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RESEARCH ARTICLE

Spectroscopic and molecular modeling investigations on heat induced behaviour of soy proteins

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ABSTRACT

Soy protein derivatives are widely used in food industry. Because structural properties of proteins vary with the environment conditions, thermal processing might be useful for modulating proteins functionality. The effect of thermal treatment at temperatures ranging from 25 to 100°C on soy proteins behaviour was tested using fluorescence spectroscopy and *in silico* techniques. Experimental tests were carried out at pH 4.5 and 7.0. Intrinsic fluorescence of proteins was monitored for probing the dynamics of Trp and Tyr microenvironments, such as to estimate the eventual conformation changes. Shifts of the maximum emission were recorded with pH change and temperature increase, and were associated to proteins structure alteration. Synchronous spectra indicated maximum and minimum fluorescence intensity of both chromophores at 70°C and 90°C, respectively, attributed to denaturation of the main soy proteins. Further molecular dynamics simulations, performed at single molecule level to complement the experimental results, indicated higher structural stability at 100°C of glycinin trimer compared to the β-conglycinin trimer.

Keywords: Soy proteins; Glycinin; β-conglycinin; Conformational changes; Thermal denaturation; Molecular behaviour

INTRODUCTION

Soybean proteins are widely used in the food industry due to the good functional properties. Many food formulations with high protein contents include soy protein isolates (SPI) or concentrates for improving technological performance and quality of the final products (Nishinari et al., 2014; Chen et al., 2013). Soybean proteins possess the ability to form gels, and have excellent water and oil holding capacities. Moreover, soy protein derivatives are used as food emulsifiers or emulsion stabilizers (Diftis and Kiosseoglou, 2006). Recently, Nishinari et al., (2014) published a comprehensive review on soy protein composition and functionality. They indicated that, in addition to the high quality proteins with well-balanced amino acid composition, soybeans contain physiologically beneficial components that exert hypocholesterolemic effects, and decrease the hyperlipidemia and cardiovascular diseases incidence. Moreover, epidemiological studies showed that consumption of soy-based foods can reduce breast, prostate, and colon cancer incidence (Tay, 2005).

Over 80% of the total protein in the SPI consists of two main globular protein fractions, namely glycinin (11S) and β-conglycinin (7S). Glycinin is a heteromultidimer with a molecular weight of 300-380 kDa, while β-conglycinin is composed by three units summarizing a total molecular weight of 188 kDa (Miriani et al., 2011). The ratio between β-conglycinin and glycinin ranges from 0.5 to 1.3, depending on soy variety.

Different processing variables such as ionic strength, concentration, pH, temperature and holding time influence the functionality of soy proteins in model or complex matrices. The thermal induced gelation mechanism between soy fractions is similar, on the other hand, each fraction has a specific denaturation temperature (Petrucelli and Anon, 1995; Guo et al., 2012). Soy proteins denaturation occurs when heated at temperatures higher than 85 °C for 30 min, leading to gelation followed by aggregation (Difti and Kiosseoglou, 2006).Wang et al., (2014) investigated the relationship between secondary structure and surface hydrophobicity of SPI subjected to heat treatment between 70 - 90 °C and found that heating increased the surface hydrophobicity, whereas aggregate formation reduced the surface hydrophobicity.

The pH changes might significantly influence proteins behaviour in terms of stability against thermal denaturation.

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The pH changes can even induce cold denaturation of the proteins (Nikolaidis et al., 2017). Moreover, the functionality related changes are more complex in case of SPI compared to the two prevalent protein fractions. Chen et al (2016) showed that native soy globulins self-assemble into aggregates, having increasing size with protein concentration increase and pH decrease. These changes affect the functional characteristics, biological activity, allergenicity and bioavailability of soy proteins (Miriani et al., 2011). The heat treatment often improves the functional properties of soy proteins. Moreover, if heating is applied to SPI under acidic conditions, desirable structural changes might occur. Liu et al., (2015) demonstrated that mild heat treatment of SPI under acidic environment resulted in superior functional properties, most probably because of the protein structural changes and hydrophobic aggregation. On the other hand, Renkema et al (2000) reported that in alkaline conditions, the presence of β conglycinin in SPI reduces the formation of aggregates during heating. Jiang et al., (2010) analysed the stability of SPI and its globulin fractions in different environment conditions and concluded that soy proteins structurally altered by pH shifting had a reduced sensitivity to thermal aggregation.

Although, the thermal denaturation mechanism of SPI has been extensively investigated, the gelation of globular proteins is still poorly understood because of its complexity (Nishinari et al., 2014).

Taking into consideration that formulation of food products requires most of the time, processing which involves temperature or pH shifting, the aim of the present study was to deepen the knowledge on the thermal behaviour of SPI over a wide range of temperatures at neutral and acidic pH conditions, using the fluorescence spectroscopy methods. Moreover, the molecular modelling approach was used to bring insights into the atomic level mechanism responsible for heat dependent behaviour of the main soy proteins. The knowledge on thermal dependent particularities of soy proteins might allow better exploitation of SPI as ingredient for different new food applications.

MATERIALS AND METHODS

Materials

SPI was purchased from Ubimedia S.R.L., Galati, Romania (82.10% protein and 9.52% moisture), acrylamide and potassium iodide from Sigma (Sigma–Aldrich Co., St. Louis, MO). All reagents were of analytical grade.

SDS-PAGE electrophoresis

SDS-PAGE was performed in a discontinuous buffer system, using stacking gel of 4.5% concentration and separating gel of 15% concentration, and on a Mini-Protean Tetra System (Bio-Rad).

An amount of 0.2 g SPI was first suspended in 5 mL ultrapure water and then dilluted 1:50 using ultrapure water. The sample was further mixed with 4x Laemmli protein sample buffer (Bio-Rad) under reduction conditions using β-mercaptoethanol and then heated in boiling water bath for 5 min (Laemmli, 1970).

The electrophoresis was run at 70 V in the stacking gel and 100 V in the separating gel, until the tracking dye reached the bottom of the gel. The gels were stained with Coomassie Brilliant Blue G 250.

