Research article

Expression, purification and thermal stability evaluation of an engineered amaranth protein expressed in *Escherichia coli*

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ABSTRACT

Background: The acidic subunit of amarantin (AAC)—the predominant amaranth seed storage protein—has functional potential and its third variable region (VR) has been modified with antihypertensive peptides to improve this potential. Here, we modified the C-terminal in the fourth VR of AAC by inserting four VY antihypertensive peptides. This modified protein (AACM.4) was expressed in *Escherichia coli*. In addition, we also recombinantly expressed other derivatives of the amarantin protein. These include: unmodified amarantin acidic subunit (AAC); amarantin acidic subunit modified at the third VR with four VY peptides (AACM.3); and amarantin acidic subunit doubly modified, in the third VR with four VY peptides and in the fourth VR with the RIPP peptide (AACM.3.4).
Results: E. coli BL21-CodonPlus (DE3)-RIL was the most favorable strain for the expression of proteins. After 6 h of induction, it showed the best recombinant protein titer. The AAC and AACM.4 were obtained at higher titers (0.56 g/L) while proteins modified in the third VR showed lower titers: 0.44 g/L and 0.33 g/L for AACM.3 and AACM.3.4, respectively. As these AAC variants were mostly expressed in an insoluble form, we applied a refolding protocol. This made it possible to obtain all proteins in soluble form. Modification of the VR 4 improves the thermal stability of amarantin acidic subunit; AAC manifested melting temperature (T_m) at 34°C and AACM.4 at 37.2°C. The AACM.3 and AACM.3.4 did not show transition curves.
Conclusions: Modifications to the third VR affect the thermal stability of amarantin acidic subunit.

1. Introduction

Hypertension is a worldwide health problem and is a major risk factor for cardiovascular diseases such as heart failure, stroke, peripheral arterial disease and myocardial infarction. Some drugs developed for hypertension treatment cause side effects including the angiotensin converting enzyme (ACE) inhibitors. They inhibit the conversion of angiotensin I to angiotensin II within the renin-angiotensin system. Because of this, several researchers have focused on the study of new alternatives including nutritive compounds that contribute to the treatment of hypertension. These include bioactive peptides that are usually composed of 2–20 amino acid residues but may be larger [1,2,3]. These structures have been isolated from different foods and are usually inactive within the

sequences of parent proteins but can be released by enzymatic hydrolysis during gastrointestinal digestion or during food preparation to subsequently acquire bioactivity [4,5]. Other strategies to obtain these products include the design of antihypertensive peptide multimers or protein engineering. Biopeptides can be introduced into proteins with nutritional or functional importance, and these new structures may be used as components in foods or additives in the formulation of new products [6,7]. An alternative to synthesizing these proteins is through recombinant systems such as *Escherichia coli* because the genetic manipulation is easy, culture is inexpensive, and expression is fast to quickly produce large amounts of protein [8].

In our group, we purified and characterized the amarantin acidic subunit (AAC) by expressing it in *E. coli*. Amarantin is important because of its nutritional value, it has an excellent essential amino acid balance; the AAC is a good candidate for modification in terms of its structure [9], it harbors four variable regions on the molecular surface (Fig. 1). The variable regions have been suggested as suitable targets for modification because they are not important in the structure
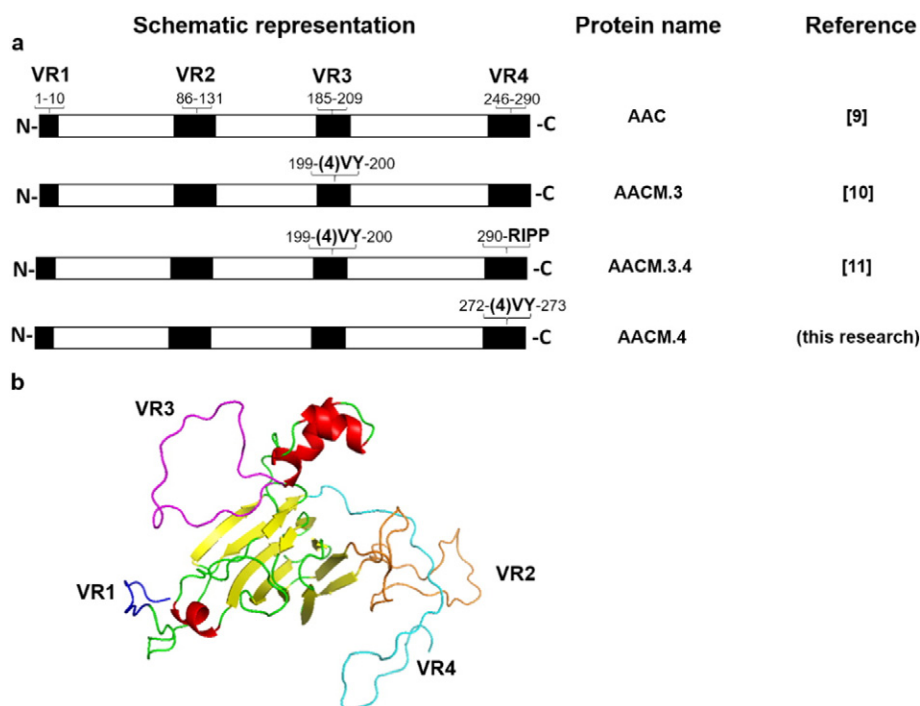
Fig. 1. Schematic representation of AAC variants and putative 3D model. (a) AAC variants previously reported and variant generated in this research (AACM.4). Four variable regions are indicated in AAC scheme by VR1, VR2, VR3 and VR4; residue numbering is based on the PDB structure of amaranth 11S proglobulin seed storage protein from *A. hypochondriacus L.* (3QAC); white and black areas represent conserved and variable regions, respectively. Numbers in the second to fourth schemes indicate insertion in each protein. (b), putative 3D model using as template 11S globulin from amaranth (PDB 3QAC). Variable regions are indicated with VR1, VR2, VR3, and VR4 that correspond with unordered structure exposed in surface.

