

Spectroscopic studies on the pH-dependent structural dynamics of γ -conglutin, the blood glucose-lowering protein of lupin seeds

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ABSTRACT

γ -Conglutin is a blood glucose-lowering protein purified from lupin (*Lupinus albus*, L.) seed. Despite various features of this protein have already been studied, no function in the seed nor any mechanism of action as a hypoglycemic nutraceutical compound have been identified so far. The lupin protein was shown to exist both in monomeric and multimeric forms as a function of pH. However, a detailed description of the pH-dependent structural dynamics of this protein, as the basis to investigate the reason/s of its functional behaviour, is not available yet. In this study, multiple and independent spectroscopic approaches, including light scattering associated to size exclusion chromatography of both untreated and covalently cross-linked protein, near and far UV circular dichroism, intrinsic and extrinsic fluorescence measurements, have been used to monitor oligomeric and conformational modifications caused by pH changes. Altogether, the results revealed a tetramer–dimer–monomer transition between neutral to slightly acidic pH and a dramatic and abrupt conformational change below pH 3.5. According to these findings, a model depicting γ -conglutin structural dynamics was drawn. This model highlights the primary role of amino acid side group electrostatic interactions in the oligomer association/dissociation equilibria and in the pH-driven collapse of the native conformation.

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1. Introduction

Lupin seed, a Mediterranean legume crop which is a source of food proteins comparable to soybean seed, is currently being reappraised for its contribution to human diet. Moreover, the blood glucose-controlling properties of lupin seeds, claimed by the traditional pharmacopoeia since long, have recently been attributed to a protein of this seed, termed γ -conglutin [1].

Lupin mature γ -conglutin is a glycoprotein made up of two disulphide-linked subunits of about 30 and 17 kDa, respectively. Two genes of γ -conglutin have been identified, but only one seems to be quantitatively expressed in the developing seed [2]. The deduced amino acid sequence of the expressed pre-protein is available (UniProtKB/TrEMBL accession number: Q9FSH9_LUPAL). From this sequence, the theoretical molecular weight is 45,446 Da and the pI is 8.24. The basic pI makes γ -conglutin a peculiar protein, since the seed proteins have usually acidic pIs. The mature native protein has been shown to undergo pH-dependent association–dissociation equilibrium between a monomeric unit and an oligomeric assembly [3]. This oligomer has been quoted both as a hexamer or a tetramer [4,5]. Various attempts to crys-

tallize the protein have unsuccessfully been carried out, therefore no 3D structure of the protein is available so far. However, a partial 3D predictive model, based on the homologous wheat xylanase inhibitor structure, has been proposed [6]. No physiological role nor catalytic activity of γ -conglutin have been unveiled as yet. As far as γ -conglutin biological activity is concerned, an insulin-binding capacity of this protein, which appeared to be strongly influenced by ionic strength and pH, has been described [1]. More recently, based on γ -conglutin effects upon cellular myocyte models, an insulin-mimetic activity of the protein has been proposed [7]. γ -Conglutin molecular and functional properties have recently been reviewed [8].

In order to get deeper insight into γ -conglutin structural features, as a pre-requisite to understand the molecular determinants which its biological activity is based upon, we have undertaken the present spectroscopic study for the determination of the pH-dependent structural dynamics of this lupin seed protein in view of its possible exploitation as a nutri-functional component.

2. Material and methods

2.1. Materials

The buffers at different pH values used in the spectroscopic studies were the following:

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- pH 2.0, 3.0, 3.25 and 3.5: 0.05 M sodium phosphate.
- pH 4.0, 4.5 and 5.5: 0.05 M sodium acetate.
- pH 6.5: 0.05 M sodium citrate.
- pH 7.5 and 8.5: 0.05 M TRIS/HCl.
- pH 9.5: 0.05 mM sodium borate.

All solutions contained suitable NaCl concentrations to balance the overall ionic strength.

