UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE CIENCIAS QUÍMICAS

PROGRAMA DE POSGRADO EN BIOPROCESOS

ESTUDIO DEL EFECTO ANTIHIPERTENSIVO DE PÉPTIDOS BIOACTIVOS PRODUCIDOS EN *CHLAMYDOMONAS REINHARDTII* EN UN MODELO DE RATAS HIPERTENSAS

ARTÍCULO DE INVESTIGACIÓN QUE
PARA OBTENER EL GRADO DE

MAESTRO EN CIENCIAS EN BIOPROCESOS

PRESENTA:

L.N. CELMA ESTEFANIA OCHOA MÉNDEZ

DIRECTORA

DRA. RUTH ELENA SORIA GUERRA

CO-DIRECTORA

DRA. PATRICIA AGUIRRE BAÑUELOS

SAN LUIS POTOSÍ, S. L. P. Junio 2016

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DR. ÁNGEL GABRIEL ALPUCHE SOLÍS

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RESUMEN EN ESPAÑOL

La hipertensión arterial (elevación crónica de la presión arterial sistólica (PAS), diastólica (PAD) o de ambas por encima de los valores considerados como normales (120/80 mmHg), es el factor de riesgo mayor sobre la morbilidad y mortalidad de causa cardiovascular, ya que produce aproximadamente 7,4 millones de defunciones a nivel mundial (Arima y col., 2011)

El principal tratamiento de la hipertensión es farmacológico y medicamentos como inhibidores de la ECA, diuréticos, beta bloqueadores y bloqueadores del canal de calcio, son los más utilizados, aunque presentan ciertas limitaciones al tener que ser usados a largo plazo, ser costoso y presentar efectos secundarios (Mancia, 2013).

Ante esta problemática, se han buscado alternativas para controlar la hipertensión arterial. Recientemente, los péptidos antihipertensivos derivados de proteínas de los alimentos han recibido una atención considerable, debido a sus buenos efectos antihipertensivos, seguridad, bajos efectos secundarios en humanos y su potencial para ser usados como alimentos saludables y preparaciones farmacéuticas. Estos péptidos normalmente tienen propiedades multifuncionales y son de fácil absorción (Iwaniak y col., 2014)

Algunas de las plataformas que se han utilizado para producir este tipo de péptidos son las bacterias, plantas, levaduras, animales, células de mamíferos y microalgas, siendo las microalgas las que presentan mayores ventajas sobre los otros sistemas de producción de proteínas recombinantes. La microalga *Chlamydomonas reinhardtii* tiene ciertas ventajas: es un organismo genéticamente caracterizado, se puede modificar el genoma de su cloroplasto, mitocondria y núcleo, es considerado GRAS (Generally Recognized As Safe) por lo cual puede usarse para el consumo humano, y por ultimo tiene la capacidad de plegar proteína humanas. El objetivo del presente trabajo es evaluar el efecto antihipertensivo de una proteína quimérica expresada en el cloroplasto de *Chlamydomonas reinhardtti* (productora del péptido VLPVP) utilizando modelos animales. Los resultados demuestran que la digestión *in vitro* realizada permite la liberación del péptido antihipertensivo VLPVP. Por otro lado, la administración oral de la biomasa liofilizada a las ratas espontáneamente hipertensas es capaz de disminuir la presión arterial sistólica a valores normales (<150 mmHg) a una dosis de 30 mg/kg de peso en 6 horas.

RESUMEN EN INGLÉS

Hypertension (chronic elevation of systolic blood pressure (SBP), diastolic blood pressure (DBP) or both above normal values (120/80 mmHg), it is the biggest risk factor for morbidity and mortality from cardiovascular causes, it causes 7,4 millions of deaths in the world (Arima et al., 2011).

The first treatment for hypertension is pharmacological and drugs as ACE inhibitors, diuretics, beta blockers and calcium channel blockers, uses frequently, although it presents limitations having to be used long term, be expensive and have side effects (Mancia, 2013).

Face with this problem, have been searched alternatives for controlling hypertension. Recently, antihypertensive peptides derived from food have received considerable attention due to the ability to lower systolic blood pressure, security, low o null side effects in humans and its capability to be used as healthy foods or drugs. These peptides usually have different functions and are easy absorbed in the intestine. (Iwaniak A. et al, 2014)

Some expression platforms have been used to produce these peptides like bacteria, plants, yeast, animal, mammal cells and microalgae. Microalgae present more advantages than other recombinant protein production systems. *Chlamydomonas reinhardtii* have some advantages, for example its genetically well characterized, can be modified the chloroplast, nuclear and mitochondrial genome, it has a GRAS (Generally Recognized As Safe) grade, which means that can be used for human consume, and can fold human proteins. The objective of this work was evaluate the antihypertensive effect of a quimeric protein expressed in *Chlamydomonas reinhardtii* chloroplast (VLPVP peptide producer) using murine models. Results have shown that *in vitro* digestion can release the antihypertensive peptide VLPVP, and oral administration of freeze-dried biomass to spontaneous hypertensive rats can reduce systolic blood pressure under 150 mmHg at 30 mg/kg of body weight at 6 hours.

RESUMEN EN EXTENSO

1. INTRODUCCIÓN

Las enfermedades no trasmisibles son causantes de más del 68% de las muertes en el mundo, representando un incremento comparado con el 60% del año 2000. Las principlaes enfermedades pertenecientes a este grupo son enfermedades cardiovasculares, cáncer, diabetes y enfermedad pulmonar crónica (WHO, 2013).

La hipertensión afecta a un billón de personas a nivel mundial, y es uno de los principales factores para la enfermedad cardiovascular causando ataques cardiacos y embolias. Es un asesino silencioso que raramente muestra síntomas, por lo cual se ha vuelto un problema de salud pública. A nivel global, las enfermedades cardiovasculares son responsables de aproximadamente 17 millones de muertes cada año lo que representa casi un tercio del total de muertes. De estas, la hipertensión contribuye a 9.4 millones de muertes mundiales cada año (WHO, 2013).

La hipertensión no es una enfermedad solitaria; usualmente se acompaña de otros factores de riesgo para la salud, los cuales incrementan la probabilidad de ataques cardiacos, enfermedad cerebrovascular y falla renal. El tratamiento solo se enfoca en aspectos farmacológicos, como inhibidores de los canales de calcio, diuréticos, inhibidores de la enzima convertidora de angiotensina (ECA), entre otros, los cuales presentan efectos adversos (como nausea, fatiga y mareos) y cambios metabólicos en concentraciones séricas de ácido úrico, glucosa y colesterol total (Higgins y Williams, 2007).

Considerando lo anterior, se han buscado alternativas para solucionar este problema de salud pública; con especial interés en los péptidos bioactivos derivados de alimentos. Algunos péptidos derivados de alimentos han mostrado tener funciones fisiológicas, pero para que muestren algún efecto necesitan ser liberados de la proteína en la cual se encuentran por medio de una hidrolisis ya sea *in vivo* o *in vitro* (Hartmann y Meisel, 2007). Las actividades de estos péptidos incluyen propiedades hipocolesterolémicas y antimicrobianas, disminución de la presión arterial (inhibiendo la ECA), antioxidante y actividad opioide (Silva y Malcata, 2005; Hartman y Meisel, 2007; Iwaniak y col., 2014).

Bajo condiciones normales, el control de la presión arterial ha sido asociado con el sistema renina-angiotensina-aldosterona, el cual juega un papel importante en su regulación. El hígado secreta angiotensinogeno (compuesto de 13 aminoácidos) el cual es convertido en angiotensina I (decapéptido) por la acción de la renina, posteriormente la ECA convierte la angiotensina I en angiotensina II (octapéptido) el cual es biológicamente

activo. Algunos péptidos naturales o sintéticos actúan al nivel del sistema renina-angiotensina-aldosterona reduciendo la presión arterial (Hong et al., 2008). VLPVP es un péptido inhibidor de la ECA el cual no es susceptible a la hidrolisis enzimática. Además se ha demostrado que es de fácil absorción a nivel intestinal (Lei y col., 2008: Dong y col., 2008).