formation [10]. Thereafter, we generated the AACM.3 protein inserting four Val-Tyr (VY) antihypertensive peptides into the AAC third variable region (VR) to improve its nutraceutical properties [11]. We also generated and overexpressed the doubly modified acidic subunit (AACM.3.4) into the third VR with four VY and a RIPP peptide at the C-terminal [12]. This last protein showed antihypertensive effect both in *in vitro* and *in vivo* trials [13]. The objective of this research was to modify AAC by inserting four VY antihypertensive peptides into the C-terminal to generate the protein AACM.4; and to compare expression levels and thermal stability of the new protein and the above mentioned variants of AAC. This might lead to the identification of a good candidate or model to establish the basis for the production of a functional food additive or to improve the nutraceutical properties in some crops in the near future.

2. Materials and methods

Fig. 1a is a schematic diagram that summarizes the proteins used in this work.

2.1. Homology modeling of AAC

In order to evaluate the spatial positioning of the variable regions, a 3D model was generated using amaranth 11S globulin (PDB 3QAC) as template [14] and RaptorX server [15] which predict secondary and tertiary structures together with disordered regions. Moreover, as VRs in 3QAC are disordered regions, which are not visible in the crystal structure, the model of AAC generated by RaptorX was optimized using ModLoop server (https://modbase.compbio.ucsf.edu/modloop/) which is a software tool used to predict disordered regions by satisfaction of spatial restraints. Then, the resulting homology 3D model suggests that the VRs are exposed on the surface (Fig. 1b).

2.2. Construction of the modified amarantin acidic subunit expression plasmid to express AACM.4

The plasmid pET-ACID-R4-6His that codes for AACM.4 was constructed from pET-AC-6His [9] which contains the cDNA that codifies amarantin acidic subunit. This plasmid is derived from pSPORT-11S, which contains the cDNA that codifies amaranth 11S globulin (GenBank accession no. CAA57633.1) [16]. pET-AC-6His was used as PCR template for carrying out site-directed mutagenesis to insert four VY biopeptides in tandem into the fourth variable region corresponding to the C-terminal region in the AAC. Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) was used to obtain the PCR products of AAC mutated into its fourth variable region. The primers for amplification were: forward 5′-TGGGTG<u>ATTAAT</u>GGAAGG AAGG-3′ (underlined corresponding to the *Ase*I restriction site that enabled cloning into the pET-32b(+) vector). Reverse: 5′-<u>CTCGAG</u> **GTAAACGTAAACGTATACGTAAAC**CCTATTGGGAAGG-3′ (the encoding sequence of the VYVYVYVY peptide inserted in bold letters, with *Xho* I restriction site underlined). This enabled cloning into the pET-32b(+) vector.

Following amplification, the PCR product was cloned into pPCR®2.1-TOPO®. *E. coli* cells harboring recombinant plasmid were selected on LB plates containing 100 μg/mL ampicillin and X-gal. The DNA fragment encoding AACM.4 was released from pPCR®2.1-TOPO vector using *Vsp*I and *Xho*I restriction enzymes. The *Vsp*I restriction site enabled cloning the region of AACM.4 into the ribosome binding site in the pET-32b(+) vector. The split by *Xho*I enabled cloning of AACM.4 onto the frame with the His tag region of the vector. The VspI/XhoI fragment was ligated into plasmid pET-32b(+) and transformed into the TOP 10 cloning host as described by Sambrook et al. [17]. The *E. coli* transformants were selected on LB plates containing 100 μg/mL carbenicillin. The positive clones were confirmed with PCR, restriction analysis and DNA sequencing using both T7 forward and reverse sequencing primers.

2.3. Transformation of expression cells

E. coli, Rosetta 2 and BL21-CodonPlus(DE3)-RIL strains were transformed with pET-AC-6His codifying AAC [9], pET-AC-M1 codifying AACM.3 [11], pET-ACID-R4-6His codifying AACM.4, and pET-AC-M3-6His codifying AACM.3.4 [12] by applying heat shock following the manufacturer's instructions. All used plasmids contained an ampicillin resistance gene (AmpR) it makes the *E. coli* strain ampicillin resistant. Subsequently, successfully transformed cells were selected on LB (Sigma) agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol.

