Calcium modulates protease resistance and carbohydrate binding of a plant defense legume lectin, *Griffonia simplicifolia* lectin II (GSII)

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Abstract

Site-directed mutagenesis previously identified the residues responsible for the biological activity of the plant defense legume lectin, *Griffonia simplicifolia* lectin II (GSII) [Proc. Natl. Acad. Sci. USA 95, (1998) 15123–15128]. However, these results were inconclusive as to whether these residues function as direct defense determinants through carbohydrate binding, or whether substantial changes of the protein structure had occurred in mutated proteins, with this structural disruption actually causing the loss of biochemical and biological functions. Evidence shown here supports the former explanation: circular dichroism and fluorescence spectra showed that mutations at carbohydrate-binding residues of GSII do not render it disfunctional because of substantial secondary or tertiary structure modifications; and trypsin treatment confirmed that rGSII structural integrity is retained in these mutants. Reduced biochemical stability was observed through papain digestion and urea denaturation in mutant versions that had lost carbohydrate-binding ability, and this was correlated with lower Ca²⁺ content. Accordingly, the re-addition of Ca²⁺ to demetalized proteins could recover resistance to papain in the carbohydrate-binding mutant, but not in the non-binding mutant. Thus, both carbohydrate binding (presumably to targets in the insect gut) and biochemical stability to proteolytic degradation in situ indeed contribute to anti-insect activity, and these activities are Ca²⁺-dependent. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Lectins are carbohydrate-binding proteins of non-immune system origin. In plants, a principal function of these proteins is defense against insect herbivores (Peumans and Van Damme, 1995). The anti-insect activity of lectins has been attributed to their binding to chitin in the peritrophic matrix, or to an interaction with glycoproteins on the epithelial cells of the insect gut that attenuates nutrient assimilation (Chrispeels and Raikhel, 1991; Eisemann et al., 1994; Peumans and Van Damme, 1995; Zhu-Salzman et al., 1998; Habibi et al., 2000; Trigueros et al., 2000). Although plant lectins have unique carbohydrate-binding specificity, those in the Leguminosae have high degrees of primary, secondary and tertiary structure similarity. Carbohydrate-binding amino acids and their flanking residues are remarkably conserved among
these plant lectins, and they all require divalent cations for interaction with carbohydrate substrates (Sharon and Lis, 1990). Structural analysis of concanavalin A (ConA, a lectin from jack bean) showed that transition metal ions, such as Mn$^{2+}$ or Zn$^{2+}$, associate with the metal binding site S1, which facilitates subsequent Ca$^{2+}$ binding to the S2 site (Bouckaert et al., 1996), and Ca$^{2+}$-lectin association, in turn, coordinates interaction between carbohydrate-binding amino acid residues in ConA and the substrate. The removal of metal ions from ConA disrupted carbohydrate binding (Kalb and Levitzki, 1968; Bouckaert et al., 1995). However, no direct experimental evidence has yet established that metal ions facilitate biochemical stability of legume lectins.

*Griffonia simplicifolia* lectin II (GSII) is a legume lectin with N-acetylglucosamine (GlcNAc) specificity. Seed GSII is a homotetramer and leaf GSII contains two different subunits. Both seed and leaf GSII and bacterially expressed leaf large subunit (rGSII) significantly delayed development of cowpea bruchid, *Callosobruchus maculatus* (F.) (Zhu et al., 1996b). Site-directed mutagenesis established that rGSII anti-insect function against cowpea bruchid is dependent on carbohydrate binding. Interestingly, all non-binding mutant proteins were also more susceptible to gut proteolysis by *C. maculatus* (Zhu et al., 1996a; Zhu-Salzman et al., 1998). It is unknown whether only the lectin-substrate interaction (mediated by hydrogen bonding and hydrophobic stacking) was affected by these site-specific mutations, or if major alterations in protein structure occurred, rendering the protein susceptible to proteolysis, and causing loss of carbohydrate binding and anti-insect activity as well.

Herein, evidence is presented that mutations that disturb carbohydrate binding of rGSII did not affect the overall structure of rGSII protein, although protein stability was substantially reduced in mutant variants that lost carbohydrate binding in insect guts. We also showed, for the first time, that Ca$^{2+}$ enhances stability of a legume lectin, implying Ca$^{2+}$ has a significant function in the biological activity of another important group of proteins.