2.2. γ -Conglutin purification

Defatted lupin flour was suspended in distilled water adjusted at pH 5.0 in the ratio of 1:10 (w/v) and stirred for 4 h at 4 °C. The suspension was centrifuged at $10,000 \times g$ for 30 min at 4 °C. The supernatant, containing the albumin fraction and other minor soluble constituents, was discarded and the pellet was resuspended in the ratio 1:20 (w/v) in 0.05 M sodium phosphate buffer (pH 7.5) containing 0.5 M NaCl. After stirring for 4 h at 4 °C, the suspension was centrifuged at $10,000 \times g$ for 30 min at 4 °C. The supernatant was subsequently desalted on a Sephadex G-50 column (GE Healthcare, Milan, Italy) equilibrated with 0.05 M sodium phosphate buffer (pH 7.5). The desalted extract was immediately fractionated on Whatman DE 52 DEAE-cellulose equilibrated with 0.05 M sodium phosphate buffer (pH 7.5); in these conditions the raw γ -conglutinin fraction was not retained on the column. The γ -conglutinin protein solution was adjusted to pH 4.5 with acetic acid, loaded on a Whatman CM-cellulose column equilibrated in 0.050 M sodium acetate buffer (pH 4.5) and then eluted by addition of 0.35 M NaCl in the same buffer. For further purification, an insulin-agarose immobilized affinity chromatography step (Sigma-Aldrich, Milan, Italy) was used [1]. γ -Conglutinin solution was loaded on the column equilibrated in 0.025 M TRIS/HCl, pH 7.2) and eluted by addition of 0.2 M NaCl to the loading buffer.

The purified protein was dialyzed at 4 °C with frequent changes of milli-Q water, freeze dried and resuspended in the appropriate buffers before use.

2.3. Light scattering system

Analysis of the oligomeric structures of γ -conglutinin were performed by using the serial system composed by a HPLC pump (515 HPLC pump, Waters), a SEC column (Superose 12, HR10/30, Pharmacia LKB), an UV spectrophotometer (Dual λ absorbance detector 2487, Waters), a multi-angle light scattering device (DAWN HELEOS, Wyatt), provided with a fast photon counter (QELS) and a differential refractometer (Waters). The column temperature was kept constant at 20 °C. Detection was based on the refractive index, the absorbance at 280 nm, the solution density, the intensity and fluctuation measurements of the scattered light intensity. The determination of molecular masses and hydrodynamic radii was performed by the ASTRA V software (Wyatt Technology). The γ -conglutinin unknown refraction index and molar extinction coefficient were calculated as described below.

2.4. Calculation of the refraction index (n) as a function of the protein concentration (C) (dn/dc index)

The variation of the refraction index n as a function of the protein concentration C is specific for each protein and it was calculated by "in batch" analysis through the refractometer (RI) interfaced with the LS apparatus, by using solutions of different concentration of γ -conglutinin, as assessed by UV absorption measurements (see below). Prior to the calculation of γ -conglutinin dn/dc index, the refractometer was calibrated using BSA, whose dn/dc index is known [13]. For the calculation of γ -conglutinin dn/dc index, the protein was suspended in 0.05 M sodium acetate (pH 4.5) containing 0.1 M NaCl,

at the concentration of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. Aliquots of 5 mL of each solution were injected in the LS/RI system and the instrument signal intensities (voltage) were used to evaluate the changes of refractive index as a function of the protein concentration in the solution. The slope of the obtained curve, calculated as 1.877 mL/g, corresponded to the dn/dc index.

2.5. Calculation of the extinction coefficient (E) of native γ -conglutinin

Freeze-dried γ -conglutinin was resuspended in 0.05 M sodium acetate (pH 4.5) containing 0.1 M NaCl, at the concentration of 5 mg/mL and centrifuged 10 min at $10,000 \times g$. γ -Conglutinin solution was then diluted ten folds in three different buffers: native buffer (0.05 M sodium acetate, pH 4.5, 0.1 M NaCl), denaturing buffer (0.05 M sodium acetate, pH 4.5, 0.1 M NaCl, guanidine 8 M) and denaturing and reducing buffer (0.05 M sodium acetate, pH 4.5, 0.1 M NaCl, guanidine 8 M, DTT 65 mM). The latter samples were maintained at 50 °C for 16 h, in order to obtain the complete denaturation and reduction of the protein. Immediately before the spectrophotometrical measurement, the samples were diluted 1:1 with native buffer. The optical absorption of γ -conglutinin solutions was then measured.

