

Effect of pH and temperature on the immunogenicity of glycinin (*Glycine max* L.)

A. DEMONTE, I.Z. CARLOS, E.J. LOURENÇO and
J.E. DUTRA DE OLIVEIRA

*Department of Food and Nutrition, Faculty of Pharmaceutical Sciences, Araraquara,
Unesp, SP, Brazil*

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Abstract. Sensitive immunologic techniques for the detection of alterations that occur in protein antigens were used to evaluate the immunogenicity of soybean glycinin after isolation, heat denaturation and pH alteration. The objective was to determine the effect of these agents on the immunogenic ability of this protein fraction. Immunologic assays performed on heat-denatured glycinin up to 80 °C in the presence of antinative glycinin serum demonstrated that glycinin retains its immunogenic properties. Above 90 °C this biological property begins to disappear, with protein insolubilization and epitope modification due to the conformational changes imposed by temperature. A reduction in immunogenicity also occurred when glycinin was taken to pH 2.0 (below its pI) and pH 11.00 (above its pI) and exposed to high temperatures in the presence of native antiglycinin serum. From these data one can conclude that, at extreme pH values, intramolecular reactions may occur which, in combination with the structural disorganization caused by high temperatures, may contribute to the reduction of immunogenicity.

Key words: Glycinin, Immunogenicity, Protein conformation, Soybeans

Introduction

Although soy consumption, either in natura or in processed foods, has perceptibly increased over the last few years in human nutrition, there is considerable evidence about its allergenicity [1]. This mainly resides in its protein fractions, a fact that may cause problems to the large population that utilizes this protein source [2]. Glycinin is the major protein reserve of soybeans. Its structure is maintained by a series of complex non-covalent and intramolecular interactions among amino acid residues arranged in a specific sequence along the various acidic and basic polypeptide chains [3–5]. It is known that the ability of antibodies to form complexes with globular proteins depends on the tertiary structure of the antigen and on the integrity of the peptide sequence. Thus, the application of chemical and physical treatments may provide a sensitive tool for the detection of structural changes produced in the antigen, reducing its antigenicity and insuring better use of the dietary protein. On this basis the effects of variations in temperature, alone or combined with changes in

pH, on the immunogenicity of glycinin in the presence of rabbit anti-glycinin serum were studied in order to determine the effects of these physical and chemical agents on the immunogenicity of the isolated protein.

Material and methods

Sample preparation. A 200-g soy (*Glycine max* L.) (var. JL-14 from the Jaboticabal Campus of the State University of São Paulo UNESP) sample was soaked in water (1:3), peeled, triturated and placed in an oven with air circulation for 15 hours at 40 °C. The sample was then finely granulated to pass a 100-mesh sieve and defatted with n-hexane (1:20) at room temperature using a Soxhlet extractor.

Isolation of the glycinin fraction. A 10-g sample of defatted meal was used to isolate the glycinin fraction according to the procedure described by Iwabuchi et al. [6]. The isolated fraction was then dialyzed for 24 hours and lyophilized.

Sephadex G-200 gel filtration. The molecular weight of glycinin was determined by gel filtration. The method of Whitaker [7] was used.

Polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis was carried out by the method of Davis [8]. Seven percent acrylamide was used.

Physicochemical alterations in the protein. Heat denaturation: 3 µg/ml of the protein solution in TRIS-HCl buffer was heated in a tube (14 × 125 mm) to the desired temperatures (70 and 100 °C) for 60 min.

Variation in pH: 1.2 mg glycinin was dissolved in 20 ml 0.2 M glycine buffer (amino acetic acid) and 0.2 M HCl to reach acid pH (2.0). The same amount of glycinin was dissolved in 10 ml 0.2 M glycine buffer and 0.2 M NaOH that the protein would reach pH 11.0. Five-ml aliquots of the solutions were then transferred to test tubes and left to stand for 1 hour before beginning the tests.

Combined effect: Variation of sample pH and heat denaturation: The above procedures were to determine the evaluation of the effects of pH and temperature on the immunogenicity of glycinin in natura.

Antiserum preparation. A group of adult white rabbits was immunized intraperitoneally at 7-day intervals over a period of 4 weeks with a solution of the antigen (1.38 mg/ml glycinin in 240 µl of a saline mixture). After this period, serum was collected and stored at -20 °C.