2. Materials and methods

2.1. Purification of rGSII proteins

Recombinant GSII proteins were obtained as described previously (Zhu-Salzman et al., 1998). Briefly, cDNAs encoding rGSII and its site-specific mutated variants were ligated into pET28 vector (Novagen), and constructs transformed into bacterial strain BL21(DE3). Bacterial cultures were grown at 37 °C until OD$_{600}$ of 0.5–1.0 was reached. Recombinant protein production was then induced with isopropyl-D-thiogalactoside overnight at 18 °C. Cells were disrupted by sonication, and recombinant proteins were purified via a Ni$^{2+}$ chelate affinity column (Amersham Pharmacia Biotech). Thrombin was used to cleave the hexahistidine tag, which also caused simultaneous elution of recombinant proteins. Purified proteins were then dialyzed against distilled water and lyophilized.

2.2. Circular dichroism and fluorescence spectra

Circular dichroism (CD) spectra of rGSII and its mutated variants were obtained on a Jasco spectropolarimeter (model J600) at room temperature. For far UV CD spectra, the instrument scanned from 250–190 nm with eight scans for each protein sample (2.5 μM). Near UV CD spectra were obtained by scanning protein samples (200 μM) from 350–250 nm four times for each sample. The path length for each sample was 0.1 cm. The solution used for baseline spectra was 20 mM sodium phosphate buffer, pH 7.0, the buffer in which all proteins were dissolved. The instrument was calibrated against (+)-camphorsulfonic acid.

Fluorescence spectra were acquired on a Hitachi F-2000 fluorescence spectrophotometer, using rGSII and variants at a concentration of 2.5 μM dissolved in 20 mM sodium phosphate, pH 7.0. At the concentration employed, protein UV absorbance at 280 nm was less than 0.06. At an absorbance of this magnitude, inner filter effects would not lead to distortions in fluorescence spectra. Solutions used for fluorescence spectra were placed in a 1-cm cuvette, stirred at 25 °C, and equilibrated in the instrument for 5 min before spectral acquisition. Trp excitation was set to 290 nm and fluorescence emission was scanned from 310 to 390 nm.

2.3. Digestion of rGSII and variants with trypsin and papain

Ten-μg of each rGSII variant were mixed with 0.2 μg trypsin or papain, and incubated at 37 °C...
for 0, 10 min, 1 h and 5 h, respectively. Time zero samples were exposed to protease for less than 15 s before addition of SDS-PAGE sample buffer. The digestion buffers were 40 mM Tris–HCl, 1 mM CaCl₂, pH 8.0 for trypsin, and 50 mM sodium phosphate, 0.5 mM EDTA and 4 mM β-mercaptoethanol, pH 6.0 for papain. The reaction products were separated on SDS-PAGE. Degradation of BSA was used as a positive control for proteolytic activity.

2.4. Urea denaturation

An 8 M urea stock solution in 20 mM sodium phosphate, pH 7.0 was prepared. A series of 31 solutions (1.5 ml) with urea concentrations ranging from 0–8 M were prepared by mixing the urea solution with proper proportions of buffer. To each solution, rGSII or variants was added to a final concentration of 1 μM. All tubes were inverted several times to ensure mixing and incubated at room temperature for 24 h. The excitation monochromator was set to 290 nm and emission was scanned from 310–400 nm at 25 °C. The λmax value was monitored and recorded as a function of urea concentration in triplicate experiments. Emission with a maximum at 353 nm was considered to represent denatured protein.

2.5. Ca²⁺ content determination

Demetalized water, obtained by mixing distilled water with chelating resin (Chelex 100, Sigma) overnight, was used to dialyse purified, bacterially expressed rGSII and its mutated variants to remove residual unbound metal ions. These proteins were lyophilized and then redissolved in 1 ml HNO₃ (69%) overnight followed by addition of 1 ml deionized water. Ca²⁺ content was determined by inductively coupled plasma-atomic emission spectrometer (ICP-AES) with a Perkin–Elmer Plasma 400 (Norwalk, Connecticut) at the Department of Food Science, Purdue University.