Heat treatment

Two different stock protein solutions of 1mg/mL were prepared in Tris buffer of pH 4.5 and 7.0 by stirring for 30 minutes at room temperature, followed by centrifugation at 4000 rpm for 10 min. In order to be heat treated, the protein solutions (0.25 mL) were filled in plastic tubes (1 cm diameter). Heat treatment was performed for 20 minutes at different temperatures ranging from 25 to 100 °C, using a thermostatic water bath (Digibath-2 BAD 4, RaypaTrade, Barcelona, Spain). After the thermal treatment, samples were immediately transferred into ice, such as to limit any further thermal denaturation.

Fluorescence spectroscopy measurements

The fluorescence of protein samples was recorded at room temperature using LS-55 luminescence spectrometer (Perkin Elmer Life Sciences, Shelton, CT, USA) equipped with Perkin Elmer FL Winlab software.

Emission spectra were collected between 310-420 nm using excitation wavelengths of 290 nm for monitoring the fluorescence associated to Trp residues, 278 nm for Tyr residues and 280 nm for both Trp and Tyr residues. Excitation and emission slit widths of 10 nm each were chosen, and all fluorescence measurements were performed using a quartz cell of 10 mm path length. Each presented spectrum is the average of three scans and is expressed as arbitrary units. The synchronous spectra were collected at excitation wavelength ranging from 240-320 nm, with intervals of 15 nm for Tyr residues, and 60 nm for Trp residues.

Fluorescence quenching experiments were conducted with fresh solutions of acrylamide (8 M) and potassium iodine (5 M) as previously described by Dumitraşcu et al., (2016). The fluorescence quenching mechanism was described by Stern Volmer equation, which was used to determine the Stern-Volmer quenching constant (*Ksv*) (Dumitraşcu et al., 2016).

Three dimensional spectra provide detailed information regarding the conformational changes in proteins and were obtained as previously reported by Dumitraşcu et al., (2016).

In silico investigations on molecular behaviour of main soybean proteins

Only the models of one oligomer representing the 7S and 11S families which are evolutionary related were considered for the molecular modeling investigations. The 3D models of the proteins were selected from RCSB Protein Data Bank. In order to estimate the heat induced behavior of the main soybean proteins the following PDB models were considered: 1IPJ which is the crystal structure of β homotrimer of β-conglycinin (Maruyama et al., 2001), and 1FXZ which is a proglycinin A1aB1b homotrimer (Adachi et al., 2001). For convenience the models were called thereafter 7S and 11S, respectively.

Molecular mechanics steps for optimizing the geometries of the proteins and molecular dynamics meant to heat and equilibrate the solvated models were performed using Gromos 43a1.ff force field and Gromacs 5.1.1 package (Abraham et al., 2015). All simulations were performed in parallelism conditions on the High Performance Computer System (HPC) from *Dunarea de Jos* University using Intel E5 2680 v3, 12-cores, 2.5 GHz.

The procedure used to solvate, optimize and equilibrate the protein models at 25 and 100°C was in agreement with Dumitraşcu et al., (2016). The final protein models were analyzed in terms of energy, structural and conformation particularities using dedicated Gromacs, PDBsum (Laskowski, 2009) and PDBePISA (Krissinel, and Henrick, 2007) (The European Bioinformatics Institute, Cambridge, UK) tools.

Statistical analysis

Statistical analysis of fluorescence data was carried out using Minitab17 software. Statistical significance was correlated to data at significance level of 0.05. Ryan Joiner test similar to Shapiro–Wilk test was employed to assess whether the residuals of samples and controls were normally distributed (normal distribution if $p > 0.05$). The influence of pH (4.5 and 7.0) and temperature values on SPI fluorescence was tested using one-way ANOVA. Post-hoc analysis via Fisher LSD Method at 95% confidence was performed when appropriate (significant if $p < 0.05$).

RESULTS AND DISCUSSION

Soy protein profile

The protein profile of SPI was investigated by SDS-PAGE electrophoresis and the obtained pattern revealed bands corresponding to the major components of soy protein isolates, represented by β-conglycinin and glycinin. Fig. 1 shows the typical protein bands corresponding to α', α, and β subunits of β-conglycinin and the acidic and basic subunits of glycinin. The three bands having the molecular weights of approximately 75 kDa, 69 kDa, and 53 kDa were associated with the trimer, β-conglycinin, and the other two having the molecular weights of approximately 35 kDa and 19 kDa were assigned to the acidic and basic polypeptides of the glycinin. The molecular weights of β-conglycinin and glycinin subunits were previously reported by a number of researchers. Molecular weights of 68 kDa and 52 kDa (Fontes et al., 1984) or 68 kDa and 48 kDa (Natarajan et al., 2006) were reported for α and β subunits of β-conglycinin. Arrese et al. (1991) found molecular weights of 36.3 and 19.3 KDa for the acidic and basic subunits of glycinin. Similar results were reported by Wu et al., (2009), Vasconcellos et al., (2014) and Chen et al., (2016). It seems that, there is a great variation in the molecular weights of β-conglycinin and glycinin components, in the band frequencies or their ratio. Liu et al., (2007) analyzed the SDS-PAGE protein patterns of 640 soybean cultivars and landraces extracts, and divided the soy proteins into two regions, over and under 44 kDa, based on their molecular weights. Furthermore, they divided each regions into four and six subunits groups based on the genetic variation of soybean cultivars.

