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Enzyme Immunoassays for Glycoalkaloids in Potatoes

by

Leslie C. Plhak ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **Enzyme Immunoassays for Glycoalkaloids in Potatoes** submitted by **Leslie C. Plhak** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Food Chemistry.**

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Abstract

New methods for the synthesis of glycoalkaloid 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 moles of alkaloid per mole of bovine albumin) than earlier methods. *Limulus polyphemus* hemocyanin (LPH)-solanidine conjugates were used to immunize rabbits and produced high sera titers ($>10^6$; 10^5 serum dilution was used for assays). Bovine serum albumin coating conjugates were used in competitive enzyme immunoassays (EI) using polyclonal antibody (PAb)-containing serum 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 because demissidine glycoalkaloids from *Solanum demissum* were also quantified. Using the same immunogen, mice were immunized for the production of monoclonal antibodies (MAb). Approximately 5000 hybridoma resulted with at least 445 producing Ab specific for solanidine alkaloids. Eight cell lines were selected and two were cloned using soft-agar. Of these, one line was carried further because of its greater ability to produce high affinity antibody (Ab). Isotyping revealed that the MAb produced by this cell line was murine IgG1. These cells were grown in serum-free media and could produce MAb in stationary tissue culture at a rate of 50-83 μg/ml/day. MAb production was not found to be related to cell growth rate. Purification of supernatants after growth of hybridoma in serum-free media was accomplished using ammonium sulfate precipitation (0-50% saturation). This yielded a highly purified MAb preparation, verified by SDS-PAGE. The MAb was used in an optimized immunoassay at a concentration og 200 ng/mL with only 10 ng/mL required for detectable signals (titer). From half absorbance levels (I_{50} values) the MAb detected α-solanine the best (0.021 μM) while α-chaconine, demissidine and solanidine were detected at levels of 0.043, 0.092 and 0.103 μM, respectively.

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List of Abbreviations

A/A_o	absorbance measurement uncorrected for preimmune serum (see section 2.3.6)
Ab	antibody
Ag	antigen
B/B_o	absorbance measurement corrected for preimmune serum (see section 2.3.6)
BSA	Bovine serum albumin (see appendix I for conjugates)
CV	coefficient of variation
EI	enzyme immunoassay
FC	FetalClone serum product from HyClone, Logan, Utah.
GA	glycoalkaloid
fwb	fresh weight basis
GC	gas chromatography
HB ELISA	commercial enzyme immunoassay from Holland Biotechnology bv, Leiden, The Netherlands
HPLC	high performance liquid chromatography
I_{50}	concentration of analyte giving 50% reduction in absorbance
Ig	immunoglobulin (can be IgG, IgM, etc.)
IR	infrared spectroscopy
LPH	*Limulus polyphemus* hemocyanin (see appendix I for conjugates)
MAb	monoclonal antibody
MW	molecular weight
NMR	nuclear magnetic resonance
PAb	polyclonal antibody
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PBS-t	phosphate-buffered saline without thimerosol
PEG	polyethylene glycol
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	thin layer chromatography
UofA EI	competitive indirect enzyme immunoassay using PAb developed during this study and 0.2 µg/mL BSA-H-9 solid-phase with 1/100,000 dilution of serum
uv	ultraviolet

Chapter 1. Introduction and Review of Literature

1.1 Overview and History of the Potato

The potato originated in the Andean Cordillera of what is now Peru and Bolivia. It is believed that it has been used as a food source for more than 13 thousand years and cultivated for 3 to 5 thousand years (Ugent, 1970; Hawkes et al., 1979; Rhoades and Rogers, 1982; Fenwick, 1986). It has been suggested that the cultivation of the potato facilitated settlement of the Andes and thereby gave rise to the great Inca civilization (Salaman, 1949). The important role of the potato during this era is evidenced by archaeological finds which show that potatoes were worshipped, placed in tombs with the dead, and depicted in the form of pottery (Salaman, 1949; Ugent, 1970; Rhoades and Rogers, 1982).

