ORAL ADMINISTRATION OF *Amaranthus hypochondriacus* L. HYDROLYZED PROTEIN ISOLATES TO RATS WITH TRANSIENT HYPERGLYCEMIA

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SUMMARY

*Type 2 diabetes (T2D) is a chronic metabolic disorder with abnormal blood glucose regulation affecting more than 15% of Mexican adults. Globally represents a significant health challenge, and by 2040 642 million people worldwide are expected to be affected by this disease; consequently, there is a need for treatments to help module sugar metabolism effectively. Food protein hydrolysates have emerged as promising candidates for blood sugar regulation since they have antihypertensive, antioxidant, and hypoglycemic activity. Therefore, this study aimed at establishing the hypoglycemic action of amaranth (*Amaranthus hypochondriacus*) protein hydrolysates (Ahm) in a transient hyperglycemia rat model. An amaranth protein isolate (APi) was hydrolyzed at pH 7.3, and 55ºC for 6, 12, and 24h with alcalase (0.8AU/ml), and their hydrolysis degree and molecular weight profile were established by the free aminoacid groups and mass exclusion chromatography, respectively. While the isolate contained 60-90% protein, the degree of hydrolysis (DH) for the three digestion times was 34%, 46%, and 53%, respectively. The enzymatic hydrolysis products fell in four peaks within the 1605 and 105Da molecular weight, with the peak amplitude varying with the degree of hydrolysis. Besides, the 24h hydrolysate contained a fraction with a molecular weight corresponding to free amino acids. Administration of 300mg/Kg body weight of the six-hour hydrolysate resulted in the best hypoglycemic effect in the animal model. This work shows that amaranth protein hydrolysates could be a promising alternative to modulate blood sugar positively.*

Introduction

The recent Covid-19 pandemic emphasized the relevance of properly managing chronic diseases such as diabetes and hypertension. Having diabetes increases the risk of developing severe illness from Covid-19 and other health complications (CDC, 2020). According to WHO 2014, 8.5% of over 18-year-old adults had diabetes, and 1.6 million deaths were directly related to diabetes in 2016 (WHO, 2020). Diabetes continues to rise worldwide; by 2040, 640 million people may have the disease. In Mexico, the incidence of diabetes in the population older than 20 years increased from 9.2% in 2012 to 9.4% in 2016 and was more prevalent among women (10.3%) than men (8.4%) (OMENT, 2016). Diabetes is an incurable chronic disease characterized by hyperglycemia, insulin resistance, and relative insulin deficiency; its causes are multifactorial, but mainly genetic and lifestyle factors are involved (Olokoba *et al*., 2012). Management of type 2 diabetes (T2D) involves both drug therapy as well as lifestyle and diet changes. In some cases, diet and lifestyle changes may be sufficient to manage the disease, but drug therapy may be necessary in others. Over the years, several drugs have been developed and used to treat T2D, including biguanidines, meglitinidines, sulfonylureas, and alpha-glucosidase inhibitors. Recently, two new therapy classes have been used to treat T2D, incretin and Dipeptidyl-peptidase IV inhibitors (DPPI). Both therapies are effective and well-tolerated but relatively expensive. The effect of proteins and certain amino acids on insulin secretion and plasma glucose levels has been known for quite some time. In the '70s, Fineberg *et al*. reported that ingesting a protein meal and arginine infusion along glucose increased plasm insulin levels and flattened the glucose response curve (Fineberg *et al*., 1970). Other studies also showed a synergistic insulinotropic and lowering glucose level effect between beef protein and glucose, beef protein and starch, cottage cheese-glucose, and egg white-glucose in healthy and T2D volunteers (Gannon *et al*., 1992; Pallotta and Kennedy, 1968). Most recent studies have confirmed the insulinotropic and glucose-lowering effects of both proteins and their hydrolysates. Administration of a 25, 30, or 55g whey protein pre-load 30min prior or with a high

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ADMINISTRACIÓN ORAL DE AISLADOS PROTEICOS HIDROLIZADOS DE *Amaranthus hypochondriacus* L. A RATAS CON HIPERGLUCEMIA TRANSITORIA

