The Lectin from *Bauhinia purpurea*: Effect of Modification of Lysine Residues on Conformation and Biological Properties

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Bauhinia purpurea agglutinin (BPA) has been previously characterized as an *N*-acetylgalactosamine binding protein. The protein has also been demonstrated to possess toxic effects on the European corn borer (ECB), a major insect pest of corn. To understand the role of specific amino acid side chains in the biological and biochemical properties of the protein, we have examined the effect of modification of lysine ϵ-NH_2 groups on the conformation and sugar binding properties, as well as the toxicity of BPA toward the corn borer. Modification through guanidination (retention of positive charge) and carbamylation (conversion to neutral residue) indicated some conformational differences between the native and the modified species, although the sugar binding, hemagglutinating, and toxic properties were indistinguishable. However, succinylation (conversion to negative charge) caused significant changes in the conformation and biological activity of the molecule. Significantly, the deglycosylated form of the protein retained complete toxicity toward the corn borer.

Lectins are proteins of nonimmune origin that recognize and reversibly bind to carbohydrate moieties of complex carbohydrates and glycoproteins without altering the covalent structure of any of the recognized glycosyl ligands (Goldstein *et al.*, 1980). Although lectins are present in all taxonomic groups of the plant kingdom, they appear to be particularly abundant in the Leguminosae family (Strosberg *et al.*, 1986). Seeds are the richest source of lectins, but other parts of the plants may also contain smaller amounts of lectins (Etzler, 1986). In recent years, there has been a great deal of speculation on the possible role of lectins in plants, and among other functions, it has been suggested that they may well serve a defensive role in protecting plants against invasion by bacteria, fungi, and insects (Janzen *et al.*, 1976; Etzler, 1986). Indeed, *in vitro* feeding studies have demonstrated the toxic effects of some lectins on insects (Janzen *et al.*, 1976; Shukle *et al.*, 1983; Murdock *et al.*, 1989; Gatehouse *et al.*, 1984). Furthermore, Czapla and Lang (1990) showed that BPA possessed larvicidal activity on the European corn borer (*Ostrinia nubilalis* [Hubner]), a major insect pest of corn.

The lectin from *Bauhinia purpurea* (BPA) was first purified and characterized as a glycoprotein by Irimura and Osawa (1972). They observed a high degree of specificity of BPA for sugars such as *N*-acetylgalactosamine (GalNAc) and galactose. The binding site specificity of BPA was also determined by Allen et al. (1980). Subsequently, Young *et al.* (1985) demonstrated that the N-terminal amino acid sequence of BPA was homologous to that of other leguminous lectins and also that the protein was tetrameric under native conditions. In a recent paper, Kusui *et al.* (1991) described the isolation, sequencing, and expression of a cDNA clone for BPA from the germinated *B. purpurea* seed cDNA library. Also, the homology of BPA to two other leguminous lectins, concanavalin A (Con-A) and pea lectin, was clearly established. In another study, Yamamoto *et al.* (1992) reported the purification of a nonapeptide involved in the carbohydrate-binding site of BPA.

In proteins, selective chemical derivatization of side chains can provide invaluable *a priori* information on the importance of a specific amino acid residue for a particular biological activity (Means and Feeney, 1990). The gene for the corresponding protein can eventually serve as a template for making rational amino acid substitutions through site-directed mutagenesis to alter the property of the molecule. In this laboratory we are interested in delineating the role of the various amino acid residues as well as the role of glycosylation in the structure–function properties of BPA. Here, we examine the effect of specific modification of the ϵ-NH_2 group of lysine residues through carbamylation, guanidination, and succinylation on some of the properties of BPA. Our experiments have an important bearing on the recently discovered insecticidal property of BPA (Czapla *et al.*, 1990). Potentially, trypsin-like enzymes that exist in the larval midgut of the corn borer (Houseman *et al.*, 1989) can cleave BPA at the lysine residues and decrease the *in vivo* half-life of the protein upon ingestion. The information gained in our study will be useful in the design of novel BPA derivatives with possibly enhanced stabilities through the substitution of lysine residues by site-directed mutagenesis.

MATERIALS AND METHODS

Affinity-purified BPA was obtained from Vector Laboratories, Inc. (Burlingame, CA). Protein concentration was determined using an extinction coefficient of 1.43 for a 1 mg/mL protein solution measured at 280 nm (Young *et al.*, 1985).

Glycosyl Composition. The carbohydrate composition of BPA was analyzed by preparing trimethylsilyl (TMS) methylglycosides as described by York *et al.* (1986). In addition, the protein was also *N*-acetylated in order to determine the amino sugar content. Briefly, BPA (500 μg) was methanolized in methanolic 1 M HCl at 100 °C for 18 h. The solvent was evaporated with air and the residue *N*-acetylated in acidic anhydride/pyridine and methanol. The solvent was again evaporated, and the TMS derivative was prepared by adding Tri-Sil (Pierce Chemical Co., Rockford, IL) and heating at 80 °C for 20 min. Analysis of the TMS methylglycosides was performed

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by GC and GC–MS using a 30-m DB1 capillary column from J&W Scientific and a Hewlett Packard GC–MS system. Quantitation and identification of individual glycosyl components were made by comparing retention times with those of authentic standards and using response factors of authentic standards. Inositol was added to both the standards and the sample as an internal standard.