2.6. Papain digestion of demetalized and remetalized rGSII proteins

Three to 5 mg of rGSII-Y134D (non-binding rGSII mutant) or rGSII-N196D (binding rGSII mutant) dissolved in water were demetalized by the addition of concentrated HCl to pH 1.2 (Kalb and Levitzki, 1968). After 30 min, the protein solutions were transferred to dialysis bags (previously boiled in 1 mM EDTA) and dialyzed against three changes of demetalized water at 4 °C for 20 h. The resulting solution was lyophilized and the final protein concentration was adjusted to 10 μg/μl. To 10 μg of each demetalized protein, 1 μl of 5 mM MnCl₂ was added and the protein was incubated at room temperature overnight. This was followed by addition of 1 μl of 5 mM CaCl₂ and a further 24 h incubation, yielding the remetalized protein. MnCl₂ or CaCl₂ alone was added and incubated for 24 h as single metal controls. Both demetalized and remetalized proteins were subjected to papain digestion for 1 h with 2 μl of 10 mM EDTA in each reaction and analysed by SDS-PAGE subsequently.

3. Results

3.1. Mutations in rGSII did not introduce major disturbances in protein folding

Mutations at D¹⁸⁸, Y¹³⁴ or N¹³⁶ (the GlcNAc-binding residues) resulted in the loss of carbohydrate-binding capacity (Zhu et al., 1996a), biochemical stability in the insect gut, as well as anti-insect activity (Zhu-Salzman et al., 1998). Rather than loss of binding activity causing loss of anti-insect activity, the commonly accepted hypothesis, it could be argued that a major disruption of the protein conformation resulted from the mutations, which could cause a loss of functionality compared to rGSII by means beyond binding disruption. To resolve this question, circular dichroism (CD) spectra were obtained for all rGSII variants (Fig. 1). CD spectra have often been used to detect substantial conformational changes in proteins, since change in protein structure is often accompanied by significant changes in the CD spectrum of the protein (Kyte, 1995). A comparison of both far (Fig. 1) and near UV spectra (data not shown) of rGSII with spectra of the mutated proteins revealed no significant differences in secondary or tertiary structure. Far UV CD spectra also indicated that rGSII consists predominantly of β-strands, a finding that is consistent with many legume lectins (Sharon and Lis, 1990).

Changes in protein structure upon mutation may have been too subtle to be observed by CD spectroscopy. Thus, methods based on measurements of Trp fluorescence were employed to look for structural variations (Lackowicz, 1983). If
substantial structural changes were occurring as the result of mutations such that Trp environments were altered, a shift toward a longer wavelength of maximum intensity ($\lambda_{\text{max}}$) would be expected. However, Trp fluorescence of rGSII and all of its variants achieved a $\lambda_{\text{max}}$ at 330 nm ($\pm$ 1 nm), indicating that the four Trp residues in the rGSII molecule were in relatively hydrophobic environments.

We also assessed whether conformational change occurred as a result of the mutations using trypsin digestion (acting specifically on Lys and Arg). Given that as many as 20 trypsin cleavage sites are dispersed throughout the rGSII molecule, substantial alteration in protein folding would most likely expose some of these sites, leading to protein fragmentation and possible subsequent degradation. Interestingly, trypsin did not hydrolyze non-binding rGSII-Y134D, rGSII-Y134G, or rGSII-N136Q (Fig. 2a), indicating that mutations at Y134 and N136 did not substantially disturb the protein structure. Replacement of D88 with N apparently induced a sufficient conformational change, exposing trypsin cleavage sites (Fig. 2a). However, all of the changes must be taken in the context of the spectroscopic data, which indicate that the general protein structure was not greatly altered. Thus, tryptic cleavage of rGSII-D88N should not have been the result of a dramatic structural collapse. It is possible that this protein retains the same secondary structure elements and overall tertiary structure as rGSII, but is less tightly packed. As a result, protease sites may be exposed, leading to protein degradation.

3.2. Non-binding insect digestive enzyme-susceptible rGSII variants are less stable than rGSII in the presence of urea

To utilize Trp fluorescence as a probe of structural integrity and stability, urea sensitivity of selected proteins (non-mutated rGSII, GlcNAc-binding rGSII-N196D, partially binding rGSII-N136D, non-binding, trypsin-susceptible
Fig. 2. Digestion of rGSII variants with (a) trypsin and (b) papain. Proteins were mixed with 0.2 μg of trypsin or papain and incubated at 37 °C for 0, 10 min, 1 h and 5 h. Samples for time zero digestion were prepared by mixing all ingredients followed by immediate boiling in protein sample buffer. Total time taken was less than 15 s. BSA was used as positive control for proteolytic activity. Relative GlcNAc binding at the physiological pH of cowpea bruchid is indicated with + (strong binding), + (partial binding) and – (no binding). The experiment was repeated two times with similar results.