Cerca del 40% de las proteínas recombinantes de uso terapéutico en el mercado son expresadas en bacterias (*Escherichia coli*) o levaduras (*Saccharomyces cerevisae*). De igual manera, existen otros sistemas de expresión disponibles como células de mamíferos, de insectos, plantas transgénicas y animales. Estos sistemas cuentan con ventajas y desventajas, así que la selección del sistema de expresión depende particularmente de la proteína. Un organismo ideal para la expresión de proteínas recombinantes debe de cumplir con: crecimiento rápido, alto rendimiento de la proteína en su forma biológicamente activa, manipulación genética simple, y fácil escalamiento, entre otros (Rasala y Mayfield, 2011).

Chlamydomonas reinhardtii es una microalga genéticamente caracterizada que ha mostrado muchas de las características mencionadas anteriormente. Sus tres genomas (nuclear, cloroplasto y mitocondrial) han sido secuenciados y los métodos de transformación genética a nivel nuclear y de cloroplastos están bien establecidos (Rasala y Mayfield, 2011).

Además *C. reinhardtii* tienen un crecimiento rápido a densidades mayores a 10^7 células/mL. Su cloroplasto ocupa cerca del 40% del volumen celular (Schotz y col, 1972) y ha mostrado que las proteínas recombinantes se acumulan a mayores niveles cuando se expresan en el genoma del cloroplasto en comparación del genoma nuclear (Daniell, 2006), lo cual hace al cloroplasto un organelo atractivo para la producción de proteínas recombinantes. Además *C. reinhardtii* tiene el grado GRAS (Generally Recognized As Safe) por la FDA.

En este estudio se analizó la capacidad antihipertensiva de una proteína quimérica expresada en el cloroplasto de *C. reinhardtii* que contiene péptidos antihipertensivos (VLPVP) en ratas espontáneamente hipertensas después de la administración intragástrica de la biomasa liofilizada.

2. OBJETIVOS

Objetivo general

Evaluar el efecto antihipertensivo de una proteína quimérica expresada en el cloroplasto de *Chlamydomonas reinhardtii* utilizando modelos animales.

Objetivos específicos

a) Realizar un ensayo de digestión gastrointestinal *in vitro.*
b) Evaluar la liberación de péptidos antihipertensivos mediante cromatografía de líquidos de alta resolución en fase reversa (HPLC).
c) Evaluar el efecto antihipertensivo mediante la administración oral de la biomasa liofilizada en ratas espontáneamente hipertensas.
d) Cuantificar niveles de ECA en plasma y en tejido de pulmón.

3. RESULTADOS

- *Efecto de la digestión intestinal simulada*

La proteína quimérica expresada en la cepa transgénica se sometió a un digestión gastrointestinal simulada, para ello, la muestra fue hidrolizada con pepsina, tripsina y quimiotripsina (relación enzima-substrato 1:50, 1:200 y 1:200 respectivamente), se tomó la biomasa liofilizada de la cepa Cr.AHP4 de *C. reinhardtii* la cual contiene el péptido VLPVP y biomasa de la cepa WT, y se simularon las condiciones gastrointestinales, iniciando con una digestión ácida por un periodo de 90 min a un pH de 2, para posteriormente ser llevada a una digestión básica por un periodo de 3 horas a un pH de 7.5. Todo el procedimiento se llevó a una temperatura de 37°C y se terminó con la inactivación de las proteasas a 90°C por 10 minutos.

Al finalizar, se tomó la muestra la cual fue centrifugada y filtrada para poder ser llevada posteriormente al análisis por medio del HPLC fase reversa, en el cual se pudo comprobar la liberación de los péptidos antihipertensivos comparando los tiempos de retención de la muestra analizada de Cr. AHP4 (45.1 ± 0.3 minutos) contra los mostrados en el análisis del estándar sintetizado químicamente que contiene la secuencia antihipertensiva VLPVP (45.3 ± 0.27 minutos).

- *Actividad antihipertensiva de la proteína recombinante después de la administración oral a las ratas espontáneamente hipertensas (SHR)*

Para evaluar la actividad antihipertensiva de la proteína recombinante, se incluyeron cuatro grupos de 8 animales cada uno: a) grupo Cr. AHP4 (biomasa liofilizada de *C.*

reinhardtii transplastomica), b) grupo WT (biomasa liofilizada de *C. reinhardtii* sin transformar), c) grupo de captopril (control positivo), y d) grupo Agua (control negativo).

Se utilizaron 3 dosis: 10, 30 y 100 mg/kg de peso corporal (PC) de proteína recombinante producida in *C. reinhardtii* transplastomica. A una dosis de 30 mg/kg en el grupo de Cr. AHP4 la presión arterial sistólica disminuyó considerablemente; alcanzando niveles <150 mmHg 6 horas después de la administración vía oral, con una reducción mayor a la observada a la dosis de 100 mg/kg. El grupo WT, como se esperaba, no mostró una disminución en la presión sistólica a ninguna de las dosis probadas. En el grupo captopril, el cual utiliza un prototipo inhibidor de la ECA, la disminución en la presión sistólica fue evidente sin embargo no alcanzo valores menores a 150 mmHg, administrándose 100 mg/Kg peso.

La máxima reducción en presión sistólica se observó 6 horas después de la administración (141.1 ± 15.1 mmHg) a una dosis de 30 mg/kg. La presión sistólica regreso a sus niveles basales 24 horas después de la administración. No hubo diferencias significantes (p>0.05) entre las horas 6 y 8 después dela administración oral. Estos resultados indican que la proteína recombinante producida en *C. reinhardtii* tiene actividad antihipertensiva como los péptidos antihipertensivos purificados de la leche.

- *Ensayo de actividad de la ECA*

La actividad de la ECA fue medida como lo describe Beneteau y col., (1986). El substrato FAPGG es hidrolizado a furilacriloifenilalanina (FAP) y glicyglicina (GG) por la ECA, resultando en una disminución de la absorbancia a 345 nm. En este análisis observamos una mayor actividad en la muestra de pulmón macerado que en las muestras de suero. Esto era lo esperado debido a que la ECA es una glicoproteína unida a la membrana principalmente localizada en el tejido endotelial de los capilares pulmonar (Ryan y col., 1976). La actividad de la ECA disminuyó en el grupo Captopril y en el grupo Cr. AHP4. En el grupo Cr. AHP4 el decremento está asociado al péptido VLPVP liberado de la proteína recombinante por las proteasas gastrointestinales de las SHR. Así, el péptido VLPVP mostro una fuerte actividad inhibitoria de la ECA resultando en un efecto antihipertensivo.

4. CONCLUSIONES

Los datos recolectados en este estudio demostraron que la cepa transplastómica de *C. reinhardtii* productora de un péptido antihipertensivo (VLPVP) es capaz de disminuir la

presión arterial sistólica en ratas espontáneamente hipertensas después de la administración intragástrica, lo cual sugiere que puede ser capaz de reducir la presión arterial en humanos como una alternativa para el tratamiento de la hipertensión, sin las desventajas presentadas por el tratamiento farmacológico.

ARTÍCULO COMPLETO

Bioactivity of an antihypertensive peptide expressed in *Chlamydomonas reinhardtii*

Celma Estefanía Ochoa-Méndez[1±], Ignacio Lara-Hernández[1], Patricia Aguirre-Bañuelos[1], Maximiliano Ibarra Barajas[2], Patricia Castro Moreno[2], Omar González-Ortega[2], Ruth Elena Soria-Guerra[1*]

[1]Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, Av. Dr. Manuel Nava 6, SLP, 78210, México

[2]UBIMED, FES-Iztacala, Universidad Nacional Autónoma de México, Av. de los Barrios 1, Tlalnepantla, 54090, México.

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***Corresponding author**
Ruth Elena Soria-Guerra
Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí
Av. Dr. Manuel Nava 6, SLP, 78210, México
Phone: (+52) 444-826-2440
Fax: (+52) 444-826-2440
E-mail: ruth.soria@uaslp.mx

ABSTRACT

In this study, we developed a transplastomic *C. reinhardtii* strain that accumulates anti-hypertensive peptides. Tandem repeats of VLPVP peptide were included. PCR analysis confirmed the presence of the transgene in the modified strains. After *in vitro* digestion of biomass of a recombinant *C. reinhardtii* strain the VLVPV peptide was identified by RP-HPLC. The highest expression line produced 0.292 mg of recombinant protein per mg of freeze-dried biomass. Intragastric administration of the genetically modified strain, at a dose of 30 mg/kg of body weight of recombinant protein, to spontaneous hypertensive rats, significantly reduced systolic blood pressure. This is the first study that indicates the potential of this microalga producing an antihypertensive peptide as a dietary supplement for hypertension patients.

