, respectively (Table 1). G. IV fraction may have between 5-6 amino acid residues more likely to inhibit DPP-IV. It was observed that Glu consisted of three fractions (Fig. 2c): Glu.I (Mr=11.2 kDa); Glu.II (Mr=0.73 kDa) and Glu.III (Mr=0.45 kDa). In turn, Glu.I, which contained low Mr proteins, did not show any DPP-IV inhibitory activity. On the other hand, Glu.II and Glu.III fractions displayed the following IC₅₀ values: 1.95 ± 0.09 and 0.12 ± 0.09 mg/mL, respectively (Table 1). This finding showed that Glu.III fraction with peptides from 3-4 amino acids had a higher DPP-IV inhibitory activity. Hatanaka reported the occurrence of DPP-IV inhibitors (IC₅₀ = 2.3 ± 0.1 mg/mL) as obtained from rice bran hydrolyzate (Hatanaka et al., 2012). This figure, when compared to the G.III fraction (Table 1), is 20 times as much as the one yielded by rice bran hydrolyzate. Lacroix and Lee-chan (2012a) were also able to obtain hydrolyzates from whey (IC₅₀ of 75 µg/mL) using Thermolysin. In their work, the

Table 1: IC₅₀ values of peptide fractions obtained by size exclusion chromatography from Alb1H48, GloH48 and GluH24 which displayed the highest inhibition of DPP-IV.

Peptide fraction	IC ₅₀ ^a (mg/mL)
Alb1H48	ND ^b
A.I	ND
A.II	8.34 ± 0.09 ^c
A.III	1.98 ± 0.01
GloH48	ND
G.I	ND
G.II	ND
G.III	5.6 ± 0.1
G.IV	0.25 ± 0.04
GluH24	ND
Glu.I	ND
Glu.II	1.95 ± 0.08
Glu.III	0.12 ± 0.006
Diprotin A	8.43 ± 0.29 µg/ml
Sitagliptin	9.41 ± 0.15 ng/ml

^a IC₅₀ was assessed by linear regression analysis of % inhibition of DPP-IV against Log of peptide concentration (mg/mL).

^b Not detected under the assay conditions.

^c Data represent mean values ± the SD of triplicate determinations.

authors stated that cow milk proteins, especially β-casein, α-S1-casein, κ-casein, β-lactoglobulin and lactotransferrin, have amino acid sequences that can inhibit DPP-IV. This whey hydrolyzate inhibits DPP-IV activity much more efficiently than that yielded by Alb1H48, GloH48 and GluH24 of amaranth grain. In our work, Glu.II fraction displayed an IC₅₀ value that ranges from 1.2 to 2.0 mg/mL, as reported by other authors (Lacroix and Lee-Chan, 2012a). In sum, we observed that peptides smaller than 10 kDa as obtained of Alb1H48, GloH48 and GluH24 could inhibit the enzyme following a dose-response pattern. Particularly, Glu.III showed the highest DPP-IV inhibitory activity, which is higher than that reported by Velarde-Salcedo et al. (2013) for amaranth protein hydrolyzates. Table 1 shows that the inhibitory activity of hydrolyzates obtained is an order of magnitude weaker than the corresponding values for tri-peptide Diprotin A (IC₅₀=8.43 ± 0.29 µg/mL) and two orders of magnitude for STG (IC₅₀=9.41 ± 0.15 ng/mL). However, we should bear in mind that we worked with semi-purified hydrolyzates and that DPP-IV inhibitory activity could improve if peptide purity were enhanced.

Inhibition kinetics of DPP-IV by peptide fractions.

Inhibition kinetics parameters of DPP-IV were assessed through a Michaelis-Menten equation and the double reciprocal plot method by Lineweaver-Burk. By doing so, the enzymatic inhibition of fractions with the highest DPP-IV inhibitory activity was assessed *in vitro*, namely, those fractions with the lowest IC₅₀ value as obtained of A.III, G.IV and Glu.III. All these hydrolyzates showed a competitive inhibition mode (Fig. 3). Table 2 shows the kinetic parameters of different fractions of protein hydrolyzates with potential inhibitory activity against DPP-IV. Since this is a competitive inhibition mode, V_{max} value is roughly the same (about 0.0047 µM/min) when a zero order kinetics is reached. In other words, the concentration of the assayed hydrolyzate remains constant whereas the substrate concentration increases in order to promote the interaction with the catalytic site. According to Yan et al. (1992), the general chemical structure of most peptides with DPP-IV inhibitory activity of competitive mode corresponds to the following parameters: Xaa-Pro, Pro-Xaa and Xaa-Ala. Different inhibition modes, however, have been reported, including non-competitive, mixed and irreversible, which indicate that the enzyme inhibition can occur in the enzyme active site or out of it. Molecular docking simulations of amaranth Glo inhibitory action have shown that DPP-IV active structure exists as a dimer with two domains forming an eight-bladed

