Self-assembly of Gliadin protein modulated by pH

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Abstract

Gliadin, a protein present in wheat, rye and barley induces zonulin releases in intestinal epithelial cells in vitro, leading to increase of epithelium permeability. It is known that this protein and some proteolytic resistant peptides are responsible of allergies, gluten sensitivity and celiac disease. Taking account the importance of pH in digestion, evaluation of gliadin under different pH conditions is presented. Different aggregates were observed in the whole range of pH and evaluated by UV/Vis and steady state fluorescence experiments in combination with dynamic light scattering. At physiological pH, a self-assembly process occurred; and vesicles were detected by transmission electron microscopy experiments. Currently, physicochemical evaluation of the self-assemble system is under progress in order to shed light into gliadin intolerance disorders.

Keywords: gliadin**,** self-assembly, coacervate, food intolerance

1. Introduction

Gliadin, a protein present in wheat, rye and barley induces zonulin releases in intestinal epithelial cells in vitro, leading to increase of epithelium permeability. (1) It is known that this protein and some proteolytic resistant peptides are responsible of allergies, gluten sensitivity and celiac disease. (2-4) Gliadin is really a complex mixture of proteins which are electrophoretic separated in four fractions: alpha, beta, gamma y omega. (5-6) It is possible to obtain gliadin from wheat gluten by ethanolic extraction and some aqueous conditions.(7) In addition, it is known that alpha gliadin is able to form nanofibrils at pH 5.0 on specific ionic strength conditions. (8) Different solvent and condition have been used to study gliadin protein (6, 9), mainly in acidic medium but few are known at physiological pH. Considering that digestion is a complex process controlled by several factors in which pH plays a crucial role, we embarked on a project to evaluated gliadin system under different pH. The aim is to shed light on the structural features that led gliadin to be proteolytic resistant and toxic. Herein, it is presented a biophysical evaluation by UV/Vis and fluorescence spectroscopy in combination with dynamic light scattering and transmission electron microscopy.

2. Material and Methods

Sample preparation

Gliadin was purchase from Sigma-Aldrich. MilliQ water was used. NaOH and HCl water solutions were filtered five times through a 0.2 μm Nylon membrane before sample preparation. Basic gliadin solutions were prepared in water at pH 11, NaOH 0.01M final concentration of protein 1mg/ml. To get reproducible results, incubation of the basic gliadin solution at 4°C during 12 hrs. was performed. After, this period of time samples were

prepared for evaluation. The pH adjustment was done with a solution of HCl 0.125M until the desired pH was attained.

UV/Vis spectroscopy

Gliadin was evaluated by UV-Vis absorption at 276nm and 350 nm, 25°C. Solutions at a constant protein concentration of 0.5mg/ml were prepared at 3, 7 and 11.

All the measurements were taken with a V-630 BIO JASCO spectrophotometer using a Peltier system as temperature controller.

Fluorescence Spectroscopy

Protein solution at pH 3, 7 and 11 was diluted to 0.05mg/ml. Emission spectra were taken in a range from 300 nm- 400 nm, employing 279 nm as the excitation wavelength. All measurements were made at 25°C with a Shimadzu spectrofluorimeter using a Peltier system as temperature controller

Dynamic Light Scattering

Dynamic Light Scattering experiments were performed using Zetasizer Nano from Malvern at a fix angle of 90 at a constant temperature of 25°C. The sensitivity of LS to particle size was confirmed by monitoring the LS intensity of latex particles with 04 μm and 0.9 μm diameter at different concentration.

Transmission Electron Microscopy

An aliquot of gliadin solution at pH 7.0 was deposited onto Copper grids (200 Mesh) coated with Formvar. After 5 minutes of interaction the excess fluid was removed. The sample was negatively stained with 2% of uranyl acetate in water. After 2 minutes, excess fluid was removed, let dry and observed in a JEOL 100CX II microscope operating at 100 kV.