Phase diagram method

Proteins denaturation at thermal treatment involves dissociation into constituent subunits, unfolding events, and exposure of hydrophobic patches on proteins surface (Petrucelli et al., 1995). The phase diagram method was employed here to estimate the dynamics of unfolding/refolding pathways of soy proteins, using combinations of temperatures and pH to identify eventual intermediate states. Fig. 2 shows the phase diagram of the heat induced structural changes of soy proteins, resulted by plotting the change of fluorescence intensity at 320 nm and 365 nm for protein solutions of pH 4.5 and pH 7.0. Both samples showed linear dependence indicating the *all-or-none* transition while heating, characterised by the presence of two conformational species, namely the native form and the denatured state. As in case of most proteins, the folding process of soy proteins occurs independently in different segments or domains of the protein, and key residues are involved in interactions responsible for the native-like fold within local domains (King et al., 2002). Glycinin is formed of twelve subunits, with six acidic subunits and six basic subunits, while β-conglycinin contains three acidic subunits. Jiang et al., (2009) demonstrated that, regardless of pH conditions and holding process, glycinin is mainly involved in forming protein polymers and aggregates. During heating in the presence of other soy protein

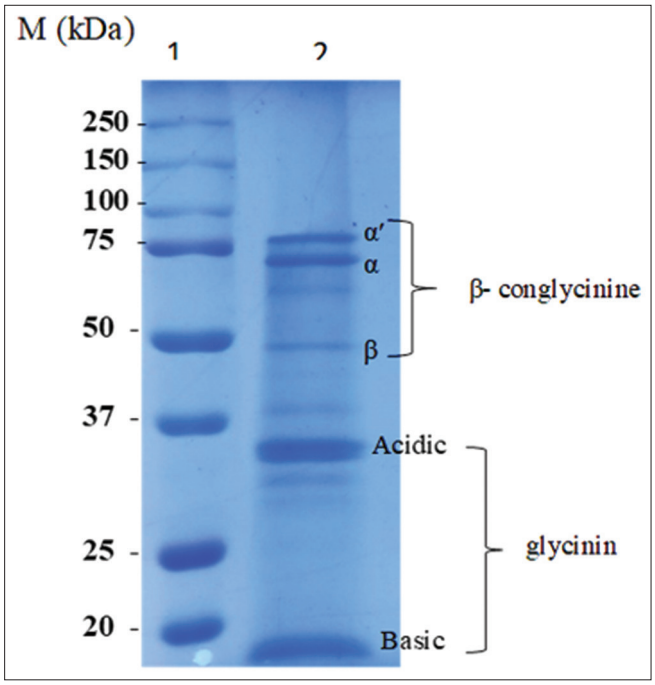

Fig 1. SDS-PAGE of soy protein isolate. Lane 1 - Molecular weight standards - Precision Plus Protein™ Dual Color Standards (Bio-Rad). Lane 2 - Soy protein isolate: α′, α, and β subunits of β-conglycinin and the acidic and basic subunits of glycinin

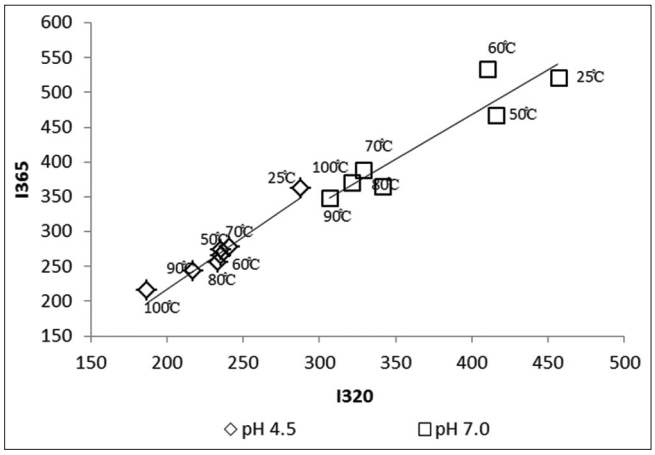

Fig 2. Phase diagram analysis of conformational changes of soy proteins at different pH values based on intrinsic fluorescence intensity values measured at wavelengths 320 and 365 nm. Temperature values, in the 25-100°C range, are indicated in the vicinity of the corresponding symbol

fractions, the basic subunits of glycinin dissociates from the oligomeric structure, preventing aggregation. Wang et al., (2014) reported secondary structure alterations and surface hydrophobicity increase upon heating of SPI. It seems that the interaction between conglycinin and the basic subunits of glycinin leads to formation of soluble complexes (Damodaran and Kinsella, 1982). Nagano et al., (1994) studied the conformational states of β-conglycinin at pH ranging from 3.0 to 9.0, and revealed that the high degree of protonation of carboxyl groups led to protein fractions denaturation at low pH, due to the changes of the balance of electrostatic forces. The pH induced changes of soy proteins were related mainly to glycinin fraction, while β-conglycinin tended to denature when decreasing the pH below 6.0 (Nagano et al., 1994). At pH 7.6 glycinin exists in hexameric form, whereas at pH 3.8 is present as trimer (Kim et al., 2016). Temperatures above 70°C lead to soy proteins unfolding with exposure of sulphydryl and hydrophobic groups. As depicted in Fig. 2, heat treatment and acidic pH caused the unfolding of globular structure of soy proteins. As a consequence of protein denaturation causing the exposure of new patches, the formation of new intra- and intermolecular hydrogen bonds and establishment of electrostatic and hydrophobic interactions different from the ones identified in the native states are favoured (Wang et al., 2014).

Intrinsic fluorescence

The fluorescence signal of a protein is derived from its aromatic aminoacids, tryptophan (Trp), tyrosine (Tyr) and phenylalanine. Since Trp fluorescence is highly sensitive to the microenvironment, is a useful spectroscopic probe to outline the local structure and rotational dynamics surrounding the residue (Lakowicz, 2004). Emission fluorescence spectra of SPI solutions at different pH values are presented in Fig. 3. It can be seen that, at room temperature, the fluorescence intensity of SPI solutions was significantly affected by pH. Higher fluorescence intensities were collected at pH 4.5 compared with those obtained at pH 7.0. Moreover, red shifts were recorded when dropping the pH from 7.0 to 4.5, indicating that fluorophores became more exposed to the solvent under acid environment.

Heat treatment is widely applied in food processing, to modify proteins, to form protein gels, or set the structure. One of the most important intrinsic factors that affect the conformational stability and surface activity of soy proteins is the hydrophobicity of the constituent amino acids that can be measured either in water or organic solvents (Damodaran and Paraf, 1997).

