Electronic Journal of Biotechnology 37 (2019) 18–24

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

The insertion of bioactive peptides at the C-terminal end of an 11S globulin changes the structural stability and improves the antihypertensive activity

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ARTICLE INFO

Article history:
Received 29 August 2018
Accepted 7 November 2018
Available online 14 November 2018

Keywords:
Amaranth
Antihypertensive peptides
Bioactive
Culture media design
Globulin
Mature seeds
Potato waste
Protein engineering
Protein expression
Protein stability
Storage protein
Thermal stability

ABSTRACT

Background: The 11S globulin from amaranth is the most abundant storage protein in mature seeds and is well recognized for its nutritional value. We used this globulin to engineer a new protein by adding a four valine-tyrosine antihypertensive peptide at its C-terminal end to improve its functionality. The new protein was named AMR5 and expressed in the *Escherichia coli* BL21-CodonPlus(DE3)-RIL strain using a custom medium (F8PW) designed for this work.
Results: The alternative medium allowed for the production of 652 mg/L expressed protein at the flask level, mostly in an insoluble form, and this protein was subjected to *in vitro* refolding. The spectrometric analysis suggests that the protein adopts a β/α structure with a small increment of α-helix conformation relative to the native amaranth 11S globulin. Thermal and urea denaturation experiments determined apparent Tm and $C_{1/2}$ values of 50.4°C and 3.04 M, respectively, thus indicating that the antihypertensive peptide insertion destabilized the modified protein relative to the native one. AMR5 hydrolyzed by trypsin and chymotrypsin showed 14- and 1.3-fold stronger inhibitory activity against angiotensin I-converting enzyme (IC_{50} of 0.034 mg/mL) than the unmodified protein and the previously reported amaranth acidic subunit modified with antihypertensive peptides, respectively.
Conclusion: The inserted peptide decreases the structural stability of amaranth 11S globulin and improves its antihypertensive activity.
How to cite: Espinosa-Hernández E, Morales-Camacho JI, Fernández-Velasco DA, et al. The insertion of bioactive peptides at the C terminal end of an 11S globulin changes the structural stability and improves the antihypertensive activity. Electron J Biotechnol 2019;37. https://doi.org/10.1016/j.ejbt.2018.11.001.

1. Introduction

Hypertension is a health problem worldwide that is mainly observed in adults. This disease is associated with sedentary lifestyle and is treated with drugs that produce side effects [1]. It has been reported that antihypertensive peptides could be an alternative treatment for mildly hypertensive patients or even a supplemental treatment [2,3]. Thus far, many antihypertensive peptides have been reported; however, few studies have been evaluated to determine their effectiveness *in vivo*. The animal model most frequently employed is the spontaneously hypertensive rats. In humans, only the IPP, VPP, and VY (Valine-Tyrosine) peptides have been evaluated [4]. Regarding the latter peptide, it was demonstrated that it can reduce blood pressure in middle-age hypertensive individuals [5]. Therefore, diverse protein-rich foods have been developed to include bio-functional peptides to be consumed and delivered or absorbed by the gastrointestinal system [3,6,7].

Because of its nutritional value, amaranth seed has attracted attention as an alternative food for future generations. Amaranth 11S globulin (A11Sg) is the most abundant globulin in amaranth seeds and has a high nutritional value. Notably, it is rich in the amino acids

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2018.11.001

lysine, methionine, serine, and cysteine, which are limited in cereals [8]. In the amaranth seeds, this protein is found as a hexameric conformation; a monomer consists of acidic and basic subunits linked by a disulfide bond [8]. It has also been demonstrated that A11Sg has high thermal stability [9].

The functionality of A11Sg has been improved by the insertion of four VY antihypertensive peptides using only the acidic subunit. However, the expression level in *E. coli* (Origami(DE3), Rosetta(DE3), Rosetta 2(DE3), or BL21-CodonPlus(DE3)-RIL strains) remains low [10,11,12]. The modified polypeptides also have a lower thermal stability [12] than that reported for the whole A11Sg, which may be a limiting factor for its application in the food industry. The aim of this study was to modify the A11Sg protein by adding four VY antihypertensive peptides in tandem at the C-terminal to study both the structural stability and antihypertensive activity *in vitro*. Furthermore, a culture medium was designed and optimized using potato waste as a low-cost alternative substrate for recombinant protein production.