Deglycosylation. Enzymatic deglycosylation of BPA, under native and denaturing conditions, was performed by digestion with the endoglycosidase, Peptide-N-Glycosidase F (Boehringer-Mannheim, Indianapolis, IN), for 18 h at 37 °C, with other conditions being as described in the kit. Denatured BPA was obtained by heating 1 mL of 5 mg/mL protein in 0.1% SDS at 100 °C for 2 min. After cooling to room temperature, *n*-octyl glucoside (0.5%) and EDTA (50 mM) were added, boiled for an additional 2 min, and returned to room temperature. The solution was then diluted to 2 mL with PBS pH 7.4 and subsequently treated with the enzyme. Following deglycosylation, the samples were exhaustively dialyzed against 0.1 M ammonium bicarbonate, lyophilized, and analyzed on a 10–30% SDS–acrylamide gradient gel (Laemmli, 1970).

Modification of Free Amino Groups. Free amino groups of BPA were modified by three independent methods.

Carbamylation (Stark, 1972). To 1 mol of the lectin in phosphate-buffered saline (PBS), pH 8.0, was added 400 mol of potassium cyanate (Aldrich Chemical Co., Milwaukee, WI) and the reaction mixture was incubated at room temperature for 48 h. Unreacted reagents were removed by extensive dialysis against PBS, pH 7.4.

Guanidination (Condrea et al., 1983). The native lectin was treated with 0.5 M *O*-methylisourea (Sigma Chemical Co., St. Louis, MO) at pH 10.8, at a final protein concentration of 1% by weight. The reaction mixture was then allowed to incubate at 4 °C for 72 h. Excess reagents were removed by dialysis against PBS, pH 7.4.

Succinylation (Klotz, 1967). To every mole of ϵ-NH_2 was added 100 mol of succinic anhydride (Sigma Chemical Co., St. Louis, MO). The addition was carried out in small aliquots with constant stirring, over a period of 2 h at room temperature. The pH of the reaction was maintained between 8.0 and 8.5 by the addition of 5 M NaOH. Following the addition of succinic anhydride, the reaction mixture was allowed to incubate at room temperature for 30–60 min. The succinylated protein was then extensively dialyzed against PBS, pH 7.4, in order to remove the unreacted reagents.

Determination of Extent of Modification by Amino Acid Analysis. The extent of modification by carbamylation and guanidination was determined by amino acid analysis as homocitrulline and homoarginine, respectively, as described below. About 2 mg of the control or modified BPA in 0.5 mL of PBS, pH 7.4, was hydrolyzed in 6 N double-distilled HCl for 18 h at 110 °C and filtered through a 0.45-μm syringe-tip filter. An aliquot of the filtrate (100 μL) was dried in a Speed Vac and solubilized in 1 mL of Beckman sodium diluent, 0.27 N Na^+, pH 2.2. The samples were then analyzed in a Beckman Gold System amino acid analyzer by injecting a 20-μL aliquot into a Beckman-Spherogel AA-Na^+ column (0.3 × 25 cm) and developing at 18 mL/h using a step gradient consisting of Beckman buffers, sodium eluent 1 (pH 3.28), sodium eluent 2 (pH 7.4), and sodium regenerate 3 (pH 10.5), over a period of 2 h. Postcolumn derivatization was performed by reaction with Beckman Trione ninhydrin at 130 °C and detected at 570 nm. Homoarginine, resulting from guanidination, eluted just after arginine with a peak absorbance similar to that of arginine, at a column temperature of 53 °C. Homocitrulline, resulting from carbamylation, eluted just before valine at a column temperature of 56 °C (Plapp *et al.*, 1971). Since homocitrulline is converted to lysine following prolonged hydrolysis, the values for 1, 2, and 3 h were extrapolated to zero time. This indicated a modification of >90% of the lysine residues. The extent of succinylation was determined by the trinitrobenzenesulfonic acid (TNBS) method of Hall *et al.* (1973). Amino acid analysis of native BPA was also performed on protein that was electroblotted onto poly(vinylidene difluoride) (PVDF) membrane (Millipore) from a 10–30% SDS–acrylamide gradient gel according to the method of Matsudaira (1987). The air-dried blotted protein band was hydrolyzed with 500 μL of 6 N HCl at 150 °C for 60 min. The free amino acids were converted to PTC amino acids on the Applied Biosystems 420A derivatizer followed by separation on a narrow-bore HPLC system using a C-18 reverse-phase column with detection at 254 nm.