A detailed analysis was performed through *in silico* approach to identify the particularities of the aminoacids responsible to the fluorescence properties of main soy proteins. The 11S model used in the investigations is a trimer and each of the three equivalent chains has four Trp residues with varying solvent accessible surface (SAS), as follows: Trp^{36} with SAS 1.93÷3.34 Å^2, Trp^{131} with SAS 9.60÷10.20 Å^2, Trp^{132} with SAS 40.49÷44.72 Å^2, and Trp^{335} with SAS 102.28÷115.04 Å^2. Upon the interfaces formation within the 11S trimer, up to 92.42% and 10.68% of the available surface of Trp^{132} and Trp^{132}, respectively, get buried. In particular, the Trp^{32} of each chain establishes non bonded contacts with Leu^{436} of the neighboring chain. Regarding

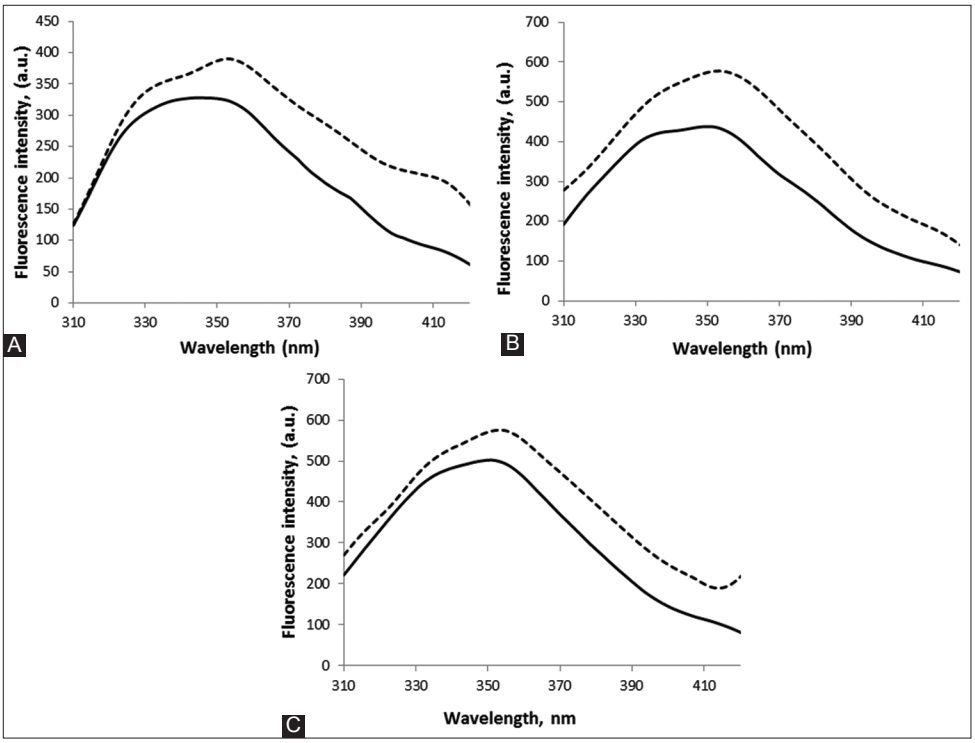

Fig 3. Fluorescence intensity of Trp (A), Tyr (B) and Trp+ Tyr (C) residues in SPI solutions at pH 4.5 (dashed line) and pH 7.0 (full line)

the Tyr residues, the following location has been identified in each chain of 11S molecule used in the study: Tyr^{65} (SAS of 51.78÷56.73 Å^2), Tyr^{73} (SAS of 1.72÷6.25 Å^2), Tyr^{85} (SAS of 43.17÷55.66 Å^2), Tyr^{115} (SAS of 39.87÷40.49 Å^2), Tyr^{134} (SAS of 6.10÷12.87 Å^2), Tyr^{164} (SAS of 7.72÷9.22 Å^2), Tyr^{176} (SAS of 16.31÷21.13 Å^2), Tyr^{315} (SAS of 138.3÷146.13 Å^2), Tyr^{355} (SAS of 43.76÷50.65 Å^2), Tyr^{364} (SAS of 2.21÷3.69 Å^2) and Tyr^{412} (SAS of 2.94÷4.30 Å^2). Out of these residues, only Tyr^{65}, Tyr^{85}, Tyr^{164}, Tyr^{315} and Tyr^{355} are buried upon the formation of the trimer, with a Tyr buried surface of up to 249.07 Å^2, representing 63.22% of the total SAS associated to Tyr residues within a single chain. The highly conserved Tyr residues located at the interfaces are involved in establishing inter-chain hydrogen bonds such as Tyr^{65}-Asn^{398}, Tyr^{85}-Met^{422} and Tyr^{355}-Gly^{128}. Unlike most of the hydrogen bonds connecting the protomers which are located close to the water molecules, those involving Tyr^{65} and Tyr^{355} are buried within the core of the interfaces (Adachi et al., 2001). Moreover, the Tyr^{65} of each chain is in direct contact with Tyr^{355} of the neighboring chain, therefore acting as potential quenchers of each other fluorescence.

β-conglycinin has one Trp residue in the α-subunit, two in α/, and none in the β subunit. The Trp residues of β-conglycinin are part of the solvent-exposed N-terminal extended domains of the α (Trp^{63}) and α/ subunits (Trp^{63} and Trp^{100}) (Keerati-u-rai et al., 2012). The homologous chains of the 7S trimer used for molecular modeling investigations have 11 Tyr residues, namely Tyr^{13} (SAS of 29.47÷38.13 Å^2), Tyr^{50} (SAS of 32.59÷34.41 Å^2), Tyr^{94} (SAS of 12.90÷22.07 Å^2), Tyr^{110} (SAS of 2.14÷4.31 Å^2), Tyr^{111} (SAS of 39.18÷55.17 Å^2), Tyr^{136} (SAS of 19.94÷29.58 Å^2), Tyr^{150} (SAS of 75.80÷84.73 Å^2), Tyr^{227} (SAS of 17.18÷25.26 Å^2), Tyr^{308} (SAS of 29.60÷36.27 Å^2), Tyr^{324} (SAS of 63.55÷68.75 Å^2) and Tyr^{389} (SAS of 25.51÷34.39 Å^2). The Tyr surface buried at the interfaces when the trimer is formed ranges from 43.11% to 46.90% of the total available Tyr surface of each protein chain. The residues buried at the interface are Tyr^{111}, Tyr^{150} and Tyr^{324}. In addition, because of the small distance between residues (3.56Å), the fluorescence of Tyr^{324} residues might be quenched by the Phe^{139} of the neighboring chains. Regardless of the temperature, the hydrophobic residues establishing contacts at the interface of the monomers belong to α helices and β barrel.