Key words:

Bioactive peptides, hypertension, spontaneous hypertensive rats, microalgae

Abbreviations:

SHR: Spontaneous Hypertensive Rats. SBP: Systolic Blood Pressure. ACE: Angiotensin Converting Enzyme. WT: Wild Type. BW: Body Weight. GRAS: Generally Recognized As Safe. TSP: Total Soluble Protein. HPLC: High Performance Liquid Chromatography. AHP: Antihypertensive Peptide

1. INTRODUCTION

Today, non-communicable diseases caused more than 68% of deaths in the world, representing an increase compared with 60% in 2000. The four major disease entities of this group are cardiovascular disease, cancer, diabetes and chronic lung disease (WHO, 2016).

Hypertension affects one billion people worldwide, and is one of the principal factors for cardiovascular disease leading to heart attacks and strokes. It is a silent killer that rarely causes symptoms, thus it has become a problem of public health. Globally, this cardiovascular disease is responsible of approximately 17 million deaths each year that represents nearly one third of the total deaths. Of these, hypertension accounts for 9.4 million deaths worldwide each year. Hypertension is not a single disease; it is usually accompanied by other health risk factors that increase the probabilities of heart attack, stroke, and kidney failure. These risk factors include tobacco use, obesity, diabetes, and high cholesterol levels (WHO, 2016). The update focuses only on pharmacological aspects of treatment, either calcium channel inhibitors, thiazide-type diuretics, angiotensin converting enzyme (ACE) inhibitors and angiotensin-II receptor inhibitors, but with adverse side effects (like nausea, fatigue, and dizziness) and metabolic changes in serum concentration of uric acid, glucose, and total cholesterol (Hartmann and Meisel, 2007).

Considering all the above, several efforts have been conducted looking to solve this problem of public health; with special interest in bioactive peptides derived from foods. Some peptides derived from foods have been found to have physiological activities, for them to show any effect; they must be released from the host protein by *in vivo* or *in vitro* hydrolysis (Higgins and Williams, 2007). A wide range of activities for these peptides including hypocholesterolemic and antimicrobial properties, blood pressure-lowering (inhibiting the angiotensin converting enzyme), antioxidant, and opioid activities has been described (Silva and Malcata, 2005; Iwaniak et al. 2014; Hong et al. 2008).

Under normal conditions the blood pressure control has been associated with the renin-angiotensin-aldosterone system, which plays an important role in regulating blood pressure. The liver secrets angiotensinogen that is converted into angiotensin I (decapeptide) by the action of renin, afterwards the Angiotensin Converting Enzyme (ACE) converts angiotensin I into angiotensin II (octapeptide) that is biologically active. Some natural or synthesized peptides acting on the renin-angiotensin-aldosterone system can reduce the blood pressure (Hong et al. 2008). VLPVP is an ACE inhibitory peptide that is not susceptible to non-enzymatic hydrolysis. This antihypertensive peptide has also been suggested to have a high oral bioavailability in humans (Lei et al. 2008; Dong et al. 2008).

About 40 % of the recombinant therapeutic proteins on the market are typically expressed in bacteria (*Escherichia coli*) or yeast (*Saccharomyces cerevisiae*). However, other expression systems are available such as mammalian tissue culture cells, insect cell culture, and transgenic plants and animals. These systems have advantages and disadvantages, thus the expression system choice depends on the particular protein. An ideal organism for recombinant protein expression would include: rapid growth, high protein yield in a biologically active form, simple genetic manipulation, and easy scale-up; among others (Rasal and Mayfield, 2011).

Chlamydomonas reinhardtii is a genetically-characterized microalga that shows many of the above-mentioned characteristics. All three genomes (nuclear, chloroplast, and mitochondrial) have been sequenced and the methods of genetic transformation are well established (Rasala and Mayfield, 2011). Moreover *C. reinhardtii* grows quickly at densities above 10^7cells/ml. Its chloroplast occupies about 40% of the cell volume (Schotz et al. 1972) and it has been shown that recombinant proteins accumulate at higher levels when expressed in the chloroplast genome in comparison to the nuclear genome (Daniell , 2006), which makes the chloroplast an attractive organelle for the production of recombinant proteins. In addition *C. reinhardtii* holds a GRAS status by the Food and Drug Administration.

In this study, we generated a *C. reinhardtii* strain transformed with a synthetic gene encoding a chimeric protein containing antihypertensive peptides (VLPVP). The anti-hypertensive activity of the transplastomic microalga was assessed in SHRs after oral administration of freeze-fried biomass.

2. MATERIALS AND METHODS

2.1 Design of the synthetic gene and plasmid construction

The sequence of the VLPVP peptide, previously reported as antihypertensive (Lei et al. 2008; Dong et al. 2008), was joined by linkers containing two amino acids; which correspond to the gastrointestinal cleavages sites of pepsin, trypsin, and chymotrypsin. A histidine tag was also included to facilitate the recombinant protein identification.

The designed gene was synthesized (GenScript Inc. USA) avoiding destabilizing mRNA structures and including the most frequently-used codons in *C. reinhardtii.* Restriction sites were added to facilitate subcloning into the p463 expression vector (Chlamydomonas connection, http://www.chlamy.org/). One positive clone, named p463-AHP, was selected by restriction analysis

and sequencing. All these procedures were performed using standard molecular cloning techniques (Sambrook and Rusell, 2001).

2.2 Chloroplast transformation by particle bombardment

The p463-AHP construct was introduced into the chloroplast of the CC-137 (mt+) *C. reinhardtii* strain by particle bombardment using the PDS-1000 / Helio (BioRad) system as previously described (Daniell et al., 2004). Transplastomic lines were selected using spectinomycin (100 mg/L).

2.3 Molecular characterization of transplastomic lines

Genomic DNA from the putative transformed colonies, developed in selective medium, was extracted according to Goldschmidt-Clermont (Goldschmidt-Clermont, 1991). PCR analysis was performed with specific primers for the *AHP* and *aadA* genes. After 5 min of initial denaturation at 95°C, the samples were carried through 35 PCR cycles under the following conditions: 95°C for 30 s, 55°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 10 min. To prove site-specific integration of the expression cassette and homoplasmy, another PCR was performed with primers annealing on the *tscA* and *ch1N* regions; which are located flanking the expected insertion site.

2.4 Immunological detection of recombinant protein in C. reinhardtii

The total soluble protein (TSP) from either wild type (WT) or transplastomic *C. reinhardtii* lines was extracted with a buffer containing 50 mM Tris–HCl (pH 8), 40 mM NaCl, 0.1% Tween 20, and 1 mM PMSF. Recombinant protein accumulation levels were determined using quantitative ELISA, as previously described (Campos-Quevedo et al., 2012), with an anti-VLPVP antibody produced in rabbit. The VLPVP peptide (produced by chemical synthesis, GenScript Inc. USA) was used as standard. The amount of the recombinant protein was expressed as mg of recombinant protein per mL of TSP.

2.5 *In vitro digestion*

Lyophilized biomass from a WT and a recombinant *C. reinhardtii* strain was digested as previously described by Reddy et al., (2011) with some modifications. Briefly, the samples were diluted in saline solution and adjusted to pH 2. Pepsin was added at a 1:50 ratio (enzyme: substrate). The reaction was incubated for 90 min at 37°C under stirring. Afterwards, the pH of the samples was adjusted to pH 7.5. Trypsin and chymotrypsin were added at a 1:200 ratio and the reaction was incubated for 180 min at 37°C under stirring (Hur et al., 2011). Digestion was stopped by boiling for 10 min.