2.4. Inoculum preparation and shake flask fermentation

Transformants expressing AAC and its variants were used to inoculate 5 mL LB broth (Sigma) supplemented with appropriate antibiotics as mentioned previously. Pre-cultures were grown overnight at 37°C on an orbital shaker at 200 rpm. Shake flask fermentations were performed in 250 mL Erlenmeyer flasks containing 42 mL of LB broth. Cultivations were inoculated with 2.5% (v/v) pre-culture. Cultures were shaken at 200 rpm in an orbital incubator at 37°C. Expression of different variants of AAC (in both strains) were initiated when cultures reached 0.5 OD at 600 nm by adding lactose as an inducer at 0.5% (w/v) according to

Morales-Camacho et al. [18]. This process was continued after induction and samples were taken at 0, 1.5, 3, 6, and 24 h to determine the best strain to express all variants of AAC; 1 mL from each culture was transferred to 1.5 mL centrifuge tube. All samples were harvested by centrifugation at $13{,}300 \times g$ for 5 min at room temperature. Supernatants were discarded and cell pellets were stored at -20°C for further analysis.

2.5. Bioreactor fermentation

To increase the production yield of AAC and its variants, the fermenter cultures were performed in a 5 L bioreactor Biostat A (Sartorius) with an 80% working volume as described in Morales-Camacho et al. [18] with some modifications. For each expression experiment, (AAC, AACM.3, AACM.4, and AACM.3.4) batch culture conditions were as follows: culture media, 4 L (200 g/L potato waste, 12 g/L tryptone, 4 g/L glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4); inoculum, 2.5% (v/v); agitation, 340 rpm; aeration, 1 vvm; pH was maintained at 7 by addition of 2 M NaOH or 5 M H_2SO_4; initial temperature, 37°C and subsequent induction temperature was adjusted to 30°C. Protein expression was induced when the culture had an OD_{600} of 0.5 with 0.5% (w/v) lactose. The cells were harvested 6 h after induction by centrifugation $13{,}300 \times g$ for 5 min at room temperature.
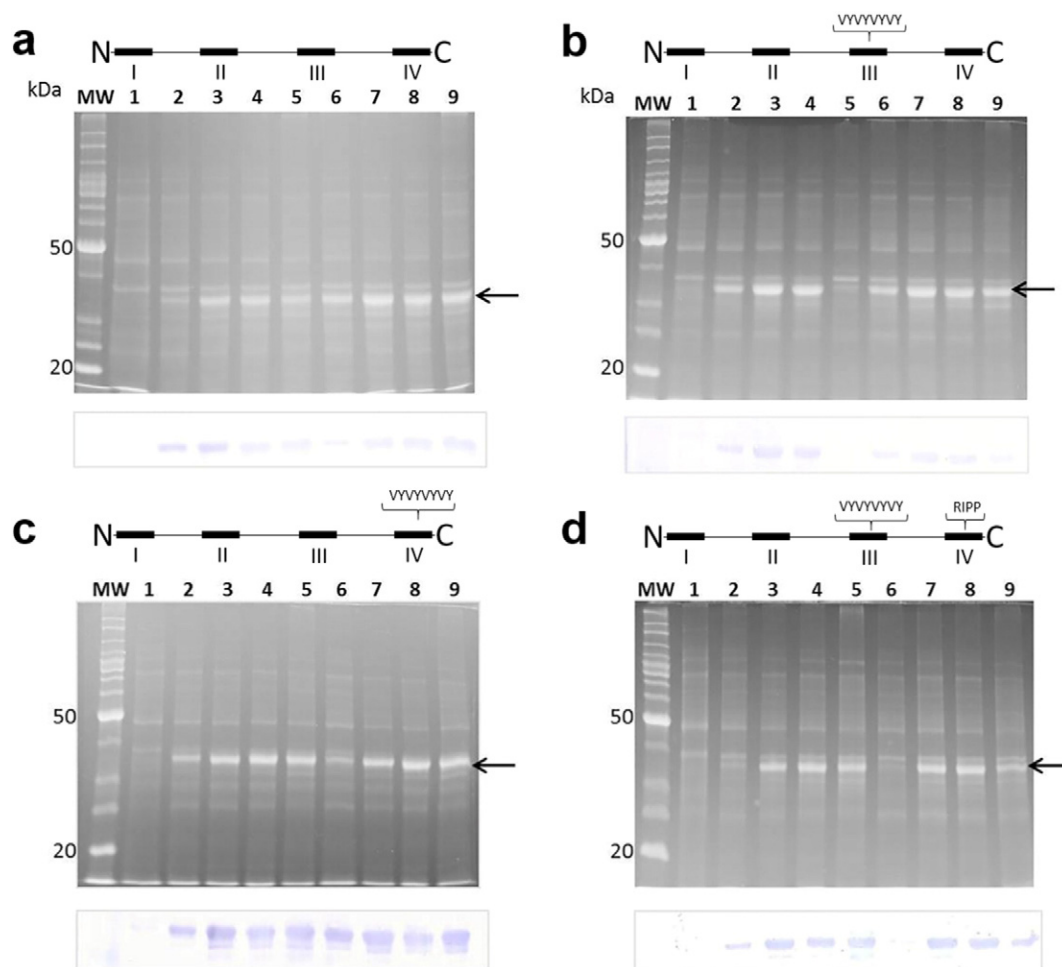