Affinity Chromatography. Four milliliters of GalNAc–agarose (Sigma Chemical Co., St. Louis, MO) was packed in a 10-mL column and equilibrated in PBS, pH 7.4. Five hundred micrograms of the native or modified BPA was applied to the column and allowed to adsorb. The column was washed with about 3 bed volumes of PBS, and the bound proteins were eluted by washing with 25 mM GalNAc in PBS. Fractions (1 mL) were collected, and the absorbance was measured at 280 nm.

Circular Dichroism (CD) Measurements. Spectra (190–250 nm) were recorded at 25 °C with a JASCO 600 spectropolarimeter using 0.1-cm cells. The protein concentration was 0.05 mg/mL in PBS, pH 7.4. Mean residue ellipticity was calculated using a mean residue weight of 110.

Fluorescence Measurements. Fluorescence spectra (300–400 nm) were recorded after excitation at 280 nm, in a thermostated 1-cm cuvette (25 °C) with a Perkin-Elmer Model LS50 luminescence spectrometer. Excitation and emission slit widths were set at 10 and 15 nm, respectively. All protein samples had an A_{280} of <0.1.

Quenching of Fluorescence of 4-Methylumbelliferyl *N*-Acetyl-β-D-galactosaminide (4-MeuGalNac). Quantitative measurements of the sugar binding ability of BPA and modified derivatives were performed by monitoring the quenching of 4-MeuGalNac (Sigma Chemical Co., St. Louis, MO) fluorescence upon titration with the protein (Delmotte *et al.*, 1975). Specifically, 500 μL of a 2–4 μM solution of the ligand in PBS, pH 7.4 (diluted from a 1 M stock in *N*,*N*′-dimethylformamide) was placed in a thermostated cuvette set to 25 °C and titrated with 10-μL aliquots of the protein in the range 1.5–28 μM, also in PBS. After each addition, the sample was excited at 313 nm and the fluorescence spectrum recorded in the 340–450-nm range. Titrations were done in triplicate, and all spectra were corrected for background as well as dilution. Extrapolation of $1/F$ (where F is the fluorescence intensity) vs $1/P$ (where P is the concentration of added protein) to infinite protein concentration allowed evaluation of F_{max}, the fluorescence intensity of the ligand when fully bound to the protein. The fraction of ligand bound, α, was calculated from the ratio of $F/\Delta F_{max}$. From a determination of α at different protein concentrations, a plot of $[P]/\alpha$ versus $1/(1-\alpha)$ was made and the dissociation constant (K_d) for the protein ligand interaction was obtained from the slope as described by Webb (1963).

Quenching of Protein Fluorescence. The quenching of intrinsic fluorescence of BPA and modified derivatives was followed by titration with aliquots of a 6 M acrylamide solution. The excitation wavelength was set at 295 nm to ensure optimal absorption by tryptophan residues. The fluorescence intensities were corrected for the self-absorption of incident light (McClure and Edelman, 1967) using a molar extinction coefficient of 0.23 for acrylamide (Parker, 1968).

The fluorescence quenching was analyzed by the modified Stern–Volmer plot (Lehrer, 1971) according to eq 1. F_0 is the fluorescence intensity at the emission maximum in the absence of quencher, ΔF is the change in the intensity in the presence of quencher, and [Q] is the quencher concentration. A plot of $F_0/\Delta F$ vs $1/[Q]$ will yield a straight line of slope $1/f_a K_q$ and intercept $1/f_a$, with K_q = intercept/slope. f_a represents the effective fraction of tryptophan residues which are accessible to the quencher at infinite concentration, and K_q is the Stern–Volmer quenching constant.

$$F_0/\Delta F = 1/f_a + 1/f_a K_q[Q] \quad (1)$$

Gel Filtration. Gel filtration experiments were performed on a 25-mL Superdex column (Pharmacia LKB) connected to a FPLC (Pharmacia LKB) and equilibrated with PBS, pH 7.4. The native or modified BPA (0.5 mg) in 0.5 mL of buffer was applied to the column and eluted at a flow rate of 30 mL/h. Fractions (0.5 mL) were collected, and the absorbance was read at 280 nm.

Hemagglutination. Native and modified BPA were 2-fold serially diluted in a V-bottomed microtiter plate. PBS, pH 7.4,

Table I. Carbohydrate Composition of BPA[a]

glycosyl residue	wt %	glycosyl residue	wt %
fucose	0.4 (0.5)	glucose	0.3 (0.9)
xylose	0.8 (1.1)	glucosamine	1.6 (3.4)
mannose	3.1 (4.9)		
galactose	trace (0.3)	total	6.2 (11.1)

[a] The values represent weight percent of total carbohydrate in the protein. The percent of mass of protein accounted for by these sugars is 6.2%. The results of Irimura and Osawa (1972) are indicated in parentheses.

and BSA were used as negative controls. Rabbit erythrocytes (4×10^5) (Colorado Serum Co., Denver, CO) in a final volume of 100 μL were added to each of the wells. Plates were incubated at room temperature and read after 5 h.