The theoretical E value was calculated according to Ref. [12] by using the equation:

$$E = \frac{n_{\text{tyr}} \cdot E_{\text{tyr}} + n_{\text{trp}} \cdot E_{\text{trp}} + n_{\text{cystine}} \cdot E_{\text{cystine}}}{\text{MW}}$$

where E ($\text{L mol}^{-1} \text{cm}^{-1}$) is 1490 for tyr, 5500 for trp and 125 for cystine.

From the sequence of γ -conglutinin (Q9FSH9.LUPAL), deprived of the signal peptide, it was possible to calculate the theoretical E value corresponding to the E value of the denatured protein. Dividing the OD value at 280 nm by the theoretical E value of denatured and/reduced γ -conglutinin, it was possible to obtain the protein actual concentration in solution. Then, by dividing the OD value at 280 nm of native γ -conglutinin by its actual concentration, it was possible to obtain the E value of native γ -conglutinin, which resulted 0.733 L/g cm.

2.6. Light scattering (LS) studies

γ -Conglutinin was dissolved in different buffer systems at the desired pH values, at the concentration of 1 mg/mL, and 200 μL solution were loaded into SEC column (1 cm $\times$ 30 cm) after centrifugation at $10,000 \times g$ for 10 min. The mobile phase consisted of the same buffer in which the protein was dissolved. The elution flow rate was 0.5 mL/min. The software ASTRA V (Wyatt Technologies, 2004) was used to extrapolate the molecular mass, the hydrodynamic radius and the percentage of the species present at the various pH values from raw data.

2.7. Chemical cross-linking of γ -conglutinin

The cross-linking of γ -conglutinin was achieved by mixing 1 mL of γ -conglutinin at the concentration of 0.8 mg/mL in 0.05 M sodium phosphate (pH 7.5) containing 0.1 M NaCl with 50 μL of 2.5% freshly prepared solution of glutaraldehyde. The reaction mixture was incubated at room temperature for 5 min, taking aliquots every 30 s to check the efficacy of the cross-linking. Each sample was stopped by adding 100 μL of 1 M TRIS/HCl, pH 8.0. At the end of cross-linking reaction, the pH of the solution was adjusted to 4.5 by adding a suitable volume of acetic acid. The better results were obtained after 1 min and 30 s incubation.

2.8. Circular dichroism studies

CD spectra were recorded at 20 °C between 240 and 360 nm using a J-810 Jasco spectropolarimeter and analysed by means of the dedicated Jasco software. The cell path was 1 cm; the scanning speed was 10 nm/min, and the band width was 2 nm. The protein solutions (2 mg/mL in the near-UV studies and 0.2 mg/mL in the far-UV studies) were prepared by dissolving γ -conglutin in water and then by dilution in the different buffer systems. CD measurements were corrected by subtracting the corresponding buffer spectrum.

2.9. Intrinsic and extrinsic fluorescence

Fluorescence was measured at room temperature in a LS 50 B Luminescence Spectrometer (PerkinElmer), by using a quartz cell (path 1 cm) with four clear faces. Tryptophan intrinsic fluorescence was monitored by taking emission spectra from 300 to 440 nm wavelengths, with excitation at 282 nm.

Emission spectra of 1-anilin-8-naphthalene-sulphonate (ANS) were taken from 400 to 550 nm wavelengths, with excitation at 390 nm. Emission and excitation band widths were set at 5 nm and the scan speed at 50 nm/min. For the intrinsic fluorescence measurement γ -conglutin was dissolved in water, and then diluted 1:1 in the buffers at different pH values at the final concentration of 0.05 mg/mL. Titration with ANS was performed by adding ANS (from a 0.02 M stock solution) to individual γ -conglutin samples diluted in the different buffer systems at the final concentration of 0.4 mg/mL. The ANS concentration ranged between 0.01 and 0.13 mM, with appropriate increments. Titration results were analysed by standard binding algorithms that allowed the estimation of the protein overall binding capacity for the probe and the apparent dissociation constant of the proteins–ANS complex. The overall binding capacity ($F_{\max}$) was then corrected for the total protein content of each sample. The protein surface hydrophobicity (PSH) index was calculated according to Ref. [14]:

$$\text{PSH} = \frac{F_{\max}}{K_d \cdot [\gamma\text{-conglutin}]}$$

where $F_{\max}$ is ANS fluorescence at saturation, K_d is the apparent dissociation constant and $[\gamma\text{-conglutin}]$ is the protein mM concentration.