Fluorescence intensities and maximum emission wavelengths (λ_{max}) of soy proteins at different temperatures and pH values are summarized in Table 1. For different combinations of pH and temperatures, shifts in the emission wavelength corresponding to the maximum intensity of Trp and Tyr fluorescence were measured. Compared to the samples at 25°C, heating the SPI solutions at 50°C induced a blue shift associated to Trp residues of 5 nm and 3 nm at pH 7.0 and pH 4.5 respectively, whereas in case of Tyr residues blue shifts of 8 nm (pH 7.0) and 3 nm (pH 4.5) were measured. These results suggested changes in the exposure extent and number of moving toward a more hydrophobic location.

Table 1(a): Characteristics of Trp, Tyr and Trp+Tyrfluorescence of SPIsolutions at pH 7.0

Temperature (°C)	Trp λ_{ex}=292 nm		Tyr λ_{ex}=274 nm		Trp+Tyr λ_{ex}=280 nm	
	λ_{max} (nm)	I_{max} (a.u.)	λ_{max} (nm)	I_{max} (a.u.)	λ_{max} (nm)	I_{max} (a.u.)
25	345	328.05±39.52^{a}	350	437.82±55.63^{a}	351	457.96±44.36^{ab}
50	340	382.27±37.36^{a}	342	438.01±24.58^{a}	351	406.27±42.69^{b}
60	341	343.18±25.71^{a}	351	397.18±8.45^{ab}	352	549.25±24.92^{a}
70	346	351.45±28.37^{a}	352	358.62±39.37^{bc}	350	420.18±57.49^{ab}
80	348	305.44±13.00^{ab}	351	329.31±25.74^{bc}	348	381.72±76.22^{b}
90	350	274.08±54.09^{ab}	350	309.13±8.56^{c}	351	339.77±58.09^{b}
100	350	311.20±44.85^{b}	349	350.53±19.02^{bc}	350	426.27±18.02^{ab}

Table 1(b): Characteristics of Trp, Tyr and Trp+Tyrfluorescence of SPIsolutions at pH 4.50

Temperature (°C)	Trp λ_{ex}=292 nm		Tyr λ_{ex}=274 nm		Trp+Tyr λ_{ex}=280 nm	
	λ_{max} (nm)	I_{max} (a.u.)	λ_{max} (nm)	I_{max} (a.u.)	λ_{max} (nm)	I_{max} (a.u.)
25	353	390.4±10.09^{a}	353	577.47±30.39^{cd}	354	575.64±20.86^{ab}
50	350	229.23±25.46^{b}	350	512.33±11.49^{e}	351	434.78±35.85^{c}
60	352	263.12±2.99^{b}	350	678.97±23.00^{a}	352	511.67±23.72^{bc}
70	348	358.51±11.17^{a}	351	645.6±35.76^{ab}	351	485.05±19.80^{bc}
80	353	244.59±3.95^{b}	350	600.9±30.7^{bc}	349	480.92±49.69^{c}
90	351	272.14±29.77^{b}	350	542.16±0.45^{de}	350	459.13±10.38^{c}
100	354	248.22±11.17^{b}	349	649.36±6.44^{ab}	349	621.16±72.43^{a}

Each value is a mean of triplicate analysis±standard deviation. Means that on each column do not share a letter are significantly different ($p<0.05$)

As stated by Tang et al., (2007), the hydrogen bonds and electrostatic interactions between water and protein molecules play important roles in the folding and maintaining the specific conformation of the native soy proteins, whereas the hydrophobic interactions are mainly associated with thermal history of the protein. Heat treatment alters the hydrophilic interactions, and the hydrophobic regions initially buried in protein core become exposed to the protein surface, available to associate with hydrophobic regions of other protein molecules to form aggregates.

On the other hand, further temperature increase up to 100 °C, caused a red shift in λmax of 5 nm for Trp residues at neutral pH, whereas for the acidic counterpart a red shift of 14 nm was recorded. Lakowicz (2004) highlighted that information acquisition related to protein structure are more accurate if they are based on the position of maximum fluorescence emission, and not on the emission fluorescence intensity. The emission maximum of Trp residues in water occurs near 350 nm and is strongly dependent upon the polarity of the environment around the side chain (Miriani et al., 2011). These results suggest that during heating, Trp residues of soy proteins at pH 7.0 become more exposed to hydrophilic environment. As in case of pH, the thermal induced changes in glycinin structure prevail over β-conglycinin. Miriani et al., (2011) showed that, among the main soy proteins, glycinin treated at 95 °C loses the highest amount of its structure, resulting in exposure of Trp containing regions to the polar microenvironment.

Keerati-u-rai et al., (2012) investigated by fluorescence spectroscopy the structural changes of SPI, glycinin and β-conglycinin fractions. The lowest structural changes were observed in SPI, and were correlated with the highest extent of protein-protein interactions as compared with isolated soy protein fractions. Chen et al., (2017) reported that the mechanism of aggregation and gelation of SPI is similar to that of β-lactoglobuline. In the first stage of denaturation, the proteins form relatively small elementary particles, that subsequently randomly aggregate until eventually a system spanning network is formed.

Aggregation and gelation mechanism of gylcinin is similar to that of SPI at neutral pH. Although, at neutral pH, β-conglycinin exerts a negligible effect during heating upon SPI denaturation, this fraction prevents precipitation of basic subunits in SPI after heat treatment (Renkema et al., 2000). Guo et al., (2012) investigated the thermal aggregation behavior of soy β-conglycinin and glycinin at pH 7.0. The authors reported that the melting temperature of β-conglycinin ranged from 68.0 to 79.4°C, and from 88.0 to 96.4°C for glycinin. At neutral pH glycinin has a denaturation temperature of about 90°C, whereas β-conglycinin already unfolds at 74 °C (Keerati-u-rai et al., 2012; Renkema et al., 2000). The authors concluded that at acidic pH conditions, the denaturation of the main SPI fractions occurs at lower temperatures. Our results are in agreement with those reported by Renkema et al., (2000).