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propeller domain with a 30-45 Å cavity between each monomer where the inhibitors bind to the catalytic site (Wiedeman, 2007). The interaction between DPP-IV and amaranth Glo peptides is mainly via hydrophobic interactions and hydrogen bonds (Velarde-Salcedo et al., 2013).

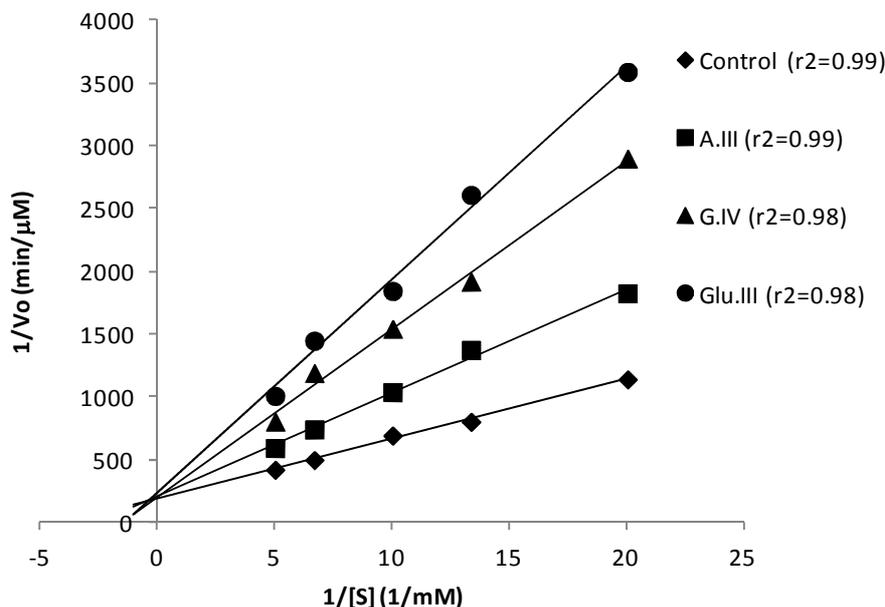


Figure 3: Lineweaver-Burk plot of the effect of amaranth peptide fractions on the rate of Gly-Pro-pNA digestion by DPP-IV. (a) A.III, (b) G.IV and (c) Glu.III were obtained by size exclusion chromatography from Alb1H48, GloH48 and GluH24, respectively. Sitagliptin was used as a control.

It is difficult to compare the inhibition kinetic parameters of DPP-IV as obtained of several proteins because there is no standardized way to express these kinetic values. Sometimes different DPP-IV inhibitory activity values have been reported for the same di-peptide, which may be ascribed not only to different experimental conditions, but also to the substrate and enzyme source used for assessing DPP-IV inhibitory activity (Lacroix and Lee-Chan, 2012a).

Table 2: Kinetic parameters of the effect of amaranth peptides fractions on the rate of Gly-Pro-pNA digestion by DPP-IV.

Kinetic parameter	Control	A.III	G.IV	Glu.III	Sitagliptin ^a
K _m or K _m ' (mM)	0.25	0.41	0.66	0.75	1.22
V _{max} or V _{max} ' (μmol/min)	0.0052	0.0049	0.0049	0.0043	0.0045
CE ^b (x10 ⁻⁶)	20.8	11.9	7.39	5.76	3.67
K _i (mg/mL)		5.61	0.21	0.11	2.86 x 10 ^{-6c}

^a Data were experimentally obtained under the same assay conditions.

^b Catalytic efficiency (min⁻¹)

^c nM

Glu.III was the hydrolyzate fraction that afforded the highest DPP-IV inhibitory activity (K_i 0.11 mg/mL), which was lower than that of STG (K_i 2.86 x 10⁻⁶ ng/mL). This explains the effectiveness of treatment with hypoglycemic synthetic drugs for T2D patients. However, when the enzyme is inhibited, there is a risk of presenting side effects, such as hypoglycemia, weight gain and gastrointestinal disorders. On the other hand, DPP-IV can also inactivate chemokines, neuropeptides and other peptidic hormones leading to a wide range of physiological effects such as angioedema, pancreatitis and ineffective disorders, not to mention its insulinotropic response (Mateucci and Giampietro, 2011). Natural compounds with DPP-IV inhibitory activity can be a complementary treatment because they increase the time of incretins with no risk of hypoglycemia, a common side effect of classic antidiabetic drugs (Nauck, 2011).