2. Materials and methods

2.1. Plasmid construction

The pET-AMAR-R5-H plasmid that expresses the AMR5 protein was constructed from the pET-AMAR-6His plasmid [13], which contains the cDNA of A11Sg (GenBank accession number: X82121.1) [8]. It was used as a PCR template to accomplish site-directed mutagenesis by inserting four VY biopeptides in tandem at the C-terminal. Platinum® Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific) was used to obtain the PCR products. The primers for amplification were as follows: forward 5'-CACCACCACCACCACCACTG-3′ (the histidine tag encoding sequence is underlined). Reverse: 5'-CTCGAG**GTATACGTAAACGTAAACGTAAAC**GCGGGCAATGCTGATTTTCC-3′ (the encoding sequence of the VYVYVYVY peptide is given in bold, and the *Xho*I restriction site is underlined).

The PCR product was recircularized by T4 DNA ligase (Thermo Fisher Scientific), and then, *E. coli* One Shot™ TOP10 cells (Thermo Fisher Scientific) were transformed by thermal shock [14]. Cells harboring the recombinant plasmid were selected on LB plates containing 100 μg/mL of ampicillin. Positive clones were confirmed by PCR, restriction analysis, and DNA sequencing using T7 sequencing primers. The new plasmid pET-AMAR-R5-6His was extracted by the alkaline lysis method [14], and then, *E. coli* BL21-CodonPlus(DE3)-RIL strain (Agilent Technologies) was transformed by following the manufacturer's recommendations. Cells harboring the recombinant plasmid were selected on LB plates containing 100 μg/mL of ampicillin.

2.2. Formulation of culture media

Seven culture media were formulated with potato waste and evaluated (Table 1). The potato waste was prepared by adding 200 g of potatoes to 800 mL of distilled water, and after boiling for 15 min, the solids were separated by filtration. Then, the filtered liquid was used to mix with the other components. The mixture was autoclaved, and phosphates were added. LB broth (Sigma-Aldrich), which is commonly used for protein expression, was employed as a control medium.

To determine the contribution of potato waste to protein expression, the formulation labeled FPW was tested by replacing the potato waste with distilled water. Experiments were performed to minimize the amount of yeast extract and lactose as an inducer in the FPW broth. First, the content of yeast extract was tested at 0, 5, 8, and 12 g/L. Second, lactose was tested at 0.1%, 0.3%, and 0.5%. Finally, to determine whether glycerol increases the recombinant protein production in *E. coli*, AMR5 was expressed in both FPW broth alone and FPW broth with glycerol at 4 g/L. All formulated media were tested under the same fermentation conditions, which are discussed in the next section. The results were subjected to analysis of variance (ANOVA) followed by Fisher's test to assess the differences among treatments.

Table 1
Components of formulated culture media, with all of them containing potato waste.

Culture medium	Yeast extract (g/L)	Glycerol (g/L)	KH_2PO_4 (mM)	K_2HPO_4 (mM)
APW	–	–	–	–
BPW	12	–	–	–
CPW	–	4	–	–
DPW	–	–	17	72
EPW	12	4	–	–
FPW	12	–	17	72
GPW	–	4	17	72
F8PW	8	–	17	72

2.3. Expression of modified protein

Protein expression was carried out in BL21-CodonPlus(DE3)-RIL *E. coli* cells harboring the pET-AMAR-R5-H plasmid. The formulated media were assessed in 250-mL Erlenmeyer flasks containing 48 mL of culture medium, which were inoculated with 2.5% (*v*/v) preculture and incubated at 200 rpm at 30°C. When OD_{600} reached 0.3–0.4, protein expression was induced by adding 0.3% lactose, and growth was continued. Next, 1-mL samples of each formulated culture were taken at 0, 1.5, 3, 6, and 24 h and harvested by centrifugation at 10,000 × *g* for 10 min at room temperature. The supernatants were discarded, and the cell pellets were stored at -20°C for further processing. All protein expression experiments were performed in triplicate. The results were subjected to ANOVA followed by Fisher's test to assess differences among treatments.

To obtain a greater amount of the AMR5 protein, the fermentation volume was increased to 400 mL of FPW broth in a 2-L Erlenmeyer flask using the same conditions mentioned previously. One milliliter of the sample was taken every hour from 0 to 6 h after the induction for protein analysis. The fermentation was stopped at 6 h, and the culture was harvested by centrifugation at 9000 × *g* for 15 min at 15°C. The supernatant was discarded, and the cell pellet was stored at -20°C for further processing. All samples were subjected to 10% SDS-PAGE [15]. The proteins were visualized with Coomassie Brilliant Blue G-250. Quantitative analysis of the AMR5 accumulation was carried out by densitometry using Image Lab 4.0 (Bio-Rad).