Fig. 2. SDS-PAGE and Western blot of recombinant proteins. (a) AAC protein and schematic representation; (b) AACM.3 protein and schematic representation; (c) AACM.4 and schematic representation; (d) AACM.3.4 protein and schematic representation. The four variable regions are shown with labels I, II, III, and IV. The modification is indicated. Lanes: MW: molecular weight; 1: 0 h of induction; 2: 1.5 h of induction; 3: 3 h of induction; 4: 6 h of induction; 5: 24 h of induction in *E. coli* Rosetta 2; 6: 1.5 h of induction; 7: 3 h of induction; 8: 6 h of induction; 9: 24 h of induction in *E. coli* BL21-CodonPlus(DE3)-RIL. The image below corresponds to Western blot of each recombinant protein. Arrows indicate the presence of AAC and their variants in each SDS-PAGE gels. 5 μg of protein was loaded into each lane.
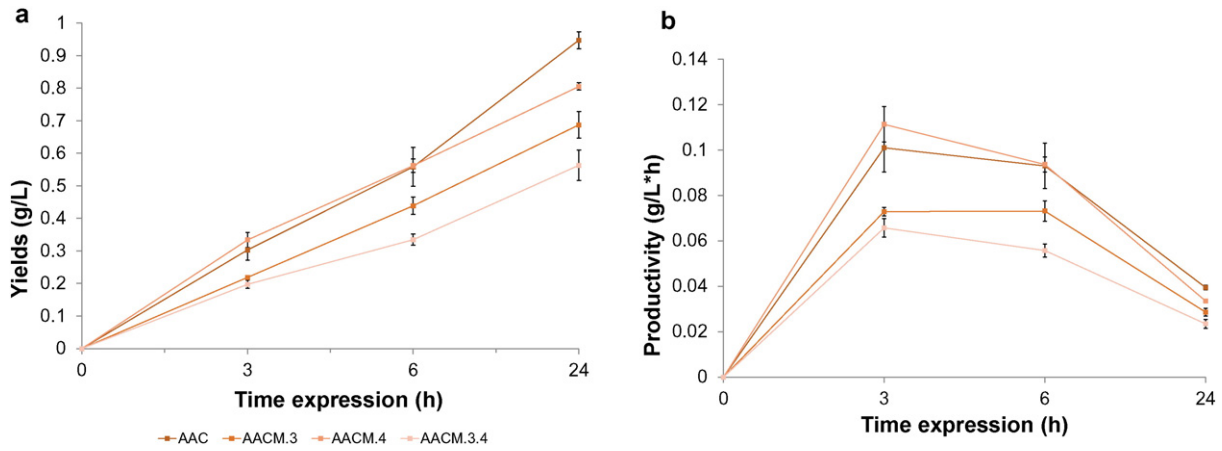
Fig. 3. Expression of recombinant proteins by *E. coli* BL21-CodonPlus(DE3)-RIL. (a) Yields achieved after induction; (b) productivity achieved in recombinant proteins after induction. Each value is the mean of triplicate experiments. ANOVA analysis evaluated significant differences in expression ($P < 0.05$).

2.6. Detection of AAC and its different variants

Cell pellets were resuspended in 0.25 mL of distilled water and 0.25 mL of loading buffer. Samples were analyzed by 12% SDS-PAGE [19] and stained with Coomassie brilliant blue G-250. Western blotting was used to detect the proteins, which were transferred to PVDF membrane using a Mini Trans-Blot cell (Bio-Rad). Membrane was incubated with a polyclonal antiamarantin as first antibody, and anti-rabbit conjugated to alkaline phosphatase as secondary antibody in a dilution 1:60,000 and 1:3,000, respectively [9].

2.7. Protein assay

Protein concentration was determined using BCA (bicinchoninic acid) assay using bovine serum albumin (Sigma) as a protein standard. The concentration of recombinant proteins was determined in cell pellets disrupted in alkaline buffer (0.2 M NaOH, 1% SDS) and by SDS-PAGE (12%) under denaturing and reducing conditions. Gels

were scanned, and the area and intensity of the bands were quantified by densitometric analysis using Image Lab 4.0 (Bio-Rad).

2.8. Experimental design

A simple factorial design for three factors was used to carry out experiments for the expression of four recombinant proteins (AAC, AACM.3, AACM.4, and AACM.3.4) in two *E. coli* strains (Rosetta 2 and BL21-CodonPlus(DE3)-RIL) at different induction times (0, 3, 6, and 24 h). All trials were performed in triplicate.

