Enzyme Immunoassay for Potato Glycoalkaloids

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New methods for the synthesis of solanidine protein conjugates were developed. The protein conjugates were synthesized from γ-chaconine or solanidine hemisuccinate and resulted in greater alkaloid to protein ratios (up to 23 mol of alkaloid/mol of bovine albumin) than earlier methods. *Limulus polyphemus* hemocyanin conjugates were used to immunize rabbits and produced high sera titers (>10^6; 10^5 serum dilution could be used for assays). Bovine serum albumin coating conjugates were used in competitive enzyme immunoassays (EI) that detected and quantified the major solanidine glycoalkaloids (α-solanine and α-chaconine) in commercial *Solanum tuberosum* cultivars. Quantitation of these glycoalkaloids in several potato samples showed high correlation between EI and high-performance liquid chromatography (HPLC). EI was found to be more comprehensive than HPLC since demissidine glycoalkaloids were also quantified.

INTRODUCTION

The potato (*Solanum tuberosum*) is an extremely valuable food source, ranked fourth by total yield per acre after wheat, rice, and corn and first by protein yield per acre (Rhoades and Rogers, 1982). While it is a nutritionally important source of high-quality protein, energy, vitamins, and minerals, the potato plant, including its tubers, contains toxic glycoalkaloids (GA).

Commerical cultivars commonly contain 20–150 μg of GA/g of unpeeled tuber (Slanina, 1990). GA are not removed or destroyed by cooking, baking, or frying but GA synthesis may be triggered by poor handling practices (Jadhav et al., 1981). The safety factor between the average GA level occurring and a toxic dose is only about 4-fold for a 500-g serving of potatoes. Therefore, it should not be surprising that a number of documented episodes of glycoalkaloid poisoning, some fatal, have been reported (Morris and Lee, 1984). It has been suggested that potato GA are one of the most serious toxic components in the human diet (Hall, 1992).

In the past, potato breeders have not concentrated on reducing or eliminating GA for a variety of reasons: (1) low levels of GA in potatoes add to their "savory" flavor characteristics (Ross et al., 1978); (2) GA are important in pest and disease resistance in potatoes (Sanford et al., 1990; Deahl et al., 1991; Osman et al., 1991); (3) GA synthesis in the plant is under multigenic control, making the elimination of GA very difficult (Sinden et al., 1984); and (4) GA analysis is difficult and expensive, inhibiting routine screening. At the retail or processing level, control of GA levels has depended on a secondary indicator, greening of potatoes, caused by exposure of tubers to light. This is unreliable, inaccurate, and wasteful since chlorophyll production does not always accompany synthesis of GA and vice versa. Other factors that are related to GA concentration include genetic factors and environmental factors such as exposure to light, temperature stress, disease or insect damage, and mechanical damage (Ross et al., 1978; Sinden et al., 1984; Olsson et al., 1986).

Although there have been numerous attempts to produce a reliable GA analysis method, there is still no simple method that meets all of the requirements of potato breeders and processors. Methods of GA analysis have been reviewed by Maga (1980), Coxon (1984), Morgan et al. (1985), and van Gelder (1991). High-performance liquid chromatography (HPLC) has been reported by several authors in the separation and quantitation of some major GA (Bushway et al., 1980, 1986; Crabbe and Fryer, 1980; Hunter et al., 1980; Morris and Lee, 1981; Carmen et al., 1986; Hellenäs, 1986; Kobayashi et al., 1989; Saito et al., 1990). GA do not have a suitable UV chromaphore, and therefore absorbance is measured at 208 nm, where many compounds absorb light. This drawback dictates the need for relatively large sample sizes and extensive sample cleanup to overcome background noise (Coxon, 1984; Morgan et al., 1985). In addition, UV absorbance will not detect alkaloids that are saturated at C5 (Figure 1). Gas chromatography has also been reported in the analysis of many GA (Herb et al., 1975; Coxon et al., 1979; King, 1980; van Gelder, 1985). This methodology has the advantage of sensitive detector systems, but problems arise from the poor volatility of GA. Derivatization complicates the analysis, and high temperatures reduce column life (Morgan et al., 1985).

The most promising test to date is the development of an immunoassay (Morgan et al., 1983). This test is accurate, sensitive, and simple, it can measure many samples simultaneously, and it correlates well with other more expensive methods (Morgan et al., 1985; Hellenäs, 1986). Immunoassays, due to their specificity, can eliminate the problem of extraction and purification, which has been a drawback of previous methods.

With the obvious need for a simple GA analysis method, such as the procedure developed by Morgan et al. (1983), it is surprising that further improvements of their enzyme immunoassay (EI) procedure have not appeared in the scientific literature. Their method of linking alkaloid to protein results in very low hapten to protein ratios. The low ratio of alkaloid to protein for an immunogen can lead to a poor immunological response (Erlanger, 1980), yielding low-affinity Ab and high background absorbances in the EI analysis. Also, there has been no investigation of the well-known effects of different hapten linking methods on a competitive EI assay for GA (Harrison et al., 1991; Sheth and Sporns, 1991; Gosling, 1990; Wie and Hammock, 1984; Vallejo et al., 1982).

The following study uses new methods to prepare solanidine–protein conjugates in an attempt to improve the competitive EI analysis of GA.