157 *2.6 Peptides identification by HPLC*

158 Samples from *in vitro* digestion along with the pure peptide (used as standard) were analyzed with
159 an HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a silica C-18 column and
160 a diode array detector. The column thermostat was set at 40°C. The mobile phase consisted of 0.1%
161 trifluoroacetic acid in acetonitrile changing to 0.1% trifluoroacetic acid in water at a flow rate of 0.5
162 mL/min. At the end of the gradient, the column was washed with 0.1% trifluoroacetic acid in
163 acetonitrile and equilibrated to the initial condition for 10 min. Detection was carried out at 215 nm.

164

165 *2.7 Anti-hypertensive activity in spontaneously hypertensive rats*

166 Male spontaneously hypertensive rats (SHR) of 12 weeks-old and about 250-300g of body weight
167 (BW) were obtained from FES Iztacala biotery. The rats were fed with standard food and accessed
168 water freely and housed in a temperature controlled room on a 12h light/dark cycle. The rats were
169 chosen when their systolic blood pressure (SBP) was higher than 150 mmHg. The experiments were
170 performed in rats after 1 week of their training in the animal facility. This study was performed in
171 accordance with UASLP-FCQ guidelines for the care and use of laboratory animals.

172 Lyophilized biomass from WT and transplastomic *C. reinhardtii* lines (10, 30 100 mg/kg of
173 body weight of recombinant protein) was given by intragastric administration (i.g.) to SHRs by metal
174 cannula. The SBP was measured by the tail cuff method with a programmed noninvasive blood
175 pressure controller (LE 5067 automatic blood pressure computer, Letica Scientific Instrument,
176 PanLab, BCN, ES). Distilled water administration (4 mL/kg of body weight, i.g.) served as the negative
177 control and captopril (100 mg/kg i.g., Sigma, St. Louis, MO, USA), a known ACE inhibitor, as the
178 positive control. The lyophilized biomass was suspended in distilled water. Blood pressure was
179 measured before administration and at 2, 4, 6, and 8 h post-administration. Before the
180 measurement, the rats were kept at 37°C for 10 min to make the pulsations of the tail artery
181 detectable.

182 Changes in SBP were calculated as the mean values of three measurements obtained before
183 and after administration. The results, expressed as mean values ± standard error of the mean (SEM)
184 for a minimum of 5 rats, were analyzed using ANOVA. The differences between the groups were
185 assessed by Dunnet test and considered significant for $p < 0.05$.

186

187 *2.8 ACE activity assay*

188 The SHRs were sacrificed 6 hours after administration, the blood and lungs were collected. The
189 serum was separated by centrifugation and analyzed immediately. The lung tissues were macerated
190 with PBS and later analyzed. The reaction was carried out as follows: 30 µL of serum or macerated

lung were added to 270 µL of assay buffer (25 mM HEPES, 300 mM NaCl, pH 8.2) and pre-incubated for 5 min at 37°C. The reaction was initiated with the addition of the synthetic substrate N-[3-(2-furyh)-acryloyl]-L-phenylalanylglycylglycine (1 mM) (FAPGG) dissolved in the assay buffer (Beneteau et al, 1986). The absorbance was registered at 345 nm in the GloMax®-Multi+ Detection System (Promega Corporation, Madison, WI, USA). Triplicate tests were performed for each sample. One unit (1 U) of ACE activity was defined as the amount of enzyme that hydrolyzes 1 µmol of FAPGG per min at 37°C. The enzyme activity was calculated as follows: $U/L = (\Delta A/min)\ X\ (V_T\ X\ 1000)/(V_S\ X\ 0.5)$ where V_T is the final assay volume, V_S is the sample volume, and 0.5 is the maximum change in absorbance at 540 nm produced by hydrolysis of 1 mmol of FAPGG. The results were analyze by ANOVA, the differences between the groups were assessed by Dunnet test and considered significant for $p < 0.05$.

3. RESULTS

3.1 Selection of antihypertensive peptides and design of the synthetic AHP gene

The VLPVP peptide was selected to design the chimeric protein. This peptide was chosen because it has proved high *in vitro* ACE inhibitory activity and high *in vivo* antihypertensive activity [7]. Six tandem repeats were joined by cleavage sites for gastrointestinal proteases (trypsin, pepsin, and chymotrypsin). The gene was synthesized (GenScript Inc. New Jersey, USA) and cloned into the unique *Nco*I site in the p463 vector. The recombinant plasmids were confirmed by restriction analysis and verified by sequencing (Fig. 1).

3.2 Characterization of transplastomic C. reinhardtii lines

Five individual putative transplastomic lines were generated by ballistic using the construction p463-AHP. After four selective rounds on spectinomycin-containing medium the resistant clones were rescued, whereas wild-type and non-transformed strains became yellowish and died.

To identify the presence of the transgene in spectinomycin-resistant clones, a PCR analysis was carried out using specific primers for the *AHP* gene. The expected 309 bp amplicon was obtained in putative transformed *C. reinhardtii* lines and the positive control, but it was absent in the wild type (WT). In Figure 2a, the five positive clones are shown. To confirm that the *AHP* gene had been correctly inserted into the plasmid genome of *C. reinhardtii*, a PCR analysis was performed using primers that align on the insertion site flanking regions (*tscA* and *ch1N* genes) as previously described [15]. The sizes of the PCR amplification, 3000 bp and 850 bp in transformed and WT strains; respectively, indicated that the transgene had been inserted in the *C. reinhardtii* chloroplast (Fig. 2b) resulting in a homoplasmic event in five lines.

3.3 Quantification of recombinant protein in C. reinhardtii

The protein expression levels in transplastomic *C. reinhardtii* lines were determined by quantitative ELISA using anti-VLPVP antibody and the corresponding peptide as standard. The accumulation levels ranged from 0.87 to 1.58 mg of AHP protein per mL of total soluble protein (TSP) (Fig. 3). The highest expression line was Cr.AHP4, which was used for the following experiments. After freeze-drying, the new quantification revealed that Cr.AHP4 produced 0.292 mg of recombinant protein per mg of freeze-dried biomass.

3.4 Effect of a simulated intestinal digestion

The resulting hydrolysates from the i*n vitro* digestion of lyophilized biomass of a WT and a recombinant Cr.AHP4 *C. reinhardtii* strain were analyzed by RP–HPLC. The VLPVP peptide included in the recombinant protein was effectively identified by comparison of retention times with the pure peptide obtained by chemical synthesis (Table 1).

3.5 Anti-hypertensive activity of recombinant protein after oral intragastric in SHRs

To evaluate the anti-hypertensive activity of the recombinant protein, four groups were included: a) Cr.AHP4 group (freeze-dried biomass from transplastomic *C. reinhardtii*), b) WT group (freeze-dried biomass from untransformed *C. reinhardtii*), c) Captopril group (positive control), and d) Water group (negative control).

Three doses were used: 10, 30, and 100 mg/kg of body weight (BW) of recombinant AHP protein produced in transplastomic *C. reinhardtii*. As shown in Figure 4B, the SBP at a dose of 30 mg/kg in the Cr.AHP4 group was significantly reduced; reaching normal levels (<150 mmHg SBP) 6 h after oral administration, with a higher reduction than the one obtained at 100 mg/kg dose. The WT and Water group, as expected, did not show a decrease in the SBP for any of the tested doses. In the Captopril group, which uses an ACE-inhibitor prototype, the maximum decrease in the SBP at a dose of 100 mg/kg did not reach less than 150 mm Hg.

The maximum reduction in SBP was observed 6 h post-administration (141.1 ± 15.1 mm Hg) at a dose of 30 mg/kg (Fig. 4B). The SBP returned to the basal level 24 h after administration (data not shown). No significant differences (p >0.05) were observed at 6 or 8 h after the oral administration. These results indicated that the recombinant protein produced in *C. reinhardtii* has antihypertensive activity as the AHP purified from natural milk.

3.6 ACE activity assay

The activity of the ACE was measured as described by Beneteau et al. (1986). The FAPGG substrate is hydrolyzed to furylacryloylfenylalanine (FAP) and glycylglycine (GG) by the ACE, resulting in a decrease in absorbance at 345 nm. In this assay we observed a higher activity in the lung macerated tissue (Fig 5B) rather than serum samples (Fig. 5A). This was expected since the ACE is a membrane-bound glycoprotein mainly located in the endothelial cells of pulmonary capillaries (Ryan et al., 1976). As can be observed in Figure 5, the ACE activity decreased in the Captopril and Cr.AHP4 groups. In the Cr.AHP4 group the decrease is presumably associated to the VLPVP peptide released from the recombinant protein by the gastrointestinal proteases of the SHR. Thus, the VLPVP peptide showed a strong ACE-inhibitory activity resulting in an antihypertensive effect.