Toxicity to European Corn Borer. Neonate larvae of *O. nubilalis* were obtained from the Pioneer Entomology Laboratory (Johnston, IA) and reared as previously described (Czapla and Lang, 1990). They were used in a topical bioassay essentially as described by Czapla and Lang (1990) but modified by Balasubramaniam *et al.* (1991). BPA and modified BPA were prepared as 2.5 mg/mL solutions in PBS, and a 100-μL aliquot of each protein was used per cell containing one larva. PBS served as the control treatment. Eight insects were used per treatment, and it was repeated on 2 consecutive days. Weight and mortality were recorded after 7 days. The effect of modified BPA on larval weight (milligrams) was evaluated using a one-way treatment design, where all five treatments were replicated in a randomized complete block. The recommendations of Milliken and Johnson (1984) for multiple range tests were employed for making comparisons among the treatment averages.

RESULTS

Carbohydrate Content. The glycosyl composition of BPA was determined by GC and GC–MS. While our results were in general agreement with the hitherto only published data of Irimura and Osawa (1972), the total carbohydrate content of 6.2% (by weight) obtained by us was considerably lower than the reported 11.1% (Table I). These differences may be attributed to the more accurate and quantitative results obtained by us with the GC–MS analysis of the sugar residues as trimethylsilyl (TMS) methylglycosides compared to phenolsulfuric acid hydrolysis and analysis by trifluoroacetylation as described by Irimura and Osawa (1972). The results clearly indicated that mannose was the major neutral sugar component and that the major amino sugar was glucosamine.

Deglycosylation of BPA. The effect of Peptide-N-glycosidase F on BPA is shown in Figure 1. Control BPA (lane 1) showed two closely migrating bands with an approximate M_r of 35K. Denatured BPA, after incubation with the endoglycosidase, migrated as a single band with a decreased relative molecular weight (lane 2). However, in the case of the native protein treated with the enzyme, in addition to the lower molecular weight band, there appeared to be a substantial amount of the native protein as well (lane 3).

Chemical Modification. There are 9 lysine residues in BPA (Kusui *et al.*, 1991). These were modified so as to retain the positive charge through guanidination, guan-BPA (conversion to homoarginine), convert to a negative charge through succinylation, succ-BPA (succinyllysine), or form a neutral moiety through carbamylation, carb-BPA (homocitrulline). In all instances amino acid analysis of the modified species indicated that >90% of the available lysine residues had been modified.

Gel Filtration. Upon gel filtration on a molecular sieve column (Superdex 75), native BPA and all the modified derivatives eluted as a single major peak in the void volume, indicative of a species possessing M_r >70K (data not shown).

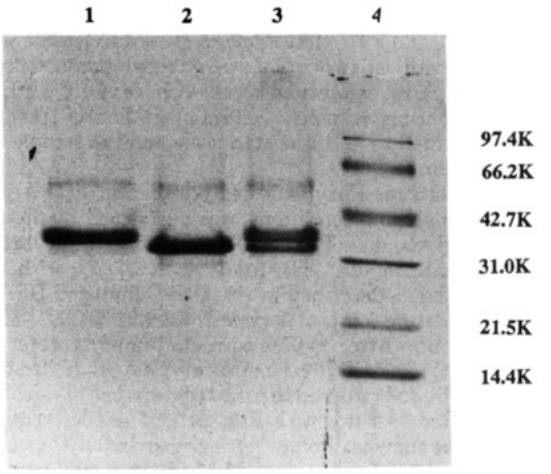

Figure 1. SDS–PAGE of native and deglycosylated BPA on a 10–30% gradient gel as described by Leammli (1970). Lane 1: 10 μg of native BPA. Lane 2: 10 μg of BPA deglycosylated under denaturing conditions. Lane 3: 10 μg of BPA deglycosylated under native conditions. Lane 4: 2.5 μL (10 μg) of Bio-Rad molecular weight standards.

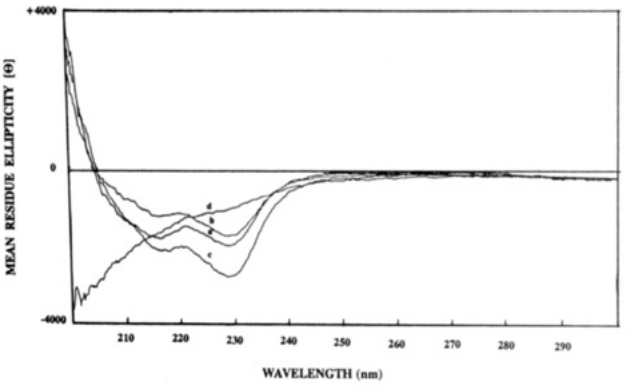

Figure 2. Far-UV CD spectra of (a) native BPA, (b) guan-BPA, (c) carb-BPA, and (d) succ-BPA as described in Materials and Methods. Individual spectra are the average of three scans.