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RESUMEN

*La diabetes tipo 2 (DMT2) es un trastorno metabólico crónico con una regulación anormal de la glucosa en sangre que afecta a más del 15% de los adultos mexicanos. A nivel mundial representa un reto importante para la salud, para el 2040 se espera 642 millones de personas afectadas por esta enfermedad en todo el mundo, en consecuencia, existe la necesidad de tratamientos que ayuden a modular el metabolismo del azúcar de manera efectiva. Los hidrolizados de proteínas alimentarias han surgido como prometedores candidatos para la regulación de azúcar en sangre, ya que tienen actividad antihipertensiva, antioxidante e hipoglucemiante. Por lo tanto, este estudio tiene como objetivo establecer la acción hipoglucemiante de los hidrolizados de proteínas (Ahm) de amaranto (*Amaranthus hypochondriacus*) en un modelo de rata con hiperglucemia transitoria. Un aislado de proteína de amaranto (APi) fue hidrolizado a pH 7.3, y 5 ºC durante 6, 12, y 24h con alcalasa (0,8 AU/ml), y su grado de hidrólisis y perfil de peso molecular fueron establecidos por los grupos de aminoácidos libres y cromatografía de exclusión de masa, respectivamente. Mientras que el aislado contenía un 60-90% de proteínas, el grado de hidrólisis (DH) para los tres tiempos de digestión fue del 34%, 46% y 53%, respectivamente. Los productos de la hidrólisis enzimática se agruparon en cuatro picos dentro de un intervalo de peso molecular de 1605 y 105Da, la amplitud del pico varió con el grado de hidrólisis. Además, el hidrolizado de 24h contenía una fracción con un peso molecular correspondiente a los aminoácidos libres. La administración de 300mg/Kg de peso corporal del hidrolizado de 6h produjo el mejor efecto hipoglucemiante en el modelo animal. Este trabajo demuestra que los hidrolizados proteicos de amaranto podrían ser una alternativa prometedora para modular positivamente la glucemia.*

ADMINISTRAÇÃO ORAL DE ISOLADOS PROTEICOS HIDROLIZADOS DE *Amaranthus hypochondriacus* L. A RATOS COM HIPERGLICEMIA TRANSITÓRIA

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RESUMO

*O diabetes tipo 2 (T2DM) é um distúrbio metabólico crônico com regulação anormal da glicose no sangue que afeta mais de 15% dos adultos mexicanos. Globalmente, representa um grande desafio para a saúde, com a expectativa de que 642 milhões de pessoas em todo o mundo sejam afetadas pela doença até 2040 e, consequentemente, há uma necessidade de tratamentos que ajudem a modular o metabolismo do açúcar de forma eficaz. Os hidrolisados de proteínas alimentares surgem como candidatos promissores para a regulação do açúcar no sangue, pois têm atividade anti-hipertensiva, antioxidante e hipoglicêmica. Portanto, este estudo tem como objetivo estabelecer a ação hipoglicêmica dos hidrolisados proteicos de amaranto (*Amaranthus hypochondriacus*) (Ahm) em um modelo de hiperglicemia transitória em ratos. Um isolado de proteína de amaranto (APi) foi hidrolisado em pH 7,3 e 55 °C por 6, 12 e 24 horas com alcalase (0,8 AU/ml), e seu grau de hidrólise e perfil de peso molecular foram estabelecidos por grupos de aminoácidos livres e cromatografia de exclusão de massa, respectivamente. Embora o isolado contivesse de 60 a 90% de proteína, o grau de hidrólise (DH) para os três tempos de digestão foi de 34%, 46% e 53%, respectivamente. Os produtos da hidrólise enzimática foram observados em quatro picos em uma faixa de peso molecular de 1605 e 105 Da, e a amplitude do pico variou com o grau de hidrólise. Além disso, o hidrolisado de 24 horas continha uma fração com um peso molecular correspondente a aminoácidos livres. A administração de 300 mg/kg de peso corporal do hidrolisado de 6 horas produziu o melhor efeito hipoglicêmico no modelo animal. Este trabalho demonstra que os hidrolisados de proteína de amaranto podem ser uma alternativa promissora para modular positivamente a glicemia.*