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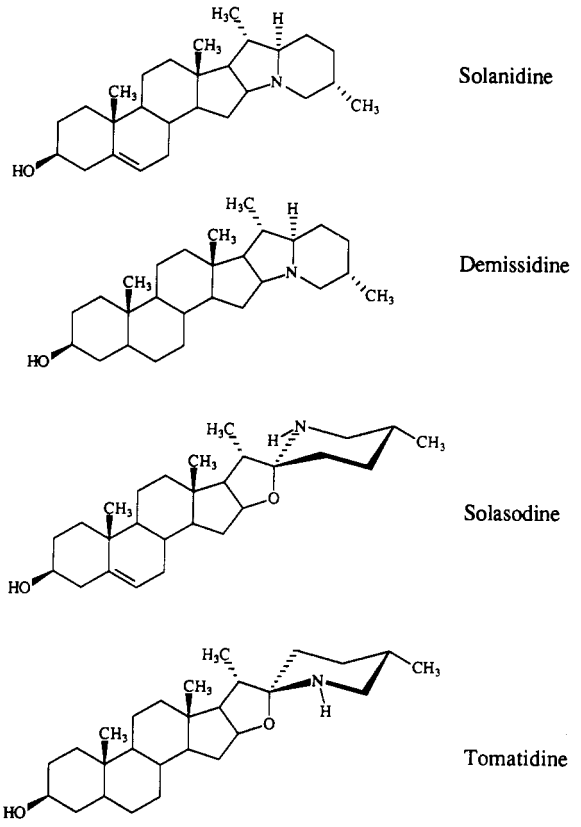


Figure 1. *Solanum* sp. alkaloids.

MATERIALS AND REAGENTS

Materials. Water used in all reactions and EI tests was purified using a Milli-Q system (Millipore Corp., Milford, MA). α-Chaconine, α-solanine, solanidine, tomatidine, demissidine, solasodine, thimerosol, Tween 20, sodium cyanoborohydride, 4-(dimethylamino)pyridine, bovine serum albumin (BSA), and *Limulus polyphemus* hemocyanin (LPH) were obtained from Sigma Chemical Co., St. Louis MO. Antimony trichloride, dioxane, sodium metaperiodate, pyridine, bromocresol green, and urea were purchased from Fisher Scientific, Edmonton, AB. Sodium borohydride was obtained from Terochem Laboratories Ltd., Edmonton, AB. Succinic anhydride was obtained from BDH Inc., Edmonton, AB. *N*-Ethyldiethanolamine, *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, and *N*,*N*-dimethylformamide were purchased from Aldrich Chemical Co., Milwaukee, WI. Freund's complete adjuvant and Freund's incomplete adjuvant were obtained from Difco Laboratories, Detroit, MI. Urea peroxide, *o*-phenylenediamine, and peroxidase conjugated goat anti-rabbit antibodies were obtained from Calbiochem Co., San Diego, CA.

Dynatech Immulon II microtiter plates and Spectrum dialysis tubing (12 000–14 000 MW cutoff) were purchased from Fisher Scientific. Sep-Paks were Waters C-18 cartridges from Millipore. A commercial ELISA kit for solanidine glycoalkaloids (Lot D400 3513A02) was purchased from Holland Biotechnology bv, Leiden, The Netherlands.

Potato samples a–d were *S. tuberosum* obtained commercially. Samples a and b were the same sample peeled and unpeeled, respectively. Sample c was a purple-skinned cultivar. Samples e and f were greened *S. tuberosum* cv. Russett Burbank (harvested at soil surface) obtained from the Department of Plant Science, University of Alberta. Sample e was stored for 12 months at 5 °C in darkness, and sample f (extremely green and sprouted) was stored for 6 months at 5 °C in darkness and then 6 months at 25 °C exposed to daylight. Sample g was plant material (leaves, stems, and flowers) from *Solanum demissum* obtained from the Department of Plant Science, University of Alberta. After treatments, all samples were cut into 0.5-cm³ pieces, freeze-dried, ground to pass through a 20-mesh screen, and stored at 5 °C until needed.

Phosphate-buffered saline (PBS) solution was prepared by dissolving NaCl (18.0 g), disodium hydrogen phosphate (2.22 g), potassium dihydrogen phosphate (0.6 g), and thimerosol (0.2 g) in 1.9 L of water and the pH adjusted to 7.3 with 1 N NaOH. The total volume was then made up to 2.0 L with water. To prepare PBST, Tween 20 (1.0 g) was added before the pH was adjusted. PBS without thimerosol (PBS-t) was prepared as PBS but omitting thimerosol.

Instrumentation. Ultrafiltration was performed using a 10-mL Amicon Model 12 ultrafiltration unit (Amicon Canada Ltd., Oakville, ON) fitted with a Diaflo PM10 ultrafiltration membrane. Centrifugation was performed in a Damon/IES Division Model HN-S II centrifuge from International Equipment Co., Needham Heights, MA. A Model 5L Virtis lyophilizer was used to freeze-dry conjugates, and a Virtis Pilot Scale Freeze Drier was used to freeze-dry potato samples, both from the Virtis Co. Inc., Gardiner, NY. Freeze-dried samples were ground in a Braun Model KSM2 coffee grinder (Braun Canada Ltd., Mississauga, ON) to pass through a screen (0.85-mm openings). Homogenization was performed using a Kinematica PT10/35 Polytron homogenizer from Brinkmann Instruments, Rexdale, ON. Microtiter plate optical densities were measured with a Model EL 309 ELISA reader from Bio-Tek Instruments, Inc., Burlington, VT. A Buchi Rotoevaporator R (Fisher Scientific) was used for the removal of solvents from samples.

Preparative thin-layer chromatography (TLC) was used to monitor the progress of chemical reactions. TLC plates were silica gel 60 F_{254} (0.2 mm thick) aluminum sheets from E. Merck, Darmstadt, Germany. Two solvent systems were used: chloroform/methanol/1% aqueous ammonia (2:2:1 v/v/v, lower layer), for the separation of α-chaconine hydrolysis products, and ethyl acetate/methanol/1% aqueous ammonia (80:20:1 v/v/v), for the separation of succination reaction products. Spots were visualized by spraying with either 25% antimony trichloride in glacial acetic acid (for the detection of alkaloid compounds) or 5% sulfuric acid in 95% ethanol (for the detection of all compounds) and charring for 2–5 min on a hot plate (Filadelfi and Zitnak, 1983). Unreacted succinic anhydride was visualized by spraying with 0.04% bromocresol green in ethanol, made green with 1 N NaOH.