The statistical software package Statgraphics 16.2.04 (Statistical Graphics Corp) was used to analyze the data. An analysis of variance (ANOVA) was used to assess the best strain to express AAC and the modified variants according to protein titers.

2.9. Analysis of soluble and insoluble fraction of all variants of AAC

Cell disruption was used to determine the fraction in which each variant of AAC was accumulated inside cells. The samples were
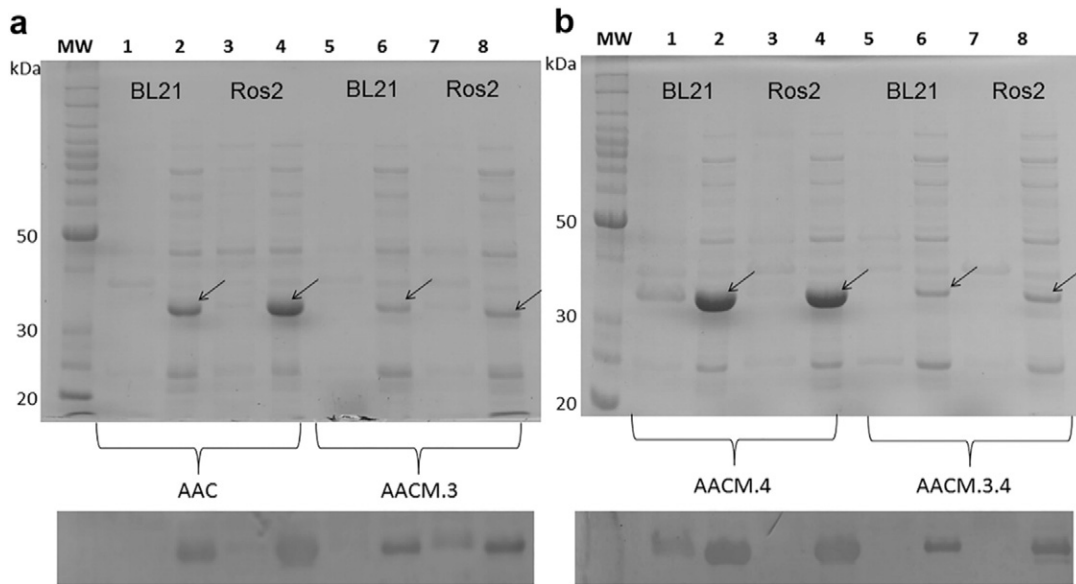

Fig. 4. SDS-PAGE and Western blot of soluble and insoluble protein fraction in AAC, AACM.3, AACM.4, and AACM.3.4. (a) Extraction of AAC and AACM.3 expressed by BL21-CodonPlus(DE3)-RIL and Rosetta 2. (b) Extraction of AACM.4 and AACM.3.4 expressed by BL21-CodonPlus(DE3)-RIL and Rosetta 2. Odd numbers in lanes represent the soluble protein fraction and even lanes are the insoluble protein fraction. Arrows indicate recombinant proteins.

harvested 6 h after induction and were used to induce cell disruption. This treatment was carried out in 5 mL extraction buffer (EB) (0.2 M NaCl, 20 mM phosphates, pH 7.5) applying sonication for 1 min at an amplitude of 60% and then, kept on ice for 3 min. This process was repeated six times. Subsequently, the disrupted cells were fractionated by centrifugation at 15,600 × g for 30 min at 4°C. The supernatants were discarded and the cell debris pellets were resuspended in 10 mL solubilization buffer (SB) (6 M urea, 0.2 M NaCl, 20 mM phosphates, pH 7.5) and shaken at 200 rpm and room temperature for 4 h. Subsequently, the samples were centrifuged at 15,600 × g for 30 min at 4°C to remove insoluble material. The supernatants were recuperated and analyzed by 12% SDS-PAGE and stained with Coomassie brilliant blue G-250 to detect recombinant proteins.

2.10. Purification of AAC and its modified variants

The insoluble crude extract of each variant of AAC was applied to an AP-2 20 × 300 mm column (Waters) packed with Protino Ni-TED resin (Macherey–Nagel) coupled to BioLogic DuoFlow™ Chromatography System (Bio-Rad) and eluted with buffer SB plus imidazole at a flow rate of 2.5 mL/min and at room temperature. Increasing concentrations of imidazole were applied during purification process to evaluate which imidazole concentration eluted each variant of AAC.

Five column volumes of SB plus 5, 25, 50, 125, 250, and 500 mM imidazole were passed through the column. All fractions collected during the purification process were analyzed by SDS-PAGE and stored at 4°C.

2.11. Protein refolding of AAC and its modified variants

The refolding protocol was established to eliminate urea and allow refolding of AAC, ACM.3, ACM.4, and ACM.3.4. First, 0.5 mL at 0.5 and 1 mg/mL for each sample of protein was dialyzed against 50 mL of refolding buffer (4 M urea, 0.2 M NaCl, 20 mM phosphates, pH 7.5) for 1.5 h in an analogue tube roller (Bibby Scientific) at 4°C. This process was repeated to diminish the urea concentration to 3, 1.5, and 0.5 M. Finally, three cycles of dialysis were applied against 50 mL of EB.