CD Spectra. To determine if the chemical modification of BPA had any effect on the secondary structure of the molecule, the CD spectra of the native and modified species were recorded in the 200–300-nm range (Figure 2). The spectra for BPA, guan-BPA, and carb-BPA showed a pronounced minimum around 230 nm, with a much smaller minimum around 218 nm. However, while the mean residue ellipticity values at 230 nm were comparable for native and guan-BPA, the value for carb-BPA at this wavelength was 35% higher. In contrast, the spectrum for succ-BPA was devoid of the characteristic minima observed for the other modified species.

Fluorescence Spectra. Conformational changes in the protein consequent upon modification were also examined by fluorescence spectroscopy. Native, guanidinated, and carbamylated BPA exhibited a fairly broad peak around 330 nm when excited at 280 nm. However, the quantum yield of fluorescence for guan-BPA was considerably lower. In comparison, the fluorescence spectrum of succ-BPA was not only quenched but the emission peak was red shifted to about 350 nm (Figure 3).

Quenching of Protein Fluorescence with Acrylamide. Another useful technique for monitoring possible localized conformational changes is to examine the microenvironment of the tryptophan residues by titration with low molecular weight quenchers such as acrylamide and KI, which quench the tryptophanyl fluorescence by a collisional mechanism with the indole ring of the excited

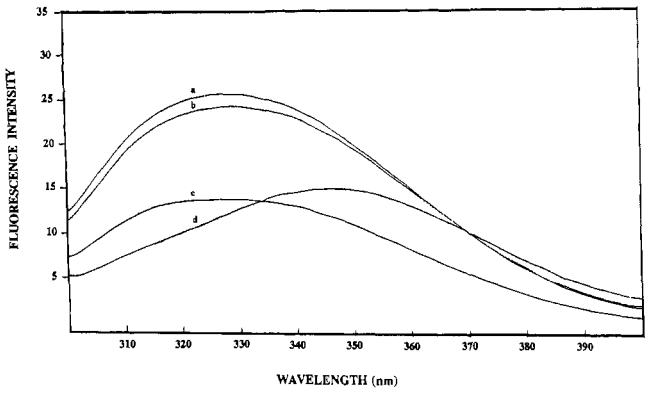

Figure 3. Fluorescence spectra of (a) native BPA, (b) carb-BPA, (c) guan-BPA, and (d) succ-BPA after excitation at 280 nm.

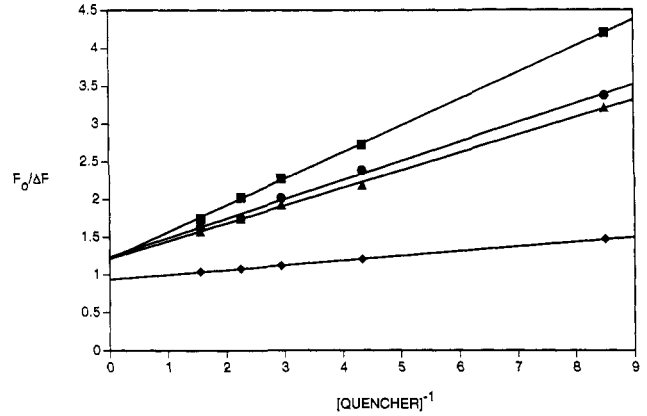

Figure 4. Quenching of the intrinsic fluorescence of BPA and modified derivatives by titration with acrylamide analyzed by the modified Stern-Volmer plot (see Materials and Methods). (■) Control BPA; (●) guan-BPA; (▲) carb-BPA; (♦) succ-BPA.

tryptophan (Lehrer, 1971). The susceptibility of the tryptophan residue to quenching, in turn, depends on its degree of exposure and the surrounding ionic environment. Quenching by I^- is influenced by the ionic environment surrounding the tryptophan residue, whereas acrylamide is an efficient quencher that is relatively uninfluenced by the ionic environment (Eftink and Ghiron, 1976).

In experiments using KI as the solute, the observed quenching of tryptophan fluorescence was nonspecific (as shown by identical changes observed in titrations with NaCl) and was entirely attributable to ionic strength effects (data not shown). Therefore, we employed acrylamide as the quencher. In these experiments, analysis of the titration data by the direct Stern-Volmer equation (F_0/F vs Q) resulted in downwardly curved plots (data not shown), indicative of a heterogeneous population of tryptophan residues differing in their ability to be quenched. Accordingly, the data were analyzed by the modified Stern-Volmer equation (see Materials and Methods) (Figure 4). The results are summarized in Table II. The f_a values for native BPA, carb-BPA, and guan-BPA were comparable and corresponded to about an 80% accessibility of the reported six tryptophans (Kusui *et al.*, 1991). In the case of succ-BPA, however, the value of f_a increased to 1, indicating that 100% of the tryptophans were now accessible to acrylamide. The Stern-Volmer quenching constant K_q varied between 3.5 and 5.2 M^{-1} for control BPA, carb-BPA, and guan-BPA but increased to 15 M^{-1} for succ-BPA.