4. DISCUSSION

Antihypertensive peptides are good candidates to substitute antihypertensive drugs, such as captopril, due to their capability in lowing blood pressure and the lack of side effects (Hong et al. 2008; Meira et al. 2012; Rosales-Mendoza et al. 2013). The VLPVP peptide was selected due to its high *in vitro* ACE inhibitory activity and high *in vivo* antihypertensive activity (Lei et al. 2008; Dong et al. 2008).

In this study, we developed a transplastomic *C. reinhardtii* strain that accumulates the VLVP anti-hypertensive peptide in the chloroplast. This platform was selected due to its capability to accumulate biomass and GRAS category (Generally Recognized as Safe), therefore it is not necessary to purify the produced recombinant protein. A PCR analysis confirmed the presence of the transgene in the modified strains. According to an ELISA assay, the highest expression line produced 0.292 mg of recombinant AHP protein per mg of freeze-dried biomass.

It is important to determine antihypertensive effects *in vivo* since it has been reported that some antihypertensive molecules have important *in vitro* activity but it can lose after intake (Lei et al. 2008). Therefore to evaluate the anti-hypertensive activity after intragastric administration in SHRs, three doses were selected: 10, 30, and 100 mg/kg of body weight (BW) of recombinant AHP protein produced in transplastomic *C. reinhardtii*. As it can be seen in Figure 4a, at a dose of 10 mg/kg, the greater decrease in SBP occurs in the Cr.AHP4 group 4 h after oral administration; reaching values of 149 ± 6.2 mmHg, however the effect was not maintained after 6 h. At a dose of 100 mg/kg (Fig. 4c), the effect in the Cr.AHP4 group can be observed at 6 and 8 h after intragastric administration reaching levels of SBP <150 mmHg. At a dose of 30 mg/kg in the same group, the SBP reached near normal levels (141.8 ± 15.1 mmHg) at 6 h (Figure 4b). This was the dose that showed the greater decrease in SBP when compared to the other doses used. In the Captopril group the

decrease was not lower than 150 mm Hg. The WT and Water groups did not show a significant decrease in the SBP at any of the doses tested.

Due to the above mentioned results, the dose of 30 mg/kg was used for further analyses in the ACE inhibition in plasma and lung. In the ACE activity assay, as can be observed in Figure 5; the difference between the Cr.AHP4 and Captopril groups is very noticeable when compared with the Water and WT groups, used as controls. These results indicate that the recombinant AHP protein has an ACE-inhibitory activity similar to captopril, with no statistical difference between these two groups. Nevertheless since the changes in the SBP in both groups are different, another action mechanism of the VLPVP peptide involved in the regulation of the SBP can be suggested.

As we have shown, the purification of the antihypertensive sequences or the use of additional enzymes to release them is not necessary; therefore, the modified transplastomic *C. reinhardtii* can be used as an alternative to treat hypertension.

Previous works have expressed bioactive peptides in other platforms, Yang et al. (2006) produced an anti-hypertensive peptide in the variable regions of glutelins. They administrated the extracted fusion protein (partial purification) and the rice seeds to SHR, their results established that there was a decrease in blood pressure at a dose of 140 μg/kg and 470 μg/kg; respectively (Yang et al. 2006). An anti-hypertensive peptide was also expressed in *E. coli* as inclusion body, thus extraction and purification processes were necessary to be administrated in SHR (Onishi et al. 2012; Huang et al. 2012; Wang et al. 2015). The advantages of our expression system are that no purification process is needed and an effect in the SBP was obtained at lower doses. The transplastomic *Chlamydomonas reinhardtii* strain producing recombinant AHP protein will be a potential agent for further development into functional foods for the prevention and treatment of hypertension. Additional clinical trial studies are required to confirm this.

5. Conclusion

The data collected in this study demonstrate that a transplastomic *Chlamydomonas reinhardtii* strain producing an antihypertensive peptide (VLPVP) can reduce the systolic blood pressure in spontaneous hypertensive rats, which suggests that it could be able to reduce blood pressure in humans as an alternative for the treatment of hypertension; however further research is required.

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REFERENCES

Beneteau, B., Baudin, B., Morgant, G., Giboudeau, J., Baumann, F.C., 1986. Automated kinetic assay of angiotensin-converting enzyme in serum. Clin. Chem. 32, 884–886.

Campos-Quevedo, N., Rosales-Mendoza, S., Paz-Maldonado, L.M.T., Martínez-Salgado, L., Guevara-Arauza, J.C., Soria-Guerra, R.E., 2013. Production of milk-derived bioactive peptides as precursor chimeric proteins in chloroplasts of Chlamydomonas reinhardtii. Plant Cell. Tissue Organ Cult. 113, 217–225.

Daniell, H., 2006. Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol. J. 1, 1071–1079.

Daniell, H., Ruiz, O., Dhingra, A., 2004. Chloroplast Genetic Engineering to Improve Agronomic Traits, in: Peña, L. (Ed.), Methods in Molecular Biology™. Humana Press, pp. 111–137.

Dong, L., Yan, S., Lin, L., Min, L., 2008. The absorption mechanism of the antihypertensive peptide, Val-Leu-Pro-Pro-Val (VLPVP), was investigated in the Caco-2 cell model. Acta Nutr. Sin. 4, 354–358.

Goldschmidt-Clermont, M., 1991. Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker of site-directed transformation of chlamydomonas. Nucleic Acids Res. 19, 4083–4089.

Hartmann, R., Meisel, H., 2007. Food-derived peptides with biological activity: from research to food applications. Curr. Opin. Biotechnol. 18, 163–169.

Higgins, B., Williams, B., Guideline Development Group, 2007. Pharmacological management of hypertension. Clin. Med. 7, 612–616.

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., Chi, L., 2008. The antihypertensive effect of peptides: A novel alternative to drugs? Peptides 29, 1062–1071.

352 Huang, L., Ma, H., Li, Y., Li, S., 2012. Antihypertensive activity of recombinant peptide IYPR expressed
353 in Escherichia coli as inclusion bodies. Protein Expr. Purif. 83, 15–20.

354 Hur, S.J., Lim, B.O., Decker, E.A., McClements, D.J., 2011. In vitro human digestion models for food
355 applications. Food Chem. 125, 1–12.

356 Iwaniak, A., Minkiewicz, P., Darewicz, M., 2014. Food-originating ACE inhibitors, including
357 antihypertensive peptides, as preventive food components in blood pressure reduction.
358 Compr. Rev. Food Sci. Food Saf. 13, 114–134.

359 Lei, L., Sun, H., Liu, D., Liu, L., Li, S., 2008. Transport of Val-Leu-Pro-Val-Pro in human intestinal
360 epithelial (Caco-2) cell monolayers. J. Agric. Food Chem. 56, 3582–3586.

361 Meira, S.M.M., Daroit, D.J., Helfer, V.E., Corrêa, A.P.F., Segalin, J., Carro, S., Brandelli, A., 2012.
362 Bioactive peptides in water-soluble extracts of ovine cheeses from Southern Brazil and
363 Uruguay. Food Res. Int. 48, 322–329.

364 Onishi, K., Matoba, N., Yamada, Y., Doyama, N., Maruyama, N., Utsumi, S., Yoshikawa, M., 2004.
365 Optimal designing of β-conglycinin to genetically incorporate RPLKPW, a potent anti-
366 hypertensive peptide. Peptides 25, 37–43.

367 Rasala, B.A., Mayfield, S.P., 2011. The microalga Chlamydomonas reinhardtii as a platform for the
368 production of human protein therapeutics. Bioeng. Bugs 2, 50–54.

369 Reddy, I.M., Kella, N.K.D., Kinsella, J.E., 1988. Structural and conformational basis of the resistance of
370 .beta.-lactoglobulin to peptic and chymotryptic digestion. J. Agric. Food Chem. 36, 737–741.

