Investigación

Spectroscopic Characterization of the Thermal Unfolding of Wheat Germ Agglutinin

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Dedicado a la memoria del doctor Raymundo Cruz Almanza, entrañable colega y ejemplo del quehacer científico

Abstract. We present a study of the thermal unfolding process of wheat germ agglutinin, a prominent member of the chitin-binding lectin superfamily. As evidenced by circular dichroism (CD) measurements, the unfolding was fully reversible at acidic conditions, indicating that the process was under thermodynamic control. Thermal CD profiles appeared to be independent on protein concentration, suggesting an unimolecular character for the reaction. This property was confirmed by dynamic light scattering experiments, which revealed that the lectin was monomeric under the conditions studied. In literature, wheat germ agglutinin always has been referred to as a homodimer. This is the first time that the agglutinin is revealed as a non-obligate homodimer that dissociates into compact native-like monomer under acidic conditions.
Key words: lectin; circular dichroism, dynamic light scattering, non-obligate homodimer.

Resumen. Presentamos un estudio del proceso de desplegamiento de la aglutinina de germen de trigo, un miembro prominente de la superfamilia de lectinas unidoras de quitina. Experimentos de dicroísmo circular (DC) evidenciaron que el desplegamiento fue completamente reversible bajo condiciones ácidas, indicando que el proceso estuvo bajo control termodinámico. Los perfiles térmicos de DC resultaron independientes de la concentración de proteína, sugiriendo un carácter unimolecular para la reacción. Esta propiedad fue confirmada mediante experimentos de dispersión dinámica de luz, los cuales revelaron que la lectina se encontraba como monómero bajo las condiciones estudiadas En la literatura siempre se ha descrito a la aglutinina de germen de trigo como un homodímero. Éste es el primer estudio que evidencia que la aglutinina de germen de trigo es un oligómero no obligado, el cual puede disociarse en monómeros con estructura tipo nativa en condiciones ácidas.
Palabras clave: lectina, dicroísmo circular, dispersión dinámica de luz, homodímero no obligado.

Introduction

Lectins encompass one of the major groups of carbohydrate-binding proteins. Initially, they were distinguished from other protein groups almost by exclusion. For instance, as late as in 1995 Lee and Lee still defined lectins as any "…carbohydrate-binding protein, excluding enzymes and immunoglobulins" [1]. Through time, however, lectins have prevailed as a coherent group as they have proved to share some key properties. Functionally, every lectin is specialized in the recognition of specific oligosaccharidic configurations. Due to the highly combinatorial capacity of oligosaccharides, the recognition of these structures by lectins represents the core of a fundamental stereochemical device for a large number of recognition-assisted processes in all live beings. Pre-formed binding sites and the non-induction of covalent distortions on the saccharide are other salient properties that all lectins present in common.

Another characteristic frequent among lectins is the trend to form homoligomers [2]. Oligomerization yields the basis for the multivalency necessary for typical lectin activities such as cell agglutination [3,4]. Saliently, the recognition sites of the majority of lectins are exclusively composed of intracatenary residues. An exception to this rule has been observed in

lectins belonging to the superfamily of chitin-binding proteins, among which wheat germ agglutinin (WGA) is one of the most prominent members. WGA is a homodimeric protein composed of 17-kDa subunits each containing 16 disulfide bridges. The monomers associate with each other in a head-to-tail fashion forming a two-fold symmetric globule (Fig. 1).

Fig. 1. Schematic representation of the 3D structure of WGA dimer complexed with GlcNAc(β1,4)GlcNAc. In the crystal structure, only two saccharides are observed (vdW-sphere representation). The figure was generated with "rasmol" using the coordinates with the PDB code 1K7U.

The amino acid sequence of the monomer shows an approximate four-fold sequence repeat defining 43-residue isostructural domains. Each of the four carbohydrate-binding sites of WGA is located at the interface of two intercatenary domains [5].

To explore the molecular basis of the formation of the intercaternary recognition-sites of chitin-binding lectins, we have undertaken a systematic characterization of the acquisition/disruption (folding-dimerization/unfolding-dissociation) processes of the native conformation of WGA. In this communication we present the results of the first stage of our study. Our goal has been to establish the conditions whereby thermal unfolding of WGA may occur under thermodynamic control. This is with the purpose of eventually characterizing the stability of the protein in a quantitative way. We found that at acidic conditions WGA undergoes a fully reversible unfolding process. Also, it was observed that WGA prevails as a native-like monomer, as inferred from circular dichroism and dynamic-light scattering measurements.

