Electronic Journal of Biotechnology 61 (2023) 45–53

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage: www.elsevier.com/locate/ejbt

Research Article

Improvement of techno-functional properties of acidic subunit from amaranth 11S globulin modified by bioactive peptide insertions

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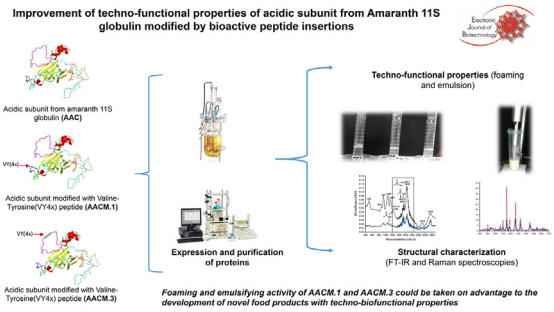
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 April 2022
Accepted 21 November 2022
Available online 25 November 2022

Keywords:
11S globulin
Amaranth
Amarantin
Bioactive peptides
Biopeptide insertions
Conformational changes
Food products
Modified proteins
Protein engineering
Seed
Techno-functional properties

ABSTRACT

Background: Proteins are often used in foods as ingredients to provide desirable appearance, texture, or stability. It is commonly used as gelling, emulsifiers, foaming, and thickeners. An awareness of its properties can be achieved by exploring the relationship between structural conformation adopted by a protein and its techno-functional properties. Here, we analyzed the techno-functional properties of the acidic subunit from amarantin AAC and modified variants AACM.1 and AACM.3 with bioactive peptides (VY 4x) insertion. The proteins were expressed at the fermenter level using *Escherichia coli.* The techno-functional properties were analyzed at different pH and compared with fresh white egg and sodium caseinate. Moreover, the structural characterization of proteins was done through infrared and Raman spectroscopies.

Results: The techno-functional properties of all proteins studied were better at pH 7.5. Solubility and emulsifying activity of AAC and AACM.3 were similar to sodium caseinate, at pH 5. AACM.3 showed similar foaming stability to the white egg, while at pH 7.5, both modified proteins showed higher foaming stability and were equivalent to the white egg. Spectroscopic techniques showed conformational differences between AAC and modified variants. AACM.3 showed higher unordered secondary content (59.6%) followed by AACM.1 (39.6%), suggesting AACM.3 adopted a structure as a molten globule by modifications done, which enhance its techno-functional properties.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.
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https://doi.org/10.1016/j.ejbt.2022.11.003

Conclusions: The biopeptide insertions promote conformational changes, such as structure molten globule (AACM.3) and compact structure (AACM.1). In both cases, the techno-functional properties were improved, it could be an advantage focusing on plant protein ingredient development to novel food product creation.
How to cite: Cruz-Morán Y, Morales-Camacho JI, Delgado-Macuil R, et al. Improvement of techno-functional properties of acidic subunit from amaranth 11S globulin modified by bioactive peptide insertions. Electron J Biotechnol 2022;61. https://doi.org/10.1016/j.ejbt.2022.11.003.

1. Introduction

Proteins from foods are the second most important macronutrient, after animal protein, which is used as a nutrient and by industrial applications for foodstuffs, however, its production requires an intensive amount of resources; an alternative for this is the use of protein from plant species because they have a good balance of amino acids, lower cost of production, and have techno-functional properties for its use [1,2].

Techno-functional properties of proteins from plants have been widely recognized, these properties affect the physicochemical characteristics of food during processing, storage, and consumption [3]. Among the most relevant techno-functional properties stand out: solubility, emulsifying, foaming, gelling, and others. Globular proteins can act as additive agents due to non-polar regions on their surfaces that promote adsorption to oil–water or air–water interfaces, which allow use as emulsifiers or foaming agents, respectively. In addition, depending on the type of globular protein used, techno-functional properties may vary. Some globulins from seeds have higher emulsifying properties than albumins due to the structure of the polypeptide chains as molecular flexibility and hydrophobicity. Also, techno-functional properties are influenced by process conditions such as temperature, pH, solvent for extraction, ionic strength, and others; which influence to undergo dissociation, unfolding, and aggregation limiting protein solubility [2,4].

The solubility is an important property, if a protein remains soluble in a solvent, it carries out other techno-functional properties. Moreover, different researches have demonstrated that techno-functional properties are the result of structural conformation adopted by proteins. Abaee et al. [5] reported that a greater disulfide bridges and hydrophobic interactions into globulins from whey the hardness of gels obtained is improved. Proteins from amaranth have demonstrated the foaming capacity similar to caseins [6]. In addition, it has been reported that globulins extracted from amaranth show emulsifying and gelling properties [7,8,9,10].

Proteins from amaranth have techno-functional properties [11]. In this context, amarantin is an 11S globulin protein from amaranth seed, this protein has been widely studied because it is the majority globulin protein in the seed and it has an excellent amino acid balance. It has a molecular mass of around 398 kDa (as hexamer form), and each monomer is composed of acidic and basic subunits with a molecular mass of around 32–34 kDa and 22–24 kDa, respectively [7]. In our group, the acidic subunit of amarantin (AAC) was modified with VY (Valine-Tyrosine) (4x) dipeptide; modifications were done through protein engineering as a strategy to produce a therapeutic product. The insertions of dipeptides were made into variable regions of AAC, then AACM.1 (modified into variable region I at sites M1-E2) and AACM.3 (modified into variable region III at sites R199-E200). The proteins were produced using *Escherichia coli* as the expression system, and both proteins showed an antihypertensive effect by the inhibition of angiotensin-converting enzyme [12].