K_d was calculated according to the equation:

$$\frac{F}{\text{ANS}} = \frac{-1}{K_d} \cdot F + \frac{F_{\max}}{K_d}$$

3. Results

3.1. Light scattering (LS) studies

LS analyses in combination with size exclusion chromatography (SEC) were used to monitor the changes in the association state of lupin γ -conglutin as a function of pH. The most representative chromatograms of the protein eluted from a Superose 12 column at various pH values are reported in Fig. 1A. A dramatic change in the chromatograms from pH 4.5 to 5.5, consisting in the marked shift of the peak to shorter elution times and thus greater molecule size, was detected. Already at pH 5.5, the disappearance of the peak at 26 min elution time was complete, while a new peak eluting at 20 min became visible. The changes at pH higher than 7.5, were less marked consisting essentially in the formation of larger aggregates with shorter elution times. However, the amounts of the aggregates were below 10% (Table 1). For this reason the chromatograms obtained at pHs 8.5 and 9.5 were not included in the figure. LS analyses of the eluted peaks allowed the determination of the molecular weights, hydrodynamic radii and a quantitative

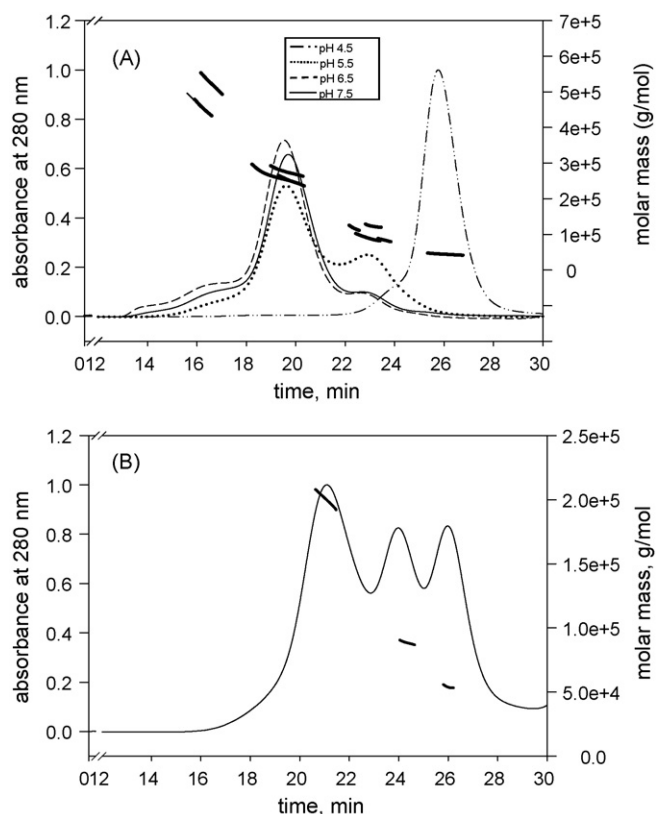


Fig. 1. The UV-recorded SEC chromatograms of (A) native γ -conglutin assemblies at pH values of 4.5 (dash-dotted line), 5.5 (dotted line), 6.5 (dashed line) and 7.5 (solid line), and (B) chemically cross-linked γ -conglutin at pH 4.5. The molecular masses of γ -conglutin assemblies, as calculated by LS, are indicated on the panels as bold line segments with the values marks on the right axes. For details see Section 2.

evaluation of the protein species separated by SEC at all pH values tested. These results are reported in Table 1. At pH 4.5, the most acidic pH value tested with SEC, γ -conglutin consisted of a single peak with a molecular mass around 50 kDa. Since this mass is very close to that deduced from the amino acid sequence (see the Section 1), we assumed that this species corresponded to the two-subunit monomeric unit of the protein. At pH $\geq$ 5.5, the monomeric form had completely disappeared with the concomitant formation of other forms with average molar mass around 100, 255 and 480 kDa, respectively. Among these forms, the predominant one was that with molecular weight around 255 kDa, which quantitatively represented almost 90% of all species present at pH 6.5 (Table 1). This form decreased at pH 8.5 and 9.5 with the concomitant increase of 100 kDa species. Hydrodynamic radii increased with the increase of the molar mass of all molecular species identified, confirming the molecular aggregation of the constituent units.