Before and after refolding, the samples were assayed by fluorescence (Supplementary Fig. 1).

2.12. Thermal stability using Thermofluor assay

The proteins AAC, AACM.3, AACM.4, and AACM.3.4 were subjected to thermofluor assay [20], implemented using an ABI 7900 Real-Time PCR (Applied Biosystems) system. Sypro Orange (Invitrogen) with emission wavelength at 625 nm was used as a fluorescent dye. Briefly,
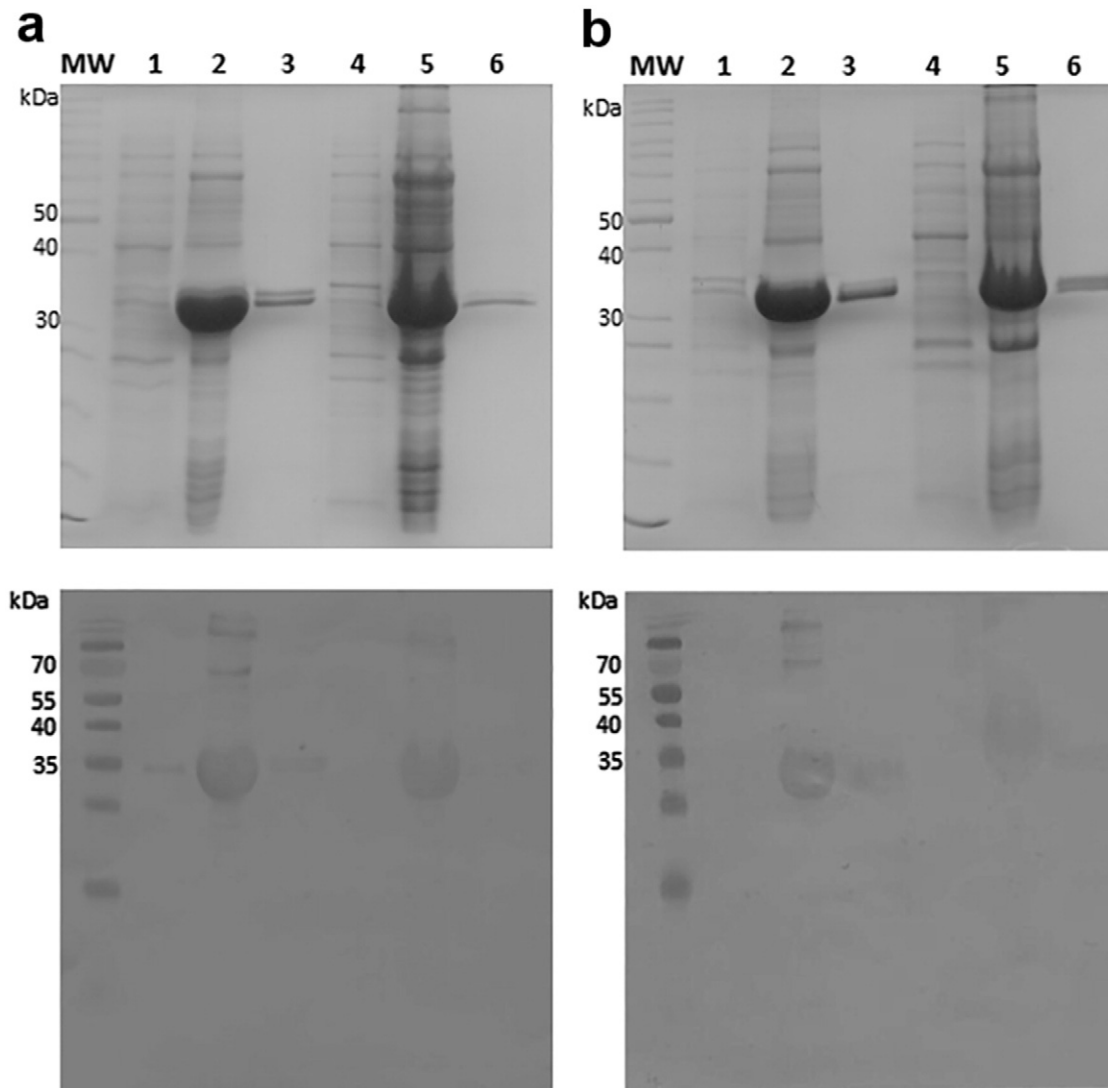

Fig. 5. SDS-PAGE and Western blot of purification and refolding process of recombinant proteins. (a) AAC and AACM.3 proteins; (b) AACM.4 and AACM.3.4 proteins. Lanes: MW: molecular weight; 1 and 4: soluble extract of each protein; 2 and 5: insoluble extract of each protein; 3 and 6: pure proteins after refolding.

20 μL of the sample (final volume) containing 1 mg/mL of the protein and 5× SYPRO orange dye in EB buffer were used. The temperature was increased from 25 to 95°C at a rate of 1°C/min. Equilibrium time was 5 s for each temperature. All experiments were in triplicate, melting curve analysis and determination of melting temperature (T_m) were undertaken using Origin software (version 6.0) (Northampton).