The aim of this research was to analyze the techno-functional properties like solubility, emulsifying, and foaming capacity of AAC protein and modified proteins AACM.1 and AACM.3 at different pH conditions (5 or 7.5). So, in this study, AAC and the modified proteins were expressed at the fermenter level using *Escherichia coli*. Moreover, structural characterization of proteins was done through infrared (FT-IR) and Raman spectroscopies in an attempt to understand the structure adopted by proteins associated with their techno-functional properties.

2. Materials and methods

2.1. Expression and purification of proteins

The pET-AC-6His plasmid was used to express AAC protein [13]. The pET-ACM1-6His and pET-AC-M3-6His plasmids were used to express AACM.1 (modified with four VY (4x) at position M1-E2) and AACM.3 (modified with four VY (4x) at position R199-E200), respectively [12,14]. *E. coli* BL21-CodonPlus (DE3)-RIL (Agilent, Santa Clara, CA, USA) was used for the expression of the recombinant proteins. The production of AAC, AACM.1, and AACM.3 were carried out at fermenter level using a 5 L bioreactor Biostat A (Sartorius, Germany) with an 80% working volume as was described by Morales-Camacho et al. [15]. Batch culture conditions for each recombinant protein were as follows: media volume, 4 L (200 g/L potato waste, 10 g/L yeast extract, 4 g/L glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol; inoculum, 5% (by volume); agitation, 200 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 the subsequent induction temperature was adjusted to 30°C. Protein expression was induced when the culture had an OD600 of 0.3 with 0.5% (*w*/*v*) lactose. The cells were harvested 6 h after the induction by centrifugation (Multifuge X3, Thermo Scientific, Madrid, Spain) 13,300 × *g* for 5 min at room temperature.

Purification was carried out as reported previously [12]. Briefly, a BioLogic DuoFlow™ Chromatography System (Bio-Rad, Hercules, CA, USA), and an AP-2 20 × 300 mm column (Waters, Milford, MA, USA) packed with Protino Ni-TED resin (Macherey-Nagel, Düren, Germany) were used. The insoluble crude extract of each recombinant protein was injected into the column and eluted with solubilization buffer (SB) (6 M urea, 0.2 M NaCl, 20 mM phosphates, pH 7.5) plus imidazole at a flow rate of 1 mL/min at room temperature. A 3- to 5-column volume of SB plus 5, 125, 250, and 500 mM imidazole was passed through the column. The collected fractions were analyzed by SDS-PAGE (12%) and stained with Coomassie brilliant blue G-250 [16]. Electrophoresis was performed using a mini-Protean Tetracell (Bio-Rad, Hercules, CA, USA) at constant voltage (100 V) for 90 min., as protein molecular weight marker was used, PageRuler™ unstained broad range protein ladder Thermofisher™, and Blu10 Plus (BLUltra) prestained protein ladder for the western blot. Finally, a refolding protocol was applied to eliminate urea and allow refolding of each recombinant protein through the dialysis process as follows: first, 2 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 analog SRT9D tube roller (Bibby Scientific, Stone,

Staffordshire, UK) 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 distilled water and freeze-dried.

2.2. Protein assay and detection of proteins

The bicinchoninic assay (BCA, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the protein concentration, and bovine serum albumin (BSA) was used as the standard protein. Western blotting was used to detect AAC and their modified variants which were transferred from SDS-PAGE gels to PVDF membranes using a Mini Trans-Blot cell (Bio-Rad, Hercules, CA, USA); membranes were incubated with a polyclonal antiamarantin antibody (dilution 1:60,000) and anti-rabbit conjugated to alkaline phosphatase (dilution 1:3,000) as first and secondary antibodies, respectively.

2.3. Techno-functional property analysis

2.3.1. Protein solubility

The solubility of AAC, AACM.1, and AACM.3 was determined at pH 5 and pH 7.5 as reported by Maldonado-Torres et al. [17] with some modifications. Briefly, each purified protein was dissolved in 2 mL of solubility buffer (SB) (ionic strength i = 0.1, 20 mM phosphates, pH 5 or pH 7.5) to reach a concentration of 0.1% of protein (*w/v*). Each sample was stirred in a vortex mixer (VX-200, Labnet) at minimal speed for 30 min at room temperature (23°C) and centrifuged at 10,000 × g for 2 min. The protein content in the supernatant was determined using BCA assay and was expressed as the percentage of soluble protein that remained in suspension.

2.3.2. Emulsifying properties

Emulsifying activity and emulsifying stability were determined as reported by Nunes et al. [18] with some modifications. Briefly, a blend of 0.1% of protein solution in SB at pH 5 or pH 7.5 for each protein (AAC, AACM.1, and AACM.3) was prepared. Emulsions were prepared by sonicating 9 mL of protein solution and 6 mL of corn oil at an amplitude of 50% for 0.5 min using an ultrasonic homogenizer Omni Sonic Ruptor 400 (OMNI International, Bedford, NH, USA). An aliquot of 5 mL was recovered and centrifuged at 2300 × g for 2 min at 23°C. Emulsifying activity (EmA) was calculated using **Equation 1**.

$$\text{EmA}\ \ (\%) = \frac{\text{ELV}}{\text{TV}} \times 100 \qquad \text{Equation1}$$

where ELV is emulsified layer volume measured immediately after the centrifugation, and TV is the total volume.