2.4. Solubilization of insoluble modified protein

To dissolve the inclusion bodies of the expressed protein, 0.8 g of cell pellet collected at 6 h was suspended in 7.0 mL of extraction buffer (EB) (0.2 M NaCl, 20 mM phosphate, pH 7.5). Cell disruption was performed using an Omni Sonic Ruptor 400 Ultrasonic Homogenizer (Omni International). Five cycles of sonication for 1 min at amplitude of 60% were applied while keeping the sample on ice for the whole time and then separated by centrifugation at 9000 × *g* for 30 min at 4°C. The supernatant (soluble protein fraction) was discarded, and the pellet was dissolved in 3.5 mL of solubilization buffer (SB) (0.2 M NaCl, 20 mM phosphate, 6 M urea, pH 7.5) by agitation for 4 h at 12°C at 200 rpm. It was then separated by centrifugation at 9000 × *g* for 30 min at 4°C. The supernatants containing the dissolved inclusion bodies were stored at 4°C. The samples were analyzed by SDS-PAGE. The protein concentration was determined by the bicinchoninic acid method using the Pierce BCA Protein Assay (Thermo Fisher Scientific) and BSA (Sigma-Aldrich) as the protein standard.

2.5. Purification of modified protein

The AMR5 protein was purified by affinity chromatography using an AP-2 20 × 300 mm column (Waters) packed with Protino Ni-TED resin (Macherey-Nagel) coupled to a BioLogic DuoFlow™ Chromatography System (Bio-Rad). The supernatant containing the dissolved AMR5 from inclusion bodies was loaded into the column that had previously been equilibrated with SB. The unbound fraction was washed with 110 mL of SB. The bound protein was eluted with SB together with imidazole at a flow rate of 2.5 mL/min at room temperature. Two column volumes of SB together with 5, 25, 50, 125, 250, and 500 mM of imidazole were passed through the column. All fractions collected during the purification process were analyzed by 10% SDS-PAGE and stored at 4°C.

2.6. In vitro refolding of modified protein

The purified AMR5 protein obtained in the 125 mM imidazole fractions was pooled and adjusted to 0.5 mg/mL. A dialysis process was performed with 1 mL of the sample against 50 mL of refolding buffer (RB) (urea, 0.2 M NaCl, 20 mM phosphate, pH 7.5). The urea concentration in RB was reduced from 3.0 M to 1.5 M and then to 0.5 M every 1.5 h. Two additional steps were performed with EB. During the dialysis process, the tubes were collocated on an analogue tube roller (Bibby Scientific) at 4°C. After dialysis, the sample was maintained at 4°C and analyzed immediately.

2.7. Spectrometric analysis

Circular dichroism (CD) spectra were recorded on a Chirascan Spectropolarimeter (Applied Photophysics), which was equipped with a Peltier device to control the temperature. CD spectra were obtained with 0.2 mg/mL of AMR5 protein at 25°C using a quartz cell with a light path of 0.1 cm, wavelength range of 195–260 nm, bandwidth of 2.0 nm, and digital resolution of 0.5 s per point. Five spectra were recorded and averaged for each experimental condition. The data were corrected by subtracting the EB buffer signal, and the results obtained in millidegree were converted to the mean residue ellipticity ($[\theta]_{mrw}$) using the equation defined by Martin and Schilstra [16], $[\theta]mrw = (S * MRW) / (10 * C * L)$, where S is the CD signal in millidegree, MRW is the mean residue weight (molecular weight divided by the number of residues), C is the concentration in mg/mL, and L is the cell path length in cm.

Secondary structure prediction was performed using the DICHROWEB server (University of London, UK) [17]. The intrinsic fluorescence of AMR5 was measured on a PC1 ISS spectrofluorometer (Champaign, IL, USA) equipped with a Peltier device and a water-jacketed cell holder for temperature control. The excitation wavelength was 295 nm, the range of the emission wavelength was 310–410 nm, and the slit width was 2 nm. AMR5 samples at 0.2 mg/mL in EB at 25°C were measured in a quartz cell with a path length of 0.5 cm. The wavelength of maximum emission (λ_{max}) was determined from the fluorescence spectra.

2.8. Thermal and chemical stability analysis

Temperature-induced conformational changes were simultaneously recorded at 208 and 222 nm at a heating rate of 1.5°C/min in the range of 20 to 80°C. After the heating ramp, the sample was cooled to 20°C, and Far-UV CD spectra were obtained to determine the reversibility of the conformational changes. The changes in the CD signal were analyzed by normalizing the transition curve to the fraction of unfolded molecules (f_D) using $f_D = (y_t - y_f) / y_u - yf)$, where y_t is the experimentally observed CD signal at a given temperature, y_f is the spectroscopic signal of the native protein, and y_u is the spectroscopic signal of the unfolded protein.