Nuclear magnetic resonance (NMR) spectra were measured on a Bruker WH-200 instrument. Infrared (IR) spectroscopy was measured in a Nicolet 7199 FT-IR spectrophotometer. Microanalyses were measured using a Perkin-Elmer 240 CHN analyzer. All NMR, IR, and elemental analyses were performed by Chemistry Services at the University of Alberta.

PROCEDURES

Preparation of Immunogen. *Production of γ-Chaconine.* α-Chaconine (155 mg, 0.182 mM) was dissolved in 25 mL of aqueous ethanolic HCl (about 0.36 M, made from 188 mL of 98% ethanol, 6 mL of water, and 6 mL of concentrated HCl) and heated, with stirring for 50 h at 50 °C. The reaction was observed by TLC. The reaction mixture was then added to 30 mL of saturated sodium bicarbonate and extracted with chloroform (40 mL followed by another 10 mL). The combined chloroform extracts were evaporated to dryness. The residue was taken up in 4 mL of methanol and 10 mL of ethyl acetate and added to the top of a wet-packed (using ethyl acetate) 2.5 cm diameter by 15.5 cm high (20 g) Kieselgel 60 (0.040–0.063 mm diameter, Merck) silica gel column. The column was washed with 50 mL of ethyl acetate followed by 400 mL of ethyl acetate/methanol/aqueous 1% ammonia (375:20:5 v/v/v) and finally 111 mL of ethyl acetate/methanol/aqueous 1% aqueous ammonia (100:10:1 v/v/v). The first 190 mL of eluant was discarded, and the next 330 mL, containing the desired (identified by TLC) γ-chaconine, was collected. Later fractions contained partially hydrolyzed α-chaconine that could be used in future hydrolyses. The γ-chaconine fraction was evaporated to dryness, dried overnight under vacuum in the presence of phosphorus pentoxide, and weighed. The 60.7 mg of γ-chaconine (MW 560 g/mol, Figure 2) recovered represented a 60% yield for the selective hydrolysis. ^{1}H NMR (200 MHz, solvent CD_3OD, δ 3.35, 4.84) δ 5.38 (br d, 1 H, 5.0 Hz, H-6), 4.37 (d, 1 H, 7.7 Hz, H-1′), 3.84 (dd, 1 H, 1.7 Hz, 12.2 Hz, H_1-6′), 3.62 (m, 3 H, H_2-6′, H-3, H-3′), 3.35 (CHD_2OD likely obscures H-4′ and H-5′), 3.13 (dd, 1 H, 7.7 Hz, 8.5 Hz, H-2′), 3.01 (m, 1

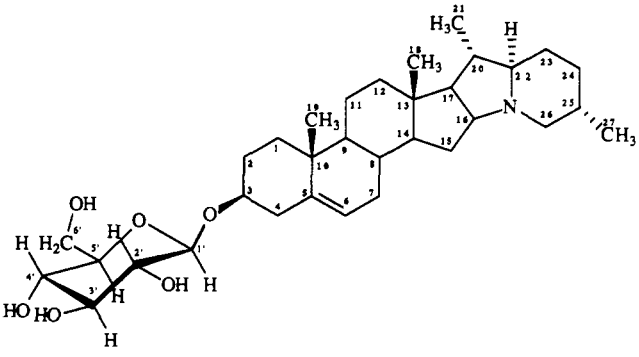


Figure 2. γ-Chaconine.

H, alkaloid), 2.87 (m, 1 H, alkaloid), 2.43 (m, 1 H, alkaloid), 2.25 (m, 1 H, alkaloid), 1.70 (m, 18 H, alkaloid), 1.24 (m, 9 H, alkaloid), 0.94 (m, 15 H, alkaloid).

Conjugation of γ-Chaconine to Protein. γ-Chaconine (16.5 mg, 0.0295 mM) was added to 2 mL of 98% ethanol, 1.5 mL of dioxane, and 1.5 mL of water. The γ-chaconine was not totally dissolved in this solvent mixture, and the solution appeared slightly cloudy; 73 mg (0.341 mM) of sodium metaperiodate was added and the reaction stirred for 1.5 h. LPH (27.4 mg) which had been dialyzed with water and lyophilized, was added in 3 mL of pH 9.00 *N*-ethyldiethanolamine buffer (26.6 mL of *N*-ethyldiethanolamine in 1 L of water and about 14 mL of 6 M HCl to pH 9.00). The protein container was rinsed with 3 × 2-mL portions of pH 9.00 *N*-ethyldiethanolamine buffer, which was added to the reaction mixture along with 100 mg (1.58 mM) of sodium cyanoborohydride (Schwartz and Gray, 1977). The reaction was heated, with stirring, for 6 days at 40 °C. At this time 60 mg (1.61 mM) of sodium borohydride was added and the reaction mixture stirred overnight at room temperature. Now the entire reaction mixture was poured into a Amicon ultrafiltration unit and filtered to about 2 mL. The filtration was repeated with 10 mL of water, followed by 10 mL of 8 M urea, 4 times 10 mL of water, 10 mL of 20% aqueous ethanol, and again 10 mL of water. The residue was then lyophilized. This reaction was also performed using BSA to obtain solid-phase protein conjugates linked in the same manner as the immunogen.

Measurement of Alkaloid in Conjugate. The percentage GA incorporation into the protein was measured using a mixed solvent hydrolysis method (van Gelder, 1984). The conjugate (1.00 mg) was added to 2 mL of 2 M HCl and 4 mL of carbon tetrachloride and stirred for 2.5 h at 90 °C under reflux. The reaction mixture was cooled and the carbon tetrachloride extracted. The remaining aqueous layer was adjusted to about pH 10 with concentrated NaOH and extracted with 5 mL of chloroform. The combined organic layers were washed with 5 mL of 1% aqueous ammonia solution and evaporated to dryness. The residue was dissolved in 5 mL of 20% (v/v) acetic acid. An appropriate amount (depending on the degree of substitution) of this solution was made up to 5 mL with 20% acetic acid and tested for solanidine using the method of Birner (1969). The results were compared with a standard curve made with pure solanidine (Sigma) from 0 to 62 μg. For ease of comparison between conjugates, the protein is assumed to have the MW of BSA (66 000 g/mol) when alkaloid to protein ratios are expressed. The LPH conjugate produced was found to contain 14 alkaloids per BSA equivalent, and 2 BSA conjugates were found to contain 6 and 23 alkaloids per BSA.