Emulsifying stability was measured following the report by Ferreyra et al. [19], and Nunes et al. [18] with some modifications, using samples prepared as described above. The aliquots (5 mL) were heated at 80°C for 30 min and then cooled at room temperature in a bath with cool water. Finally, all samples were centrifuged at 2300 × g for 2 min at 23°C. Emulsifying stability (EmS) was calculated using **Equation 2**.

$$\text{EmS}\ \ (\%) = \frac{\text{REL}}{\text{TV}} \times 100 \qquad \text{Equation2}$$

where REL is the volume of the remaining emulsion layer and TV is the total volume.

2.3.3. Foam properties

Foam activity and foam stability of AAC, AACM.1, and AACM.3 proteins were determined using the sample of each protein (1 mg/mL) on SB, 5 mL of each sample was sonicated in graduated cylinders at an amplitude of 50% for 30 s at 23°C, and all trials were done at pH 5 or 7.5. Foam capacity was determined using **Equation 3.**

$$\text{FC}\ \ (\%) = \frac{\text{TVF}}{\text{IV}} \times 100 \qquad \text{Equation3}$$

where, TVF is the total volume of liquid plus foam (after sonication), and IV is the initial volume in the liquid phase.

To evaluate the foam stability, the samples sonicated were maintained at room temperature (23°C) for 30 min. The foam stability in each sample was determined using **Equation 4**.

$$\text{FS}\ \ (\%) = \frac{\text{TVFf}}{\text{TVFi}} \times 100 \qquad \text{Equation4}$$

where, TVFi is the total volume of liquid plus foam at beginning of the assay, and TVFf is the total volume of liquid plus foam remaining after 30 min of the sonication process.

In all assays of techno-functional properties, egg white (fresh) and sodium caseinate (Sigma, St. Louis, MO, USA) were used as controls.

2.4. Fourier transform infrared spectroscopy (FT-IR)

The infrared spectra of proteins were obtained by using a Fourier transform infrared (FT-IR) spectrometer, Bruker Vertex 70v (Bremen, Germany) equipped with an attenuated total reflectance (ATR) accessory. Spectral measurements were recorded in the wavenumber range between 400 and 2500 cm^{-1} with a resolution of 4 cm^{-1}. Spectrum acquisition of each sample was repeated three times using 2 mg/mL of each protein dissolved in SB at pH 5 or 7.5 using 180 scans for each protein. The secondary structure content of proteins was obtained through the second derivative analysis of the amide band ranging from 1600 to 1700 cm^{-1} as reported by Szefczyk et al. [20].

2.5. Raman spectroscopy

Raman spectra of proteins were recorded on a DXR SmartRaman (Thermo Fisher Scientific, Waltham, MA, USA) over the spectral range 800–1600 cm^{-1}, using a diode laser as an excitation source at 785 nm with a power of 10 mW at room temperature, the spectra were obtained at 4 cm^{-1} resolution and 100 scans for each protein. Each protein (0.04 mg) was spotted on monocrystalline silicon substrates allowing drying at room temperature.

2.6. Statistical analysis

All experiments were performed in triplicate. The results reported are presented as mean ± standard deviation (SD). ANOVA analysis and Tukey tests were conducted for mean comparison, and a p-value at 0.05 was considered for significant difference between the results obtained. The software package GraphPad Prism 7 was used to analyze the experimental data.

3. Results and discussion

3.1. Protein expression: Detection and purification

As previously reported, all proteins were expressed majority as insoluble form, and then, each protein was purified through solubilizing buffer using 6 M urea. The titers obtained for each protein were very similar to those previously reported [12]. The AAC is the acidic subunit of 11S globulin from amaranth which in primary structure contains three Cys residues, also the modified proteins (AACM.1 and AACM.3) have the same amino acid residues [12]. Using IMAC chromatography was possible to purify AAC, AACM.1,

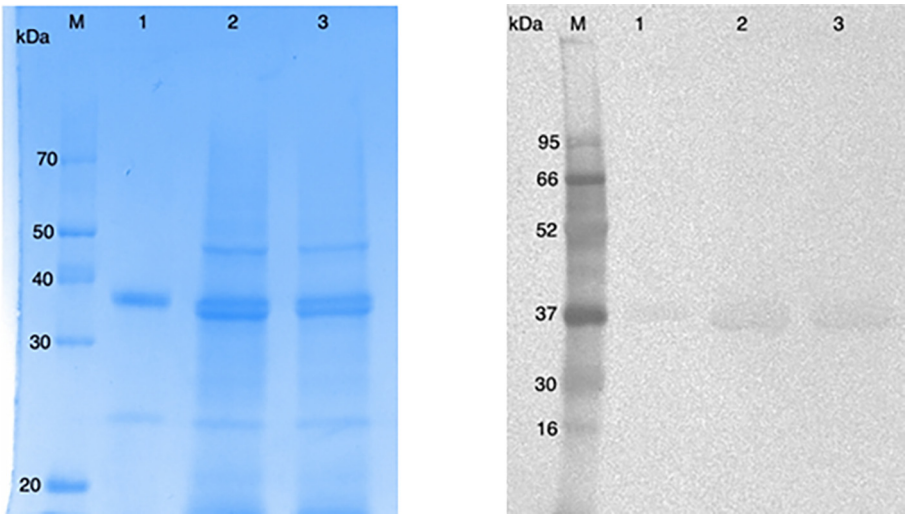

Fig. 1. Purification and detection of AACM.1, AAC, and AACM.3 proteins. **(a)** SDS-PAGE; **(b)** Western blot. Lanes: M: molecular weight marker; 1: AAC; 2: AACM.1; 3: AACM.3. Approximately 10 ug of recombinant protein were loaded into each lane. Molecular weight marker used in SDS-PAGE was PageRulerTM Broad Range Unstained Protein Ladder 26630 and in the Western blot was used Blu10 Plus (BLUltra) Prestained Protein Ladder.

and AACM.3 proteins with an acceptable-level purity, as can be seen in Fig. 1.