Results and discussion

The commercial presentation of WGA comes as a mixture of three closely related agglutinins which differ in several sequence positions [6]. The separation of these isolectins was carried out by cationic-exchange liquid chromatography. Under the conditions used (20 mM formic acid, pH 4.0), the isolectins were purified to homogenity in a single step, as evidenced by native-PAGE electrophoresis (data not shown). In this study, all experiments were performed using WGA variant 1 (WGA1), the most abundant isolectin.

Figure 2 shows the CD spectra of WGA1 obtained at room temperature and different conditions of acidity. The fact that all spectra are quite similar indicates that the protein preserves the same overall secondary and super-secondary structures in the pH range of 2-5. Overall, WGA1 exhibits a typical
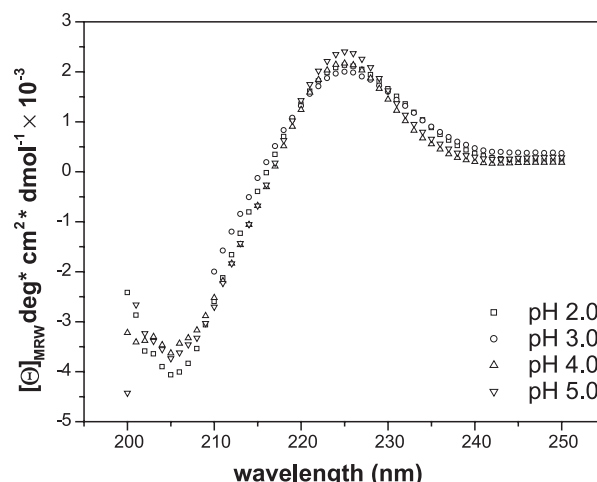

Figure 2. Far-UV circular dichroism spectra of WGA1 at room temperature and different pH values.

CD spectrum of proteins having the so-called toxin-agglutinin fold [7]. The positive band centered at ~225 nm is characteristic of cystine residues immersed in an asymmetric environment. Its relatively high intensity is due to the high density of WGA1 disulphide bridges (16 per monomer), as well as the lack of secondary-structure repetitive elements.

To test the degree of reversibility of the thermal unfolding of WGA1, it was submitted to a heating/cooling cycle in the temperature range of 20-90 °C. Figure 3A shows the spectra recorded at 90 °C under the different acidic conditions sampled. The spectra displayed the characteristic shape of unfolded proteins, indicating that the protein had lost the native conformation at this high temperature. A closer inspection of Figure 3A reveals that the spectra showed subtle but consistent differences. The signal at 225 nm (maximum of the native positive band) decreased as the acidity of the solution
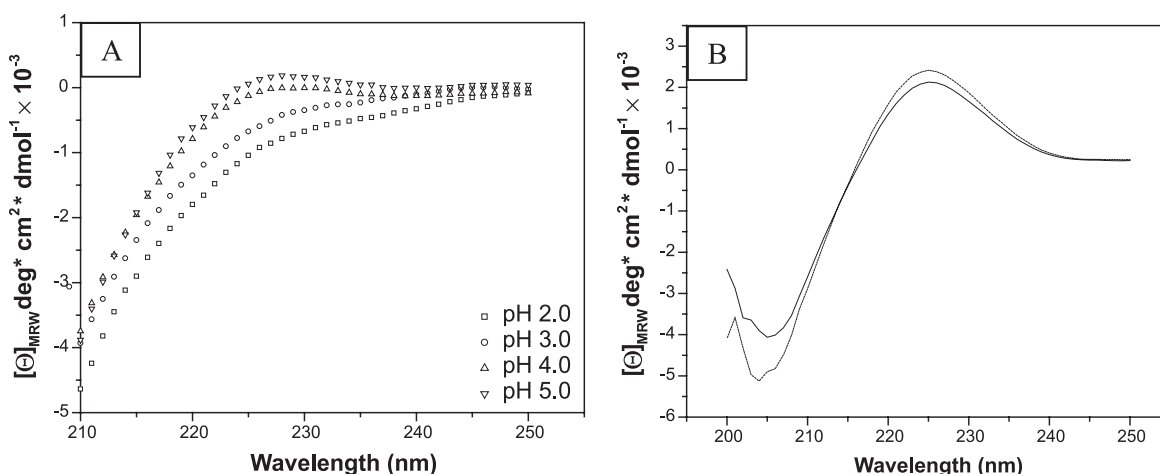