3. Results and Discussion

3.1 UV-Vis and Fluorescence Spectroscopy

Gliadin was found to be soluble in alkaline medium (pH 11.0) up to 1 mg/ml. Once it was completely dissolved evaluation of the effect of pH at a constant concentration was performed and monitored by UV/Vis and fluorescence. At a concentration of protein of 0.5 mg/ml, the system was stable under acidic and basic conditions, however between pH 4-8 a formation of a milky-oily solution was observed leading to a significant increment of absortion at 350 nm by Uv-Vis spectroscopy (Fig. 1A). No precipitation was observed in the experimental time scale.

Gliadins are proteins with high content of Tyr (tyrosine) aminoacid, only some isofomrs of alpha gliadin posses a Trp (tryptophan) in the primary structure with a relation of 9 Trp:1 Tyr(10-11).Taking advantage of this knowledge fluorescence experiments of gliadin were performed at pH 3.0, 7.0 and 11.0. Although gliadin system is highly heterogeneous in protein composition, qualitative information about the average environment of the fluorophores Tyr anf Trp in the protein mixture can be obtained by steady state fluorescence. In proteins that contain both Tyr and Trp residues, fluorescence from Tyr is barely detectable because Trp emission is strong and normally blue-shifted in the folded state (12) however considering the system has high content of tyrosine we decided to use excitation at 279 nm. The general shape of the emission spectra of gliadin at 279 nm (Fig. 1B) was similar to emission spectra at the same wavelenght of the unfolded state of RNA T1 which contains 9 Tyr and 1Trp. (13) At pH 3.0 a maximun emission peak at 342 nm and a shoulder at 309 nm was observed. Increasing the pH to 7.0 led to a decrease of the fluoresence intensity detecting two peaks, one at 309 nm and the maximun emission was observable at 339 nm. Finally, at pH 11.0 a considerable decrease of fluorescence intensity of both peaks occurred and a red-shift of the maximun to 348 nm was observed. From these results, it is possible to speculate that the peak at 309 nm can be the result of Tyr fluorescence which intensity is highly pH dependent. The emission peak at around 339-348 nm was found to be modulated by pH not only in the position of the maximun of emission but also in the intensity which is a typical behavior of Trp. (12) A quenching effect by hydroxyl ions at pH 7.0 which became significant at pH 11.0 was found (14) for both emission peaks. Interesting, at pH 11.0, emission of Tyr was not zero, showing that some of them are not exposed to be deprotonated.

Finally, treatment of gliadin solution with 8 M urea, led to an increase on the fluorescence emission of the maximun and a red-shift to 360 nm; one shoulder at 335 nm and decrease on the fluorescence intensity of the peak at 309 nm was observed. Probably, chemical denaturation process by urea was not complete, observing two types of emitting Trp, ones fully exposed to the solvent and others more buried (15). Currently, new experiments are being carried out to validate our hypothesis.

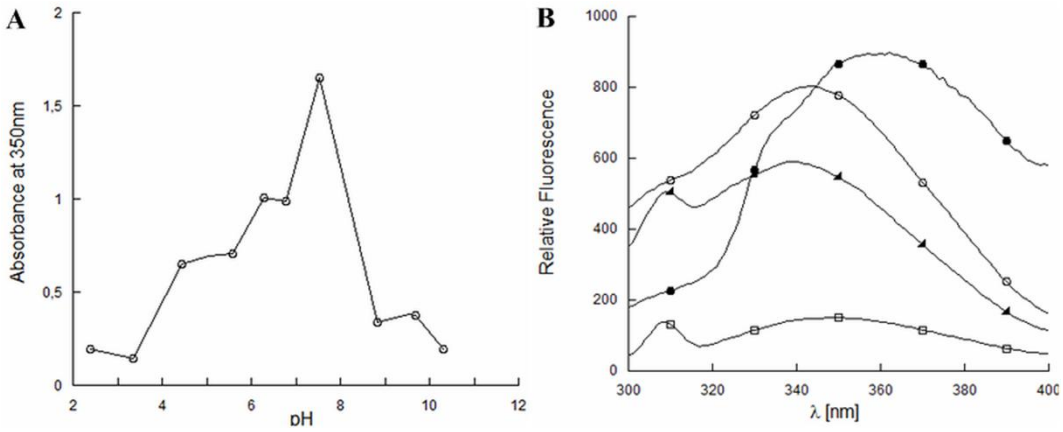

Fig. 1. A) Absorption experiment of Gliadin solution depending on pH. Protein concentration was 0.5 mg/ml. B) Relative Fluorescence Emission Spectra of gliadin depending on pH: 11(□),pH 7(▲),pH 3(○), urea 8M, pH 7.0 (●). Protein concentration was 0.05 mg/ml, excitation wavelenght at 279nm.