Sugar Binding. Affinity chromatography was used to analyze the ability of the native and modified lectins to bind to GalNAc-agarose (Figure 5). Binding was not observed only in the case of succ-BPA.

Table II. Fluorescence Quenching Parameters for Titration with Acrylamide[a]

protein	K_q, M^{-1}	f_a
control BPA	3.5	0.8 (5)
carb-BPA	4.9	0.8 (5)
guan-BPA	5.2	0.8 (5)
succ-BPA	15.0	1.0 (6)

[a] The number in parentheses represents the average number of tryptophan residues available to the quencher.

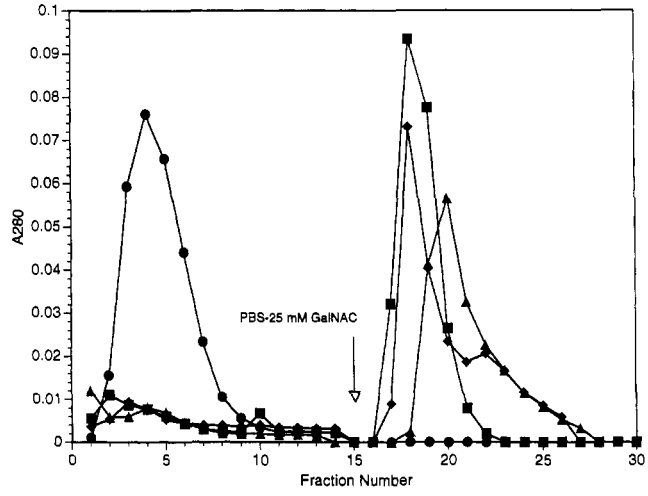

Figure 5. Binding of BPA and modified BPA to *N*-acetylgalactosamine (GalNAc) measured by affinity chromatography on a column of GalNAc-agarose. The arrow indicates the point at which elution with the free sugar was commenced. (■) Control BPA; (♦) guan-BPA; (▲) carb-BPA; (●) succ-BPA.

Continuous Titration of Fluorescence Quenching. Although the sugar binding specificity of BPA has been previously established (Irimura and Osawa, 1972; Allen *et al.*, 1980), hitherto there has been no quantitative measure of its affinity for the ligand. Here the binding of BPA and derivatives to GalNAc was examined by their ability to quench the fluorescence of 4-MeuGalNac, which exhibits a peak at 373 nm when excited at 316 nm (Delmotte *et al.*, 1975). The inset to Figure 6 shows the results from a typical titration experiment in which the ligand was titrated with native BPA (see Materials and Methods). About 35% quenching of the fluorescence was observed at the highest concentration of BPA (16.7 μM). Similar quenching profiles were observed for guan-BPA and carb-BPA (data not shown), and the dissociation constants (K_d), calculated from the slope of the curve as depicted in Figure 6, were comparable with that of the native protein (Table III). No quenching was observed with succ-BPA, indicating the inability of this derivative to bind to the sugar.

Hemagglutination. In earlier studies BPA has been shown to bind to Makela group 2 sugars and to cause the hemagglutination of rabbit erythrocytes that express these sugars on their surface (Irimura and Osawa, 1972). Therefore, we examined the ability of modified BPA to hemagglutinate rabbit erythrocytes. Native, carbamylated, and guanidinated BPA all exhibited the same relative hemagglutinating activity (Table IV). In a parallel experiment, inhibition of the hemagglutinating activity was observed with GalNAc (data not shown). However, no hemagglutination was observed even with a 250-fold excess of succ-BPA and the deglycosylated species.

Toxicity to European Corn Borer. The results from feeding studies on ECB are summarized in Table V. Native BPA, carb-BPA, guan-BPA, and BPA deglycosylated

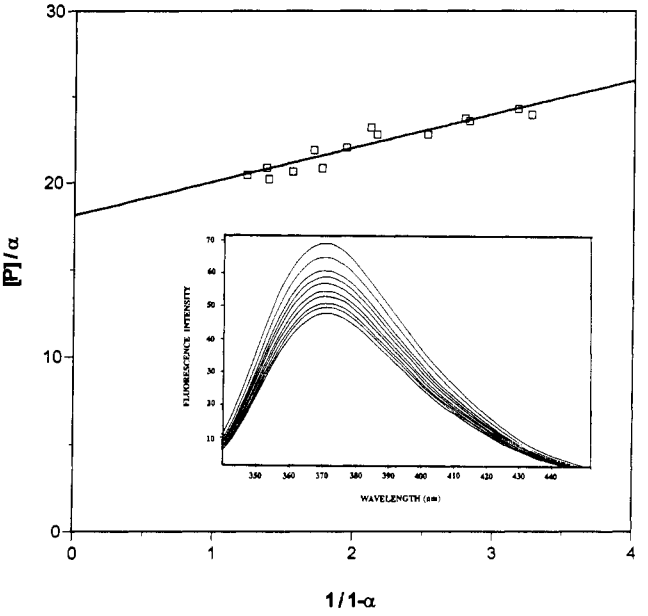

Figure 6. Graphical display of the binding data for control BPA, as described in Materials and Methods. The dissociation constant was obtained from the slope. (Inset) Quenching of the fluorescence spectrum of 4-MeuGalNac in PBS, pH 7.4, following the sequential addition of 10-μL aliquots of 100 μM BPA. The most quenched curve represents a protein concentration of 16.7 μM. Similar profiles were obtained for guan-BPA and carb-BPA (data not shown).