Effect of amaranth protein hydrolyzates in streptozotocin-induced diabetic mice Single-administration study

Alb1H48, GloH48 and GluH24 were administered to STZ-induced diabetic mice in an OGTT. A 300 mg/kg dose of GluH24 could inhibit DPP-IV activity because it managed to reduce plasma glucose level in the hyperglycemia of diabetic mice. This effect was similar to that observed in the control group STG (Fig. 4a). Additionally, the oral administration of 300 mg/kg of Alb1H48 and GloH48, which had shown DPP-IV inhibitory capacity *in vitro*, had no effect on the plasma glucose level in the biological assay. Fig. 4b shows the area under the OGTT curve; STG and GluH24 were clearly capable of reducing the plasma glucose level (p<0.05) in diabetic mice. Similarly, GluH24 oral administration not only increased plasma insulin level (p<0.05; Fig. 4c), but also diminished plasma glucagon level (p<0.05; Fig. 4d). Plasma insulin and glucagon levels are directly linked to GLP1; this behavior was also similar to the one afforded by STG, which is a strong DPP-IV inhibitor.

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Four-week chronic daily dosing study

On assessing Alb1H48, GloH48 and GluH24 (300 mg/kg) administration in a chronic daily dosing study, it was observed that daily Alb1H48 and GloH48 intake did not produce any effect on plasma glucose levels in STZ-induced diabetic mice. In the same way as SAS, it was only GluH24 that reduced plasma glucose levels in STZ-induced diabetic mice ($p < 0.05$; Figs 5a and 5b). Plasma insulin levels were twice as high (2.25 mg/mL) when compared to the one observed in SAS (1.25 mg/mL). However, plasma glucagon levels, unlike SAS, did not diminish in FWCDSS; on the contrary, they rose (548 to 983 pg/mL, respectively). There was no significant difference ($p < 0.05$) with the STZ-induced diabetic mice control group. By looking at this result, it seems that DPP-IV chronic inhibition by GluH24 peptides triggered a plasma glucose level reduction because plasma insulin increased. Simultaneously, GluH24 induced not only plasma insulin secretion, but also plasma glucagon secretion. Therefore, we have hypothesized that in order to compensate plasma glucose level reduction, glucagon is released. This research, in the same way as in Velarde-Salcedo's et al. (2013), found that hydrolyzates, as obtained of all amaranth protein fractions, have *in vitro* DPP-IV inhibitory activity. Those authors reported that peptides released from amaranth Glu, using simulated gastrointestinal digestion, conserved the *in vitro* DPP-IV inhibitory activity. Furthermore, we found out that the GluH24 showed the highest inhibitory capacity so far recorded against DPP-IV in STZ-induced diabetic mice as a model system of diabetes. This is the first effort to assess the effect of amaranth protein hydrolyzates on the DPP-IV inhibitory activity using STZ-induced diabetic mice. It is clear, however, that further studies on this topic must be performed in order to shed light on this issue.