2.13. 2-D electrophoresis

AAC and all modified variants were subjected to isoelectric focusing using 7 cm immobilized strips of 3–10 pH gradient (Bio-Rad) in a Protean i12 IEF system (Bio-Rad) to determine the isoelectric point (pI) of the purified proteins [11].

3. Results and discussion

3.1. Expression and detection of AAC and its variants

Rosetta 2 (Novagen) and BL21-CodonPlus(DE3)-RIL (Stratagene) *E. coli* strains were used to evaluate the expression of the AAC and their modified variants at flask level. SDS-PAGE data showed that the titers of all variants of AAC were different in both strains. There was an accumulative effect in all recombinant proteins for up to 6 h of expression; the presence of these proteins was confirmed by Western blot (Fig. 2). The AAC and AACM.4 were expressed at high titers in both strains at 24 h following induction in both strains (Fig. 2a and c).

Meanwhile AACM.3 and AACM.3.4 were expressed at lower titers (Fig. 2b and d). The AACM.3 was not detected at 24 h in Rosetta 2 (Fig. 2b lane 5). The ANOVA data on densitometric values revealed that the *E. coli* BL21-CodonPlus(DE3)-RIL was better for the expression of all recombinant proteins.

The expression levels of AAC and AACM.4 expressed by BL21-CodonPlus(DE3)-RIL did not show statistically significant differences (SSD) ($P < 0.05$) after 6 h. In contrast, proteins modified in the third VR (AACM.3 and AACM.3.4) did not show SSD after 3 h of expression; at 6 h SSD was clear. At 24 h after induction, all variants showed SSD ($P < 0.05$) (Fig. 3a). According to the results, the level of expression for recombinant proteins can be summarized as:

$$AAC \geq AACM.4 > AACM.3 \geq AACM.3.4 \qquad [\text{Equation 1}]$$

According to titers and productivity (Fig. 3b), AAC and AACM.4 are proteins that manifest greater *in vivo* or kinetic stability than AACM.3 and AACM.3.4. This might be because of modifications in the third VR. The cultures were fractionated to define where the recombinant proteins were accumulated at 6 h after induction. Soluble and insoluble fractions are presented in Fig. 4. The AACM.4 protein expressed by *E. coli* BL21-CodonPlus(DE3)-RIL manifested a higher proportion in soluble form (approximately 5% of total protein titer) than the other proteins. Most recombinant proteins were expressed in insoluble form; the results were similar to those in the literature [9, 11,12].
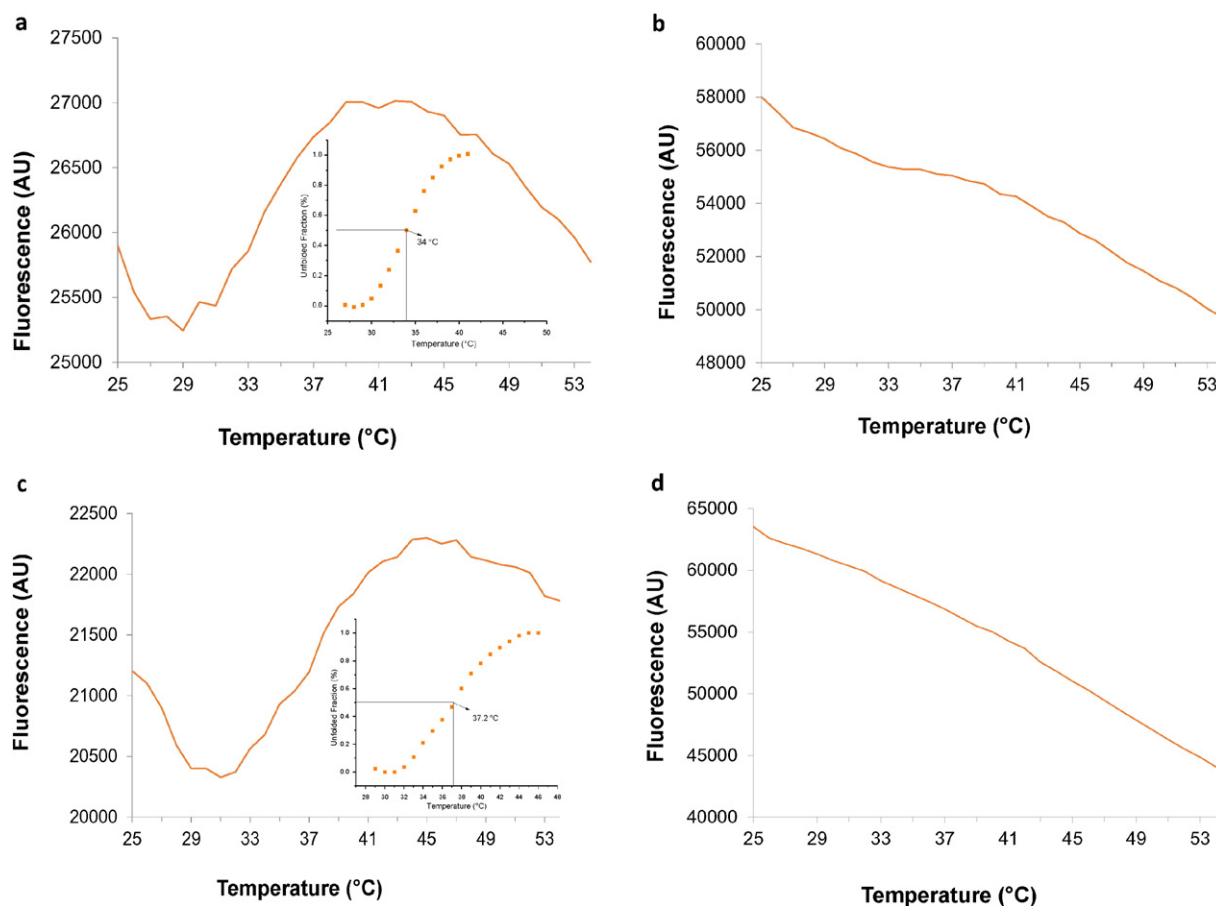

