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Overexpression of a modified protein from amaranth seed in *Escherichia coli* and effect of environmental conditions on the protein expression

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ABSTRACT

Amaranth seeds are considered as an excellent complementary source of food protein due to their balanced amino acid composition. Amarantin acidic subunit has the potential as a functional and nutraceutical protein, and it is structurally a good candidate for modification. The aim of this work was to improve its functionality, then the primary structure was modified into the third variable region of 11S globulins, by inserting antihypertensive peptides: four Val-Tyr in tandem and Arg-Ile-Pro-Pro in the C-terminal region. Modified protein was expressed in *Escherichia coli* Origami (DE3) and was purified. The culture conditions, including the culture media, temperature, agitation speed and air flow were tested in order to obtain an increased expression levels of the modified protein. A 2^3 factorial design was used for evaluate the effect of environmental conditions on modified protein production. The results indicated that the yield of modified protein could be increased by up 3-fold in bioreactor as compared with flask. In addition, the temperature, the agitation speed and the oxygen were significant factors on the expression of the antihypertensive protein. The maximum production was 99 mg protein-L^{-1}. The hydrolyzed protein showed a high inhibitory activity of the angiotensin converting enzyme (IC_{50} = 0.047 mg mL^{-1}).

1. Introduction

The modern diet together with a sedentary lifestyle has produced an epidemic of nutritionally related diseases. Hypertension is one of these important diseases in our society, given its high prevalence and its role in cardiovascular diseases, including coronary heart disease, peripheral arterial disease, end-stage renal disease and stroke (Glasser, 2001; Seppo et al., 2003; Madureira et al., 2010). About one billion people are now suffering from hypertension and it is expected to increase 60% by 2025 worldwide (Akama et al., 2009; Nakahara et al., 2010).

Drugs that inhibit the renin-angiotensin system (important regulator of blood pressure), either by inhibiting angiotensin-converting enzyme (ACE) or by blocking angiotensin (AT1) receptors, are widely used in the treatment of hypertension. ACE inhibitors have a dual effect on this system: they inhibit the production of the vasoconstrictor angiotensisn II and they inhibit the degradation of the vasolidator bradykinin (Seppo et al., 2003; Madureira et al., 2010; Chobanian et al., 1990); *in vitro* inhibition of angiotensin II formation has been used for screening therapeutic agents such as ACE inhibitors against hypertension. Chemically synthesized hypotensive drugs, such as captopril, propranolol, and losartan are still broadly used to treat and prevent hypertension. Nevertheless, these drugs are reported to have many side effects such as dry cough, taste disturbances, skin rashes, and many other dysfunctions of human organs (Fitzgerald and Meisel, 2000).

In this regard, the influence of nutritive compounds on prevention and treatment of hypertension has considerably attracted the attention during the last decade. Among these compounds are peptides derived from food proteins that exert antihypertensive activity (Hu et al., 1999; Vercruysse et al., 2005; Erdmann et al., 2008). For example, various studies on bioactive peptides in amino acid sequences of natural proteins such as milk, egg, fish, soybean, spinach, and many other sources have been reported (Akama et al., 2009; Prak et al., 2006; Murray and FitzGerald, 2007; Hong et al., 2008). These protein-derived bioactive peptides are inactive within the sequences of the parent proteins but can be released by enzymatic proteolysis during gastrointestinal digestion. Once liberated in the body, bioactive peptides many act as regulatory compounds with hormone-like activity; they usually contain 2–20 amino acids residues per molecule, but in some cases may consist of more than 20. Because of their health-enhancing potential and safety profiles

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bioactive peptides may be used as components in functional foods or nutraceuticals (Erdmann et al., 2008).

Antihypertensive peptides can be introduced into food proteins and/or concentrated for the purpose of treatment of a disease or disorder, for example in those people with cardiac or renal insufficiency or diabetes (Seppo et al., 2003). An antihypertensive effect of bioactive peptides were demonstrated by studies *in vivo* in spontaneously hypertensive rats (Nakamura et al., 1995; Muguerza et al., 2006; Quirós et al., 2007) and in human subjects (Hata et al., 1996; Seppo et al., 2002; Tuomilehto et al., 2004; Bütikofer et al., 2008). In particular, the dipeptide Val-Tyr (VY) is one of the biopeptides tested *in vivo* that has hypotensive effect in humans and it easily can be absorbed into the human circulatory blood system (Kawasaki et al., 2000; Matsui et al., 2002). Moreover, Matsui et al. (2005) showed that this small peptide (VY) exerts an antiproliferative effect on vascular smooth muscle cells, which suggests the possibility of developing novel medicinal foods containing this peptide to prevent certain diseases. Other researchers have been focused on the two antihypertensive peptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP); their antihypertensive effects in rats (Nakamura et al., 1995), and in humans (Aihara et al., 2005; Mizuno et al., 2005) have been evaluated in several clinical trials. These studies suggested that continuous intake of VPP and IPP might have the potential to improve arterial stiffness as well as central blood pressure and peripheral brachial blood pressure (Mizuno et al., 2005; Nakamura et al., 2009).

Moreover, in the last years, various expression systems using mammalian cells, yeast cells or prokaryotic cells have been established for the production of recombinant proteins (Yokoyama, 2003; Kyle et al., 2009). Moreover, several studies have reported that *Escherichia coli* is an attractive host for large-scale production, as it grows in low-cost media, offers good genetic stability, has abundant available plasmids and permits scale-up of the production process without loss of yield (Romanos, 1995; Hartmann et al., 2008; Riley et al., 2009; Moers et al., 2010).