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glycemic index meal attenuated the glycemic response and increased insulin levels in T2D volunteers (Jakubowicz *et al*., 2014; Ma *et al*., 2015; Ma *et al*., 2009). Similarly, in healthy young adults, whey protein pre-loads (10 to 40g) before a pizza meal modulated the glycemic response dose-dependently (Akhavan *et al*., 2010). Ingestion of vegetable protein also influenced the postprandial glucose excursion and hormonal response. The consumption of soy protein isolate (20 and 40g) before a 75g glucose load lowered the glucose response and increased insulin secretion in young subjects (Kashima *et al*., 2016). Moghaddam *et al*. also reported that soy protein (0-30g) elicited a dose-dependent decrease in the glycemic response to a 50g glucose load (Moghaddam *et al*., 2006). The consumption of other vegetable proteins, including lupin, millet, pea, oat, and rice, also prompts a glucose-lowering and insulin-rising effect (Chhavi and Sarita 2012; González-Santiago *et al*., 2017; Park *et al*., 2008; Smith *et al*., 2012; Tan *et al*., 2018). Amelioration of the postprandial glucose elevation by proteins involves multiple factors but is generally accepted that delaying gastric emptying and stimulation of insulin secretion by biopeptides, generated from its digestion, and their influence

on the incretin hormone system (GIP and GLP-l) are important. Furthermore, the enzyme dipeptidyl peptidase-4 (DPP-IV) rapidly degrades GIP and GLP-1 rendering them inactive (Marathe *et al*., 2013; Moghaddam *et al*,. 2006). Inhibition of DPP-IV increases the half-fife of the incretin hormones resulting in an insulinotropic effect and a lower blood glucose level.

Several studies have demonstrated that the enzymatic hydrolysis of various protein sources generated DPP-IV inhibitors (DPPI). Digestion of tuna cooking juice with protease XXIII or orientase-produced peptides with DPPI activity capable of passing through the digestive tract (Huang *et al*., 2012). In the same way, hydrolyzing common carp protein with papain, neutrase, trypsin, and pepsin produced the DPPI peptide with the highest inhibition obtained with papain (Zhang *et al*., 2020). Furthermore, the trypsin hydrolysis of a whey protein concentrate rich in β-lactoglobulin resulted in potent DPPI inhibitory peptides with an IC50 of 44.7μM (Silveira *et al*., 2013). The digestion of vegetable proteins can also produce peptides capable of inhibiting DPP-I. For example, in 2012, Hatanaka *et al*. generated DPPI peptides from rice bran using two commercial enzymes. They also reported that the peptides produced by the two enzymes differed in their inhibitory activity (Hatanaka *et al*., 2012). In 2015, Soriano *et al*. prepared amaranth (*Amaranthus hypochondriacus*) albumin 1, globulin, and glutelin hydrolysates that competitively inhibited DPP-IV in vitro. The same study demonstrated that the acute and chronic administration of a glutelin-derived peptide (GLU24) significantly increased plasma insulin and improved glucose tolerance (Soriano Santos *et al*., 2015).

Although, as mentioned above, specific food protein-derived peptides improve glucose tolerance and insulin secretion by inhibiting DPP-IV and the incretin effect, isolation of these peptides from grain protein hydrolysates requires a great effort, and the yields are often low (<1%). Therefore, a simpler, straightforward procedure to produce protein hydrolysate with hypoglycemic properties would be beneficial. This work aimed to determine the glycemic effect of *A. hypochondriacus* crude protein hydrolysate with varying hydrolysis degrees in normal fasted rats with transient hyperclycemia.

Materials and Methods

Research strategy

The study was divided into two phases; first, an amaranth protein isolate was prepared and digested with alcalase to obtain hydrolysates with 33, 45, and 55% degrees of hydrolysis. Second, the glycemic response to three doses of each hydrolysate in normal rats with transient hyperglycemia was evaluated using the oral glucose tolerance test (OGTT).

Seeds and defatted flour preparation

The National Institute of Forestry Agriculture and Livestock Research (INIFAP), located in Zapopan Jalisco, kindly donated *A. hypochondriacus* seeds of the revenge variety.

Fine amaranth flour prepared by milling dry seeds in a cyclonic mill with a 1mm opening screen and sieved through a 0.425mm mesh screen was defatted for 24h with hexane in a Soxhlet apparatus. The residual solvent was evaporated at 25°C, and the fat-free flour was stored at 4°C in a glass container. Later on, the proximal chemical composition of the flour was determined using the AOAC methods (AOAC 1990).