Quenching experiments

Fluorescence quenching is an important tool to measure the extent and accessibility of protein binding sites to small molecules (Rhodes et al., 2014). Quenching experiments with acrylamide and KI were performed to acquire information regarding the solvent accessibility and the polarity of the medium close to Trp residues from SPI solutions. Quenchers can slightly modify the geometry of the soy proteins structure, due to hydrophobic contacts of the fluorophore within the protein interior (Sułkowska, 2002). Quenching experiments generated linear correlations and the resulted Stern Volmer constants (*Ksv*) of Trp residues are presented in Table 2. The *Ksv* values are dependent on the quencher ability to reach the encounter distance of the fluorophore. From Table 2 it can be seen that in native state, quenching at pH 4.5 with acrylamide generated the highest *Ksv* value ($5.85 \pm 0.08 \cdot 10^3 mol^{-1}L$), whereas the lowest was obtained when quenching with KI ($3.68 \pm 0.22 \cdot 10^3$ $mol^{-1}L$). In agreement with the observations of the intrinsic fluorescence measurements, these results indicate that under acidic conditions, Trp residues of the soy proteins are more exposed to solvent compared to neutral pH. Acrylamide possess the ability to quench Trp residues that are totally or partially exposed to solvent, therefore the *Ksv* values are in all cases higher compared with KI. When quenching with acrylamide the SPI solutions of pH 4.5, the lowest *Ksv* value was obtained at 70°C ($3.43 \pm 0.01 \cdot 10^3$ $mol^{-1}L$), while for pH 7.0, the lowest *Ksv* value was obtained at 80°C ($2.45 \pm 0.36 \cdot 10^3$ $mol^{-1}L$). These results suggest that Trp accessibility is dependent on medium composition. Unlike acrylamide, KI has the ability to quench only the Trp residues partially exposed or located at the surface of the protein. On the other hand, quenching experiments with KI at neutral pH revealed in most cases that Trp residues are more exposed to protein surface than under acidic conditions.

Synchronous fluorescence spectra

Synchronous fluorescence spectra provided further information regarding the changes that occur within protein structure during heating, as revealed by the microenvironment of the chromophore residues. Measurements performed at differences between excitation wavelength and emission wavelength ($\Delta\lambda$) set at 15 nm and 60 nm reveal characteristics associated to Tyr and Trp residues. The synchronous fluorescence spectra of SPI solutions are presented in Fig. 4.

At neutral pH, the maximum emission of intrinsic fluorophores was recorded at 286 nm and 279 nm for Tyr and Trp residues, respectively (Fig. 4A). No shifts in the maximum emission were recorded when heating the protein, important variations in fluorescence intensity being recorded only at temperatures above 80°C. On the other hand, at 25°C the SPI solutions of pH 4.5 showed λ_{max}, corresponding to the maximum fluorescence intensity, at 280 nm for Tyr residues, and at 278 nm for Trp residues (Fig. 4B). As indicated by the significant shift of λ_{max}, heating resulted in changes of the microenvironment of Tyr residues. Red shifts of 6 nm were obtained at 60°C, while further temperature increase caused a small blue shift of 2 nm. Peaks position associated to Trp residues within soy proteins at pH 4.5 remained unchanged during heating. For both intrinsic fluorophores, the maximum fluorescence intensity was measured at 70°C, while the minimum at 90°C, temperatures that are associated with denaturation of β-conglycinin and glycinin. Dobson (2003) showed that as pH approaches 4.2, thermal denaturation of SPI occurs at lower temperatures, charge repulsion is lower and protein aggregation is higher. When no heat treatment is applied, the Tyr residues of soy proteins are more exposed to a polar location at neutral pH compared with acidic pH, while Trp residues location remains unaffected. On the other hand, heat treatment induced significant conformational changes at pH 4.5, as revealed by Tyr residues reposition to a more hydrophobic environment.

Three dimensional spectra

Using a given excitation wavelength, the fluorescence intensity of a sample can be recorded over a range of emission wavelengths to construct the excitation-emission matrix (Ranamukhaarachchi et al., 2017). Three dimensional fluorescence spectroscopy was used to acquire additional insights into conformation and structure of soy

Table 2: Stern–Volmer quenching constants ($\times 10^3$ mol L^{-1}) corresponding to Trp residues of SPI at different temperatures and pH

Temperature (°C)	Acrylamide		KI	
	pH 4.5	pH 7.0	pH 4.5	pH 7.0
25	5.85±0.08^{abc}	5.08±0.84^{a}	3.68±1.22^{a}	5.44±0.39^{a}
50	7.62±0.97^{a}	6.62±0.93^{a}	3.22±0.34^{a}	6.79±0.93^{a}
60	6.27±0.34^{ab}	5.25±0.85^{a}	4.93±1.29^{a}	6.38±0.74^{a}
70	3.43±0.01^{c}	4.40±1.61^{a}	4.73±0.21^{a}	5.26±1.92^{a}
80	4.16±0.86^{bc}	2.45±0.36^{a}	4.95±0.15^{a}	5.83±0.08^{a}
90	5.51±0.59^{abc}	4.82±1.57^{a}	2.99±0.25^{a}	6.08±0.70^{a}
100	4.13±0.88^{bc}	6.06±0.78^{a}	4.49±0.15^{a}	4.88±0.53^{a}

Each value is a mean of triplicate analysis±standard deviation. Means that on each column do not share a letter are significantly different ($p<0.05$)