3.2. Techno-functional properties

Solubility is the most important techno-functional property of proteins because it affects other techno-functional properties limiting its incorporation into foods [21]. Purified proteins AAC, AACM.1, and AACM.3 were dissolved in SB buffer for techno-functional analysis. Techno-functional properties were done at pH 5 and 7.5, which are typical conditions for different foodstuffs, such as milk and dairy products, bakery products, meats products, and others [22]. All recombinant proteins showed lower solubility at pH 5, while at pH 7.5, AACM.3 showed higher solubility (93.07%), followed by AACM.1 (25.21%), and AAC protein (3.4%) (Table 1). Solubility of proteins is influenced by different factors such as pH, ionic strength of the solvent, and temperature; these factors modify the structure adopted which is positively influenced by polar groups exposed. Solubility at pH 5 of AAC, AACM.1, and AACM.3 proteins was lower because the denatured state of proteins was promoted by closeness with the isoelectric point, which was around at pH 6, which is according with reported for amaranth protein treated by pH shifting and ultrasound, which at pH 5 showed lower solubility (26.1%) and at pH 7 and 9, solubility was increased (33.3 and 55.7, respectively), and this behavior was attributed to the proximity to the pI (Isoelectric point) [23]. The results about solubility at pH 7.5 are given by differences in structural conformation adopted by each protein; AACM.3 protein could have adopted a structure as molten globule and, in this way, their polar groups could be exposed, which promotes more interaction with the solvent; by another hand, AACM.1 and AAC proteins adopted more closed structural conformations with a lower content of polar groups exposed [3,24,25]. Different studies reported that globulins from different amaranth cultivars have shown lower solubility at pH 5 (≈5–9.6%) and it increased by either an increase or decrease of the pH value (10 to 15 times higher) [26]. So, AAC and their modified versions maintain the solubility of wild-type globulin fraction.

Emulsifying activity is a techno-functional property of materials to keep stable food systems formulated by immiscible components (oil–water) like dressing, creams, margarines, and others. Assays for emulsifying activity and emulsion stability showed better results for recombinant proteins at pH 7.5; AACM.1 and AACM.3 proteins showed significant differences ($p < 0.05$) between pH 5 and pH 7.5 in contrast with AAC protein which not showed significant difference ($p < 0.05$) between pH 5 and pH 7.5. White egg and caseinate were used as controls in order to reach truly comparable data with proteins commonly used in industry, caseinate, and white egg showed considerably higher values (around 3 times) than AACM.1 and AACM.3 at pH 7.5. This relatively poor emulsifying capacity also has been reported previously [6], in which casein showed a higher emulsifying capacity in comparison to albumin and globulin isolates from amaranth. Also, the results observed for AAC and their modified version at pH 5 were similar to that reported for proteins isolated and obtained by isoelectric precipitation from six amaranth cultivars that showed the lowest emulsifying activity at pH 5 because it was around the isoelectric region [27]. Emulsion activity values (20–26%) showed by different legume flours (soybean, lentil, pea, and others) rich in albumins and globulins are higher than AAC and their modified versions, while emulsion activity showed by AAC and their modified versions is similar to reported for cereal flours (wheat, oat, and barley) rich in prolamins and albumins showed 5–17% [28]. In both cases (legume and cereal flours), the protein content in slurries was between 4 and 13.5 times higher than AAC and their modified versions. Also, the results obtained are in accordance with the emulsifying activity of four protein isolates from amaranth reported by Das et al. [29], who observed lower activity around pH 4–5 and better emulsifying capacity when the pH of mixture was shifted towards the extreme of acidity and alkalinity (pH 3 and 9, respectively).

The results showed that AACM.1 and AACM.3 were better emulsifiers than AAC, maybe because differences in conformation adopted by modified proteins increase the surface hydrophobicity, which exposes hydrophobic amino acids to the surface and these establish non-covalent interactions within the oil phase while hydrophilic amino acids establish non-covalent interactions within the aqueous phase [30]. The emulsifying activity of AACM.1 and AACM.3 is relevant because it is the result of pure proteins, unlike other studies in which protein isolates were used; Nunes et al. [18] reported 54.8% of emulsifying activity in protein isolate from Baru nut which according to its electrophoretic profile was composed by five proteins ranging from 46.6 to 73.4 kDa.