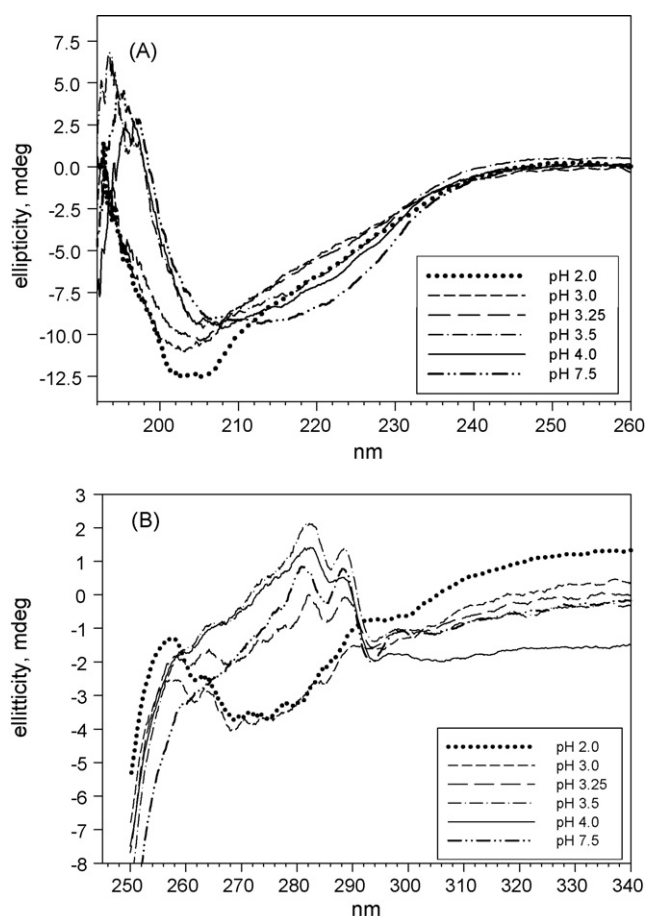
However, since these mass evaluations did not allow the unequivocal identification of the association state of the oligomeric protein forms, chemical cross-linking experiments of the protein at neutral pH by using glutaraldehyde were carried out. After chemical conjugation, aimed at fixing the prevalent protein species in their structural assembly, the pH was dropped to 4.5 in order to suppress any non-covalently bound form and HPSEC–LS analysis was performed under these conditions. The results are shown in Fig. 1B. Three distinct peaks appeared in the chromatograms for which LS analysis revealed a molar mass of $200,100 \pm 600$, $88,570 \pm 410$ and $53,990 \pm 310$ Da, respectively. Larger aggregates, which, as stated before, were always present in low amounts (Table 1), were not present, since they were removed by centrifugation prior to HPSEC–LS analysis. As a matter of facts, the analysed material rep-

Table 1

Molecular species present at various pH values and main features.

	pH					
	4.5	5.5	6.5	7.5	8.5	9.5
Molecular mass, Da	49,991 ± 250	–	–	–	–	–
Hydrodynamic radius	2.91 ± 0.06	–	–	–	–	–
%	94.5	–	–	–	–	–
Molecular mass, Da	93,450 ± 1200	90,270 ± 640	117,400 ± 2600	123,100 ± 1700	95,600 ± 560	99,340 ± 3400
Hydrodynamic radius	2.89 ± 0.01	3.23 ± 0.08	3.21 ± 0.13	4.31 ± 0.04	3.58 ± 0.12	3.72 ± 0.08
%	5.5	37.7	3.0	6.2	23.6	24.8
Molecular mass, Da	–	256,000 ± 600	260,100 ± 1000	275,300 ± 1100	244,200 ± 900	241,500 ± 8400
Hydrodynamic radius	–	5.18 ± 0.02	5.29 ± 0.01	5.50 ± 0.01	5.23 ± 0.09	5.11 ± 0.08
%	–	57.0	89.0	83.2	65.4	56.2
Molecular mass, Da	–	452,300 ± 6600	474,300 ± 5600	521,100 ± 3700	501,800 ± 4000	438,900 ± 3000
Hydrodynamic radius	–	6.40 ± 0.04	7.31 ± 0.03	7.51 ± 0.03	7.55 ± 0.13	7.15 ± 0.13
%	–	5.0	6.0	9.0	8.2	12.6

(–) Not present.