The equilibrium constant (Keq) was calculated as Keq = fD / fN. The melting point temperature (Tm) was determined when Keq = 1. The estimated change in the enthalpy of unfolding at Tm (ΔH_m) was calculated using the van't Hoff equation, d(In Keq) / d(1/T) = -ΔH/R. The Tm and ΔH_m are apparent values because the protein showed no reversibility after thermal unfolding.

Chemical-induced conformational changes were monitored by intrinsic fluorescence using the AMR5 protein at 0.2 mg/mL (final concentration) and urea concentrations ranging from 0.5 to 7.0 M with 0.5 M intervals. The protein/denaturant solutions were pre-equilibrated for 1 h at 25°C, and then, fluorescence spectra were obtained. The spectral center of mass (SCM) was calculated using $SCM = \Sigma \lambda * I_\lambda / \Sigma I_\lambda$, where λ is the wavelength of emission, and I_λ is the fluorescence intensity at different wavelengths. The estimated unfolding midpoint ($C_{1/2}$) was estimated by assuming a two-state model. The equilibrium constant of reaction (Keq), Gibbs free energy at zero concentration of denaturant (ΔG_{H2O}), and slope (m) were calculated as reported previously [18].

2.9. ACE inhibitory activity

One milligram per milliliter of the refolded AMR5 protein was digested with trypsin and chymotrypsin (E/S = 1/200 (*w*/w)) for 18 h at 37°C adjusted at pH 8. After enzymatic digestion, the sample was boiled to stop the enzyme reaction and centrifuged. The obtained peptides were used to perform the angiotensin-converting enzyme (ACE)-inhibitory activity assay. The antihypertensive property of AMR5 was then measured by a spectrophotometric assay [10]. The samples were prepared in triplicate. The ACE inhibitory activity was calculated as the peptide concentration needed to inhibit 50% of the original ACE activity (IC_{50}).

3. Results and discussion

3.1. Insertion of VY peptides into the A11Sg sequence

The A11Sg protein is classified as an 11S globulin and harbors five hypervariable regions [19,20,21,22]. The fifth variable region, located at the C-terminus, was modified by the insertion of four VY peptides in tandem (Fig. 1a) by site-directed mutagenesis in the pET-AMAR-6His expression vector. The insertion of the antihypertensive peptide was confirmed by performing PCR of the transformed *E. coli* BL21-CodonPlus(DE3)-RIL strain using a specific primer that matches with the sequence codifying four amino acids VY and by sequencing the pET-AMAR-R5-6His plasmid (Fig. S1 a–c). To release the inserted antihypertensive peptides during the digestive process (specifically, in the duodenum by chymotrypsin and trypsin enzymes), one arginine was added followed by four VY peptides after the residue A465 (Fig. 1b). In this way, chymotrypsin cleaves between arginine and valine, whereas trypsin cleaves between tyrosine and valine, thereby releasing the VY peptides (Fig. 1c). A histidine tag is placed after the last VY peptide and before the stop codon.

3.2. Design and optimization of a medium using potato waste

A culture medium was designed to cover the nutritional requirements of *E. coli* to produce recombinant proteins at low cost. Seven culture media were tested, which contained potato waste complemented with one or more of the following ingredients: yeast extract, phosphates, and glycerol (Table 1). The media labeled as BPW, CPW, and DPW were formulated to assess the effect of one component combined with potato waste, and the APW was used as a control.

E. coli growing in the DPW broth containing phosphates showed the highest protein production at 6 h. This may be related to both the nutritional action of phosphate on the metabolism of *E. coli* and a buffer effect that prevents acidification of the medium during