Fig. 3. A. CD spectra of WGA1 at 90 °C and different pH values. **B**. CD spectra of WGA at room temperature, pH 2.0, before (solid line) and after (dashed line) the heating/cooling cycle.
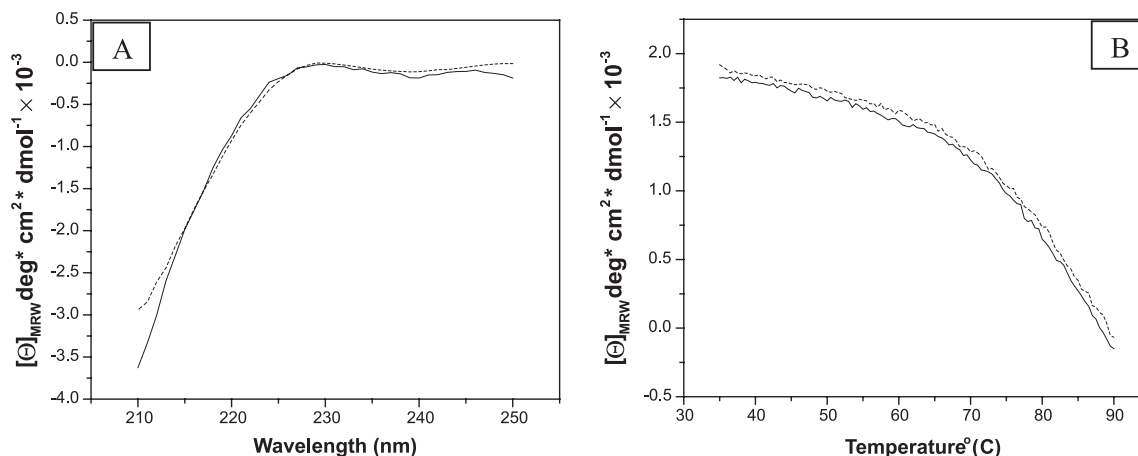
Fig. 4. A CD spectra at 90 °C and **B** CD signals at 225 nm as a function of temperature at WGA1 concentrations of 5.8 (solid line) and 53 (dashed line) μM (pH 2.0).

increased, resembling more closely the signal of a fully unfolded protein. As expected for soluble proteins, these results indicated that the stability of WGA1 increased as the pH approached its isoelectric point (pI ~7.6). Figure 3B shows that the CD spectra obtained before and after the heating/cooling cycle fully overlapped. Identical results were observed throughout the pH range considered (data not shown). Thus, these results show that WGA1 underwent a fully reversible thermal unfolding process under acidic conditions.

To gain insight into the unfolding mechanism of WGA1, thermal profiles were obtained at a fixed wavelength (λ = 225 nm), heating the solution at a constant speed of 1 °C/min and using different protein concentrations. It was systematically observed that neither the unfolding profile nor the shape of the spectrum at high temperatures depended on protein concentration (Fig. 4). As the concentrations differed up to nearly one order of magnitude, these observations suggest that the main unfolding transition observed in the CD profiles was an unimolecular reaction [8]. In other words, WGA1 dimer should be dissociated before the native secondary structure begins to be lost. As can be seen in Figures 4A and 4B, the signals recorded at 90 °C in the spectrum and scan modes were very similar. This agreement indicates that the unfolding process occurred under equilibrium conditions.