The Europeans, after immigrating to the Americas 500 years ago, introduced many New World foods to Europe, including potatoes. For 50-100 years after its introduction, the potato in Europe was mainly used as an ornamental plant in botanical gardens. There are several possible reasons why it took this long for people to accept it as a food. Firstly, people associated the potato with Old World members of the Solanaceae family, including the deadly nightshade, belladonna, henbane and mandrake. These plants were known, primarily by herbalists, for their narcotic and hallucinogenic effects. In addition, the potato was considered evil because the edible portion of the plant grows underground and it is not mentioned in the Bible (Rhoades and Rogers, 1982). It was reputed to have "unwholesome" aphrodisiac effects and was believed to cause leprosy, dysentery, syphilis, tuberculosis, rickets and leishmaniasis (Salaman, 1949; Rhoades and Rogers, 1982; Niederhauser, 1992).

During the 18th century, cultivation of the potato plant for use as a food, spread throughout Europe and Russia. Some believe that the potato was the fuel for the Industrial Revolution in Europe (Niederhauser, 1992). The Potato War, *Kartoffelkrieg*, was fought by Prussians and Austrians in 1778-79. The name originated after the contending armies ate all the

potatoes along the battle lines in Bohemia and then called off the fighting (Rhoades and Rogers, 1982).

In 1845 and 1846 *Phytophthora infestans* (or late blight) caused stored tubers to rot. While these disasters struck most of Europe, it was the Irish that suffered the greatest consequences. The result, over 1 million deaths and 1.5 million emigrants, is what became known as the Irish Potato Famine. The sociological and political impacts of these events are well documented by Salaman (1949).

Primarily through European colonialism, the potato began to reach the rest of the world. During the past 50 to 100 years its cultivation has spread to much of the developing world. In 1982, the potato was produced by 130 of the world's 167 independent countries (Rhoades and Rogers, 1982). Based on yield, the potato ranks 4th in the world, after wheat, corn and rice. It is valued at over $100 billion (US) annually, a value greater than all of the gold and silver taken by the Spanish during their 300 years of rule in the Americas (Rhoades and Rogers, 1982; Niederhauser, 1992).

On a dry-weight basis, the protein content of the potato approaches 10%, comparable to wheat and higher than most rice and corn varieties. If multiple harvesting is taken into account, the potato yields more protein and energy per land area than any other crop. It has relatively good protein quality, having substantial levels of lysine while only moderately limiting in the sulfur-containing amino acids (Woolfe, 1987), and it is a good source of ascorbic acid, thiamin, iron, magnesium and phosphorus (Jadhav and Salunkhe, 1975).

With its near perfect composition, an ability to grow in most climates and an important role in the world's food supply (Rhoades and Rogers, 1982; Rhoades and Johnson, 1991), the potato has become the subject of experiments in "designer foods". Genetic engineering has produced potatoes that produce higher quality proteins (with greater proportions of limiting-amino acids) and bactericidal proteins for improved resistance to insects, bacterial wilt and fungal disease (Vayada and Park, 1980; Daviss, 1990). Potatoes have also been fused with tomatoes to produce fruit and tubers on the same plant (Rhoades and Rogers, 1982).

While the potato is an extemely valuable food source, the potato plant, including its tubers, contains naturally-occurring toxic compounds. One class of toxic constituents found in *Solanum* sp. is the glycoalkaloids (GA).

1.2 Potato Glycoalkaloids

The first GA was discovered and named solanine by Defosses in 1820. When first reported, Baup (1826) predicted that this compound would "find a use in medicine". More than a century later, GA have been the focus of much research activity. In addition to the toxic effects of GA, they are important as starting materials for the production of steroidal pregnane hormones (Bradley et al., 1979). GA have also been reported to be effective in the treatment of skin cancers (Cham et al., 1987; Cham and Meares, 1987), to have antihepatotoxic effects (Lin and Tome, 1988), immunomodulating properties (Bähr and Hänsel, 1982), hyperglycemic effects (Satoh, 1967) and cardiotonic activity (Nishie et al., 1976).