371 Rosales-Mendoza, S., Paz-Maldonado, L.M.T., Govea-Alonso, D.O., Korban, S.S., 2013. Engineering
372 production of antihypertensive peptides in plants. Plant Cell. Tissue Organ Cult. 112, 159–169.

373 Ryan, U.S., Ryan, J.W., Whitaker, C., Chiu, A., 1976. Localization of angiotensin converting enzyme
374 (kininase II). II. Immunocytochemistry and immunofluorescence. Tissue Cell 8, 125–45.

375 Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. CSHL Press.

376 Yang, L., Tada, Y., Yamamoto, M.P., Zhao, H., Yoshikawa, M., Takaiwa, F., 2006. A transgenic rice
377 seed accumulating an anti-hypertensive peptide reduces the blood pressure of spontaneously
378 hypertensive rats. FEBS Lett. 580, 3315–3320.

Schötz, F., Bathelt, H., Arnold, C.G., Schimmer, O., 1972. The architecture and organization of the *Chlamydomonas cell*. Results of serial-section electron microscopy and a three-dimensional reconstruction. Protoplasma 75, 229–54.

Silva, S. V., Malcata, F.X., 2005. Caseins as source of bioactive peptides. Int. Dairy J. 15, 1–15.

Wang, X.L., Ma, S.-N., Yuan, Y.-H., Ding, Y., Li, D.-S., 2015. Expression and purification recombinant antihypertensive peptide ameliorates hypertension in rats with spontaneous hypertension. Protein Expr. Purif. 113, 30–34.

WHO. A global brief on hypertension [WWW Document], n.d. URL http://www.who.int/cardiovascular_diseases/publications/global_brief_hypertension/en/ (accessed 4.14.16).

TABLES

Table 1. Retention time of antihypertensive peptide VLPVP.

FIGURE LEGENDS

Fig. 1. Schematic diagram of the p463-AHP construction. The synthetic gene (*AHP*) include six tandem repeats of the VLPVP peptide separated by protease cleavage sites. The *AHP* gene is drived by the rbcL promoter. Also contain the *aadA* gene which confer resistance to spectinomycin in transformed *C. reinhardtii* lines.

Fig. 2. (A) PCR analysis to identify the presence of the transgene in spectinomycin resistant lines of *C. reinhardtii*. (B) PCR analysis to confirm that the *AHP* gene had been correctly inserted into the plasmid genome of *C. reinhardtii*. Lines: MW: Molecular weight (1 Kb); +: Positive control (p463-AHP); 1-5: Transformed lines (Cr.AHP 1 to 5); WT: Wild type.

Fig. 3. Recombinant protein produced by transplastomic *C. reinhardtii* lines. The values are presented as mg of *AHP* protein per ml of total soluble protein (TSP).

Fig. 4. Antihypertensive effect of a single oral administration of a recombinant antihypertensive protein produced in *C. reinhardtii* containing six VLPVP peptides. Each point is the mean of the changes of systolic blood pressure (SBP) of five spontaneously hypertensive rats, from time 0 to 8h. The vertical bars represent standard errors. (A) Oral administration of 10 mg/kg, (B) 30 mg/kg and (C) 100 mg of recombinant AHP protein produced in transplastomic *C. reinhardtii* (Cr.AHP4) per kg of body weight (BW). WT group (untransformed *C. reinhardtii*). Captopril group (positive control: 100 mg/kg of BW); and Water group (negative control: 4 ml/kg BW). Differences between the groups were assessed by Dunnet test. a) $p < 0.05$ versus Water; b) $p < 0.05$ versus Captopril; c) $p < 0.05$ versus Cr.AHP4 .

Fig. 5. (A) Plasma and (B) Pulmonary ACE activity after 6h of oral administration of recombinant protein Cr.AHP4 in spontaneously hypertensive rats. All data are mean + SEM for three replicates. One unit (U) of ACE activity was defined as the amount of enzyme that hydrolyzes 1 µmol of FAPGG per minute at 37°C. Differences between the groups were assessed by Dunnet test and were considered significant when $p < 0.05$.

Table

Table 1

Sample	Retention Time (min)	SD ±
VLPVP pure peptide	45.30	0.27
Lyophilized biomass of Cr.AHP4	45.1	0.30
Lyophilized biomass of WT	No peak was detected at this time	-

Fig. 1

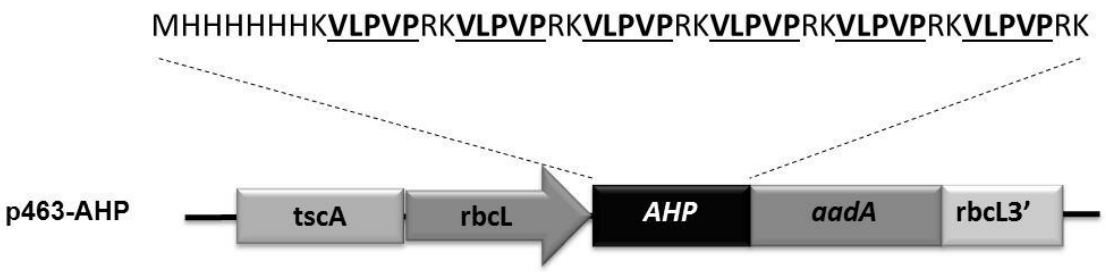

MHHHHHHK**VLPVP**RK**VLPVP**RK**VLPVP**RK**VLPVP**RK**VLPVP**RK**VLPVP**RK
p463-AHP
tscA
rbcL
AHP
aadA
rbcL3'