There has been reported that once the emulsions are formed using proteins, they tend to decrease due to changes in the protein

Table 1
Techno-functional analysis of AAC, AACM.1, and AACM.3 proteins.

pH	Protein	Solubility (%)	Emulsifying activity (%)	Emulsion stability (%)	Foaming activity (%)	Foam stability (%)
5	AAC	1.36 ± 2.36^{b}	4.45 ± 1.44^{b}	53.72 ± 5.78^{a}	126.67 ± 11.54^{c}	<1%^{c}
	AACM.1	<0.05%^{c}	5.48 ± 0.76^{b}	55.23 ± 8.71^{a}	146.67 ± 11.54$^{a, c}$	72.22 ± 25.45^{a}
	AACM.3	1.46 ± 1.93^{b}	4.83 ± 0.28^{b}	50.97 ± 4.40^{a}	180.00 ± 20.00$^{a, b}$	93.33 ± 11.54$^{a, b}$
	Caseinate	0.10 ± 0.01^{b}	1.54 ± 0.42^{a}	11.32 ± 4.99^{c}	153.33 ± 11.54^{a}	<1%^{c}
	White egg	92.72 ± 3.49^{a}	36.67 ± 2.88^{d}	36.61 ± 5.85^{b}	190.00 ± 10.00$^{b, d}$	92.96 ± 6.11^{a}
7.5	AAC	3.40 ± 0.64^{d}	5.35 ± 0.42^{b}	43.50 ± 9.41$^{a, b}$	206.67 ± 23.09$^{b, e}$	100.00 ± 0.003^{b}
	AACM.1	25.21 ± 0.37^{c}	16.66 ± 3.63^{c}	73.61 ± 6.65^{e}	200.00 ± 40.0$^{a, b, d, e}$	84.13 ± 16.72$^{a, b}$
	AACM.3	93.07 ± 4.96^{a}	18.33 ± 2.40^{c}	80.16 ± 8.60$^{d, e}$	230.00 ± 26.45$^{e, d}$	91.07 ± 7.78^{a}
	Caseinate	52.19 ± 3.98^{b}	55.00 ± 2.00^{e}	90.91 ± 4.00^{d}	253.33 ± 15.27^{d}	39.98 ± 5.77^{c}
	White egg	100.61 ± 6.44^{a}	55.00 ± 3.00^{e}	92.93 ± 8.61^{d}	236.67 ± 11.54$^{d, e}$	83.33 ± 14.86^{a}

The measurements were performed by triplicate; results were reported as mean value and ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$). Casein and white egg were used as controls.

conformation adopted, which drive to phase separation, and the emulsion system decreases over time because proteins form non-covalent bonds by protein–protein interactions and protein-solvent interactions (hydrogen, hydrophobic, and electrostatic bonds), due to the conformational changes which decrease emulsion stability. Therefore, the conformational structure adopted by AACM.1 and AACM.3 proteins allows similar emulsion stability. This suggests that modifications by peptide insertions done promote favorable interactions for emulsion stabilization. It is interesting to note that the emulsion stability of AAC and their modified variants was better than caseinate and white egg controls at pH 5, similar results to those reported for protein isolates extracted from Yellowfin tuna showed higher emulsion stability from pH 2 to pH 10 [31]. The emulsifying capacity of AAC at pH 7 was similar to values reported for isolates from Baru (53.9%) and soybean (47.5%) [18], while these values were lower than AACM.1 and AACM.3 (73.61 and 80.16%, respectively).

Foam activity is a property required to consider a protein as a food ingredient. Table 1 shows the results obtained about foam properties; AAC and the variants showed good foam activity in spite of the low solubility, perhaps the sonication that was applied to obtain the foam had a great effect so that the protein dissolved and could interact with the aqueous phase and the air. AAC does not show foam stability, different from the modified variants at pH 5. The results obtained for AAC, AACM.1, and AACM.3 were according to different studies in which the foam properties of protein isolates from amaranth have been reported [6,32]. As can be seen in Table 1, foaming activity and foam stability for AAC and their modified variants at pH 7.5 were better (200–230%) than that reported by Das et al. [29] who analyzed foam activity of protein isolated from amaranth obtained by extraction at alkaline pH (9–12) and pH treatments (3 to 9), and they reported higher foam activity at pH 9 extraction ranging from 66.15 to 86.18%. However, the results for foam stability were similar to those reported in this study for AAC and their modified variants (84.1–100%). Also, foaming activity and foam stability showed by AAC and their modified variants were higher than reported for protein isolates from quinoa seed, which showed 58.37% for foaming activity and 34.83% for foam stability [33]. In addition, similar results were reported for the foaming activity of soybean, pea, lentil, and beans flours ranging from 166 to 203%; although for same materials, their foaming stability were lower ranging from 72.9 to 83.5% [28].

The foaming activity for AAC, AACM.1, and AACM.3 is a consequence of the protein conformation, AAC is the acidic subunit of 11S globulin from amaranth and its molecular weight is lower than protein isolates obtained in other studies. So, these modifications allowed to AAC undergo rapid conformational change and rearrangement at the interface during bubbling but its rearrangements were not sufficient to maintain the foam (at pH 5). The insertions of biopeptides (VY 4x) done in AACM.1 and AACM.3 allowed higher foaming stability at pH 5, namely AACM.1 and AACM.3 undergo rapid conformational changes and rearrangements which allow them to do interactions at the interface air–water maintaining a cohesive film, increasing foam stability. Then, AACM.1 and AACM.3 proteins could be used as foaming additives because the results obtained were similar to casein and ovalbumin controls. Finally, AACM.3 protein showed better results in both pH conditions.