GA are nitrogen-containing steroidal compounds. As plant sterols, they are considered to be important in regulation of membrane fluidity and integrity and also in natural defense. GA can also be considered as saponins. Saponins are triterpenoid or steroidal glycosides of plant origin that have the ability to form a soapy lather when agitated with water. Some saponins also have hemolytic activity, cholesterol-binding properties, bitterness and toxicity (Price et al.,1987). GA are phytoalexins. Phytoalexins are low molecular-weight, antimicrobial compounds that are synthesized by, and accumulate in plants after exposure to microorganisms (Jadhav et al., 1991). Finally, GA are allelochemicals, secondary metabolites produced by one species and able to affect the growth or behavior of another species (Rosenthal, 1986).

Comprehensive reviews of GA have been published by Jadhav and Salunkhe (1975), Jadhav, Sharma and Salunkhe (1981), Kuc (1975), Maga (1980), Osman (1980 and 1983), Roddick (1974), Sharma and Salunkhe (1989) and Van Gelder (1991).

1.2.1 Structure and Biosynthesis

Solanum sp. GA contain a C_{27} steroidal alkaloidal aglycone, and a carbohydrate moiety. Their chemistry, including composition, structural determination, reactions and physical constants has been reviewed by Prelog and Jeger (1953), Schreiber (1968) and Ripperger and Schreiber (1981).

Aglycones vary in their basic skeletal structure (e.g. solanidane vs. spirosolane systems), in their saturation at C_5, or their isomerization at C_{22}. In 1981, 75 different aglycones having the C_{27} cholestane skeleton were described in *Solanum* (Ripperger and Schreiber, 1981). Only about 7, however, are of significant importance in the tuber-bearing species (Van Gelder, 1991). These are illustrated in Figures 1 and 2.

GA are biosynthesized from acetyl-CoA, *via* several intermediates including mevalonate, farnesyl pyrophosphate, squalene and cholesterol (Schreiber, 1968; Goodwin, 1980; Sharma and Salunkhe, 1989; Heftmann, 1983). It is relatively certain that all steroids, in both animals and plants, are synthesized through the same biochemical pathways to cholesterol (Schreiber, 1968). Conversion of cholesterol to a N-containing steroid, however, is less understood. Heftmann (1967) proposed that cyclization of the cholesterol side-chain may be occurring prior to the formation of 27-hydroxycholesterol, followed by replacement of the hydroxy group with an amino group. L-arginine has been postulated as the source of the amino group (Kaneko et al., 1976).

The carbohydrate moieties of GA are mono- or disaccharides, or branched tri- and tetrasaccharides, usually consisting of D-glucose, D-galactose, L- rhamnose, or D-xylose units (Table I). The more common GA sugars include β-solatriose, β-chacotriose, β-commertetraose and β-lycotetraose (Figure 3). β-Solatriose possesses the structure O-α-L-rhamnopyranosyl-(1→2_{gal})-O-β-D-glucopyranosyl-(1→3_{gal})-β-D-galactopyranose. β-Chacotriose has the structure O-α-L-rhamnopyranosyl-(1→2_{glu})-O-α-L-rhamnopyranosyl-(1→4_{glu})-β-D-glucopyranose.

Figure 1. ***Solanum*** **sp. alkaloids having a solanidane skeleton.** a) solanidine, b) demissidine, c) leptinidine, d) acetylleptinidine.