Fig. 6. Different melting curve profiles obtained for each recombinant protein by thermofluor assay. (a) Transition curve of the AAC protein; (b) transition curve of AACM.3 protein; (c) and (d): transition curves of AACM.4 and AACM.3.4 proteins, respectively. The insets in (a) and (c) correspond to the plot of T_m values for AAC (34°C) and AACM.4 (37.2°C). Each value is the mean of triplicate experiments.

3.2. Bioreactor fermentation, purification and refolding of AAC and their modified variants

Expression in *E. coli* BL21-CodonPlus(DE3)-RIL was scaled-up in a bioreactor to increase the production yield for all the recombinant proteins. The titers at 6 h after induction were AAC, 1.6 g/L; AACM.4, 1.55 g/L; AACM.3, 1.37 g/L and AACM.3.4, 1.32 g/L. These results are 3-fold higher than at the flask level because the available oxygen was increased by agitation and aeration. The cells harvested 6 h after induction were disrupted to extract proteins. SDS-PAGE and Western blot data are shown in Fig. 5. Recombinant proteins were expressed as insoluble aggregates, which may be due to an increased translation rate leading to protein misfolding and poor solubility [21]. Furthermore, the insoluble fractions were used to purify the recombinant proteins by IMAC. The AAC eluted at 5 mM imidazole. The AACM.3.4, AACM.3 and AACM.4 eluted at 50 and 125 mM imidazole, respectively. Next, purified proteins were refolded by applying dialysis to eliminate urea. The percentages of refolded proteins according to initial concentration were 20% AAC, 18% AACM.3, 36% AACM.4, and 30% AACM.3.4. These differences may be due to different conformations occurring in recombinant proteins because of modifications with the biopeptides. Fig. 5 shows the SDS-PAGE and Western blot of all refolded pure proteins.

3.3. Thermal stability and 2-D electrophoresis

The T_m provides a quantitative indication of protein stability. A more stable protein will require higher temperatures to unfold [22]. All proteins were subjected to a thermofluor assay in EB, and T_m values were $34.0 \pm 0.11°C$ for AAC and $37.2 \pm 0.2°C$ for AACM.4. This may mean that AAC exposes its hydrophobic regions at lower temperatures than AACM.4. Thus, AACM.4 is a more thermostable protein. By other hand the absence of thermal transition curves in the VR3 mutants may indicate that these proteins are unfolded, but the fluorescence spectra (Supplementary Fig. 1) indicated that they don't have the same maximum emission wavelength after the urea were eliminated, so they are partially folded. Thus, modifications in the third VR may generate conformational changes that only enable solubilization of these proteins by the refolding protocol such that AACM.3 and AACM.3.4 remained as molten globule (Fig. 6). The fact that the molten globules (VR3 mutants) were the lowest expressed by *E. coli*, it's probably related to kinetic or *in vivo* stability.

Tandang-Silvas et al. [14] reported that thermal stability increases by diminishing the loop length, but for AACM.4 the insertion of bioactive peptides into the fourth VR increases the loop. Here, thermal stability is greater than that of the AAC. Perhaps the insertion of bioactive peptides in AACM.4 enhances thermal stability because it generates interactions that stabilize the protein.

The 2-D electrophoresis demonstrated that the pI of AAC is 6, whereas AACM.3, AACM.4, and AACM.3.4 are 6.1, 5.93, and 6.3, respectively. These results are close to those calculated *via* the ProtParam tool.

In conclusion, there are differences in the expression and recovered percentage based on the refolding protocol of recombinant proteins. This may be due to structural modifications due to the insertions done in the molecule. They are also reflected in properties such as thermal stability.

It is well known that mutations by amino acid insertions in secondary structure elements tend to be more susceptible to destabilization of proteins [23]. Nevertheless, in this research variable regions (unstructured regions) were chosen to carry out mutations to decrease this possibility; even so, insertions in third variable region (VR3) destabilize the structure.

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2016.04.001.

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