Fig. 2

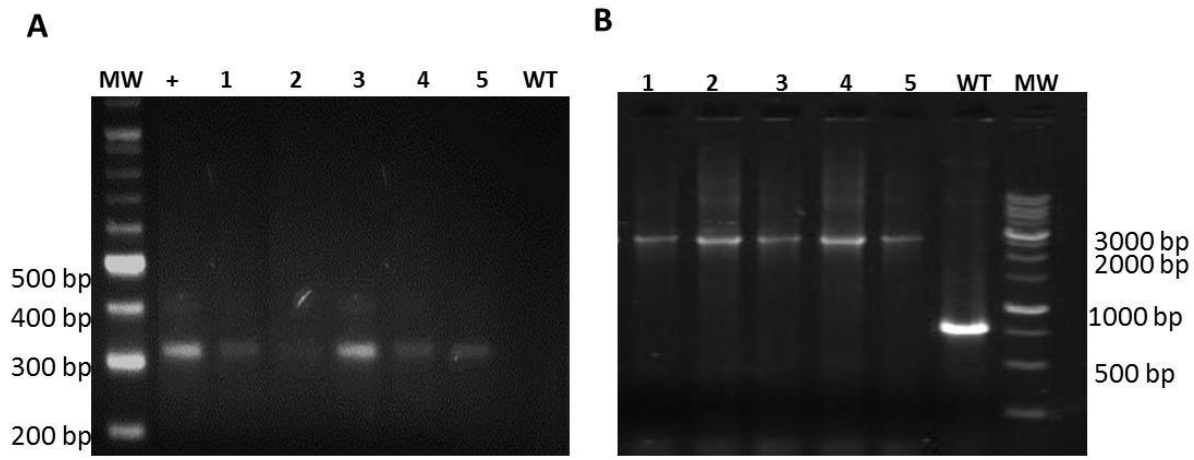
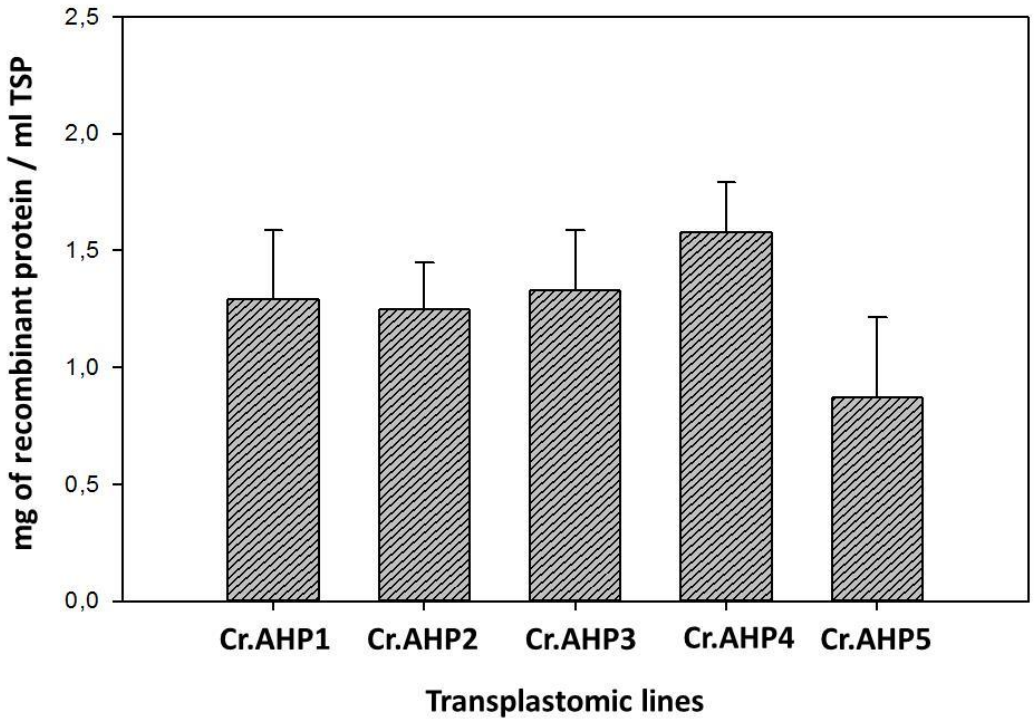
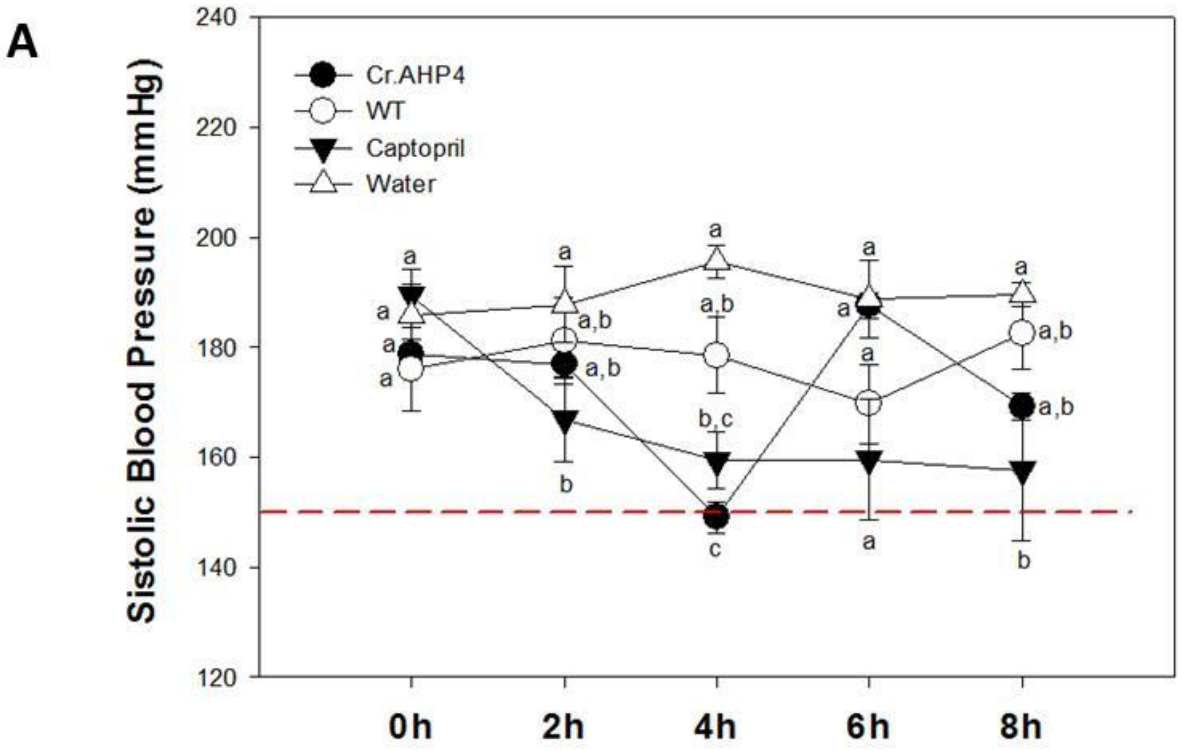

A
MW
+
1
2
3
4
5
WT
500 bp
400 bp
300 bp
200 bp
B
1
2
3
4
5
WT
MW
3000 bp
2000 bp
1000 bp
500 bp

Figure 3


2,5
2,0
1,5
1,0
0,5
0,0
mg of recombinant protein / ml TSP
Cr.AHP1
Cr.AHP2
Cr.AHP3
Cr.AHP4
Cr.AHP5
Transplastomic lines

44 **Fig. 4**

45



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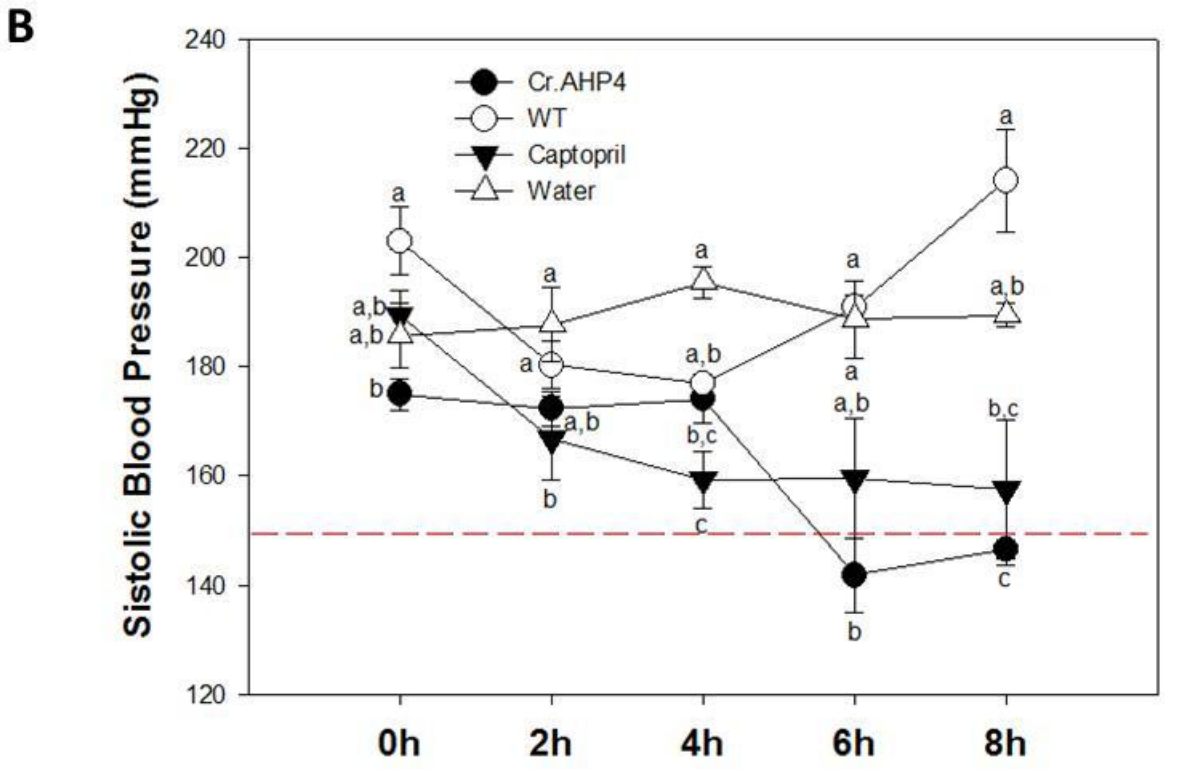
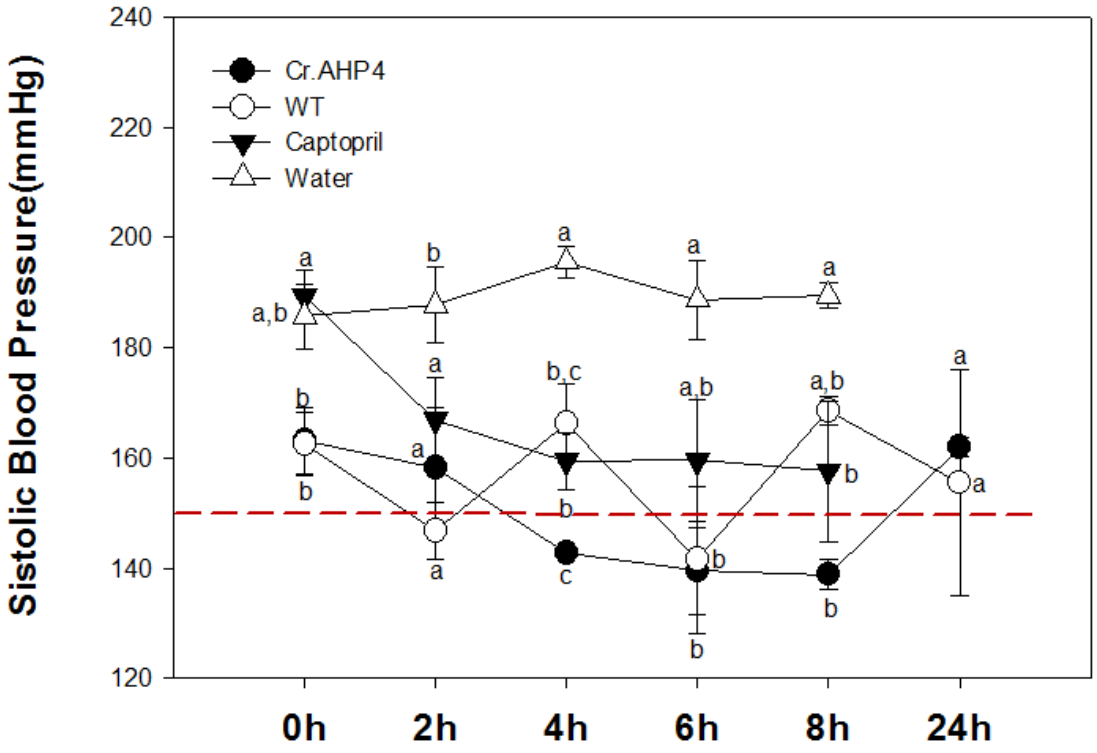