3.3. Spectroscopic characterization

FT-IR spectroscopy was employed to analyze the main bands of AAC and modified variants in order to observe the structural differences at pH 5 and pH 7.5. In Fig. 2 are highlighted with a solid line the regions of the spectra with the most important for the structural study of proteins (1200–1850 cm^{-1}), as can be seen, the spectra of AAC protein showed more intensity at pH 5 while for modified proteins, the spectra pattern is very similar. The associated bands for amide I, amide II, and amide III are around 1636, 1558, and 1234 cm^{-1}, respectively (Fig. 2a). The spectral behavior of AAC, AACM.1, and AACM.3 is similar at pH 7.5, but now, a shift was present, the amide I band is centered around 1653 cm^{-1} (Fig. 2b), the band associated with amide II is around 1549 cm^{-1}, and the band for amide III is at 1246 cm^{-1}.

In Table 2 are listed the bands observed in the FT-IR spectra of AAC and their modified variants, the band assignments shown in Table 2 were based on the literature. As can be seen in Table 2, the maximum values of bands observed for AAC, AACM.1, and AACM.3 proteins at pH 5 and pH 7.5 have been assigned to more common vibrations of different functional groups presented in protein structure. The band at 501–523 cm^{-1} can be attributed to polysulfides by S—S stretching, while vibration at 862–876 cm^{-1} is stretching vibration resulting from the C—O bridge into the carboxylic acid group [34]. The frequencies observed around 930 to 1150 cm^{-1} are the C—H bending, C—O—H stretching, and C—S stretching associated with organic compounds, which have aromatic compounds in their structure [35]. Different groups have reported that aromatic amino acids are in hydrophobic regions of folded proteins, these regions could be exposed to aqueous solvent as it modified its folded structure by factors like temperature, pH, and others [36,37]. So, the differences observed at 984 and 1150 cm^{-1} (denoted as NS in Table 2) are the result of conformational changes adopted by proteins which modify the vibrations of aromatic residues by the pH effect.

The absorption of proteins is given by the amide region; first, the amide III (1200–1400 cm^{-1}) which is in-phase in-plane by N—H bending and C—N stretching influenced with a small contribution from the C—O bending and C—C stretching; the differences into the range for amide III are the result of side-chain structure [38]. The amide II (≈1550 cm^{-1}) is the second region which is out-of-phase in-plane by N—H bending and C—N stretching

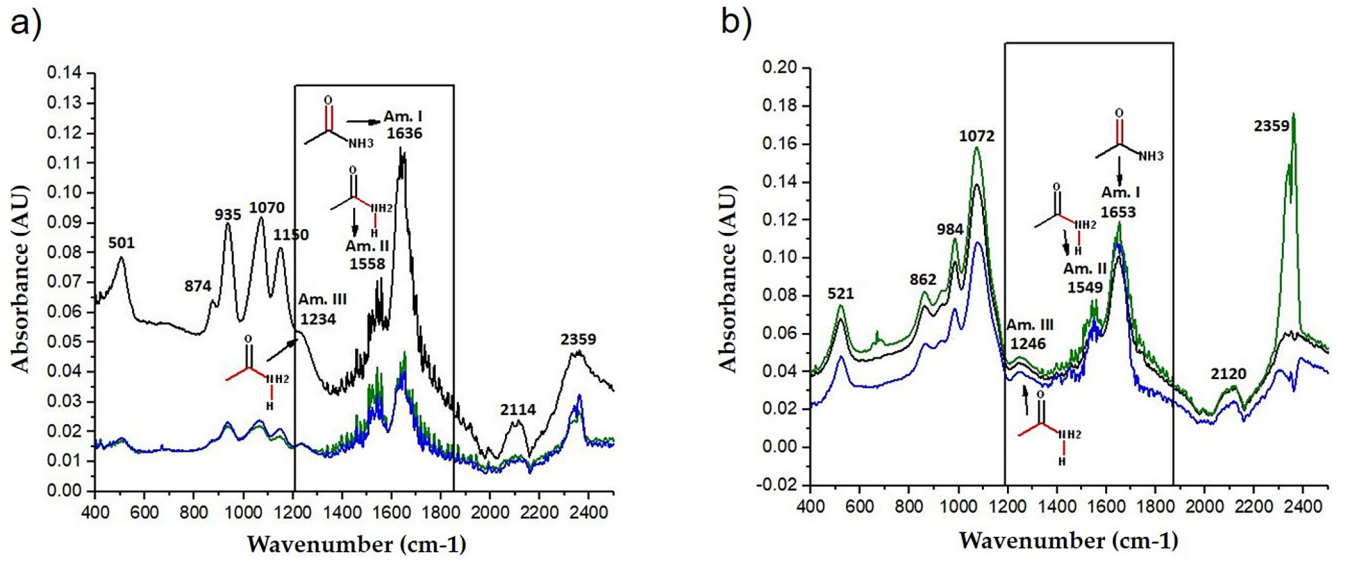

Fig. 2. FT-IR spectra of AAC and their modified variants. **(a)** proteins at pH 5; **(b)** proteins at pH 7.5. Lanes: black: AAC protein; green: AACM.1 protein; blue: AACM.3 protein. Arrows indicate band corresponding to Amide I (Am. I), Amide II (Am. II), and Amide III (Am. III). Numbers into graph are a reference of wavenumber.

Table 2
Characteristic bands (wavenumber*) observed by FT-IR spectroscopy for AAC, ACM.1, and AACM.3.