Immunization of Rabbits. Preimmunization blood samples (ear, 5 mL from each rabbit) were taken from two rabbits (3-month, male, Flemish Giant × Dutch Lop Ear). Then the rabbits were immunized with 1.0 mg of conjugate (LPH-G-14, glycosidic linked LPH conjugate containing 14 GA per BSA equivalent) in 2 mL of sterile PBS-t/Freund's complete adjuvant (1:1). Injections were made as 0.5 mL subscapular and 0.5 mL intramuscular, two sites each. Four boosts were made monthly, in a similar manner but using Freund's incomplete adjuvant. Blood samples (ear, 5 mL each) were taken 2 weeks after each boost, allowed to clot at room temperature for 1–2 h, and centrifuged (1000 rpm), and the serum was collected and tested for antibody (Ab) titer. Three days after the final boost, rabbits were completely bled (cardiac puncture, approximately 120 mL from each rabbit), and the immune sera obtained were stored at −20 °C until required.

Preparation of Solid-Phase Conjugate. *Production of Solanidine Hemisuccinate.* Formation of solanidine hemisuccinate was performed using a modified version of the procedure of Abraham and Grover (1971). 4-(Dimethylamino)pyridine (26 mg, 21.5 mM) was added to 10.00 mL of pyridine. Succinic anhydride (422 mg, 422 mM) was then added and dissolved, followed by 40.2 mg of solanidine (10.1 mM). The mixture was heated under reflux, with stirring, for 22 h at 57 °C. The reaction was followed by TLC. The reaction was stopped by the addition of 20 mL of H_2O, and the mixture (pH 6 to avoid hydrolysis) was extracted with 3 × 25 mL of methylene chloride; the organic extracts were combined and rotoevaporated (once, then coevaporated with 2 mL of toluene) to dryness, dried overnight under vacuum in the presence of phosphorus pentoxide, and weighed. The 48.3 mg of solanidine hemisuccinate (MW 498 g/mol) recovered represented a 96% yield. TLC revealed the absence of unreacted succinic anhydride. An infrared spectrum made of the product in methylene chloride showed a strong absorption band in the 1650–1750-cm^{-1} region, indicating that the hemisuccinate had been formed.

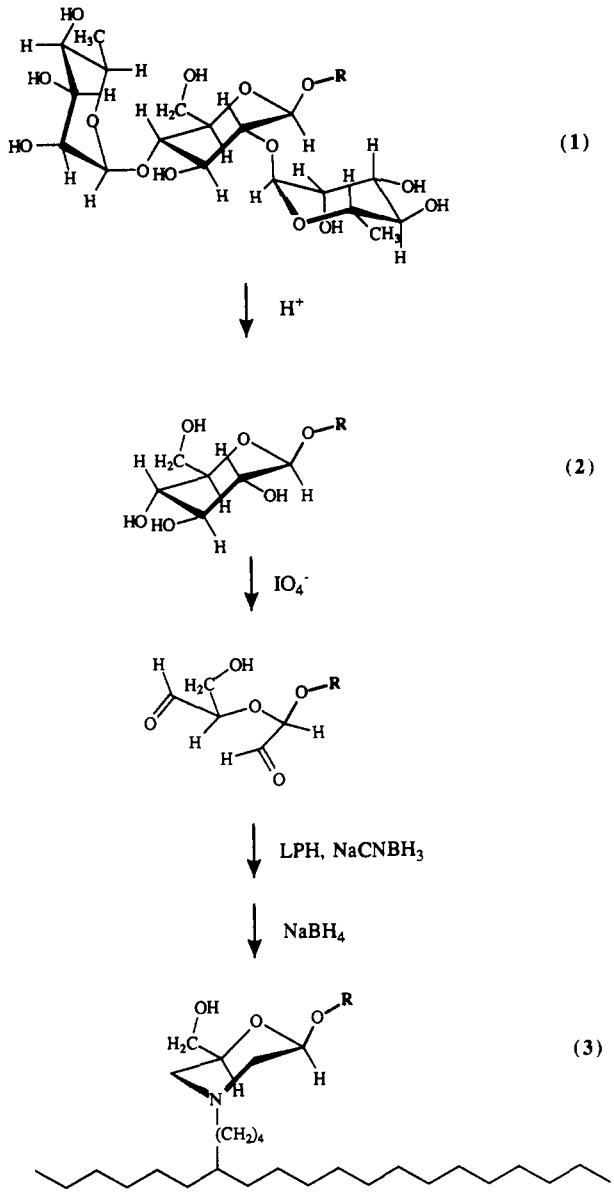
Conjugation of Hemisuccinate to Protein. Solanidine hemisuccinate (4.1 mg, 8.2 mM) was stirred overnight at 4 °C with *N*-hydroxysuccinimide (30.8 mg, 268 mM) and *N,N'*-dicyclohexylcarbodiimide (30.1 mg, 146 mM) in 1 mL of anhydrous dimethylformamide to form an active ester (Hassan et al., 1988). BSA (215.7 mg) was dissolved in 2 mL of PBS. The active ester reaction mixture was filtered through glass wool into the BSA solution. This reaction mixture was stirred 24 h at 4 °C. The entire reaction mixture was then dialyzed with 1 L of 8 M urea for 24 h, 4 L of 50 mM ammonium carbonate for 24 h, and, finally, 4 L of 25 mM ammonium carbonate for 24 h. All dialyses were performed at room temperature. The contents of the dialysis tubing were lyophilized and weighed. The dry product weighed 203.1 mg. To obtain only soluble conjugate, product (122.8 mg) was purified further by resolubilizing in approximately 20 mL of 50 mM ammonium carbonate and centrifuged until the supernatant was clear, and the supernatant was then dialyzed against 4 L of 25 mM ammonium carbonate for 18 h and lyophilized. The purified product weighed 30.2 mg.