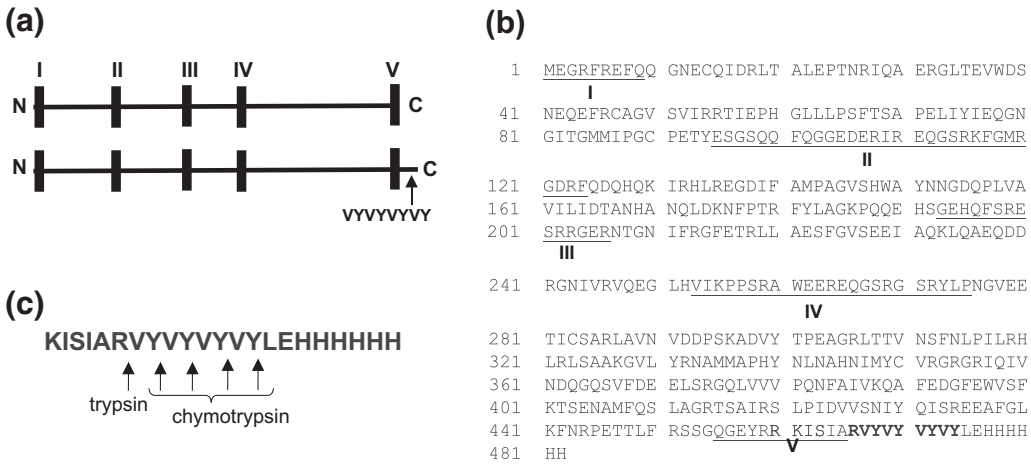

Fig. 1. Schematic representation and amino acid sequence of the modified AMR5 protein. (a) Schematic representation of the insertion of four VY peptides in tandem at the C-terminal end of the amaranth 11S globulin. The roman number represents the variable region. (**b**) Amino acid sequence of AMR5. The underlined letters indicate the variable regions. The bold letters indicate the inserted amino acids. (**c**) Cleavage sites of trypsin and chymotrypsin that release the VY antihypertensive peptides.

fermentation. These two factors improve the utilization of the nitrogen source in the potato waste, whereas this was not possible in the APW. The recombinant protein in the BPW broth was gradually accumulated in *E. coli* from 6 to 24 h, even more than that in the DPW broth, which shows that the protein degradation rate at 24 h is lower. The yeast extract is a nutritive substrate that provides carbohydrates, amino acids, peptides, minerals, and vitamins that contribute to protein synthesis [23]. The protein expression in the CPW broth was very low and similar to that in the APW broth, meaning that glycerol does not have an effect on protein accumulation (Fig. S2 a–d).

The combination of two components and potato waste resulted in significantly higher recombinant protein production in all media after induction for 3 H. *maximum* protein accumulation occurred at 24 h. In the FPW broth, *E. coli* produced the highest amount of recombinant protein, 40% more than that in commercial LB broth, whereas the GPW broth had similar levels as those of the LB broth according to densitometric analysis (Table 2, Fig. S2 f–h). The EPW broth had very low protein expression, which was even less than that of the BPW or DPW broth, which contained only one component (Fig. S2e). This indicates that a combination of phosphates and yeast extract contributes to both the growth of *E. coli* culture and recombinant protein production.

The potato waste in the FPW broth was replaced with distilled water, and the trial with this medium showed that the infusion from potato waste contributes to protein production. The accumulation was two times higher at 6 h after induction in *E. coli* growing in the media with potato waste than that with distilled water (Fig. S3).

The FPW broth was the best-formulated media to obtain high recombinant protein production. Thus, other experiments were performed to optimize the formulation of the FPW broth. *E. coli* expressing the AMR5 protein was grown in the FPW broth using different amounts of yeast extract. The results showed that 8 g/L of yeast extract (F8PW) produced a similar amount of recombinant protein as that obtained using 12 g/L of yeast extract (Fig. S4).

Lactose was used as inducer at a concentration of 0.1%, 0.3%, and 0.5%, which showed that 6 h after induction, the protein expression level with 0.3% lactose is similar to that obtained with 0.5% lactose (Fig. S5). The addition of glycerol to the FPW broth was also tested, but this carbon source did not change the protein accumulation level at 6 and 24 h (Fig. S6). Terrific broth has been used to produce the recombinant A11Sg [13] or its acidic subunit [24], which results in less protein production than that used to produce AMR5 using the F8PW broth. This suggests that the designed medium could be used to produce recombinant proteins while reducing the cost of production. Thus, the F8PW broth was used to produce the AMR5 protein.

3.3. Expression of AMR5 protein

The AMR5 protein expression was carried out in the BL21-CodonPlus (DE3)-RIL strain of *E. coli* harboring the pET-AMAR-R5-6His vector, which was grown in the F8PW broth in a flask. In the 10% SDS-PAGE, one band of size 56 kDa corresponding to the recombinant modified protein was detected at 3 h after induction, and higher protein production occurred

Table 2
Protein expression in *E. coli* using different formulations of potato waste-based culture media.

Culture medium	3 h (mg/L)	6 h (mg/L)	24 h (mg/L)
APW	n/d	n/d	45.0 ± 6.7
BPW	n/d	126.4 ± 27.4	355.9 ± 39.6
CPW	n/d	n/d	46.6 ± 7.3
DPW	117.1 ± 25.3	440.1 ± 34.7	234.1 ± 27.8
EPW	n/d	45.9 ± 7.3	162.9 ± 38.7
FPW	187.3 ± 23.5	655.5 ± 39.8	529.1 ± 35.8
GPW	140.5 ± 29.1	477.6 ± 34.2	426.1 ± 37.6
LB broth	164.8 ± 31.2	458.9 ± 32.1	435.5 ± 39.6

n/d: not detected.

