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Bioactivity of an antihypertensive peptide expressed in *Chlamydomonas reinhardtii*

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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 and quantified by 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 to spontaneous hypertensive rats at a dose of 30 mg/kg of body weight of recombinant protein significantly reduced systolic blood pressure. At the same dose, the recombinant protein exerts an ACE-inhibitory effect. This is the first study that indicates the potential of this microalga producing an antihypertensive peptide as a dietary supplement for hypertension patients.

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. The bioactive peptides were first reported in the 1950s in casein derived from milk (Hayes et al., 2007). Bioactive peptides are defined as peptides with hormone or drug-like activity that can regulate physiological functions (Fitzgerald and Murray, 2006). A number of bioactive peptides have been isolated, purified, and identified from a variety of food sources such as milk, plants, meat, fish, and eggs; among others (Meisel, 2004). Such peptides are inactive in their parent protein sequences and the corresponding biologically active peptides can be released by three ways: enzymatic digestion during passage through the gastrointestinal tract, during processing of foods, and proteolytic activity by enzymes derived from microorganisms (Moller et al., 2008).

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.

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A wide range of activities for these peptides has been described including antihypertensive, hypocholesterolemic, antimicrobial, antioxidant and immunomodulatory activities (Iwaniak et al., 2014; Hong et al., 2008; Silva and Malcata, 2005). The most extensively studied group among these food-derived bioactive peptides is the potentially antihypertensive peptides; in particular peptides with angiotensin-I converting enzyme (ACE) inhibitory activity.

The ACE, which is part of the renin angiotensin system, converts angiotensin-I into angiotensin-II regulating the peripheral blood pressure by vasoconstriction. Inhibition of the ACE exerts an antihypertensive effect through a decrease of angiotensin II and an increase of bradykinin (Fitzgerald and Meisel, 2003).

Angiotensin-I converting enzyme-inhibitors are presumably competitive substrates for the ACE. The primary structural feature governing this inhibitory response is the C-terminal tripeptide sequence and thus these peptides may interact with the active site of the ACE (Hong et al., 2008). The ACE preferentially interacts with substrates and inhibitors containing hydrophobic amino acid residues in the three C-terminal positions. In general aliphatic, basic, and aromatic residues are preferred in the penultimate positions; while aromatic proline and aliphatic residues are preferred in the ultimate positions (Cheung et al., 1980).

The bioactive peptides such as ACE-inhibitory peptides must reach their target organ intact to exert their effects *in vivo*. Degradation of peptides in the acidic environment of the stomach, alkaline conditions of the small intestines as well as hydrolysis by the brush border peptidases can either activate or deactivate ACE-inhibitory peptides before they reach the portal circulation. Therefore, only those ACE-inhibitors not affected by the action of angiotensin-II and gastrointestinal enzymes or those converted to stronger ACE inhibitors exert antihypertensive effects *in vivo* (Korhonen and Pihlanto, 2003).

The VLPVP peptide, derived from b-casein hydrolysate, is produced by an extracellular proteinase from *Lactobacillus helveticus* (Maeno et al., 1996). This is a potent anti-hypertensive peptide with ACE inhibitory activity. It has been reported as not susceptible to non-enzymatic hydrolysis and it is reasonably stable to enzymatic degradation by Caco-2 cells. The VLPVP peptide is transported through Caco-2 cell monolayers mainly via the paracellular pathway (Lei et al., 2008). Due to the above, it has been suggested that the VLPVP peptide has a high oral bioavailability in humans although it is yet to be clinically determined (Lei et al., 2008; Dong et al., 2008).

The demand for AHP peptides in the food industry has drawn great attention from consumers, food scientists, and nutritionists (Yamamoto et al., 2003). Most of the AHP has been obtained by enzymatic hydrolysis; however, the content of AHP in natural food proteins is low. There are only a few reports available regarding the commercial production of AHP. The industrial production of proteins by DNA recombinant technology has been the most promising method for mass production of ACE inhibitory peptides.

About 40% of the recombinant 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 (Rasala 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 10^7 cells/mL. Its chloroplast occupies about 40% of the cell volume (Schötz 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 intragastric administration of freeze-dried biomass.

2. Materials and methods

2.1. Strains and media

Chlamydomonas reinhardtii strain 137c (mt+) was obtained from the Chlamydomonas Center (http://www.chlamy.org/). All algal cultures were grown to the late logarithmic phase in tris-acetate-phosphate (TAP) medium under fluorescent white light (80 μmol m^{-2} s^{-1}) at 25 °C on a rotary shaker at 100 rpm.

2.2. 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.

The designed gene was synthesized and optimized for expression in *C. reinhardtii* chloroplasts by GenScript Inc. (Piscataway, NJ, USA). Restriction sites were added to facilitate subcloning into the p463 expression vector (Chlamydomonas Center, http://www.chlamy.org/). *Escherichia coli* Top10 was used for recombinant DNA manipulations. 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 Russell, 2001).

2.3. Chloroplast transformation by particle bombardment

For chloroplast transformations, a 100 mL culture of *C. reinhardtii* was grown to the late logarithmic phase. Cells were harvested by centrifugation and resuspended in 12 mL of TAP medium. These cells were then spread onto 6 TAP/agar plates without antibiotics. Chloroplast transformations were performed by particle bombardment using the PDS-1000/He (Bio-Rad, Hercules, CA, USA) with DNA-coated gold particles (1 μm) coated with 10 μg of the p463-AHP plasmid. Particle bombardment parameters were as follows: chamber vacuum at 28 in Hg, helium pressure at 1100 psi, and distance of 9 cm. After two weeks, the particle-bombarded plates were streaked on TAP plates containing 100 μL/mL of spectinomycin; two weeks later the transformed algae colonies appeared. Primary transformants were streaked 4–5 times on TAP plates containing 100 μL/mL of spectinomycin until identification of homoplasmic lines.

2.4. Molecular characterization of transplastomic lines

Genomic DNA from the putative transformed colonies, growing in selective medium (100 μL/mL spectinomicyn) along with an unmodified strain (wild type) growing in TAP medium, was extracted according to Goldschmidt-Clermont (Goldschmidt-Clermont, 1991). PCR analysis was performed with specific primers