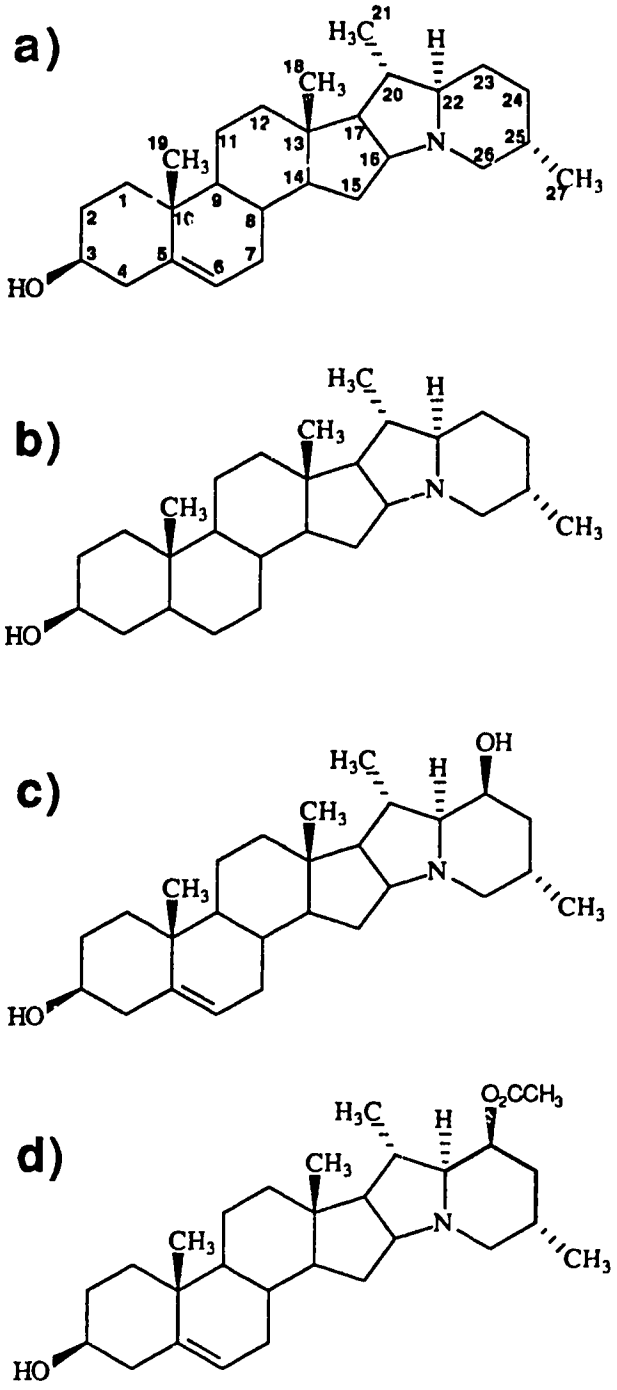


Figure 2. *Solanum* sp. alkaloids having a spirasolane skeleton. a) tomatidenol, b) tomatidine, c) solasodine.