Proteins at pH 5			Proteins at pH 7.5			Assignment	Ref
AAC	AACM.1	AACM.3	AAC	AACM.1	AACM.3		
501	517	501	521	519	523	S—S stretching	[34]
874	876	872	862	862	866	C—O stretching	[34]
935	935	935	935	930	932	C—H bending	[35]
NS	NS	NS	984	984	984	C—H bending	[38]
1070	1063	1065	1072	1072	1076	C—O—H stretching	[38]
1150	1148	1150	NS	NS	NS	C—O stretching	[38]
1234	1234	1238	1246	1246	1242	N—H bending, C—N stretching (Amide III)	[39]
1558	1541	1541	1558	1558	1549	N—H bending, C—N stretching (Amide II)	[39]
1636	1653	1653	1653	1653	1641	C=O stretching (Amide I)	[39]
2114	2100	2118	2120	2122	2116	Multiple bindings	[35]
2361	2358	2363	2351	2359	2392	Multiple bindings	[35]

NS: Not showed.
* Wavenumber (cm^{-1}).

vibrations, moreover with smaller contributions by C—O bending, C—C and N—C stretching vibrations. Finally, the amide I region (≈1650 cm^{-1}) is primarily the result of the C=O stretching vibration with minor influence by C—N stretching and N—H bending which are out-of-phase and in-plane, respectively [39]. The multiple binding signal resulting are maybe by an additive effect, the multiply bonded C=O group shows an absorption band close to 2000 cm^{-1}, although band intensity could be influenced by other groups as thiols and sulfides which show vibration at 2400 cm^{-1}, the complexity of the bands being dependent on the structure of the compound [34], in this case from structure adopted by each protein.

Deconvolution of FTIR spectra of AAC and their modified variants was done using the amide I region (1600–1700 cm^{-1}) in both pH conditions (5 and 7.5); the proteins at pH 5 showed similar structure content at pH 5 with ≈30.5% sheets, ≈19% helical content, 10.6% and 39.6% turns and unordered structure, respectively. The results at pH 7.5 revealed higher helical content in AAC protein followed by AACM.1, while AACM.3 is the protein with higher unordered content close to 60% (Table 3). The results for structure content are in agreement with previous report, which was reported for AACM.3 protein, a higher unordered content at pH 7.5 [12,15]. So, is assumed that AACM.3 protein adopted a molten globule state, which is an intermediate state between native and compact structure, thus the molten globule increases the flexibility of conformation, allowing an influx of water to obtain a structure highly hydrated; in this way is possible that AACM.3 exposed some side hydrophobic groups that improved its techno-functional properties [40].

In Table 4, each band is listed and its association with different vibrations among them C—C, S=O, O=S = O, C—H or groups is mentioned. At pH 5, Raman scattering was not possible may be due to poor solubility. Raman spectra obtained for AAC and modified variants at pH 7.5 are shown in Fig. 3; several bands were observed and the Raman shift was indicated in each spectrum. The Raman spectra of AAC and their modified variants were sufficiently different from each other in detail to allow unambiguous assignments. The Raman shift bands from 940 to 970 cm^{-1} are usually referred to C—C and N-Cα—C stretching, which could be the signal of the peptide backbone, the differences in band displacement that show the conformational differences between each protein [24,41]. The higher signal intensity was observed for AACM.1 protein at 1151 assignment to S=O=S (Fig. 3b). This could be due to a greater number of interactions of this type (S=O=S) because of the modifications done which bring on as consequence a protein with a higher number of contacts which could promote a more compact structure [12,42].

Table 3
Secondary structure percentage content by deconvolution of FTIR spectra of AAC, AACM.1, and AACM.3.

Secondary structure	Proteins at pH 5			Proteins at pH 7.5		
	AAC	AACM.1	AACM.3	AAC	AACM.1	AACM.3
Sheets	30.36	30.56	30.71	32.95	31.50	24.53
Helix	19.01	19.19	19.07	22.25	19.02	11.12
Turns	10.67	10.62	10.70	9.84	9.82	4.70
Unordered	39.96	39.64	39.52	34.97	39.67	59.65

Table 4
Raman shift and assignments of AAC and modified proteins.

Raman Shift (cm^{-1})			Assignment	Ref.
AAC	AACM.1	AACM.3		
NS	NS	885	Tyrosine	[24]
902	904	NS	–	
935	937	943	C—C bridges	[24]
970	NS	976	N—Cα—C of the peptide backbone	[43]
NS	NS	1004	Phenylalanine	[24]
1066	1067	1071	S=O	[44]
1101	1103	1105	C—N	[24]
1150	1151	1155	O=S=O	[44]
1189	1191	1185	–	
1266	1267	1262	Amide III (C—N stretching)	[43,44]
1328	1330	1331	Tryptophan	[24]
1451	1453	1454	C—H bending	[43]
1545	1547	1550	N—H bending	[43]

NS: Not showed – Not reported.