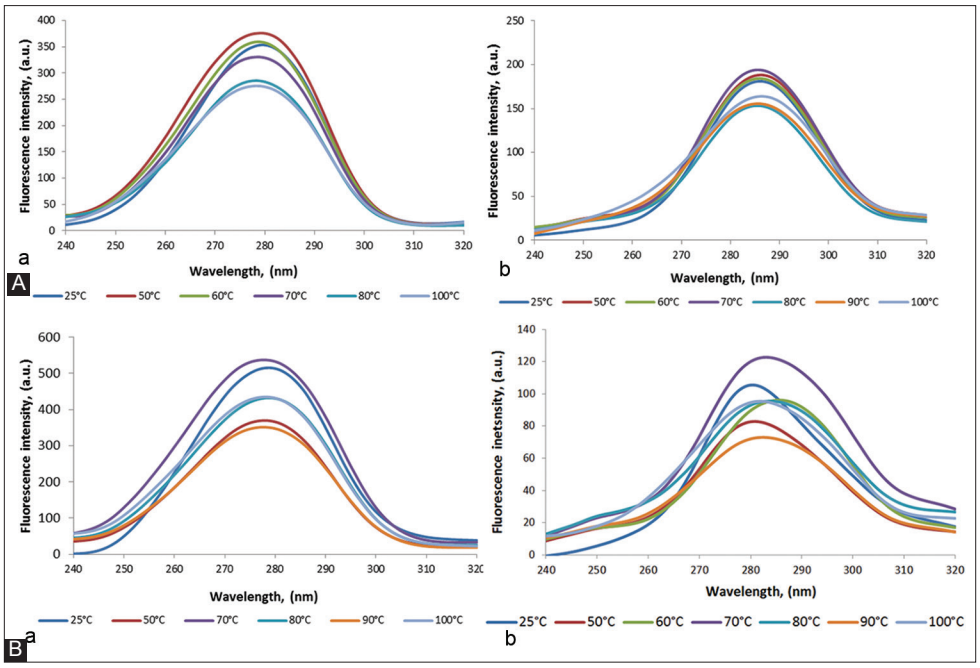

Fig 4. Synchronous fluorescence scans of SPI at pH 7.0 (A) and pH 4.5 (B) using Δλ of 60 nm (a) and 15 nm (b)

Table 3(a): 3D spectral characteristics of SPI solutions at pH 4.5 for different temperatures

Temperature (°C)	Peak 1 $\lambda_{ex}/\lambda_{em}$ (nm)	FI[a] (a.u.)	Peak 2 $\lambda_{ex}/\lambda_{em}$ (nm)	FI (a.u.)
25	230/346	532.66±54.7	280/352	456.15±55.07
50	230/349	452.81±14.21	280/352	374.00±57.42
60	230/349	530.2±81.92	280/351	371.85±33.57
70	230/344	477.65±47.54	280/351	364.75±17.37
80	230/349	458.44±82.24	280/351	344.38±16.27
90	230/346	354.90±41.11	280/350	393.93±91.13
100	230/349	473.18±72.29	280/351	322.17±58.62

Table 3(b): 3D spectral characteristics of SPI solutions at pH 7.0 for different temperatures

Temperature (˚C)	Peak 1 $\lambda_{ex}/\lambda_{em}$ (nm)	FI[a] (a.u.)	Peak 2 $\lambda_{ex}/\lambda_{em}$ (nm)	FI (a.u.)
25	230/347	876.89±1.18	280/352	805.07±70.70
50	230/346	678.75±35.69	280/349	649.45±4.19
60	230/340	911.40±39.72	280/350	612.00±1.98
70	230/353	820.74±25.95	280/350	668.76±23.74
80	230/346	740.49±30.26	280/350	505.25±67.33
90	230/348	603.59±17.08	280/350	516.09±50.53
100	230/350	465.34±28.14	280/350	489.19±145.11

a-fluorescence intensity.Each value is a mean of triplicate analysis±standard deviation.

proteins under different pH and temperature conditions. The corresponding results are presented in Table 3. Peak 1 gives information related to Tyr fluorescence, whereas Peak 2 refers to the fluorescence of both Trp and Tyr residues (Abdelhameed et al., 2017).

The highest peaks were obtained at pH 7.0 (Table 3b), regardless of tested temperature. Heating at 60°C caused a blue shift of 7 nm, while further temperature increase up to 70°C generated a red shift of 13 nm, indicating that Tyr residues moved from hydrophobic toward aqueous locations. On the other hand, in case of the SPI solutions of pH 4.5 (Table 3a), Peak 1 recorded at 60°C was characterized by a red shift (compared with the non-heated sample) of 3 nm, while at 70°C, the maximum emission was blue shifted by 5 nm. Regarding Peak 2, no significant shifts in the maximum emission were recorded regardless of pH and temperature treatment. In agreement with previous discussed findings, these results can be attributed to soy protein fractions glycinin and β-conglycinin that behave

differently depending of heat treatment and medium composition.

Thermal dependent particularities of 11S and 7S models

Single molecule level investigations using Gromacs software were carried out to estimate the thermal dependent behavior of main soybean proteins, glycinin and β-conglycinin, which account for about 40% and 30% of the total soybean protein, respectively (Maruyama et al., 2001). The 7S and 11S models were equilibrated at 25 °C and 100 °C, and the increase of the total energy values was observed (Table 4), as a result of the accelerated thermal motion at high temperature.

Temperature increase from 25 °C to 100 °C caused a slight reduction of the hydrophobic surface of about 6% and no significant changes in the secondary structure elements of 11S molecule (Table 4). Although an increase of the total number of strands was observed when raising the temperature up to 100°C, about 19% of the strands lost two to four residues. Two of the Ile^{201}-Gly^{204} sequences organized as helices in the protomers within the 11S structure at 25 oC turned into β-turns at 100 °C, whereas some other helices became two residues shorter. Anyway, the main structural elements appeared to be well packed within the two β-barrel and two extended helix domains (Fig. 5), as described by Adachi et al., (2001). The structural stability of the protein at heat treatment is partially due to the intra-chain disulfide bond between Cys^{12} and Cys^{45} and the Cys^{88} - Cys^{298} disulfide bonds connecting the acidic and basic chains at the interface between protomers.

Heat related structural rearrangements resulted in over 6% reduction of both hydrogen bonds (Hb) involved in stabilizing the protein structure and established between amino acids and surrounding water molecules (Table 4).