Measurement of Solanidine in Conjugate. The percentage solanidine incorporated into the protein was measured using elemental analysis by comparing the percentage of nitrogen in the product to that found in pure BSA. Anal. Found for pure BSA: N, 15.57. Anal. Found for ester-linked BSA conjugate: N, 14.82. Therefore, this ester-linked conjugate was found to contain approximately 9 molecules of alkaloid per BSA.

GA Extraction from Potato Samples. GA were extracted from 0.2 (for potato plant material) and 1.0 g (for potato tuber) of freeze-dried powder using 3 × 15 mL methanol and homogenized after each addition for 1 min. An additional 5 mL of methanol was used to rinse the homogenizer probe, and the supernatants were pooled. Triplicate potato crude extracts were stored at −15 °C until required for analysis.

High-Performance Liquid Chromatography. Crude extracts were purified using Sep-Pak cartridges (Saito et al., 1990). Sep-Paks were conditioned with 10 mL of methanol and then 10 mL of water. Crude extract (5 mL) was mixed with 8 mL of water and added to the cartridge, washed with 5 mL of 40% aqueous methanol, eluted with 15 mL of methanol, rotoevaporated at 30 °C, and taken up in 1 mL of methanol. Samples with high GA concentrations [i.e., >20 mg/100 g, fresh weight basis (fwb)] were applied to the Sep-Paks as 1/5 dilutions to reduce the quantities of contaminating substances which otherwise overloaded the Sep-Paks. Purified sample solutions were injected through a 20-μL loop for analysis on a 300 × 3.9 mm μBondapak NH_2 column (Phenomenex, Torrance, CA), operated at 25 °C. The mobile phase was acetonitrile/20 mM potassium dihydrogen phosphate (75:25 v/v) pumped by a Beckmann Model 110A/332 pump (Beckmann Instruments Inc., Fullerton, CA) at a flow rate of 1.0 mL/min. The pressure was approximately 1000 psi. Detection was achieved using a Bio-Rad UV monitor, Model 1305 (Bio-Rad Laboratories Ltd., Mississauga, ON) at 208 nm. The output was monitored on a Hewlett-Packard 3388A integrator

Scheme I. Preparation of Immunogen (See Scheme II for Definition of R Group)

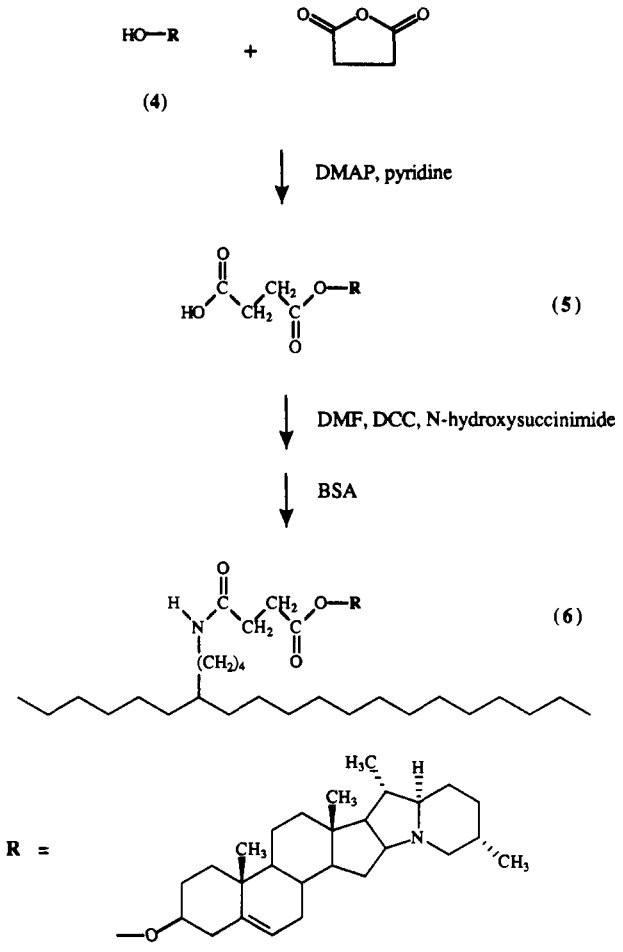


(Hewlett-Packard, Avondale, PA). Sample peak heights were compared to a linear standard curve that was constructed using standards at concentrations from 0 to 100 μM. Samples were analyzed at least in triplicate.

Enzyme Immunoassay. Ab titer was determined using the following checkerboard EI: Solid-phase BSA conjugate was dissolved at a concentration of 10 μg/mL in PBS; 1/5 dilutions were made with PBS so that the final dilution was 0.00064 μg/mL. Each row of a microtiter plate was filled with 200 μL of one of the above solutions and the final row with 200 μL of PBS. The plates prepared in this manner were stored for 18 h at 4 °C with humidity (placed in a plastic bag containing a damp paper towel and closed). The next day the solution was removed from the plate with a sharp shake of the wrist and each well coated with 200 μL of 1% BSA in PBS. This solution was left for 1 h at room temperature and then removed as before. The wells were next washed with 3 × 200 μL of PBST for 5 min at 25 °C each wash.

Rabbit serum was diluted 1/500 with 0.05% BSA in PBST. Serial dilutions (1/5) to 1/12 500 000 and a blank of 0.05% BSA in PBST were prepared and set aside. Aliquots (100 μL) of methanol were then added to each well of the coated microtiter plates, immediately followed by 100-μL aliquots of the diluted serum such that each column of the microtiter plate contained a different serum dilution. The plate was incubated for 2 h, with shaking, at room temperature with humidity. Wells were then emptied and washed as before with 3 × 200 μL of PBST. Goat

Scheme II. Preparation of Solid-Phase Conjugate



anti-rabbit Ab-peroxidase conjugate was diluted 1/3000, as required by the manufacturer, and added to each well in 200-μL quantities. This was incubated for 2 h at room temperature with shaking and humidity and washed as before. A solution of peroxidase substrate, o-phenylenediamine (0.4 mg/mL), and urea peroxide (1 mg/mL) in 0.1 M citrate buffer (pH 4.75) was added to each well (200 μL) and the absorbance ($A_{405nm} - A_{650nm}$) measured after 30 min at room temperature (Sheth and Sporns, 1990). The titer was defined as the minimum serum dilution that gave an absorbance reading that was 3 times greater than the background levels (no serum).