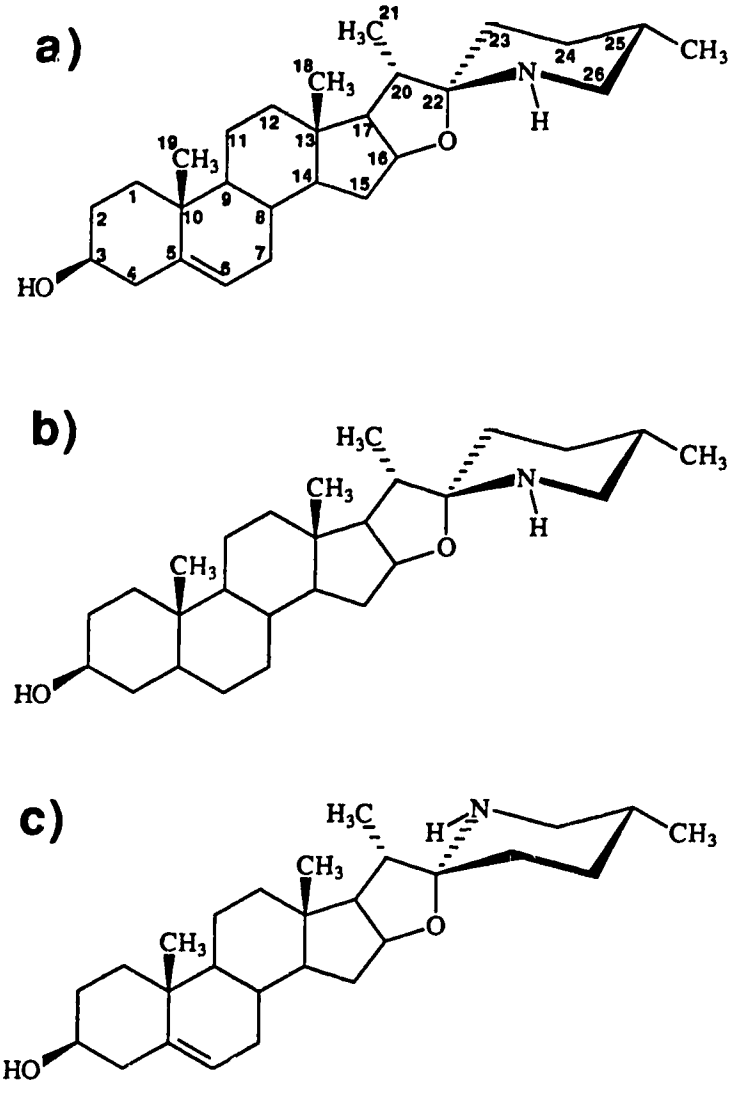
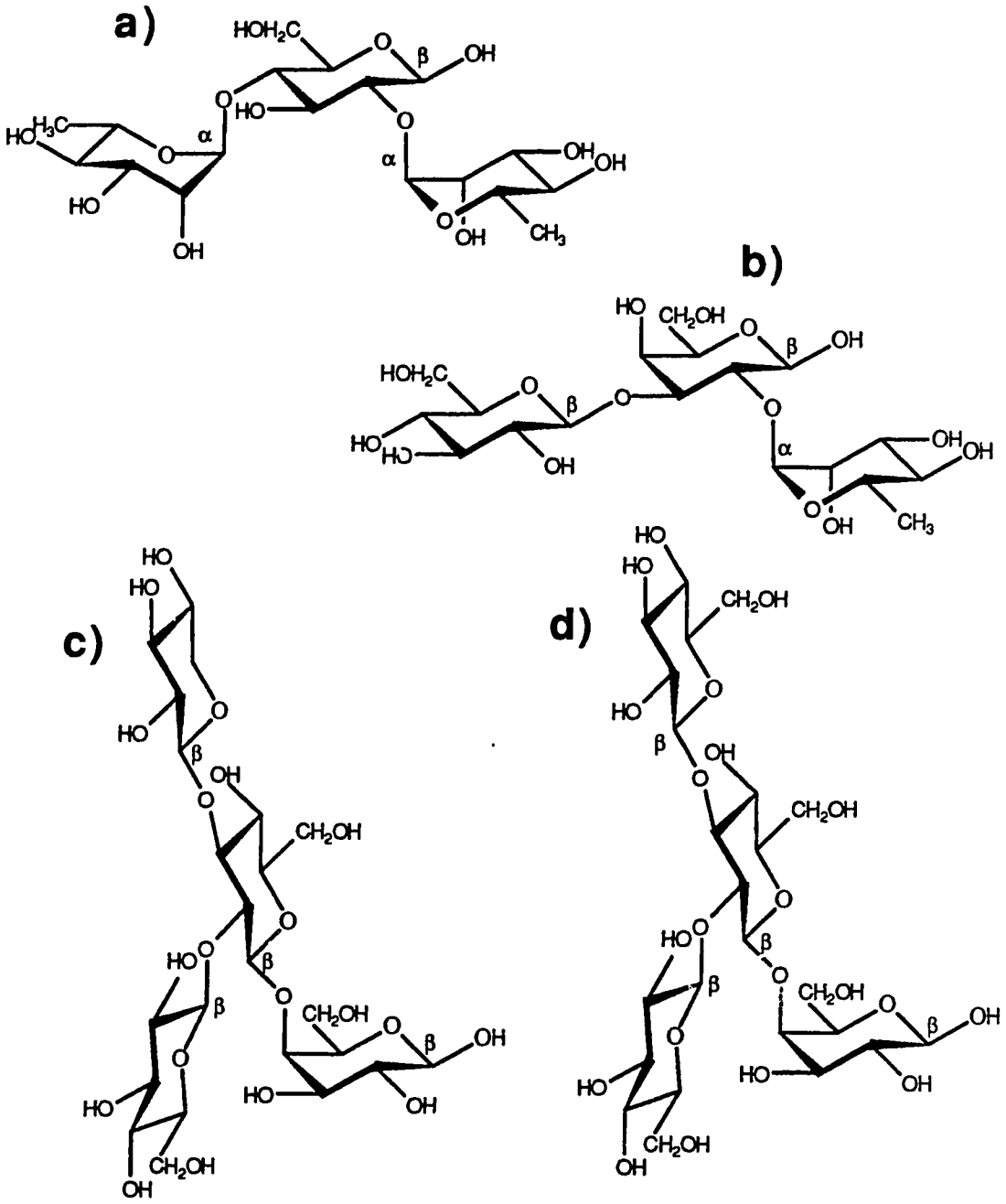