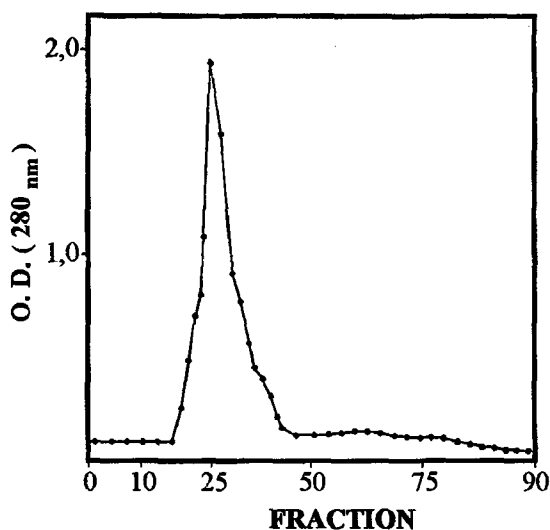


Figure 1. Glycinin separation by filtration through Sephadex G-200 resin, (1) absorbance at 280 nm; (2) 100 mg of the glycinin sample isolated with TRIS-HCl buffer, pH 8.0, containing 10 mM mercaptoethanol; (3) 49 × 2.5 cm column; flow of 15 ml/hour; 4.5 ml fractions (4) elution with 2.6 mM KH_2PO_4 , pH 7.6, and 32.5 mM K_2PO_4 , 0.4 M NaCl, 10 mM 2-ME, pH 7.6.

Immunoenzymatic assay (ELISA). ELISA was performed by using the method of Engwall et al. [9]. Absorbance was read (492 nm) using a Multiskan ELISA reading spectrum.

Results

Figures 1 and 2 show a single electrophoretic band in glycinin isolated under non-denaturing conditions. The immunoenzymatic test (Figure 3) showed a significant effect both on the reduction of the optic dispersion values measured and on antiserum titer with glycinin exposure to temperatures above 80 °C in the presence of anti-glycinin serum. These results indicate that temperature is a fundamental physical agent for the quantitation of changes in protein structure that might influence the immunologic reactivity of a protein.

Figure 4 shows the effects on immunologic reactivity determined by ELISA when glycinin was submitted to extreme basic pH and to elevated temperatures in the presence of anti-glycinin serum in natura. A marked decrease in the pattern of reactivity was observed compared to the control (protein in natura, temperature of 25 °C).

Figure 5 presents the values of the immunoenzymatic tests for the protein

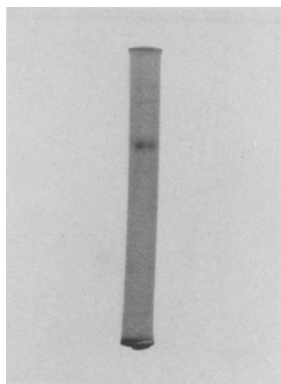


Figure 2. Disk electrophoresis of glycinin under nondenaturing conditions on 7% polyacrylamide gel, pH 8.9; 50 μ g protein was applied to the top of the polymerized rod.

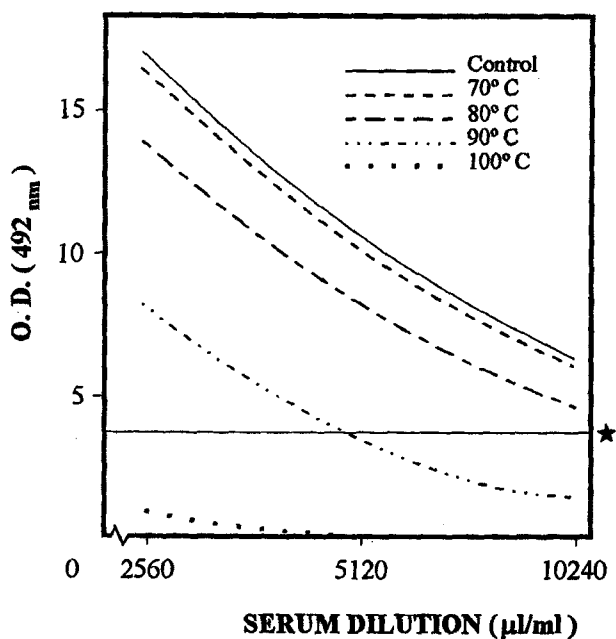


Figure 3. Immunoenzymatic test (ELISA) using anti-glycinin serum and glycinin subjected to changes in temperature.

taken to extremes of acid pH (2.0). A decrease of about five-fold in the pattern of reactivity was observed when the sample was subjected to pH 2.0 and 100°C compared to the glycinin control, pH 7.6, 25°C.

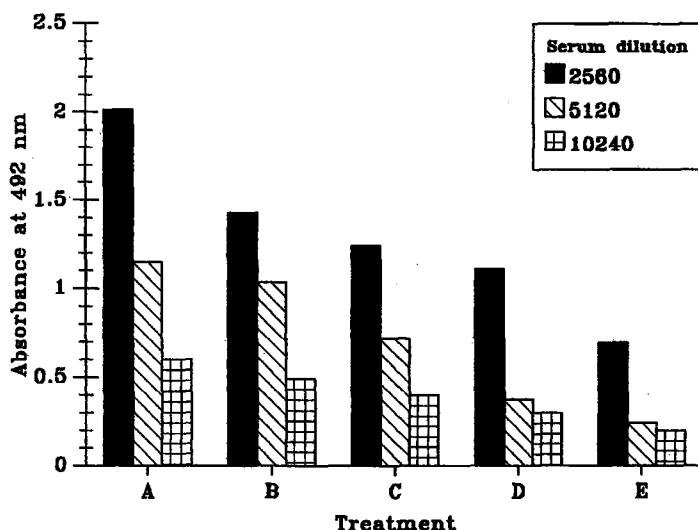


Figure 4. Immunoenzymatic test (ELISA). The glycinin antigen was submitted to extremes of basic pH and high temperatures in the presence of anti-glycinin serum at the extraction pH (7.6) and at room temperature (25°C). (A) pH 7.6, 25°C; (B) pH 11.0, 70°C; (C) pH 11.0, 80°C; (D) pH 11.0, 90°C; (E) pH 11.0, 100°C.