rGSII-D88N and non-binding, trypsin-resistant rGSII-Y134D was measured. The λ_max of each of the proteins shifted to 353 nm when dissolved in 6 M urea for 24 h, indicating that they were denatured. However, significant differences were observed among the various proteins in the way that λ_max for Trp changed in the presence of urea increasing in concentration from 0–8M (Fig. 3). The λ_max of rGSII reached the midpoint of its total change (Δλ_max) at ca. 4 M urea, as did rGSII-N196D (data not shown). In contrast, rGSII-D88N and rGSII-Y134D reached the midpoint of Δλ_max at less than 3 M urea. rGSII-N136D, a mutant that does not have binding activity at neutral and basic pHis but a partial binding at acidic pH (Zhu-Salzman et al., 1998), displayed somewhat unusual behavior compared to the other proteins. It appeared to undergo a two-step conformational change, with the first step completed at approximately 2 M urea, indicating partial denaturation at relatively low urea concentrations. The second step reached half of the Δλ_max at a urea concentration greater than 5 M (Fig. 3). Under the conditions of the urea denaturation experiments, the results revealed that the GlcNAc-binding null rGSII-D88N and rGSII-Y134D were less stable than rGSII or its comparable binding variant rGSII-N196D. The partial binding rGSII-N136D differed from the others in that it appeared to undergo one unfolding event at low urea concentration and another event at a much higher urea concentration. Not surprisingly, papain, a cysteine protease with wide specificity as the major digestive enzyme in insect guts (Kitch and Murdock, 1986; Zhu-Salz-
3.3. Ca\(^{2+}\) plays a key role in modulating protein stability of rGSII

Although structural changes in mutant proteins that lost GlcNAc-binding ability seem to be very localized, they nevertheless cause loss of rGSII stability in insect guts. Recently, we showed that this stability is independent of carbohydrate binding (Zhu-Salzman and Salzman, 2001). Based on sequence alignment with legume lectins whose tertiary structures are known, D\(^{88}\), Y\(^{134}\) and N\(^{136}\) of rGSII are all implicated in Ca\(^{2+}\) binding (Weis and Drickamer, 1996). Ca\(^{2+}\) is known to increase the resistance of many other proteins to external proteolysis by reducing the fraction of protein population that is partially unfolded (Levine and Williams, 1982). Thus, we tested whether Ca\(^{2+}\)-lectin interaction was altered in non-binding mutants. Indeed, inductively coupled plasma-atomic emission spectrometry (ICP-AES) showed that rGSII variants that were more susceptible to urea treatment and to insect gut enzyme digestion had lower Ca\(^{2+}\) content than those that were more resistant to urea denaturation and to insect digestion (Table 1). Further, demetalization rendered rGSII-N196D (the binding rGSII variant) susceptible to papain digestion (Fig. 4). When both Mn\(^{2+}\) and Ca\(^{2+}\) were added back to demetalized rGSII-N196D as well as to demetalized rGSII-Y134D (the non-binding rGSII variant), substantial resistance to proteolysis was restored in rGSII-N196D, but not in rGSII-Y134D (Fig. 4). This resistance could not be achieved by addition of either Mn\(^{2+}\) or Ca\(^{2+}\) separately, indicating that they function coordinately in forming a specific protein structure essential for biochemical stability and carbohydrate-binding activity. Although Mn\(^{2+}\) is a necessary component of legume lectins, it has no direct contact with amino acid residues that underwent site-specific mutation (Weis and Drickamer, 1996). In addition, no substantial sec-
Table 1
Ca²⁺ content of rGSII and variants. Each protein sample (5–10 mg) was dissolved in 2 ml HNO, and applied to ICP-AES assay. The ppm values detected from 1.4 mg of lectin correspond to the number of Ca²⁺ ions per protein molecule. Standard Ca²⁺ solutions, 1 and 4 ppm, were used to measure the accuracy of the machine calibration.