3.2. Dynamic Light Scattering and Transmission Electron Microscopy

Light scattering intensity is proportional to the size of the particles present in solution. (16) In order to determinate the presence of aggregates dynamic light scattering experiments of the gliadin solution at pH 3.0, 7.0 and 11.0 were performed. It was observed that at a constant protein concentration (0.5 mg/ml) the system formed aggregates of different sizes depending on pH. At pH 11.0, the system was mainly formed by oligomers (maximum value 200 nm of diameter) coexisting with a few big clusters (5 μm). Decreasing the pH to neutrality led to formation of big aggregates of 1.5 μm of diameter. Due to the solution appearance, these aggregates could be a coacervate. At pH 3.0 the system was formed mainly by the 1.5 μm aggregates coexisting with small oligomers of 35 nm diameter.

In order to evaluate the stability of the aggregates, a dynamic light scattering experiment varying protein concentration was performed at pH 7.0. As presented in Fig. 3, the size of the aggregates decreased by decreasing protein concentration.

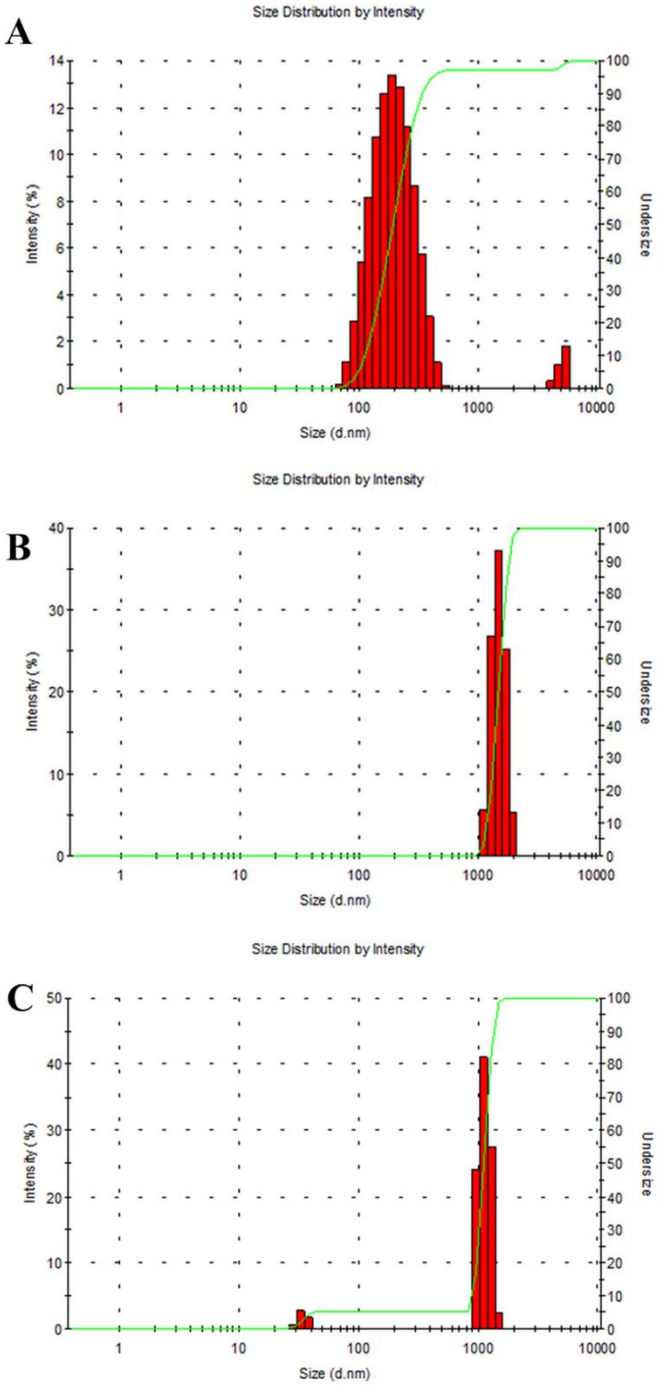

Fig.2. Dynamic Light Scattering of gliadin water solution at 0.5 mg/ml (A) pH 11, (B) pH 7, (C) pH 3.0