47

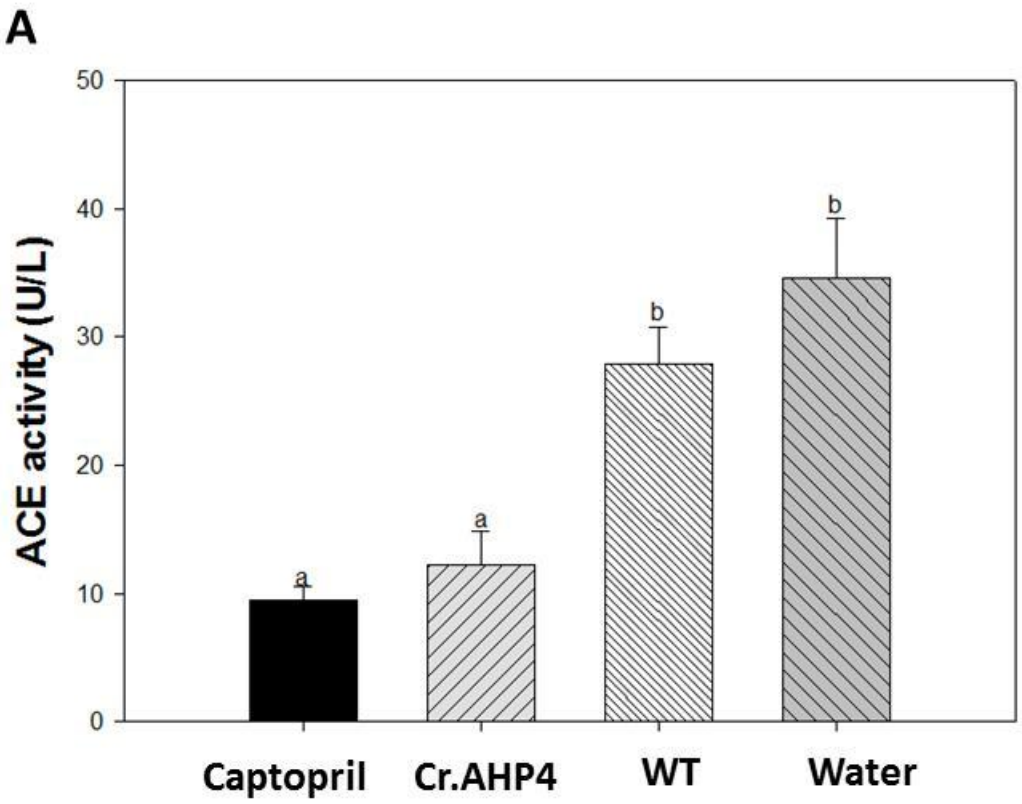
C


Sistolic Blood Pressure(mmHg)
240
220
200
180
160
140
120
Cr.AHP4
WT
Captopril
Water
0h
2h
4h
6h
8h
24h

Fig

67

68 **Figure 5**



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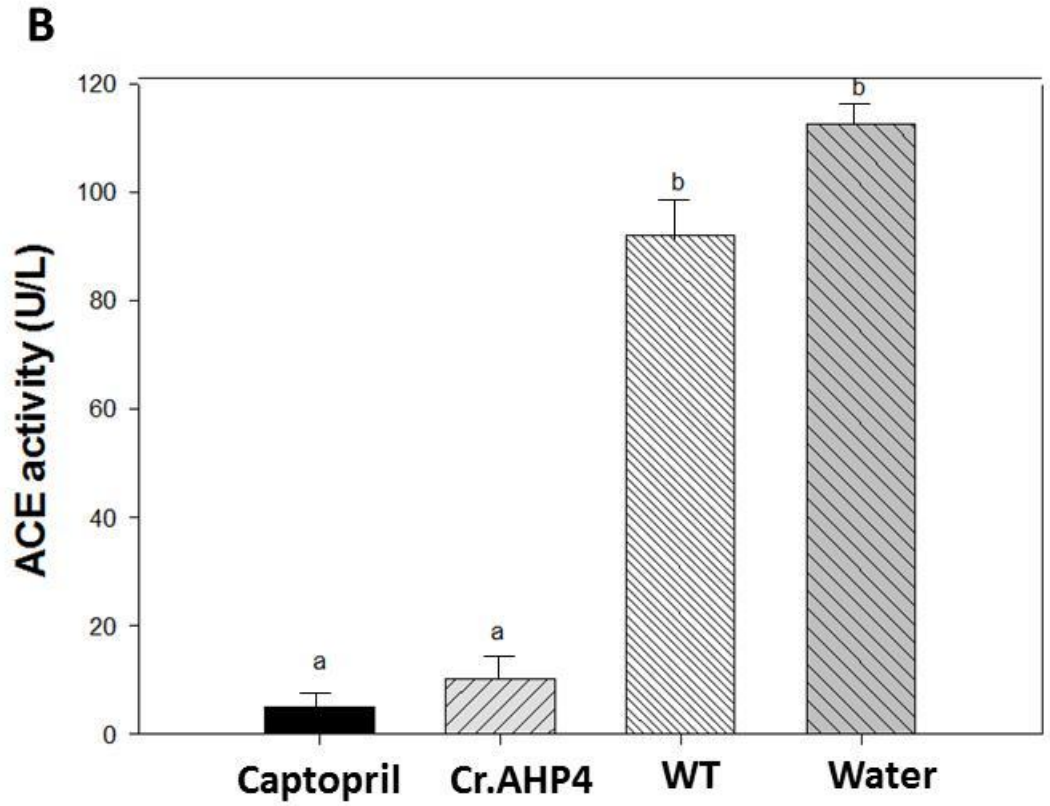
B
ACE activity (U/L)
120
100
80
60
40
20
0
a
a
b
b
Captopril
Cr.AHP4
WT
Water