To examine the aggregation behavior of WGA1, its hydrodynamic radius (R_H) as a function of temperature was determined by dynamic light scattering measurements. As seen in Figure 5, the molecular size kept roughly constant up to 70 °C. The average R_H obtained in this temperature region was 2.41 nm, a value that corresponds to an estimated molecular weight of ~22.1 kDa. This size is somewhat larger than that of the monomer (17.1 kDa), but significantly smaller than that of the dimer (34.2 kDa). Thus, DLS measurements confirmed the conclusion of the unimolecular character of the unfolding reaction. As far as we know, this is the first report that documents conditions under which WGA is not a dimer,

but a compact monomer with native-like secondary structure. Above 70 °C, R_H increased sharply. Since this abrupt change was observed at the same temperature region as the change in CD signal (Figure 4B), it follows that the lost of secondary structure and the overall expansion of the monomer occured concomitantly, suggesting that the unfolding reaction may follow a simple two-state mechanism.

Concluding remarks

Although WGA is a lectin that has been profusely characterized, studies aimed at unveiling the molecular basis of its stability have not been undertaken. In spite of its relatively high molecular complexity (16 disulphide bridges and 4 structural domains per monomer), it was shown in this work that WGA
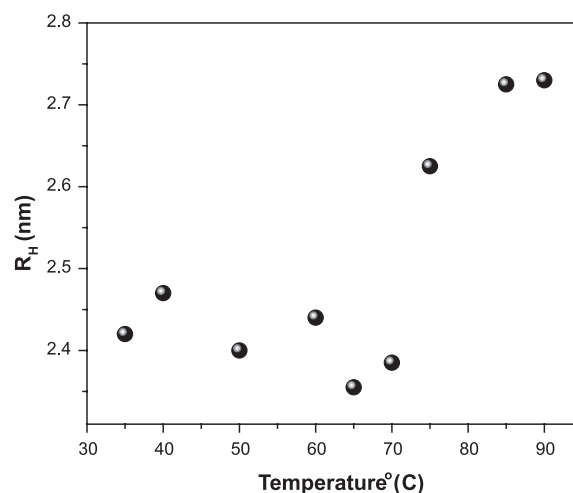

Fig. 5. Hydrodynamic radius (R_H) of WGA1 as a function of temperature (pH 2.0).

undergoes a fully reversible thermal unfolding process at acidic conditions. Moreover, it was evidenced that WGA is a non-obligate homodimer, as under those conditions it dissociated forming native-like monomers. These properties make it an excellent model system for the study of the structural and energetic principles that govern the assembly of the intercatenary binding subsites of chitin-binding proteins in particular, and the formation of homodimers in general.

Materials and methods

WGA was purchased as a mixture of three variants from Sigma Chemical Co. All other reagents were of analytical quality. Protein concentrations were determined spectrophotometrically by using an absorption coefficient of 1.27 ml(mg cm)$^{-1}$ at 280 nm (5). The molecular mass of WGA monomer was taken as 17.1 kDa.

Purification. Separation of WGA variants was carried out by cationic-exchange in an HPLC equipment. A sample of WGA was applied to a column of Mono-S HR 5/5 (Amersham Pharmacia Biotech) equilibrated with 20 mM formic acid buffer (pH 4.0) and washed with the same buffer. The variants were eluted at the rate of 1ml/min with a gradient obtained by the mixing of two solvents both containing 20 mM formic acid (pH 4.0): **a**, 0 mM LiCl; **b**, 750 mM LiCl. Variants 1, 2 and 3 eluted reproducibly at 33%, 53% and 63% of solvent **b**. Proteins fractions were concentrated and diafiltrated extensively in an Amicon-stirred cell through polyethersulfone ultrafiltration discs (cutoff 10 kD, PM10). 30 mM glicine/HCl buffer was used for experiments at pH values of 2 and 3, while 30 mM acetates buffer was used for experiments at pH values of 4 and 5.

Circular dichroism. Far-UV CD spectra were recorded on a JASCO J-720 spectropolarimeter equipped with a peltier thermoelectric device for temperature control. This instrument was calibrated with (+)-10-camphorsulfonic acid. Three scanning acquisitions were accumulated and averaged to yield the final spectrum. CD signals are reported as mean residue ellipticity, $[\Theta]_{mrw}$, using a value of 110 for the molecular weight of a mean residue.

Dynamic light scattering. DLS experiments were performed with a DynaPro-801 molecular sizing instrument (Protein Solutions Co.) as described previously (Arreguín-Espinosa *et al.,* 2001). The hydrodynamic radius (R_H) was estimated on the basis of an autocorrelation analysis of scattered light intensity data.

Acknowledgements

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