The pH dependence of the aggregation indicated that the negative charge due to ionization of tyrosine, carboxylic side chains and the C-terminal carboxylic was important for the formation of intermediate oligomers and their stabilization in solution. The positive charge induced by lowering the pH to 3.0 led to formation of oligomers (dimers or trimers probably) and stabilization of the big oligomers. It is known that alpha gliadin is monomeric

at pH 3.0 and oligomerize into fibrils at pH 5.0. (8) At neutral pH intermediate oligomers self-assembled into the 1.5 µm aggregates probably by electrostatic complementarity at the surface.

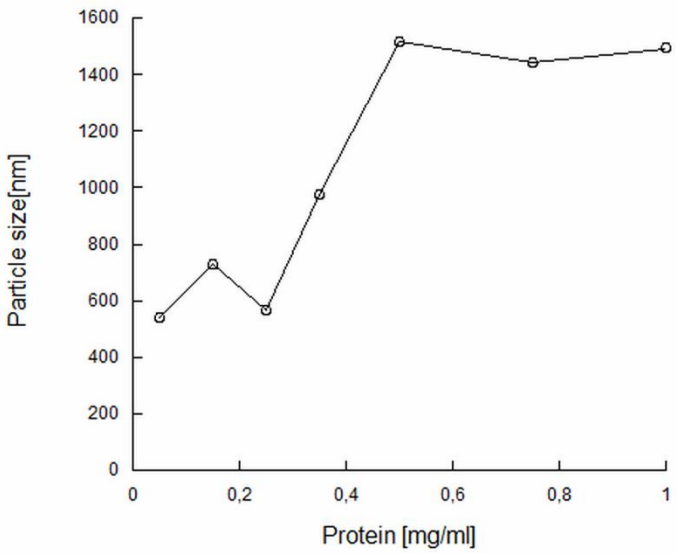

Fig. 3. Particle Size distribution at different gliadin concentration, pH7.

To validate the coacervate hypothesis at pH 7.0, TEM experiment of gliadin at different concentration has been performed (Fig. 4). It was possible to visualize microspheres and nanospheres depending on protein concentration which dimensions were in order of those obtained by DLS. There are some previous evidence that gliadin was able to form vesicles or coacervates at low pH at different salt concentration (17), and in the presence of organic solvents (18) .In addition it was reported a coacervate formation of alpha gliadin and Arabic gum at low pH. (19)

In general, the loss of solvation arises from the interaction of complementary macromolecular species. The formation of such macromolecule-rich fluids is well-known in mixtures of complementary polyelectrolytes, like proteins, leading to condensed soft matter phases with particular properties. (20)