On the other hand, amaranth seeds (*Amaranthus hypochondriacus*) have been considered as an excellent alternative or complementary source of food protein due to their balanced amino acid composition. However, their potential as a source of bioactive peptides has been little explored (Gorinstein et al., 2002; Vecchi and Añón, 2009). Amarantin is an 11S globulin, and it is the most predominant storage proteins in seeds of amaranth, its amino acid composition is close to the optimum amino acid balance required in the human diet, and has remarkable heat stability and emulsifying properties (Romero-Zepeda and Paredes-López, 1996). This protein is a homohexameric molecule with a molecular mass of 398 kDa and it has two disulfide liked subunits: acidic (32–34 kDa) and basic (22–24 kDa).

Research of our group has been focused on the expression of recombinant amarantin in different expression systems (Medina-Godoy et al., 2004); expression in maize plants and in seeds of transgenic tobacco, resulting in important increases of seed protein content (Rascón-Cruz et al., 2004; Valdez-Ortiz et al., 2005). Recently, Luna-Suárez et al. (2008) expressed in *E. coli*, purified and characterized a His-tagged version of the acidic subunit from 11S amaranth seed protein and they showed that this protein was as stable as the complete amarantin. In addition, the results obtained suggest that the acidic subunit has the potential as a functional and nutraceutical protein, and it is structurally a good candidate for modification. Moreover, using protein engineering, further characteristics could be incorporated into this high-nutritional protein, such as bioactives peptides or modified amino acid sequence, to enhance functional and nutraceutical properties.

Thus, the objective of the present study was to improve the nutraceutical properties of the amarantin. Therefore, the primary structure of this acidic subunit was modified with two antihypertensive peptides in two sites: in the globulins 11S III variable region were inserted two amino acids in tandem of four (Val-Try), and in the C-terminal of the acidic subunit were inserted four amino acids (Arg-Ile-Pro-Pro). The modified protein was expressed in *E. coli*. We also purified and determined the ACE inhibitory activity *in vitro* of this protein. Moreover, we evaluated the influence of the environmental factors (temperature, agitation, oxygen) on the expression of the modified amarantin acidic subunit.

However, an efficient production system in a bioreactor was necessary, while there have been no such report for the amarantin production.

2. Materials and methods

2.1. Strains and plasmids

E. coli TOP10 (Invitrogen, Carlsbad, CA) was used for plasmid routine transformation and propagation. *E. coli* Origami (DE3) (Novagem, Markham, Ontario, Canada) was used for the expression of the modified amarantin acidic subunit. Origami host strains are K-12 derivates, which carries mutations in both the thioredoxin reductase (*trxB*) and the glutathione reductase (*gor*) genes, which enhance disulfide-bond formation in the cytoplasm of *E. coli* (Berrow et al., 2006). The plasmid pET-AC-M1 (Luna-Suárez et al., 2010) which contains the encoding sequence of the amarantin acidic subunit modified in the third variable region with the insertion of the codons for four VY biopeptides in tandem, was used as source of DNA template for PCR. Cloning vector pPCR® TOPO 2.1 (Invitrogen, Carlsbad, CA) was used to clone the PCR products, according to manufacturer's instructions. Expression plasmid pET-32b(+) (Novagen, Markham, Ontario, Canada) was used for modified amarantin acidic subunit expression. Expression of amarantin acidic subunit was under the control of a T7 promoter induced with the isopropyl-beta-D-thiogalactoside (IPTG).

2.2. Construction of the modified amarantin acidic subunit expression plasmid

The expression plasmid pET-AC-M1 (Luna-Suárez et al., 2010) was used as PCR template to construct plasmid pET-AC-M36H plasmid, specific oligonucleotides were designed for PCR amplification that include the region of the acidic subunit and a sequence of six histidines just before the peptide RIPP on the COOH terminal. The primers for amplification were: forward 5′-GGGTGATTA*ATG*GAAGGAAGGTTTAGAGAGTTTCAAC-3′ (*VspI* restriction site is underlined and start codon is in italics) and reverse 5′ AT*GGA*GTGTGGTGGTGGTGGTGGTG**TCTTAAGGAGGA***ATC* CTATTGGGAAGGTAC-3′ (*EcoRI* restriction site is in italics, stop codon is underlined in italics, the 6-His encoding sequence inserted is underlined, and the RIPP peptide encoding sequence inserted is in bold letter). After amplification, the PCR product was ligated into pPCR®2.1 TOPO®. *E. coli* cells harboring recombinant plasmid were selected on LB plates containing 100 μg ml^{-1} ampicillin and X-gal. The DNA fragment enconding amarantin acidic subunit M36His was released from pPCR®2.1 TOPO® vector using *VspI* and *EcoRI* restriction enzymes. The *VspI*/*EcoRI* fragment was ligated into plasmid pET-32b(+) and transformed into the TOP 10 cloning host. *E. coli* transformants were selected on LB plates containing 100 μg ml^{-1} carbenicillin. The positive clones were confirmed by PCR amplification, restriction analysis and DNA sequencing.

2.3. Transformation of expression cells

E. coli Origami (DE3) cells were transformed with plasmid pET-AC-M36His following instructions provided by the manufacturers,