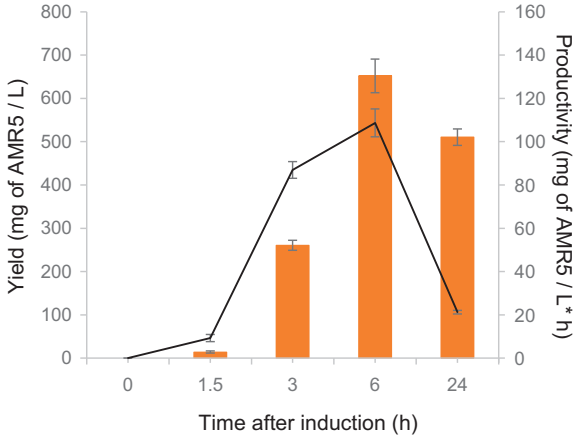

Fig. 2. Yield and productivity of the AMR5 protein expression at the flask level. Each value is the mean of triplicate experiments.

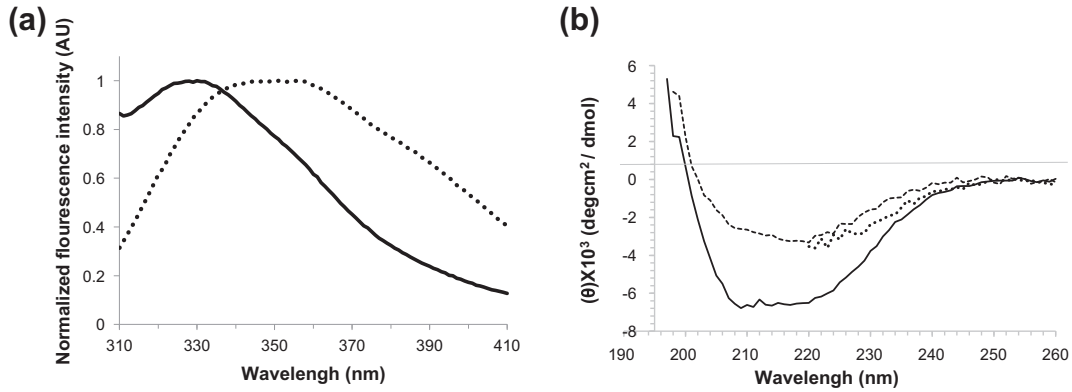

Fig. 3. Espectrometrical analysis of refolded AMR5. (**a**) Normalized fluorescence-emission spectra. Continuous line: *In vitro* refolded protein. Dotted line: unfolded protein in 6 M urea solution. Excitation wavelength: 295 nm. (**b**) Circular dichroism spectra. Solid line: *in vitro* refolded protein at 25°C; dashed line: *in vitro* refolded protein after a heating cycle measured at 25°C; dotted line: 6 M urea-unfolded protein.

at 6 h (Figs S2 and S3). A decrease in the accumulation level was observed at 24 h (Fig. 2). According to the densitometric analysis, the protein expression level at 6 h after induction was 652 mg/L, which is higher than the levels of 76 mg/L achieved with the unmodified recombinant A11Sg [13], 55 mg/L of the unmodified A11Sg acidic subunit (AAC) [25], and 550 mg/L of VY containing the A11Sg acidic subunit [12]. The best productivity was obtained at 6 h, which was higher than that reported for AAC and the VY-containing A11Sg acidic subunit [11,12,24,26]. The higher AMR5 protein production may be due to three factors: the expression strain, the F8PW broth, and that the expressed protein is the whole molecule of amaranth 11S globulin. In the fermentation scaled up to 400 mL, the protein accumulation behavior was similar to this level (Fig. S7).

3.4. Purification of protein aggregates and in vitro refolding

The cells were sonicated to extract the recombinant protein, and the insoluble protein fraction was separated from the soluble protein fraction. SDS-PAGE revealed that recombinant protein was expressed as inclusion bodies (**Fig. S8**). Most reports agree that 11S globulins tend to form inclusion bodies or show low recombinant protein solubility when expressed in the cytoplasm of *E. coli* [13,27,28,29,30]. In this case, the AMR5 insoluble expression may be influenced by both the high translation rate, which leads to misfolding protein, and the reducing environment of the cytoplasm of *E. coli*, which does not readily form disulfide bonds [31,32]. Thus, the insoluble protein fraction was solubilized with 6 M urea and passed through the Ni-TED resin to purify the protein by affinity chromatography. After washing with imidazole, the protein was eluted with 125 mM imidazole. The purified protein (Fig. S8) was refolded by the dialysis method, which recovered 50% of the recombinant protein in the soluble form.