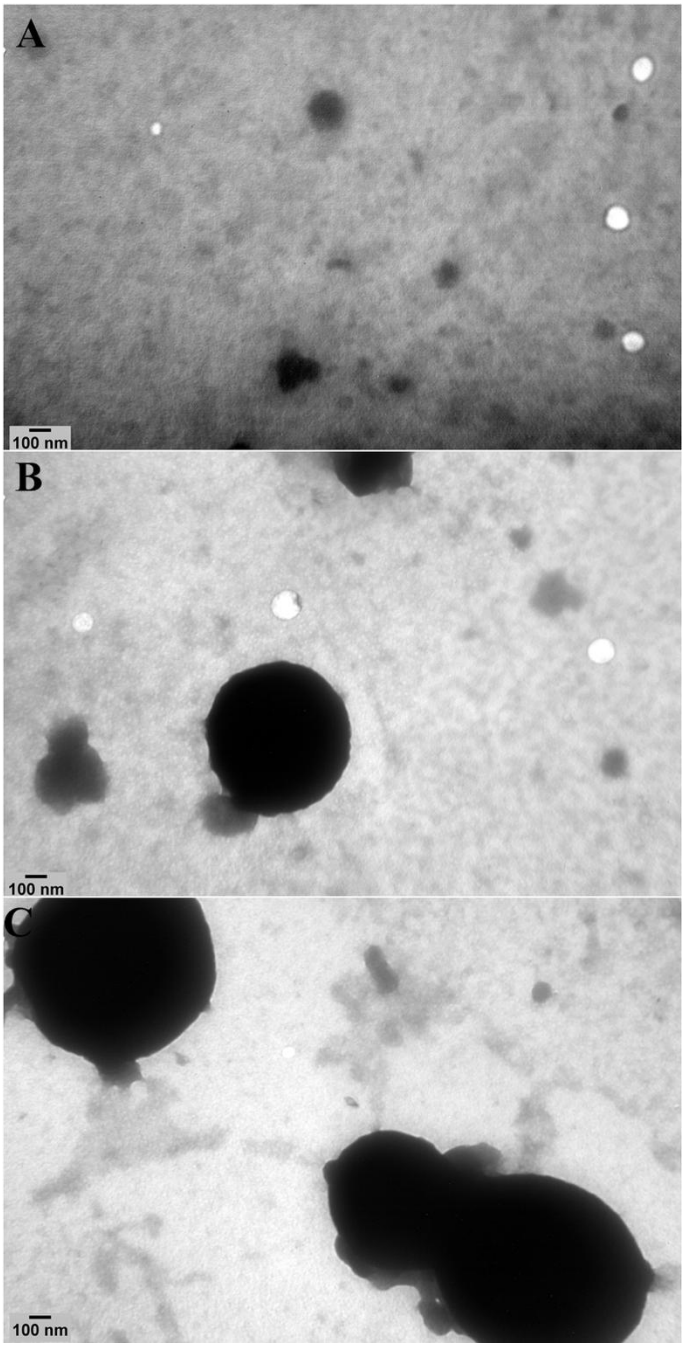

Fig. 4.TEM micrographs of gliadin solution under different concentration, pH 7.0 (A) 0.05, (B) 0.2, (C) 0.5 mg/ml of protein

4. Conclusion

Digestion is a complex process where pH among other environmental factors is determinant. Concerning of pH, the process start in the mouth, pharynx, and esophagus, where pH is typically about 6.8. In the stomach's high acidity denaturation of proteins for

further digestion in the small intestine occurs. Finally, in the small intestine, the duodenum provides critical pH balancing to activate digestive enzymes. The liver secretes bile into the duodenum to neutralize the acidic conditions from the stomach and the pancreatic duct empties into the duodenum, adding bicarbonate to neutralize the acidic chime, thus creating a neutral environment. (21)

Our findings showed that gliadin was able to form aggregates of different size between pH 3.0 to 11.0 which were evidenced by UV/Vis and DLS. Tyr and Trp amino acids seemed to be partially folded at the tested pH. At pH 7.0, the system self-assemble into spherical aggregates which dimension depended on concentration as revealed by TEM and DLS. From TEM and the solution appearance, it was hypothesized a coacervate formation. The charges on the surface of the formed aggregates must be sufficiently large to cause significant electrostatic interactions and vesicle formation. A number of naturally occurring proteins, such as hydrophobins (22), oleosins (23), latherin(24) and ranaspumin(25) are known to stabilize interfaces. In this context, it is known that isolated alpha gliadin is able to form self-assembly systems like fibrils, but up to our knowledge it is the first example of vesicle formation related to gliadin protein. Currently, our research efforts are directed towards understand the mechanism involved in the coacervation process and the relevance in gliadin intolerance disorders.

5. Acknowledgments

We thank the National Council of Scientific and Technical Research of Argentina (CONICET, PIP 114-200801-00090), National Agency for Promotion of Science and Technology (ANPCyT, PICT-2008-0083) and National University of South (ZQ/024) for supporting our research work. MGH is very grateful for her CONICET fellowship

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