**Fig. 2.** CD spectra of γ -conglutin in the far (A) and near UV (B) at pH 2.0 (dotted line), pH 3.0 (medium dashed line), pH 3.25 (long dashed line), 3.5 (dash-dotted line), pH 4.0 (solid line) and 7.5 (dash-dot-dotted line).

resented about 70% of the original cross-linked protein material. According to these quantitative results, the attribution of tetramer, dimer and monomer to each respective peak was consistently assumed.

3.2. Circular dichroism (CD) studies

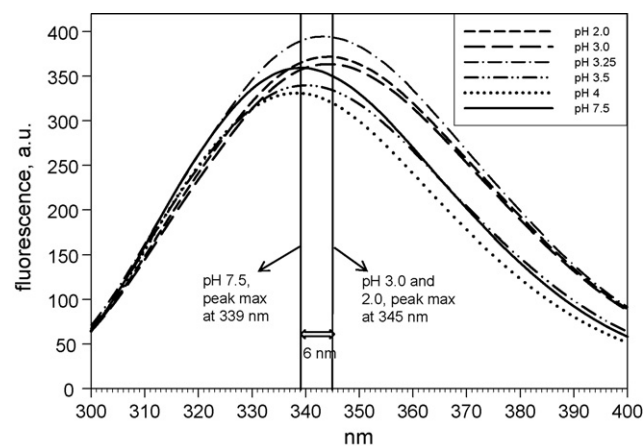
CD spectra of γ -conglutin in the far and near UV as a function of pH are reported in Fig. 2A and B, respectively. In these analyses, as well as in fluorescence studies which will be described below, the pH values tested included also lower values than those used

in HPSEC–LS analyses, thus monitoring the secondary and tertiary structures changes, rather than protein aggregation. The far UV–CD spectra at pH 7.5 reflected the predominance of β -structure with respect to α -helix, as already noted in previous studies [9] and in agreement with the predicted partial 3D structure of γ -conglutin [6]. Nonetheless, remarkable changes in the shape of the spectra, when the pH was lowered below 4.0, were observed. In particular, relevant changes of the spectral behaviour were visible below pH 3.25, where the secondary structure of the protein seemed to be strongly affected. Indeed, a similar shift of the spectra minima from 220 to 200 nm can be observed when a β -structure is converted in an irregular form [10].

This behaviour was confirmed by the near UV–CD spectral analyses, where the aromatic amino acids environment gives information on the alterations of the protein tertiary structure. Indeed, the main spectral changes occurred at acidic pH values and were dramatic at pH 3.0 and 2.0, as in the near UV spectra. This confirmed that a dramatic conformational transition took place when the protein was exposed to pH values below 3.5.

3.3. Intrinsic fluorescence studies

Intrinsic fluorescence analyses at the same pH values tested in CD were undertaken to monitor structural changes of the aromatic amino acid environment, thus related to the protein overall conformation, too. A marked red shift in the intrinsic fluorescence spectra was induced by the pH drop (Fig. 3). The greatest differences were between pH 7.5 and 2.0 with as much as 6 nm wavelength differ-

**Fig. 3.** Intrinsic tryptophan fluorescence spectra of γ -conglutin solutions at pH 2.0 (medium dashed line), pH 3.0 (long dashed line), pH 3.25 (dash-dotted line), pH 3.5 (dash-dot-dotted line), pH 4.0 (dotted line) and pH 7.5 (solid line).

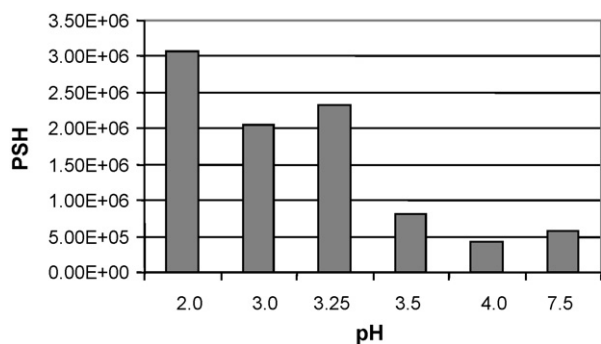


Fig. 4. Histograms of γ -conglutinin PSH at different pH values. PSH values were calculated as described under Section 2.

ence of the maxima. Once again, the great transition in the shape of the spectra occurred at $\text{pH} \leq 3.25$. These results confirmed the remarkable conformational changes at strongly acidic pH values and a substantial identity of the spectra at less acidic to neutral pH values, as observed with CD analyses. All these findings shed light onto the effect of the complete protonation of all amino acid side groups which likely led to the disruption of internal salt bridges, as it will be discussed below.