3.5. Spectrometric analysis

Fluorescence spectra were obtained for the AMR5 protein solubilized with 6 M urea and the dialyzed protein, thereby resulting in λ_{max} of 351 and 330 nm, respectively (Fig. 3a). This means that the protein adopted a tertiary structure after *in vitro* refolding, thus suggesting that the tryptophan amino acids are present in a hydrophobic environment. 11S globulin extracted from amaranth seed showed λ_{max} in the range of 337–341 nm [33]. Glycinin (an 11S globulin) extracted from soybean seed showed λ_{max} of 340 nm [34]. This indicates that tryptophan residues in AMR5 are present in a more hydrophobic environment than those in the native 11S globulin extracted from amaranth and soybean.

AMR5 CD spectra showed a positive band around 197 nm, a negative band at 208 nm, and a weak shoulder around 222 nm (Fig. 3b). Secondary structure prediction using the CDSSTR algorithm from the DICHROWEB server estimated 15% α-helix, 31% β-sheet, 12% turns, and 42% unordered structure. This indicates that AMR5 acquired a predominantly β-sheet conformation after *in vitro* refolding. This is similar to the crystal structure of A11Sg expressed in *E. coli*, which has 10.1% α-helix and 30.3% β-sheet conformation [35]. Marcone et al. [36] reported the CD spectra prediction of an amaranth seed globulin fraction containing 11S and 7S proteins, which indicated 8% α-helix, 55% β-sheet, and 14–22% turns. These results show that the salt-soluble

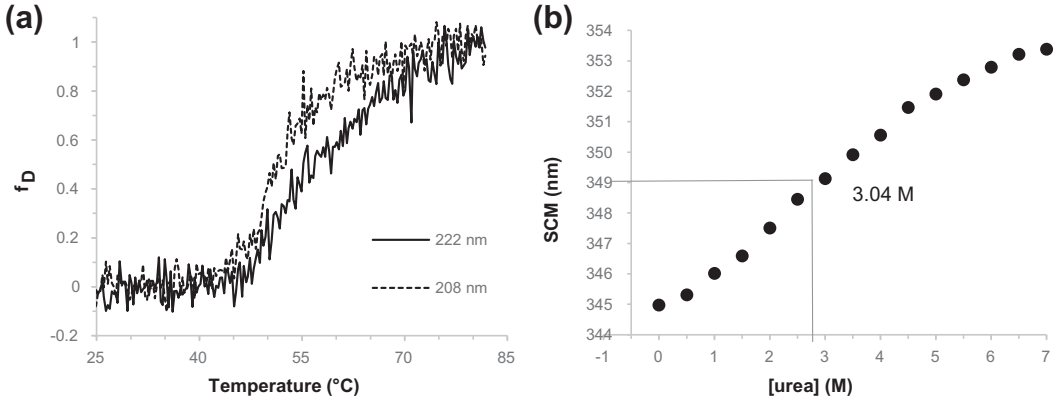

Fig. 4. Thermal and chemical unfolding of AMR5. (**a**) Thermal unfolding followed by CD. Unfolding data were simultaneously followed at 208 and 222 nm, obtaining the apparent Tm of 52.3°C and 56.9°C, respectively. f_D: denatured fraction. (**b**) Urea-induced denaturation profile monitored by fluorescence spectroscopy. Apparent concentration midpoint ($C_{1/2}$) = 3.04 M. Excitation wavelength at 295 nm.

globulins of amaranth seeds are rich in β-sheet structures. Similar data were reported by CD experiments on glycinin from both soybean seeds and expressed in *E. coli* [37]. The refolded AMR5 protein shows a similar trend of forming a structure rich in β-sheet, similar to both native and crystallized recombinant A11Sg. The AMR5 protein can therefore be classified as a β-I protein according to the CD spectra and secondary structure content [38].

3.6. Thermal and chemical unfolding

The temperature-induced unfolding of the AMR5 protein was studied by measuring the change of the CD signal at 208 and 222 nm as the temperature increased from 20 to 80°C (Fig. 4a). The refolded AMR5 spectra were not recovered after heating the sample to 80°C and cooling back to 20°C. In fact, protein aggregation was observed in the CD cell. Therefore, the temperature-induced unfolding of this protein is irreversible.

The unfolding transitions were different when both the apparent Tm (52.3 and 56.9°C at 208 and 222 nm, respectively) and the cooperativity of the transitions were estimated (apparent ΔH_m: 50.9 and 30.1 cal/mol at 208 and 222 nm, respectively). The difference in both parameters ($\Delta Tm = 4.6$°C and $\Delta H = 20.8$ cal/mol) clearly indicates that unfolding is not a two-state process, that is, an intermediate is involved in the unfolding transition.