The interaction between equivalent faces of two different trimers having higher amounts of hydrophobic residues exposed might was proposed for the formation of 11S hexamer (Adachi et al., 2001). Therefore, considering the slight decrease of the trimer hydrophobic surface with

Table 4: Energy, surface and secondary structure descriptors of the 7S and 11S trimers equilibrated at different temperatures

	7S		11S	
	25°C	100°C	25°C	100°C
Energy descriptors				
Total energy, kJ mol^{-1}	(-6070.84±2.81)·10^3	(-4582.22±3.13)·10^3	(-1872.58±2.91)·10^3	(-1408.79±2.29)·10^3
Potential energy, kJ mol^{-1}	(-8232.53±2.97)·10^3	(-7287.96±3.341)·10^3	(-2552.29±2.98)·10^3	(-2259.57±2.44)·10^3
Surface descriptors				
Volume, nm^3	187.64±5.03	185.58±5.27	177.33±3.23	176.74±3.61
Total surface, nm^2	494.94±3.93	501.00±5.80	402.45±3.02	387.16±3.09
Hydrophobic surface, $Å^2$	233.79±2.94	240.23±2.82	201.72±2.59	188.42±2.65
Structure descriptors				
Main secondary structure elements, %	Strand–40.1; αhelix–14.2;3-10 helix–1.4	Strand–38.4;α helix–10.3;3-10 helix–1.8	Strand–33.1;α helix–11.0;3-10 helix–1.2	Strand–33.2; α helix–11.0;3-10 helix–0.9
Hb within protein	862±15	807±17	911±15	850±16
Hb protein-water	1962±31	1805±40	1540±17	1433±25

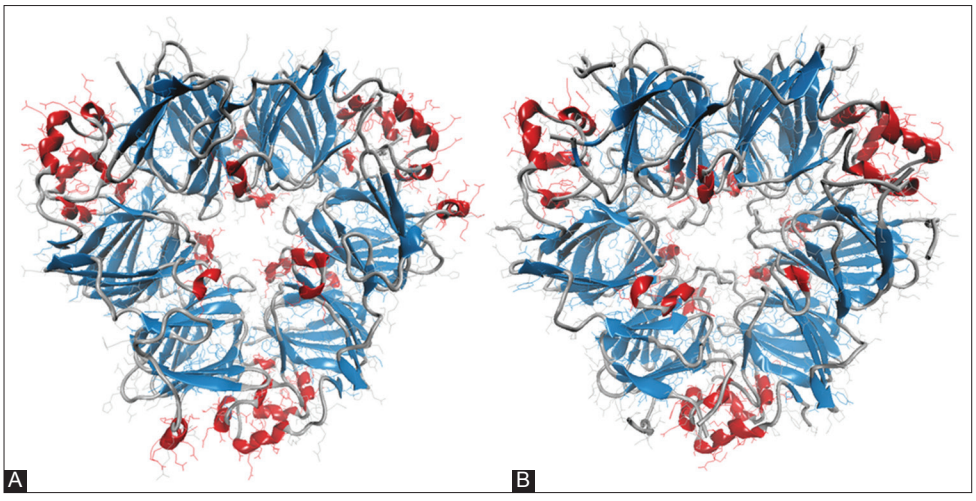

Fig 5. Overall folding of 7S (A) and 11S (B) molecules from soy represented in Catmull-Rom New Cartoon style through VMD software. The secondary structure of the proteins is represented as follows: strand motifs in blue and the helical content in red

the temperature increase (Table 4), we might expect lower stability of the hexamer at 100°C.

β-conglycinin is a trimeric glycoprotein with α/, α and β subunits, and there is a large diversity of molecular species with various subunits composition (Maruyama et al., 2001).The three types of subunits share a core region of absolute high homology (76-90%), which was reported to be mainly responsible for featuring proteins behaviour at thermal processing (Maruyama et al., 2001). The highest thermal stability was reported for β subunits (Maruyama et al., 1998), considered as model in the present study.

The secondary structure elements of the 7S model equilibrated at different temperatures have been carefully checked, such as to identify the major motifs that play the key role in molecular behavior at heating. As in case of 11S, the secondary structure of 7S is dominated by the strands based motifs, regardless of the thermal treatment (Fig. 5). Compared to the 11S, 7S exhibited lower thermal stability, with increasing exposure of the hydrophobic surface at high temperature (Table 4). The heat treatment resulted in significant reduction of the total amount of amino acids involved in defining both strands and helices. A close analysis of the strands affected by heat within each monomer indicated their location into the core domain of the amino-terminal modules, namely N terminal residues 12-139 and C terminal residues 217-349 (Maruyama et al., 2001). Anyway, the overall architecture of the core β barrels is not affected. Regarding the loop domains acting like hooks between monomers (Maruyama et al., 2001), only Tyr^{150}-Gly^{153} helix was destabilized due to changes in the orientation of their side chains, which further affected the contacts established with the residues of the neighboring monomer.

CONCLUSIONS

Fluorescence spectroscopy based measurements were performed to analyse the influence of temperature and pH on the conformation of soy proteins in solution. Phase diagrams were linear regardless of the pH value of SPI solutions. Fluorescence intrinsic data revealed that at 50°C, Tyr residues are more buried within the hydrophobic core of the protein compared to Trp residues. Heating caused the exposure of both chromophores to the solvent and favored the solvent accessible tertiary structure of the protein. Although the *in silico* tests performed on the 7S and 11S protein models indicated rather good conservation of the structural elements which are well packed within the β-barrel and helix extended domains, increasing exposure of the hydrophobic surface of 7S trimer was observed at high temperature. Quenching experiments with acrylamide and potassium iodide showed that Trp residues within soy proteins are more exposed to solvent at neutral pH compared to the acidic one. Further tests will be performed to estimate the impact of preliminary treatment of soy proteins under different temperature-pH conditions on the ability of the proteins to bind and hold different biological active compounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Authors' contributions

Loredana Dumitraşcu was involved in acquisition of data, analysis and interpretation of data and drafting the manuscript; Nicoleta Stănciuc and Iuliana Aprodu were involved in planing the experiments, interpretation of data and drafting the manuscript; Leontina Grigore-Gurgu was involved in acquisition and analysis of data. All authors read and approved the final manuscript.

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