Protein isolate preparation (APi)

A protein isolate was prepared at 25°C from the defatted flour using the acid-base method described by Gueguen and Barbot in 1988, with some modifications (Gueguen and Barbot 1988). The pH of a flour-water dispersion (1:10 flour to water w/v) was adjusted to 11.0 with 1M NaOH and stirred for 30min. Afterward, the supernatant was recovered by centrifuging the suspension for 2min at 9000g and 4°C, its pH adjusted to 4.9 with 1M HCl, mixed for 30min, and centrifuged for 20min at 9000g and 4°C. After discarding the aqueous supernatant, the protein pellet was dissolved in distilled water (1:5 w/v), adjusted its pH to 7.0, and lyophilized. 12g of protein isolate was prepared using slight pH and agitation time variations. Protein isolates were further defatted with ethanol to increase their purity.

Hydrolysates preparation (AHm)

For this study, APi containing 69-79 and 80-90% protein was enzymatically hydrolyzed for 6, 12, and 24h at 55°C and constant pH. Sufficient APi was dispersed in 0.1M phosphate buffer to achieve a 5mg /ml protein concentration, and the pH adjusted to 7.8. Next, was added 0.8UA/ml of alcalase (a protease from *B. licheniformis*), allowing the reaction to proceed with gentle stirring and maintaining the pH at 7.8 by adding 2.0 M NaOH.

APi and AHm characterization

Protein content

APi and AHm protein levels were established by determining their nitrogen content using the micro Kjeldahl technique (AOAC, 1990) and a 5.7 nitrogen-to-protein conversion factor (De León *et al*., 2005).

APi protein electrophoretic characterization

Polyacrylamide gel electrophoresis (SDS-PAGE) was used to characterized APi. The SDS-PAGE consisted of concentrating (10% acrylamide) and separating gel (12% acrylamide) under non-reducing and reducing conditions with 2-β mercaptoethanol (2 ME). Besides, was used a Thermo Fisher 6-180 KDa molecular weight marker and a BIORAD miniplate kit, model Miniprotean II, with Laemmli's buffer system (1970). After two hours in the electrophoresis apparatus at 90v, the gels were removed, stained with bright Coomassie blue for one hour, destained for one hour in a 50% methanol and 5% glacial acetic acid solution, and photographed with a Kodak brand SP2600 ultraviolet camera.

AHm degree of hydrolysis (DH)

AHm degree of hydrolysis was measured in triplicate using the method OPA (ortho-phenyl aldehyde) described by Nielsen *et al*., this technique establishes the proportion of cleaved peptide bonds over the total number of bonds (Nielsen *et al*., 2001).

Weight distribution by exclusion chromatography

AHm molecular weight distribution was established by exclusion chromatography using an ÄKTA FPLC system (GE Life Sciences) operated by Unicorn 5.11 software, a Superdex Peptide 10/300 column with an optimal separation capacity of 100 to 7,00Da, and NaCl- TRIS pH 8.0 buffer as mobile phase.

Before the assay, the column was washed and equilibrated with the mobile phase, then ran a standard sample containing Cytochrome C (12,38Da 104 aminoacids (aa)), Aprotinine (6,512Da 58 aa), Vitamin B12 (1,355Da 12), and Glycine (75.07Da 1) followed by 100 μL of a 0.05M AHm solutions. The hydrolysates solutions were prepared by dissolving 50 mg of each dry AHm in 1ml SDS-10% ME (2-mercaptoethanol).

Glycemic response

Experimental setup

Three oral glucose tolerance test (OGTT) were carried out, one for each AHm (33, 45, 52% DH) and three doses (300, 600, and 900mg/kg body weight), using three normal fasted rats per treatment. In one of the three OGTTs, a negative (water, CTR-), a positive (Sitagliptin 580mg/kg BW, CTR+) control, and an unhydrolyzed APi (900mg/kg BW) treatment were included.

Animals and housing

Thirty-six male Wistar rats weighing 150g, supplied by the University of Guadalajara Bioterium, were housed in Poly sulfone cages, five to six per cage, and placed in a room with controlled temperature and humidity (24°C and 60% RH) and a 12h dark-light cycle. Animals had free access to water and a standard rodent chow diet (Lab Diet, PMI Nutrition International, MO USA) for the study duration. All animal protocols adhered to the International Guidelines for the Care and Use of Laboratory Animals and the Mexican Official Standard 062 (Díaz *et al*., 2021). The University of Guadalajara ethics committee reviewed and approved all experimental procedures.