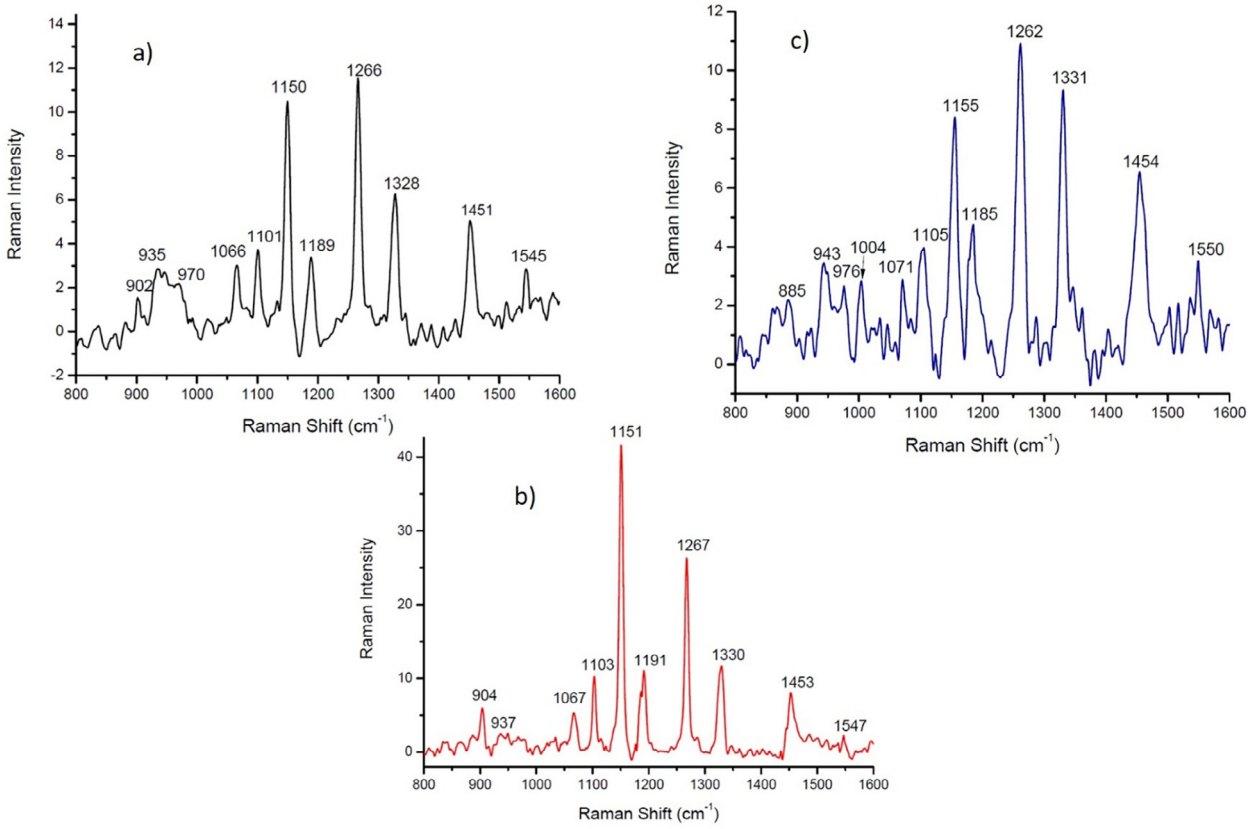

Fig. 3. Raman spectra of AAC and the modified variants. **(a)** AAC; **(b)** AACM.1; **(c)** AACM.3.

The spectrum of AACM.3 protein showed bands corresponding with all aromatic residues (tyrosine, phenylalanine, and tryptophan at 885, 1004, and 1331 cm^{-1}, respectively), which are frequently in the buried hydrophobic core of proteins. AAC and AACM.1 spectra proteins showed the band corresponding with tryptophan at 1545 and 1547 cm^{-1}, respectively (Fig. 3) while the spectrum of AACM3 showed the tryptophan band displaced at 1550 cm^{-1}, this could be because AACM.3 protein adopted a

more extended conformation as molten globule that increase the exposure of different hydrophobic groups generating more contacts with the solvent as previously mentioned [45]. Other seed globular proteins have been shown to take a molten globule state between β3 of β-conglycinin from soybean [46], also chenopodium which is an 11S globulin from quinoa seeds [47]. Raman spectroscopy results are in agreement with that obtained by FTIR.

4. Conclusions

By Infrared and Raman spectroscopy, it was possible to become aware of the structural conformations adopted by AAC, AACM.1, and AACM.3 due to biopeptide insertions and, to pH conditions tested in this research. Both modified proteins improved their techno-functional properties; AACM.3 protein adopted a conformation as a molten globule state, and AACM.1 adopted a more compact conformation. The techno-functional properties of AAC, AACM.1, and AACM.3 were strongly pH-dependent. Foaming and emulsifying activity of AACM.1 and AACM.3 could be taken on advantage to the development of novel food products. The findings of this study have the potential to generate ingredients with techno-biofunctional properties (due to the insertion of biopeptides). However, further studies must be focused to determine the hydrophobicity surface of proteins in order to study and understand the way in which our biofunctional proteins act to generate their techno-functional properties.

Author contributions

- Study conception and design: S Luna-Suárez.
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- Analysis and interpretation of results: Y Cruz-Morán; JI Morales-Camacho; S Luna-Suárez; RJ Delgado-Macuil.
- Draft manuscript preparation: JI Morales-Camacho; F Rosas-Cárdenas.
- Revision of the results and approved the final version of the manuscript: S Luna-Suárez, JI Morales-Camacho, F Rosas-Cárdenas.

Financial support

This work was supported by Secretaría de Investigación y Posgrado-IPN, México [Grant number 20221763 and Consejo Nacional de Ciencia y Tecnología (CONACYT) [Grant number 256478].

Conflict of interest

The authors declare no commercial or financial conflict of interest.

Acknowledgments

YCM thanks Consejo Nacional de Ciencia y Tecnología (CONACYT) for MSc degree scholarship.

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