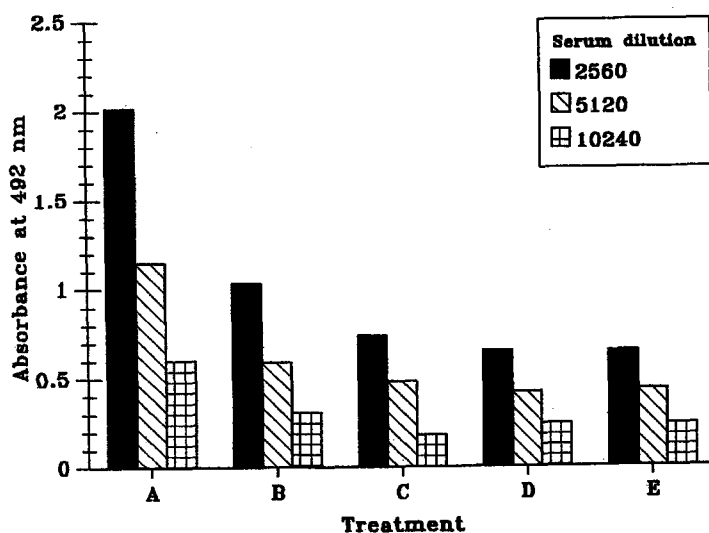


Figure 5. Immunoenzymatic test (ELISA). Glycinin was submitted to extremes of acid pH and high temperatures in the presence of the anti-glycinin serum at the extraction pH (7.6) and at room temperature. (A) pH 7.6, 25°C; (B) pH 2.0, 70°C; (C) pH 2.0, 80°C; (D) pH 2.0, 90°C; (E) pH 2.0, 100°C.

Discussion

When soy glycinin was subjected to elevated temperatures, a decreased immunologic reactivity was observed, especially at 100 °C. Possibly this was because at this temperature, conformational alterations occur in the secondary and tertiary structure together with destruction of the quaternary structure, in addition to protein aggregation as described by Catsimpoolas et al. [10] and Wolf et al. [11]. Heating may lead to ester impediment with a decrease in the number of peptide bonds susceptible to immunochemical reactivity. On this basis, one can conclude that the antigenic sites are located in the individual subunits and, therefore, may be affected by the conformational changes imposed on the protein.

Thus, temperature may produce two types of events: rupture of the oligomeric structure with liberation of subunits and these subunits may remain soluble or form soluble clusters and then insoluble clusters [12]. This conversion may be caused by polymerization through disulfide bonds and may particularly affect the immunogenicity of the intact protein in the form of epitope modification.

The efficacy of humoral immunity depends both on antibody affinity for the antigen and on the amount of antibody produced and, therefore, the events occurring in serum may reflect the cell dynamics of the immune response. The anti-glycinin serum was able to detect structural changes in the homologous antigens of the protein in which was observed the extent of the interaction of denatured molecules with native antibodies. This was confirmed by the blot (Figure 3) and by the immunoenzymatic tests (Figures 4 and 5) in which was observed a reduction in the number of bands (Figure 3) and of antibody titers (Figures 4 and 5), which were quite perceptible at high temperatures and changes in pH.

Proteins are amphoteric substances and, therefore, are subject to electrostatic forces of repulsion and attraction. In the case of glycinin taken to pH 2.0, the hydrophobic interactions that occur in the molecule may lead to a decrease in the number of sites available for the antigen-antibody reaction. On the other hand, the decreased immunologic reaction at pH 11.0 may be attributed to a rupture of S–S bridges and a consequent decrease in the hydrophobicity surface of basic polypeptides of the glycinin structure, an opposite effect to that of acidic polypeptides [13, 14]. Thus, one may assume that immunogenicity may depend both on native conformation and on the conservation of the secondary and tertiary structures of the protein. Although the change in pH favors protein-solvent interaction by cleavage of S–S bonds and reduces the titers of the immunologic reaction, these protein alterations do not always guarantee the nutritional utilization of the protein. They may reduce digestibility or even form non-bioavailable compounds.

These data lead to the conclusion that immunologic reactions depend on the conservation of the native conformation of glycinin. In the presence of dissociating conditions such as increased temperatures, the protein may undergo strong structural disorganization leading to decreased immunologic activity. The same may occur at pH ranges that might contribute to the rupture of disulfide bonds. These findings may be considered of importance for the quality control of products isolated from soy and used in human nutrition.

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