Oral glucose tolerance test (OGTT)

After a 24h adaptation period, animals were randomly assigned to each treatment and deprived of food for 14h. At the end of the fasting period (time zero), was drew blood by vein tail puncture and immediately after administered, by gavage, 2ml of water or 2ml of water containing Sitagliptin, AHm (300, 600, and 900mg/kg body weight), or APi (900mg/kg body weight APi. Thirty minutes later, animals were forced-fed a glucose load (2g/Kg body weight) and drew four blood samples, one every 30min, for glucose analysis. Blood glucose level (mg/100ml) was measured with a glucometer (Kit Accu Check Active, Roche, Inc), plotted against time, and the area under the curve (AUC) calculated using the trapezoidal rule.

Data Analysis

Data for each OGTT was processed separately. The mean and standard deviation for the AUC were calculated and an ANOVA carried out to establish the treatment factors' effect and the significance of the differences among treatments with Tukey's test at $p < 0.05$.

Results and Discussion

Proximal composition

As seen in Table I, removing fat from amaranth flour increases the protein content from 13.11 to 21.5%, on a dry basis. This result is expected because eliminating one of the components will increase the remaining components.

AHm molecular weight distribution

In the initial phase of the study we found out that the extraction conditions (pH, particle size, and extraction time) affected the isolate's protein content. The protein isolates prepared in this phase contained between 54 and 90% protein. Isolates prepared using a 9-10 solubilization pH and 4.5-5.7 precipitation pH resulted in relatively low protein content isolates (54 to 69%). Using similar conditions, other researchers obtained isolates with similar protein content (53 – 67%) (Fidantsi and Doxastakis, 2001; Salcedo-Chávez *et al*., 2002). Increasing the solubilization pH to 11 while maintaining the precipitation pH at 4.9 resulted in isolates with a significantly higher protein content (80-90%). This result agrees with those reported previously (Avanza 2006). Besides, (Paredes-López *et al*., 1988) pointed out that the best recovery is achieved when proteins are solubilized and precipitation at pH 11.0 and 4.5-5.5, respectively (Paredes-López *et al*.. 1988). These conditions are suitable for both raw and defatted flour.

TABLE I
PROXIMAL COMPOSITION OF DEFATTED AMARANTH FLOUR ON AS IS BASIS [1]. VALUES REPRESENT THE RESULT OF ONE ASSAY

Component	Percentage (%)
Moisture	5.6
Ash	3.7
Nitrogen	3.3
Protein	21.5[2]
Crude Fiber	7.3
Crude Fat	3.5
Nitrogen Free Extract	60.7

[1]: Values represent the result of one assay. [2]: N × 5.85.

Protein fractions in APi by SDS-PAGE

Results of the APi SDS-PAGE electrophoresis under reduced and native conditions showed that the isolate was made out of protein fractions with molecular weights varying from 18 to 180 KDa 1. These results are similar to those previously reported (Martínez and Añón 1996; Silva-Sánchez *et al*., 2008).

Amaranth hydrolysates and Degree of Hydrolysis (DH)

The degree of hydrolysis obtained by treating APi with alcalase for 6, 12, and 24h (Table II) was like that reported by other authors (16-60%) (Ramírez-Torres *et al*., 2017; Sabbione *et al*., 2016; Soriano Santos *et al*.. 2015; Tovar-Pérez *et al*., 2009). APi appeared to contain small quantities of endogenous proteases since a sample incubated without alcalase had 3-4% DH. In 2012, Ventureira *et al*. (2012) reported the presence of aspartate proteases in A. hypochondriacus seeds protein isolates (Ventureira *et al*., 2012). This protease is coextracted along