3.4. Protein surface hydrophobicity (PSH) measurements

PSH was determined by using the hydrophobic probe ANS in titration assays at the pH values considered in this study. The results are shown in the histogram of Fig. 4. No significant variations were observed between pH 4.0 and 7.5. At pH 3.5 only a slight increase in PSH value was detected, while at lower pH values the surface hydrophobicity increased of about 4–6-fold, thus showing a dramatic increase of bound ANS and confirming the collapse of the protein at these acidic pH values.

4. Discussion

Although lupin γ -conglutinin is deposited into the protein storage vacuoles as all legume seed storage proteins, a storage role of this protein has been ruled out due to its unusual resistance to endogenous proteolysis at seed germination [9]. No physiological function of this protein has been identified as yet, though sequence and structural homology with xyloglucan-specific endo- β -1,4-glucanase inhibitor proteins (XEGIPs) and *Triticum aestivum* xylanase inhibitor I (TAXI-I) suggest an involvement of the protein in the defence response mechanisms [6]. However, an unexpected role of the orally administered lupin protein in lowering the blood glucose concentrations was evidenced in both animal [1] and humans (Duranti et al., in preparation). This finding has triggered further studies on the possible mechanism of action which have recently led to hypothesise an insulin-mimetic activity of the protein in cellular models [7]. Since pH vary from acidic to neutral values in different tissues and organs of human body, deeper knowledge of the relationships between structure and function of γ -conglutinin, including the assembly status of the protein at pH values which can be encountered during the gastro-intestinal transit of the protein upon oral administration in monogastric animals.

In previous papers, γ -conglutinin at neutral pH was referred to as both a tetramer [5] and an hexamer [4] and a pH-dependent reversible association/dissociation equilibrium of γ -conglutinin has already been described [5]. The unequivocal attribution of γ -conglutinin assembly status was among the goals of the present work. To this purpose, we used light scattering analysis of the protein as a function of pH. However, while the estimation of the monomer size was in agreement with previous studies, the assessment of

multimer molar masses was hindered by the intrinsic limit of this technique which calculates the weighted average size of the protein species present in a given solution. The possible presence of different forms which the chromatographic separation may have not completely resolved and the possible equilibria among interchanging aggregates during the chromatographic separation, especially at neutral pH values, may have affected the reliability of the quantitative results. To overcome these difficulties, we adopted a strategy of chemical cross-linking of the protein aggregates in order to fix the prevalent species in a given condition. After cross-linkage, by shifting the pH to acidic values to cause dissociation of all non-covalently linked subunits, only soluble and specific aggregates would have been detectable. This was indeed the case with the clear SEC separation of three peaks and their LS analysis which, by assessing the homogenous size of each protein form, allowed us to conclude that γ -conglutinin oligomers consisted of dimers and tetramers of the 50 kDa unit.

In this work, independent spectroscopic approaches have shown that a pH value of 3.5 is crucial for γ -conglutinin native conformation. Indeed, spectroscopic analyses by showing the loss of secondary structure elements, the changes in the local aromatic amino acid environment and the increase of surface hydrophobicity, demonstrated the collapse of the molecule at strongly acidic pH values. Interestingly, no relevant spectral change was observed in the pH range where the oligomeric transition takes place, i.e. 5.0–6.0, thus indicating that γ -conglutinin association/dissociation equilibria between monomer and oligomers do not appear to involve any structural variation which can be measured with the used spectroscopic approaches.