Table III. Dissociation Constant (K_d) for the Interaction between BPA, Modified BPA, and GalNAc

protein	K_d, M
control BPA	$(1.9 \pm 0.2) \times 10^{-6}$
carb-BPA	$(2.5 \pm 0.4) \times 10^{-6}$
guan-BPA	$(1.5 \pm 0.3) \times 10^{-6}$

Table IV. Hemagglutination of Rabbit Erythrocytes by Control and Modified BPA[a]

protein	titer, μg
control BPA	0.195
carb-BPA	0.195
guan-BPA	0.195
succ-BPA	>50

[a] The titer in μg indicates the lowest amount of protein that caused hemagglutination.

Table V. Effect of BPA and Modified BPA on ECB Neonate Larvae after 7 Days

treatment	wt ± SEM[a]	mortality,[b] %
PBS	6.6 ± 2.7	0
control BPA		100
carb-BPA		100
guan-BPA		100
succ-BPA	8.7 ± 2.2	0
deglycosylated BPA (native)		100
deglycosylated BPA (denatured)		100
deglycosylation buffer (see Materials and Methods)	4.2 ± 1.1	0

[a] Weight is the average of live insects at 7 days from three replications ± standard error of mean (SEM). [b] Mortality is the number of dead insects for all replications divided by the total insects tested × 100 (n = 16).

under native as well as denaturing conditions caused 100% mortality of the neonate ECB larvae. Control larvae that were fed PBS grew to an average weight of 6.6 mg. Succinylated BPA did not have any effect on the larvae, and the weight gain was comparable to that of the control larvae (8.7 mg). In order to eliminate any artifactual effects arising from the deglycosylation buffer containing detergents (see Materials and Methods), the bioassay included larvae fed on the deglycosylation buffer. Although mortality was not observed, some weight loss was indicated.

DISCUSSION

The glycosyl composition of BPA indicates it to be a glycoprotein with an overall carbohydrate content of about 6.2% and a specific glycosyl composition that is quite similar to that reported by Irimura and Osawa (1972). The occurrence of two closely moving bands for BPA on SDS–PAGE (Figure 1, lane 1) has been suggested to be due to the heterogeneity of the carbohydrate chain on BPA (Kusui *et al.*, 1991) which has five potential glycosylation sites at the asparagine residues. If so, removal of the carbohydrate residues should result in a homogeneous protein population. This is supported by our enzymatic deglycosylation experiments (Figure 1). Under native conditions, in addition to the doublet for native BPA, there is a band of lower molecular weight indicative of the deglycosylated species. However, the deglycosylation is only partial and did not go to completion even when the digestion time was increased from 18 to 36 h, perhaps due to the inaccessibility of the carbohydrate residues on the protein (Figure 1, lane 3). Significantly, denaturation, and subsequent treatment with the enzyme, resulted in a single band with a lower relative molecular weight than control BPA (Figure 1, lane 2), suggesting that deglycosylation was complete. Although complete deglycosylation was obtained chemically with trifluoromethanesulfonic acid (TFMS) (Tams and Welinder, 1990) (data not shown), the product was only partially soluble in buffer at neutral pH.

Specific amino acid residues are involved in carbohydrate recognition and binding of lectins (Goldstein and Poretz, 1986). The hemagglutination property of BPA is also dependent on the recognition and binding of specific carbohydrate residues on the surface of red blood cells (Irimura and Osawa, 1972). While the hemagglutinating activities of guan-BPA and carb-BPA were indistinguishable from that of native BPA, no such activity was observed with succ-BPA or the deglycosylated protein. Furthermore, it is tempting to speculate that the larvicidal effect of BPA, specifically on the corn borer, may be directly related to its ability to recognize and bind to a carbohydrate-containing ligand on the cells lining the midgut wall. Precedence for such a mechanism comes from the demonstration of specific binding of the lectin from *Phaseolus vulgaris* to the epithelial cells of the bruchid beetle midgut, culminating in the mortality of the insect (Gatehouse *et al.*, 1984). It follows, therefore, that the abolition of carbohydrate binding activity would also result in a loss of insecticidal activity, as is the case with succ-BPA. More importantly, however, our results suggest that the carbohydrate residues on BPA may not be involved in this interaction since the deglycosylated protein apparently retains complete toxicity. Inasmuch as this appears to be contradictory to the observed lack of hemagglutination activity of deglycosylated BPA, a plausible explanation for this is that the surface glycosyl composition of the erythrocytes is quite different from that on the insect midgut cells. Indeed, it is quite well-known that not all cells or tissues perform glycosylation the same way (Parekh *et al.*, 1989).