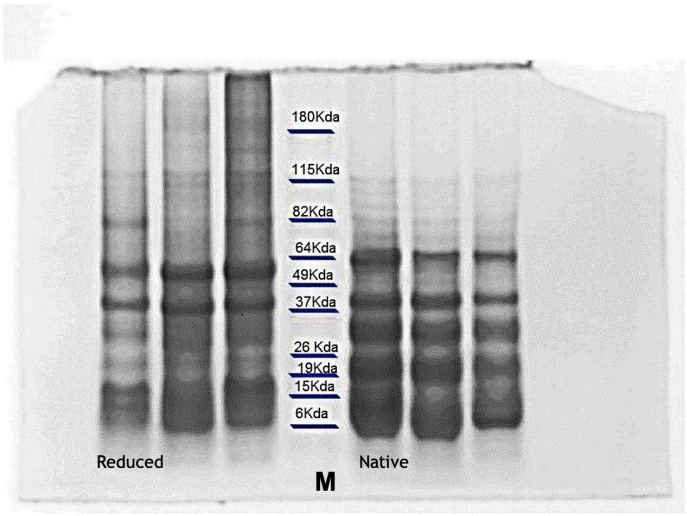

Figure 1. Electrophoresis pattern of total proteins of *Amaranthus hypochondriacus* APi. Lanes 1, 2 and 3 reduced; Lane 4. Molecular markers; Lanes 5, 6 and 7 no reduced.

TABLE II
DEGREE OF HYDROLYSIS FOR AHm USED IN THE OGTT TEST [1]

Reaction time (h)	Degree of Hydrolysis (%)
6	33.4 ± 1.5
12	45.2 ± 2.3
24	52.0 ± 1.9

[1]: Values represent the mean and standard deviation of three measurements.

other proteins (Huffaker 1990; Schaller 2004).

AHm molecular weights distribution by mass exclusion chromatography (FPLC)

As seen in the FPLC chromatograms (Figure 2), the enzymatic digestion of APi resulted in AHm with fractions of varying molecular weights depending on the reaction time. This technique made it possible to resolve polypeptides with an interval of 1 to 14 amino acid residues corresponding to a molecular weight of 105.10 to 1605,71Da. Compounds with molecular weights of 628.98, 547.47, 177.06Da for 33GH; 1058.08, 492.93, 165.18 for 42DH; and 1008, 468.96, and 165.10 for 55DH were also generated. The condition used to analyze the hydrolysates' molecular weights profile allowed unequivocally to identify 4, 4 and 3 proteins of specific size corresponding to a 33, 42 and 55DH, respectively.

It can be anticipated that the action of gastric enzymes on AHm, orally administered to animals, orally administered to animals, would generate biological activity peptides of lower molecular weights, as reported by other authors (Tovar-Pérez *et al*.. 2009). On the other hand, knowing the amino acid residues of the hydrolysates could allow us to improve APi hydrolysis and generate di, tri, and poly peptides with better hypoglycemic activity (Guadix Escobar *et al*., 2000).

The analysis of the OGTT, expressed as AUC, of the animals with transient hyperglycemia and treated with AHm hydrolyzed for 6, 12, or 24 h showed that blood glucose increased at the 600 and 900mg/Kg of body weight (BW) doses, compared to the control group. In contrast, Sitagliptin had a constant hypoglycemic effect (Table III). The observed increase in blood glucose of animals administered the 600 and 900mg/Kg BW doses suggests that the free (gluconeogenic) amino acids present in AHm and produced by digestion are rapidly absorbed into the bloodstream and used for glucose formation (Nelson *et al*., 2008). On the other hand, with 300mg AHm/Kg BW dose, a decrease in glucose