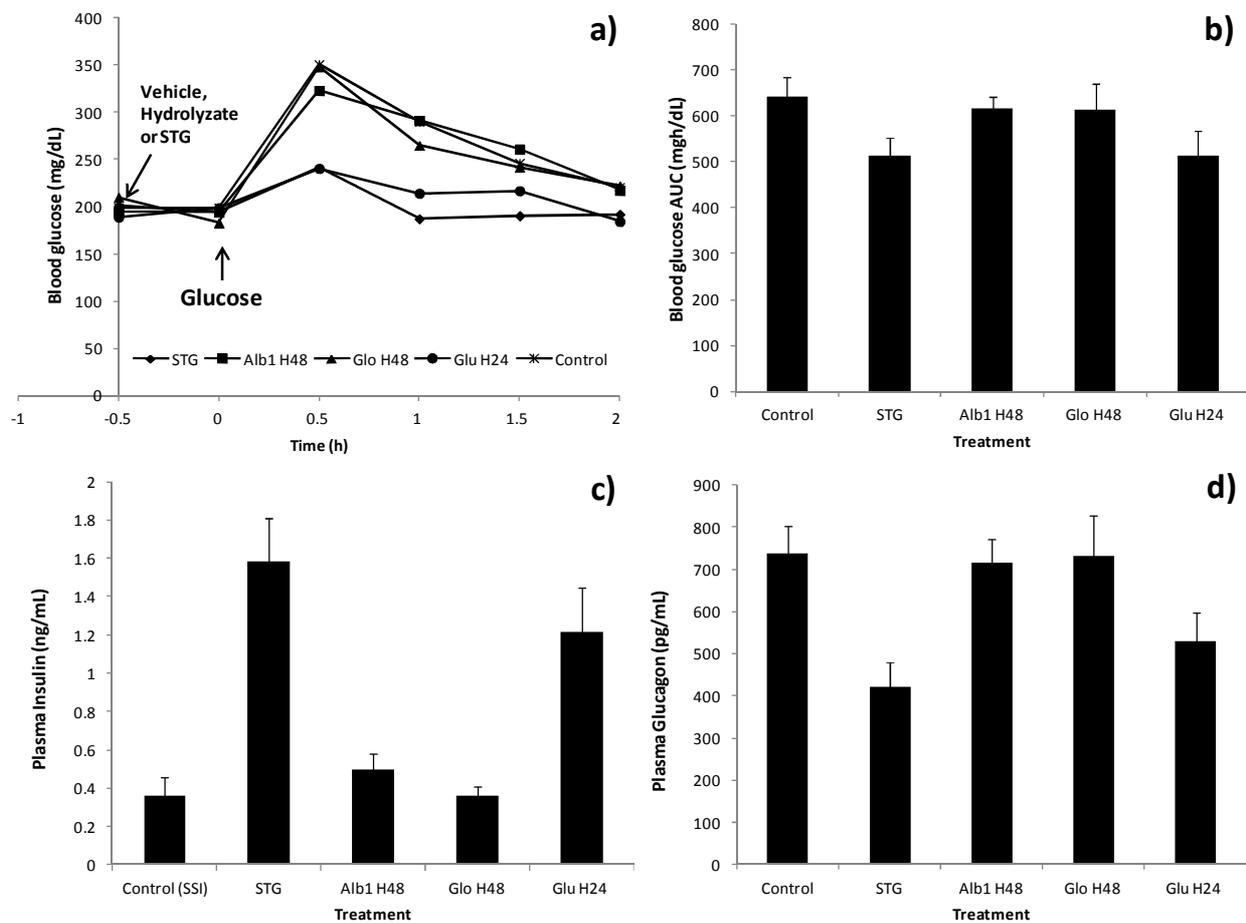


Figure 4: Single administration study to assess the effect of Alb1H48, GloH48 and GluH24 in plasma glucose, plasma insulin and glucagon levels during the oral glucose tolerance test (OGTT) in diabetic mice. (a) Time course of changes in plasma glucose level and (b) the area under the plasma glucose concentration –time curve (AUC) during the OGTT. Plasma (c) insulin and (d) glucagon levels at minute 10 during the OGTT. The values represent the mean \pm SD for five animals in each group. $p < 0.05$ vs control group.