For the determination of GA in samples, an indirect competitive EI was performed using microtiter plates coated with solanidine–BSA conjugate in PBS and blocked, as described above. All incubations were also performed as above; however, during the incubation with diluted serum (1/100 000), the 100 μL of methanol was replaced with 100 μL of methanol containing GA (methanol-diluted potato extracts or standard solutions). Standards and samples were always analyzed together on the same plate, using replicates within each plate and replicate plates. Each plate included both sera blanks and sample blanks, where sera blanks used 1/100 000 dilution of preimmunization sera and 100 μL of methanol, and sample blanks contained immune serum but no GA. Results are expressed as

$$B/B_0 = \frac{(A_{405nm} - A_{650nm})_{sample} - (A_{405nm} - A_{650nm})_{serum\ blank}}{(A_{405nm} - A_{650nm})_{sample\ blank} - (A_{405nm} - A_{650nm})_{serum\ blank}}$$

Inhibition curves using 10^{-6}–10^{2} μM GA in methanol were analyzed using SOFTmax, version 2.01 (Molecular Devices Corp., Menlo Park, CA), to determine the four parameters defining the sigmoidal curves and the sample GA determined from the equation of the sigmoidal curve. To compare the sensitivities of the Ab to various GA, I_{50} values were used; these are the concentration of GA (micromolar) required to reduce the absorbance by 50%, or when $B/B_0 = 0.500$. Analysis of HPLC eluate was performed by constructing inhibition curves of

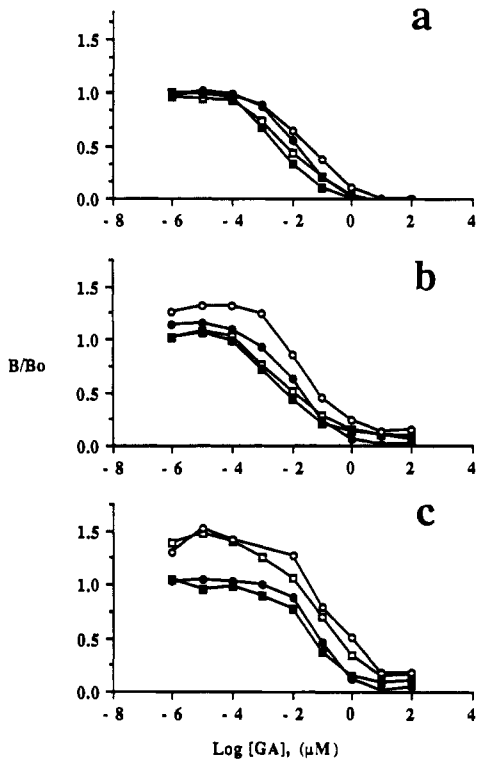


Figure 3. Competitive EI for α-chaconine (squares) and solanidine (circles) in solution using (a) 0.08 μg/mL BSA-H-9, (b) 2.0 μg/mL BSA-G-6, and (c) 0.40 μg/mL BSA-G-23. Serum was diluted 100 000 times. Solid or empty symbols represent the two rabbit sera used.

Table I. Comparison of I_{50} Values for Various GA

GA	I_{50}, μM GA	GA	I_{50}, μM GA
tomatidine	>100	α-solanine	0.10
solasodine	>100	α-chaconine	0.09
solanidine	0.16	demissidine	0.31

α-chaconine in acetonitrile/20 mM potassium dihydrogen phosphate (75:25 v/v).

Commerical Solanine ELISA Kit. Potato extracts were analyzed per the manufacturer's (Holland Biotechnology) directions. One kit (containing 12 strips of 8 wells each) allowed us to analyze 96 samples, including standards. The number of replicate determinations for each potato sample was therefore limited to three.

RESULTS AND DISCUSSION

The molecular weight of GA (<1000) is not enough to elicit a significant immune response (Erlanger, 1980); therefore, the first requirement for the preparation of an EI for GA was to chemically bond the alkaloid to proteins. The periodate cleavage and reductive condensation method used by Morgan et al. (1983), adapted from Butler and Chen (1967), was attempted but rejected. Reproducing their methodology led to extensive gel formation. This likely occurred due to protein cross-linking by the two oxidized sugars on solanine. Also, we believe that excess periodate used in this procedure would produce considerable formaldehyde, which would then compete with the sugar dialdehydes for the amino groups of the protein. Morgan et al. (1983) did report very low incorporation of alkaloid into their conjugate, with alkaloid to protein ratios on a mole basis of 3 to 1, when determined using acid hydrolysis and gas–liquid chromatographic determination of liberated solanidine. Conjugates having 15–25 groups per BSA equivalent are considered optimal for obtaining a good immune response (Erlanger, 1980).

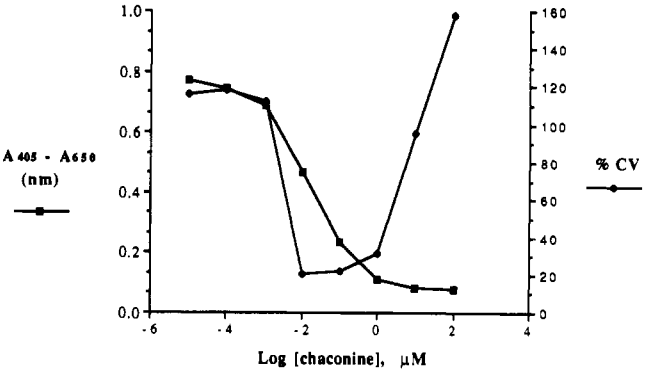


Figure 4. Inhibition curve for α-chaconine in solution and the coefficient of variation associated with each average point.