Previous calorimetric data showed that the Tm of the A11Sg protein was in the range of 95–98°C [9]. Other seed-derived 11S proteins presented a Tm in the range of 87–94°C [39]. The Tm of mature glycinin was 89°C according to CD spectrometry [40]. The Tm of the AMR5 protein is lower than that reported above, which can be explained by the expression of the 11S globulins in *E. coli* producing a trimeric conformation [27,41] that reduces the glycinin thermal stability to 74–78°C [29]. In seed plants that present vacuolar processing, the distinct 11S globulins are accumulated in a hexameric conformation, which has higher thermal stability [42].

Thus, it is likely that both the expression in *E. coli* and the peptide insertion into the C-terminal end could be involved in the reduction in the thermal stability of AMR5. However, AMR5 is more stable than other variants such as AAC and AACM.4 (modified A11Sg acidic subunit with four in-tandem VY peptides in the variable region IV), for which the Tm is 34.0 and 37.2°C, respectively [12]. The basic subunit may contribute to the structural stability of the whole molecule of A11Sg, as in AMR5.

Urea-unfolding experiments carried out in the range of 0.5–7.0 M were monitored by intrinsic fluorescence. We observed a red-shift signal when the urea concentration increased, thus indicating that some of the tryptophan residues are progressively exposed to the solvent. $C_{1/2}$ was 3.04 M, noncooperative unfolding was observed, and the CD spectra of AMR5 with 6 M urea showed a residual structure when compared with the thermal unfolded spectra (Fig. 4b). A similar $C_{1/2}$ value was reported for 11S globulin extracted from amaranth seeds [33]. Nevertheless, the apparent ΔG_{H2O} and *m* values obtained for AMR5 in the present work (1530 cal/mol and 500 cal/mol*M respectively) are half of those reported for globulin-p.

The *m* value obtained for AMR5 was compared with that obtained for the unfolding of other proteins such as pepsinogen (380 amino acids, $m = 7500$ cal/mol $*$ M), and the result clearly shows that the cooperativity in the unfolding of AMR5 is very low. Parametric equations relating the *m* value to the change in accessible surface area upon unfolding (ΔASA) and protein size [43] indicate that transition with an *m* value of 500 cal/mol $*$ M corresponds to a $\Delta ASA \approx 1200$ Å. This value corresponds to the complete unfolding of ≈23 residues in a cooperative way. The insertion of the antihypertensive peptides or a lack of *in vivo* processing toward the hexameric state may be responsible for the decrease in stability and cooperativity.

3.7. ACE inhibitory effect

The ACE IC_{50} value of the digested AMR5 protein was 0.034 mg/mL, which is 17 times higher than that of alcalase-digested amaranth globulins [44], 14 times higher than that of acidic A11Sg, and 1.8 times higher than that of the modified A11Sg acidic subunit with four in-tandem VY peptides in the variable subunit region III (AACM.3) [10]. It is 1.3 times higher than that of the A11Sg acidic subunit modified in variable regions III and IV (AACM3.4) with four in-tandem VY peptides and one IPP, respectively [11]. In AMR5, the number of antihypertensive peptides is same as that of AACM.3 and one less than that of AACM.3.4. However, the IC_{50} value of AMR5 was higher than that of AACM.3.4. This may be caused by the antihypertensive action of the peptides GR [45] and EW [46] that are delivered by trypsin and chymotrypsin during digestion *in vitro* (according to a simulation *in silico*). These peptides are also reported to be ACE inhibitors. Considering that VY peptides were inserted at the C-terminal end of the polypeptide chain, the proteases may have easy access to the cleavage site, thereby releasing the VY peptide units that consequently increase the antihypertensive effect. In the near future, this mutated protein could be considered as a food supplement for its high antihypertensive effect.

In conclusion, the insertion of antihypertensive peptides at the C-terminal of A11Sg changes its structural stability. However, it improves its antihypertensive functionality. The developed F8PW broth is also an excellent culture medium for recombinant protein production with a high level of accumulation that is even higher than that obtained with commercial LB broth.

Financial support

This work was supported by Secretaría de Investigación y Posgrado-IPN, México (20144006 and 20171468) and Consejo Nacional de Ciencia y Tecnología, México (103399 and 256478) (CONACYT).

Conflict of interest

The authors declare no commercial or financial conflict of interest.

Acknowledgments

We thank the Secretaría de Investigación y Posgrado-IPN and Consejo Nacional de Ciencia y Tecnología (CONACYT) for providing scholarship to EEH to obtain his PhD degree. We also thank Tinixcatl-Hernández C. and López y López V. E. for their technical assistance.

Supplementary material

https://doi.org/10.1016/j.ejbt.2018.11.001

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