According to the present results, it can be concluded that the electrostatic interactions are crucial for γ -conglutinin structure. Globally, relevant structural changes take place between pH 4.5 and 6.5 and below 3.5. Interestingly, these pH ranges are close or even correspond to the pK_a values of the histidine, glutamate and aspartate side groups, i.e. 6.0, 4.25 and 3.85, respectively. Therefore, while protein oligomerisation appears to be influenced by histidine residues protonation/deprotonation, monomer trans-conformations are likely dominated by glutamic and/or aspartic acid residues protonation/deprotonation. On the basis of these conclusions, an association and trans-conformation model for γ -conglutinin molecular species as a function of pH is depicted in Fig. 5. In this scheme, the overwhelming global positive charge of the molecule at pH 4.5 does prevent any aggregation of γ -conglutinin monomeric units; however, a small shift to less acidic pH values, i.e. 5.5, completes the dissociation of glutamate residue side groups and begins the positive charge loss of histidine residues, thus limiting charge repulsion and allowing some crucial salt bridges to be formed. This leads to the formation of a transient dimer. The complete dissociation of histidine side groups, taking place at $\text{pH} \pm 6.5$, still decreases the residual positive electrostatic repulsions between dimers and leads most molecules to adopt a compact tetrameric structure, or greater associations, where intra- and inter-subunit salt bridges stabilize the positive and negative charges present on the molecule in those conditions. On the other hand, at pH values lower than 3.5 complete protonation of the aspartic and glutamic acid residues disrupts the internal salt bridges, thus causing a relevant rearrangement of the native protein conformation which involves also its secondary and tertiary structures, as observed with the spectroscopic analyses.

In a previous work, where the susceptibility of γ -conglutinin to proteolytic enzymes as a function of pH was analysed [11], a pH lower than 4.0 was shown to be necessary to completely proteolyse the protein. Conversely, the protein resulted to be completely resistant to various proteolytic enzymes and protease mixtures at greater pH values. On the basis of former and present results, it may be concluded that γ -conglutinin has a very compact structure

γ -conglutin ionisation status/monomer				
pH	≤ 3.5	4.5	5.5	≥ 6.5
Asp	16 ⁰	16 ⁻	16 ⁻	16 ⁻
Glu	9 ⁰	6 ⁻	9 ⁻	9 ⁻
His	19 ⁺	19 ⁺	19 ⁺	19 ⁰
Lys	18 ⁺	18 ⁺	18 ⁺	18 ⁺
Arg	11 ⁺	11 ⁺	11 ⁺	11 ⁺
Total	48 ⁺	26 ⁺	23 ⁺	4 ⁺

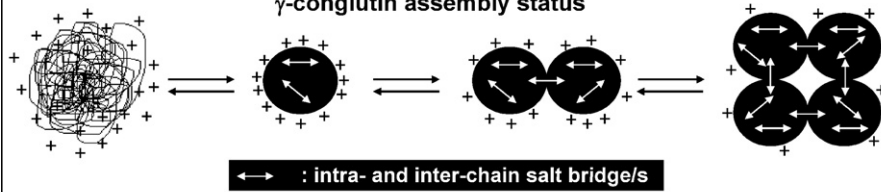
γ -conglutin assembly status				
				
↔ : intra- and inter-chain salt bridge/s				

Fig. 5. Schematic drawing of γ -conglutin pH-dependent structural changes. Figures refer to the number of each ionisable amino acid residue of the protein sequence. See Section 4 for explanations.

and that a trans-conformation induced by a low pH is required to make the protein susceptible to enzymatic hydrolysis. Whether the observed transitions are functional to the biological activity of the protein either inside the seed or as blood glucose-lowering agent and to the overall stability of the proteins in its transit through the gastro-intestinal tract it still has to be proved.

The present results also shed light on γ -conglutin binding properties to insulin, already described in a previous paper [1]. Indeed, insulin with a *pI* of 5.4 presents negatively charged side groups at neutral pH values where the binding with a positively charged γ -conglutin preferably takes place, while the interaction was shown to be suppressed at pH below 4.2, corresponding to the pK_a of the insulin glutamate four residues. This confirms the role of the electrostatic attraction as a main driving force in the interaction of the two proteins. Whether and to what extent this interaction is related to the glucose lowering capacity of γ -conglutin is still to be assessed.

In conclusion, the results of this work highlighted the strong pH-dependence of γ -conglutin association/dissociation equilibria, by providing the molecular determinants of this transition which drive the assembly status of the protein. Moreover, the present results shed light onto the electrostatically-driven conformational collapse of the molecule at pH lower than 3.25. These findings do contribute to a deeper understanding of the molecular features of this pro-

tein as the basis for unveiling its biological role and mechanism of action.

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