Table II. Comparison of [GA] Obtained by Three Methods

sample[a]	[GA],[b] mg/100 g, fwb		
	UofA EI	HB ELISA	HPLC
a	1.2 (0.56)	0.67 (0.26)	0.97 (1.7)
b	4.7 (1.4)	2.7 (0.23)	2.7 (0.64)
c	8.0 (1.9)	7.1 (1.9)	6.0 (0.85)
d	8.0 (1.7)	11 (3.2)	5.4 (0.25)
e	9.9 (3.3)	12 (1.8)	8.3 (0.86)
f	56 (10)	55 (3.2)	57 (7.2)
g	123 (13)	148 (31)	9.7 (7.7)

[a] See Materials and Reagents. [b] Assumed 20% moisture for all samples. Values in parentheses are standard deviations.

Our approach was to start with α-chaconine (1) rather than α-solanine (Scheme I). α-Chaconine contains two identical α-L-rhamnose sugar units, which are considerably more acid labile than the β-D-glucose sugar (Capon, 1969). A selective hydrolysis of this molecule and purification by column chromatography yielded γ-chaconine (2), confirmed by TLC and NMR. Using periodate oxidation of this molecule and selective reductive condensation (using sodium cyanoborohydride at pH 9) with proteins (Lane, 1975; Schwartz and Gray, 1977), a large amount of alkaloid could be covalently bound to the protein through a glycoside (3). BSA and LPH conjugates containing up to 23 and 14 molecules alkaloid per BSA equivalent, respectively, were obtained.

To use Ab effectively in competitive EI, the Ab should have an affinity for the solid-phase GA similar to its affinity for the soluble GA originating from the sample. Bridge heterology (the use of two different conjugation methods for the immunogen and the solid-phase conjugate) can improve the balance of Ab affinity for the solid-phase and soluble analyte (Harrison et al., 1991; Sheth and Sporns, 1991; Gosling, 1990; Wie and Hammock, 1984; Vallejo et al., 1982). In this study, the GA used for immunization was linked to LPH through a glycosidic bond. To test whether using a different linking arm for the solid-phase conjugate had an effect on the EI, this conjugate was produced either through a glycosidic bond or through an ester linkage, using BSA as protein. Ester-linked conjugates (Scheme II) were prepared by producing solanidine hemisuccinate (5) from solanidine (4) and through an active ester condensation, linking to BSA.

The titers of three solid-phase BSA conjugates were compared in checkerboard EI: solid-phase BSA-H-9 (ester-linked conjugate containing 9 GA per BSA), solid-phase BSA-G-6 (glycosidic-linked conjugate containing 6 GA per BSA), and BSA-G-23 (glycosidic-linked conjugate containing 23 GA per BSA). Checkerboard assays revealed that titers were of greater magnitude when the ester-linking arm was used for solid phase (approximately 1/10 000 000 serum dilution) as compared to when the glycosidic-linking

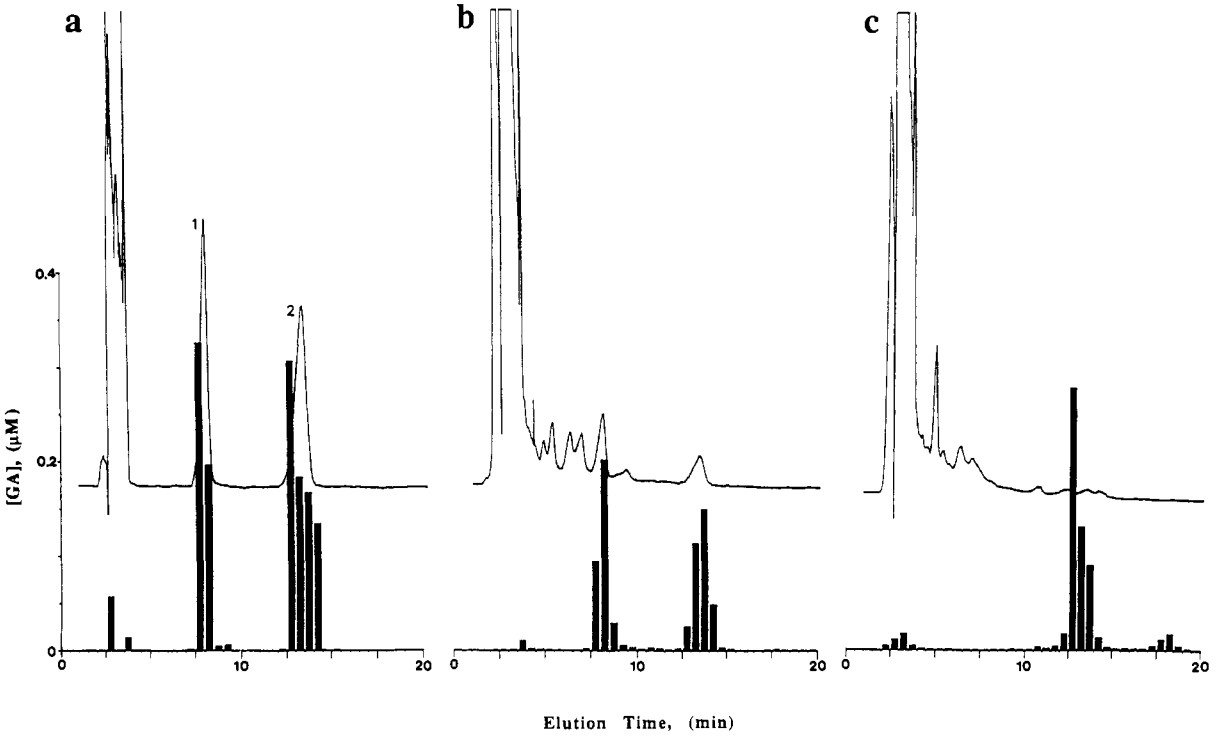


Figure 5. HPLC chromatograms of (a) a standard solution containing 100 μM each of solanidine (eluting at solvent front), α-chaconine (peak 1), and α-solanine (peak 2); (b) commercial potato tuber sample b; and (c) *S. demissum* leaf extract. Superimposed bar graph illustrates EI detection of aliquots collected.