However, the results from chemical modification can often be misleading, owing to possible conformational changes or other subtle effects accompanying the modification. This issue was addressed by circular dichroism

and fluorescence quenching studies. The fluorescence emission maximum of native BPA occurs at ~300 nm. Although the protein contains 12 tyrosines and 6 tryptophans (Kusui *et al.*, 1991), the fluorescence spectrum is dominated by tryptophan emission. Compared to the emission maximum of ~355–360 nm for free tryptophan in aqueous solution, it appears that, on an average, the tryptophans in BPA occur in the interior of the molecule. Carbamylation and guanidination of the protein do not change the λ_{max} of emission, indicating that the tryptophans are still in the interior of the molecule. However, the quantum yield of fluorescence is much lower. In contrast, the emission spectrum and λ_{max} of emission of succ-BPA clearly indicate that the tryptophan residues are now exposed to the aqueous environment, possibly as the result of a gross conformational change.

A more sensitive assessment of the microenvironment of the six tryptophan residues was obtained by quenching of the protein fluorescence with acrylamide. Although there was no change in the apparent accessibility of five of these tryptophans for native BPA, guan-BPA, and carb-BPA, there was some increase in the value of the quenching constant of the modified derivatives to an average of ~5.0 M^{-1}. Assuming that the quenching constant represents a crude measure of the ease with which residues are quenched, the results suggest that the local environment of the quenchable tryptophans in these derivatives has undergone a subtle conformational change. However, in the case of succ-BPA, all tryptophans are now accessible. In addition to the exposure of the "buried" tryptophan, the Stern–Volmer constant of 15 M^{-1} attests to the relatively increased quenching efficiency.

The conformational change accompanying modification is also reflected in the CD spectrum of succ-BPA, which is now typical of a protein with no residual secondary structure (Figure 2). However, this total loss of secondary structure is not seen in the case of guan-BPA and carb-BPA. There is an overall similarity of the profiles, with differences in the values of the mean residue ellipticity clearly indicative of conformational changes. However, there may also be a contribution from errors in the estimation of the concentration of the modified proteins.

Another key determinant in the biological activity of oligomeric proteins is the subunit structure. BPA is reported to be a tetramer at pH 7.0 (Irimura and Osawa, 1972). The retention of biological activity of guan-BPA and carb-BPA suggested that the tetrameric subunit structure was retained in these derivatives. Furthermore, as in the case of native BPA, they eluted in the void volume during gel filtration on Superdex-75, which has an exclusion limit of M_r 70K. Dissociation to a monomeric subunit (M_r ~ 34K), such as is often encountered upon succinylation (Lundblad, 1991), would be expected to retard the protein on the column. Surprisingly, however, the similar elution profile of succ-BPA suggested that it was eluting as a 70K protein. This ambiguity may be ascribed in part to changes in the hydrodynamic properties of the protein such as an increase in the Stoke's radius (Habeeb, 1967). Sedimentation velocity experiments to verify this were inconclusive, owing to aggregation under the conditions employed (data not shown). Interestingly, Con-A, to which BPA exhibits a high degree of homology, is also a tetramer under native conditions and contains 9 lysine residues (Becker *et al.*, 1976). Succinylation of Con-A also leads to the formation of a dimer in which several properties, including hemagglutination, are considerably altered (Gunther *et al.*, 1973).

In summary, it is evident that modification that retains a positive charge (guanidination) or forms a neutral side chain (carbamylation) causes no appreciable change in the properties of BPA. This agrees with the general observation that modifications that have the least effect on side-chain character will also cause the least perturbation in protein structure and properties (Means and Feeney, 1990). On the contrary, succinylation causes a drastic reversal of the side-chain character of lysine, and this is reflected in the concomitant changes in the physicochemical properties. Equally importantly, our experiments suggest that the carbohydrate residues are not critical to the larvicidal action of BPA. This is a potentially important finding that awaits validation in the recombinantly expressed protein.

ABBREVIATIONS USED

BPA, *Bauhinia purpurea* agglutinin; carb-BPA, carbamylated BPA; Con-A, concanavalin A; ECB, European corn borer; GalNAc, *N*-acetylgalactosamine; guan-BPA, guanidinated BPA; 4-MeuGalNac, 4-methylumbelliferyl *N*-acetyl-β-D-galactosaminide; PBS, phosphate-buffered saline; PVDF, poly(vinylidene difluoride); succ-BPA, succinylated BPA; TMS, trimethylsilyl; TFMS, trifluoromethanesulfonic acid; TNBS, trinitrobenzenesulfonic acid.

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