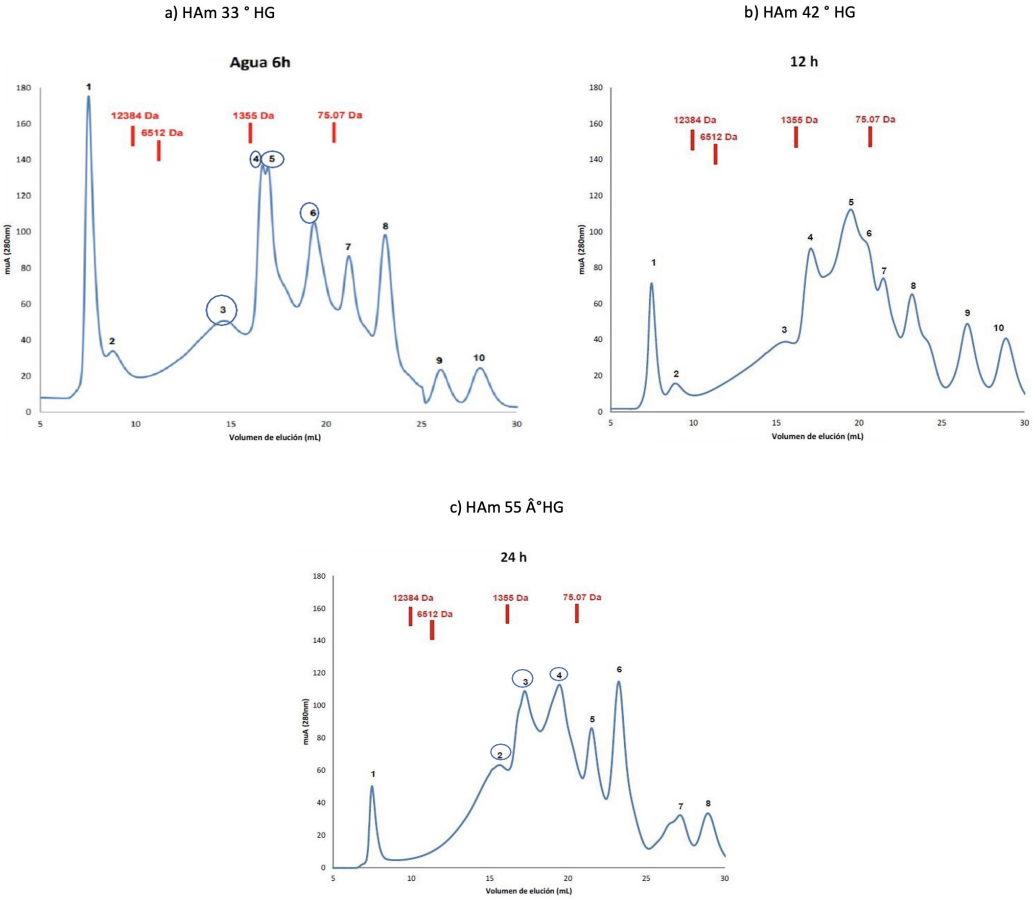

Figure 2. Chromatograms of *A. hypochondriacus* HMa obtained by high pressure size exclusion chromatography employing a ÄKTA FPLC equipment and a Superdex peptide column, a) Four peaks (Da): 3-1605.71, 4-628.98, 5-547.47, 6-177.06.Ham; b) Four peaks: 3-1058.08, 4-492.93, 5-165.18, 6-105.15; c) Three peaks: 2-1008.02, 3-468.96, 4-165.10Da.

TABLE III
HYPOGLYCEMIC EFFECT OF AHm, USING THE OGTT IN WISTAR RATS

Treatment	Dose (mg/Kg BW)[1]	Area under the curve		
		34% Hydrolysis	45% Hydrolysis	52% Hydrolysis
Sitagliptin	580	10745 ± 173 ^{c}	10745 ± 173 ^{e}	10745 ± 173 ^{b}
AHm	300	10945 ± 109 ^{c}	11675 ± 372 ^{de}	11985 ± 803 ^{b}
AHm	600	12535 ± 271 ^{b}	13040 ± 193 ^{b}	13130 ± 1891 ^{ab}
AHm	900	12865 ± 556 ^{b}	11915 ± 360 ^{cd}	13225 ± 1562 ^{ab}
APi	900	15215 ± 264 ^{a}	15215 ± 264 ^{a}	15215 ± 264 ^{a}
Water		12670 ± 573 ^{b}	12670 ± 573 ^{bc}	12670 ± 573 ^{ab}

AHm: Amaranth protein hydrolysates; OGTT: Oral glucose tolerance test; Values followed by different letters in a column are significantly different (p>0.05).
[1]: In mg of hydrolyzate per Kg of body weight.

levels was observed, which was more evident with the AHm 34DH. With this hydrolysis time, peptides with hypoglycemic activity and behaving similarly to Sitagliptin were produced (Table III). Our results with the administration of 300mg/Kg BW of the 34DH AHm hydrolysate agree with those of Soriano *et al*., 2015, who reported that oral administration of 300mg/Kg BW of a purified globulin fraction to diabetic mice had a hypoglycemic effect. The hydrolysate process used in our study to obtain peptides with biological activity could be more straightforward and less expensive than the isolation of specific peptides proposed by other investigators. Nonetheless, additional work is needed to optimize the process and corroborate the results of this study.

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