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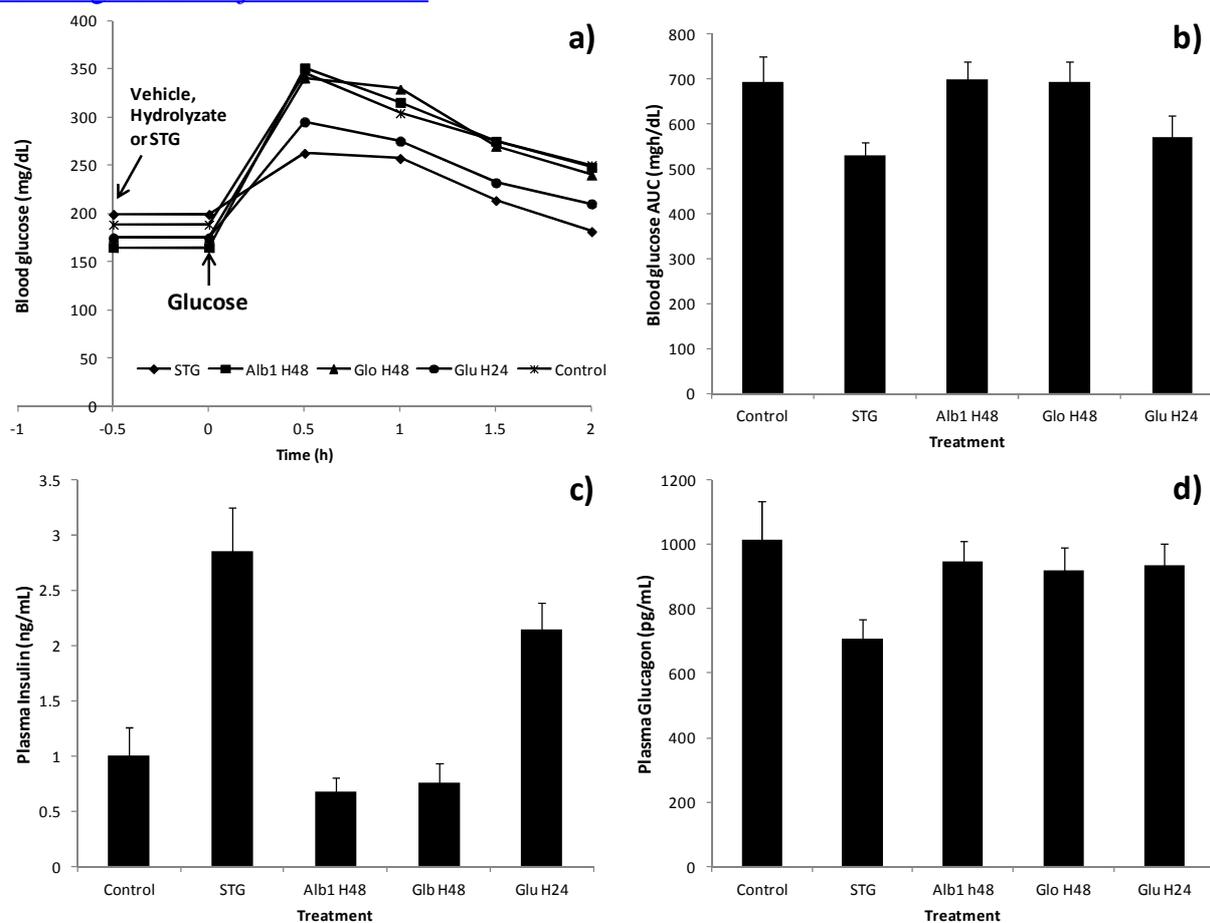


Figure 5: Four-week chronic daily dosing study to assess the effect of Alb1H48, GloH48 and GluH24 in plasma glucose, plasma insulin and glucagon levels during the OGTT in diabetic mice. (a) Time course of changes in plasma glucose level and (b) the AUC during the OGTT. Plasma (c) insulin and (d) glucagon levels at minute 10 during the OGTT. The values represent the mean \pm SD for five animals in each group. $p < 0.05$ vs control group.

Conclusions

Hydrolysis with alcalase of Alb1 and Glo as obtained from amaranth grain is limited during the first hours of treatment because it is hindered by peptides displaying Mr similar to ATSI. This does not occur in Glu, which allows a faster hydrolysis. Glu releases peptides capable of inhibiting DPP-IV from the first hour of treatment whereas Alb1 and Glo inhibit this enzyme more slowly during the same period. When peptides of about 7.9 kDa disappear, DPP-IV inhibitory activity increases. To the best of our knowledge, this is the first report on the effect of amaranth protein hydrolyzates capable of inhibiting DPP-IV in STZ-induced diabetic mice as a model system of diabetes. The fractions A.III, G.IV and Glu.III isolated from amaranth grain protein hydrolyzates afforded the lowest IC_{50} values for each hydrolyzate and competitively inhibited DPP-IV *in vitro*. Of all the amaranth protein hydrolyzates assessed, GluH24 showed the highest DPP-IV inhibitory activity reported up to now for an *in vitro* assay as well as for a SZT-induced diabetic mice model system. The assessment of the oral administration of GluH24 (300 mg/kg body weight), using one SAS or the same concentration during FWCDDDS, caused a significant improvement in the oral glucose tolerance test. GluH24 significantly increased plasma insulin in SAS and FWCDDDS in the same way as it was observed for the mice group that was administered STG ($p < 0.05$). However, GluH24 diminished plasma glucagon level in the SAS whereas in the FWCDDDS, PGL increased. Diabetes is a very complex disease; hence for a fuller understanding of this condition, further studies on the effect of amaranth protein storage hydrolyzates obtained with other enzymes, other hydrolyzates doses, other periods of treatment, among others, are needed in order to find out their impact on biological model systems.

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