Table I. Common GA and their composition.

Glycoalkaloid	Alkaloid	Glycoside	Sugars
α-chaconine	solanidine	β-chacotriose	D-glucose, 2 L-rhamnose
α-solanine	solanidine	β-solatriose	D-galactose, D-glucose, L-rhamnose
dehydrocommersonine	solanidine	β-commertetraose	D-galactose, 3 D-glucose
demissine	demissidine	β-lycotetraose	D-galactose, 2 D-glucose, D-xylose
commersonine	demissidine	β-commertetraose	D-galactose, 3 D-glucose
leptinine I	leptinidine	β-chacotriose	D-glucose, 2 L-rhamnose
leptinine II	leptinidine	β-solatriose	D-galactose, D-glucose, L-rhamnose
leptine I	acetylleptinidine	β-chacotriose	D-glucose, 2 L-rhamnose
leptine II	acetylleptinidine	β-solatriose	D-galactose, D-glucose, L-rhamnose
β-solamarine	tomatidenol	β-chacotriose	D-glucose, 2 L-rhamnose
α-solamarine	tomatidenol	β-solatriose	D-galactose, D-glucose, L-rhamnose
α-solamargine	solasodine	β-chacotriose	D-glucose, 2 L-rhamnose
α-solasonine	solasodine	β-solatriose	D-galactose, D-glucose, L-rhamnose
α-tomatine	tomatidine	β-lycotetraose	D-galactose, 2 D-glucose, D-xylose

Figure 3. Common carbohydrate moieties for GA. a) β-chacotriose, b) β-solatriose, c) β-lycotetraose, d) β-commertetraose.



Glycosylation has been demonstrated to occur enzymatically, in a stepwise manner (Jadhav and Salunkhe, 1973; Lavintman et al., 1977). Solanidine glycosyltransferase has been isolated and characterized by Stapleton et al. (1991).

Different aglycone-carbohydrate combinations give rise to a diverse group of GA. Table I illustrates the most common GA and their composition. Two of the more common GA in commercially cultivated potatoes (*S. tuberosum*) are α-chaconine and α-solanine. These both have the same aglycone, solanidine, but differ by their sugar substitutions and usually exist in a 60:40 ratio, respectively (Maga, 1980). Hydrolytic enzymes are present in potato tissue that can remove sugars by a stepwise mechanism (Jadhav et al., 1981).

GA synthesis or accumulation seems to be associated with high metabolic activity within the plant. GA levels are highest in the unripe fruits, flowers, sprouts, and leaves and lowest in the stems, roots and tubers (Wood and Young, 1974; Van Gelder, 1991). The distribution of GA that generally occurs within the potato plant and various tuber tissues is summarized in Table II. Commercial cultivars typically contain 2 to 15 mg GA/100 g, fresh weight basis (fwb), for unpeeled tubers (Slanina, 1990). The safety question arises when certain conditions allow or even stimulate GA synthesis and accumulation.

1.2.2 Factors Affecting Accumulation

Several factors are related to GA accumulation in tubers: genetic factors, environmental conditions during growth and development, storage temperature and time, exposure to light, disease or insect damage, or mechanical stress (Ross et al., 1978; Sinden et al., 1984; Olsson, 1986).