<table>
<thead>
<tr>
<th>Protein</th>
<th>GlcNAc binding</th>
<th>Ca²⁺ ion/lectin molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>rGSII</td>
<td>++</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>rGSII-D88N</td>
<td>–</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>rGSII-Y134D</td>
<td>–</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>rGSII-N136D</td>
<td>+</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>rGSII-N196D</td>
<td>++</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Control Sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 ppm CaCl₂</td>
<td>n.a.</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>4 ppm CaCl₂</td>
<td>n.a.</td>
<td>4.3 ± 0.9</td>
</tr>
</tbody>
</table>

*a* ++, + and — indicate strong, partial or no GlcNAc binding. *b* n.a.: not applicable.

Fig. 4. Mn²⁺ and Ca²⁺ are essential for resistance to papain proteolysis. rGSII-Y134D (non-binding rGSII mutant) or rGSII-N196D (binding rGSII mutant) was demetalized according to Kalb and Levitizki, 1968. MnCl₂ (1 μl of 5 mM) was added to 10 μg of each demetalized protein and incubated at room temperature overnight, followed by addition of CaCl₂ (1 μl of 5 mM) and incubation at room temperature for 24 h. MnCl₂ and CaCl₂ were also applied alone as controls. All protein samples were subjected to papain digestion with 2 μl of 10 mM EDTA in each reaction and to SDS-PAGE. The experiment was repeated two times with similar results.

4. Discussion

Circular dichroism, fluorescence spectra and trypsin digestion here indicate that no major changes in secondary or tertiary structure resulted from those site-specific mutations to rGSII that modulate carbohydrate-binding and anti-insect activities. Analogous results were observed when mutations to Phaseolus vulgaris leucoagglutinin (PHA-L) resulted in disturbance of many biochemical functions without undue modification of structural integrity (Mirkov and Chrispeels, 1993). Structural perturbations detected by urea denaturation, although sufficient for papain degradation, must have been localized or greater structural variation would have been observed. Destabilization of mutant rGSIIs was likely caused by an inability of Ca²⁺ to form coordination bonds with carbohydrate-binding residues. Such an interaction between Ca²⁺ and the legume lectin thus appears necessary both for carbohydrate binding and for biochemical stability, two independent properties of rGSII (Zhu-Salzman and Salzman, 2001). However, Ca²⁺ binding must not be sufficient for full biological functionality of rGSII. Replacing N¹³⁶ with D eliminated carbohydrate binding at neutral and basic pHs but did not affect Ca²⁺ content (Table 1). Previous experiments showed that the side chain of the D residue could partially form hydrogen bonds with the carbohydrate substrate but only under acidic pH condition. The slightly acidic physiological pH of cowpea bruchid enables partial carbohydrate-binding activity of rGSII-N¹³⁶D, resulting in partial anti-insect activity (Zhu-Salzman et al., 1998). Obviously, this can only occur when Ca²⁺ holds residue 136 in the proper position. Specific interactions of GSII with glycoconjugates in insect guts through carbohydrate binding must play an important role in realizing its anti-insect function. The ability to survive insect digestive systems is also clearly essential for realizing such function. Therefore,
biochemical stability and carbohydrate binding, both directly controlled by the binding of Ca\(^{2+}\), are integral to GSII function as an anti-insect protein.

Although resistant to trypsin, mutated rGSII proteins that lost carbohydrate binding are susceptible to cysteine proteases, the major digestive enzymes of cowpea bruchids (Kitch and Murdock, 1986; Zhu-Salzman and Salzman, 2001). Cowpea bruchids apparently utilize digestive enzymes not only for nutrient assimilation but also for protection against protein toxins. Therefore, development of stability against degradation by pest metabolic systems represents an effective defensive evolution in host plants (Michaud, 1997). Unlike plant protease inhibitors that can be defeated by inhibitor-insensitive proteases insect herbivores produce after being challenged by these protease inhibitors (Bolter and Jongsm, 1995; Giri et al., 1998), plant lectins are insensitive to insect digestive enzymes and are able to survive and reach the sites of action in insects. Therefore, overproducing or switching to more effective proteases by insects may not represent a potential mechanism for detoxification of lectins. Increased protein stability also would render less potent toxins more effective as insecticides. The advantage of less potent toxins is that they pose less selection pressure and perhaps make the ‘resistance mechanism’ less susceptible to rapid development of new insect biotypes. Combinations of defensive proteins such as GSII together with modulators of digestive proteolysis may represent an effective strategy to enhance host plant resistance against insects.

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References