arm was used (approximately 1/2 000 000). Since the Ab was raised against a conjugate containing a glycosidic linking arm, LPH-G-14, one might expect the Ab to have a greater affinity for the same type of solid phase. Surprisingly, the Ab showed a greater affinity for the alkaloid linked in a different way from the immunization conjugate, regardless of the ratio of GA to BSA. One explanation may be that the hemisuccinyl group contains a flexible methylene chain, allowing favorable orientation of the GA within the Ab binding site. Another reason may relate to differences in the ability of the conjugates to bind to the microtiter plates.

Figure 3 compares the inhibition curves when the three solid-phase conjugates were used in competitive EI with α-chaconine or solanidine. Conjugates were applied to the microtiter plates at a concentration determined by the checkerboard to give a value of B_0 of approximately 1.0 when the serum dilution was 1/100 000. This was achieved when plate coating was performed using 0.08, 2.0, and 0.40 μg/mL for BSA-H-9, BSA-G-6, and BSA-G-23, respectively. The background absorbance was greater when the solid-phase conjugate contained the same linking arm as the immunogen, shown by the higher B/B_0 values at high concentrations of competing GA when BSA-G-6 and BSA-G-23 were used. This indicates that some of the Ab has affinity for the linking arm. Using a different linking arm during assay therefore allows the Ab to be more sensitive to soluble GA (containing glycosidic groups) in the analyte. The ester-linked conjugate was found to be easier to make and more consistent between rabbit sera, and use of this coating gave less variation due to the GA used in the assay. Also, high absorbances (higher than B_0) at very low concentrations seemed to be a problem for the glycosidic-linked conjugate. For these reasons, further experimentation was performed using the ester-linked conjugate, BSA-H-9.

Ab cross-reactivity data are shown in Table I. Four-parameter sigmoidal equations were computed and I_{50} values calculated. The Ab showed very little affinity for the two spiral alkaloids tested, tomatidine and solasodine, determined by very high I_{50} values. This is not unusual, given the difference in chemical structure from solanidine, especially in the portion of the molecule most distant from the linking site (Figure 1). The solanidine alkaloids, α-solanine, α-chaconine, and solanidine, showed similar ability to compete (I_{50} values were not significantly different, $P > 0.10$). This group of alkaloids, particularly α-solanine and α-chaconine, make up the majority of GA in commercially grown potatoes. These two GA differ only by their sugar moieties. The antibody showed a slightly lower affinity for demissidine ($P < 0.10$). This alkaloid is saturated at C5 but this bond is somewhat distant to the expected Ab binding site.

EI analysis of standard solutions was performed repeatedly, and the sigmoidal calibration curves were calculated. The coefficient of variation (CV) in EI is very high for extremely low and extremely high values of concentration (Bunch et al., 1990); to determine which part of the curve has the lowest error, predicted concentration values of the standard solutions were calculated. Figure 4 was constructed by determining the CV for predicted values and plotting against concentration of the standard. This revealed that the EI was most accurate (10–20% CV) between 0.01 and 1 μM GA. All further determinations were therefore made using sample dilutions that gave at least two results in this range. Depending on the sample, total sample dilution ranged from 1/100 to 1/25 000.

Table II compares the results when potato samples were analyzed by our EI (UofA EI), HPLC, and the Holland Biotechnology (HB ELISA) EI. Correlation among the immunological methods was good ($r = 0.971$). Correlation among all methods when only commercial potato tubers were used was also good ($r = 0.978$–0.988). The leaf extract from *S. demissum* was the only sample analyzed that gave significantly less GA when analyzed by HPLC when compared to the results obtained by immunological methods. This species is known to contain glycosylated

demissidine alkaloid (Schreiber, 1968). HPLC, using 208 nm for detection, would not measure GA having saturation at C5. Eluate fractions were collected and analyzed by EI. This revealed the presence of two non-UV-absorbing GA at 13 and 18 min (Figure 5). Although the identities of these two compounds were not confirmed, *S. demissum* has been reported to contain demissine or commersonine, demissidine substituted with lycotetraose and commertetraose, respectively (Schreiber, 1968; van Gelder, 1991).

Although this test can be used to quantitate GA common in commercial cultivars of potatoes, the current trend in breeding practices will require that other, less common, GA also be measured. Greater use of "wild" species of potatoes in breeding may mean that other (e.g., spiral and leptine) alkaloids will become more prevalent in commercial potatoes (Gregory, 1984; van Gelder and Scheffer, 1991). There is no convenient method available at this time to screen for all types of alkaloids simultaneously. Current research efforts in this laboratory are therefore aimed at acquiring additional Ab (both polyclonal and monoclonal) preparations for analysis of these groups of potato alkaloids.

ABBREVIATIONS USED

BSA, bovine serum albumin; EI, enzyme immunoassay; fwb, fresh weight basis; GA, glycoalkaloid; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; LPH, *L. polyphemus* hemocyanin; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween 20; PBS-t, phosphate-buffered saline without thimerosol; TLC, thin-layer chromatography.

ACKNOWLEDGMENT

This work was supported by a Natural Science and Engineering Research Council (NSERC) of Canada grant. Preliminary studies were supported by the Central Research Fund of the University of Alberta. The advice and assistance of a number of employees from ChemBiomed Ltd., Edmonton, Canada, in the initial stages of this project are also gratefully acknowledged.

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Received for review June 17, 1992. Accepted September 10, 1992.

Registry No. Solanidine, 80-78-4; α-solanine, 20562-02-1; α-chaconine, 20562-03-2; demissidine, 474-08-8; γ-chaconine, 511-36-4; solanidine hemisuccinate, 71143-15-2.