1.2.2.1 Genetic variation

GA have been demonstrated in over 300 species of the botanical families Solanaceae, Apocynaceae, Buxaceae, and Liliaceae (Schreiber, 1979; Hardman, 1987; Gaffield, et al., 1991). Within the Solanaceae, the *Solanum* (potato) and *Lycopersicon* (tomato) genera are of the greatest

Table II. Concentration of GA in various tissues of the potato plant (*S. tuberosum*). From Wood and Young (1974), Slanina (1990) and Van Gelder (1991).

Plant Tissue	[GA], mg/100 g, fwb
Flowers	215 - 500
Sprouts	195 - 1770
Leaves	40 - 100
Fruits	42
Roots	18 - 40
Stems	3
Whole Tuber	2 - 15
Tuber Skin (2 - 3% of tuber)	30 - 64
Tuber Peel (10 - 15% of tuber)	15 - 30
Peel and Eye (3 mm disk)	30 - 50
Tuber Flesh	1.2 - 6

importance. *Solanum* includes more than 200 species, of which about 160 are tuber-bearing. In Peru, eight species are cultivated. Only one, *S. tuberosum*, is grown in Canada and the U.S.A. (Rhoades and Rogers, 1982). There is some evidence (Johns and Alonso, 1990) that during the domestication of *S. tuberosum*, selection for reduced toxicity occurred.

Wild or so-called "bitter" varieties of *Solanum* are often employed in breeding programs to gain better yield, hardiness, disease resistance, solids content or chipping quality (Sinden et al., 1984; Sanford et al., 1990; Deahl et al., 1991; Osman et al., 1991; Flanders et al., 1992). Wild types generally contain much higher GA levels, up to 100 mg/100g , fwb, for unpeeled tubers (Osman et al., 1978). In the past, reduction or elimination of GA during breeding has not been a high priority because GA synthesis in the plant is under polygenic control (Sinden et al., 1984) and GA are important in pest and disease resistance in potatoes (Sanford et al., 1990; Deahl et al., 1991; Osman et al., 1991). In addition, some wild species contain different types of GA that are not quantitatively detected by the commonly used methods of analysis (Gregory et al., 1981; Coxon, 1984; Gregory, 1984) and therefore routine screening is not always included in breeding programs.

The problem of GA heritability can be illustrated by the cultivar Lenape, developed in the late 1960's (Akeley et al., 1968). *S. tuberosum* was bred with the wild variety *S. chacoense*, a genetic source of higher yields (due to its resistance to the Colorado potato beetle), higher solids content and chipping quality (Sinden et al., 1984). In 1970, Lenape was rapidly withdrawn, just prior to its release, when it was discovered to have 27 to 65 mg GA/100 g (Zitnak and Johnston, 1970; Sinden et al., 1984). The resistance of *S. chacoense* to the Colorado potato beetle is believed to be directly related to its high content of leptine GA (Sinden et al., 1986) and/or the presence of glandular trichomes (Collins, 1987; Flanders et al., 1992).

S. vernei is another wild species used in breeding programs for a source of resistance to potato cyst nematodes and its high solids content. Van Gelder and Scheffer (1991) demonstrated that solasodine GA were inherited in hybrids from this wild species crossed with *S. tuberosum*.

GA have also been implicated to be important in potato leafhopper resistance (Tingey, 1984). In a study by Sanford et al. (1990) selection for leafhopper resistance led to an increase in foliar GA. Leafhopper resistance is also associated with the presence of hairs and/or glandular trichomes on leaves (Flanders et al., 1992).

There is a great potential to use wild *Solanum* species as genetic sources for higher yields, hardiness, resistance or quality. In order to do so, however, breeders must be certain that offspring do not inherit the capacity to produce high levels of GA. In order to ensure a safe potato, therefore, a rapid, simple, reliable, inexpensive and comprehensive method of GA analysis is required for routine screening of breeding lines (Gregory, 1984).

1.2.2.2 Growth, development and storage of tubers

Location, climate, altitude, air pollution, soil type, soil moisture, the presence of fertilizer, infestation by pests (microbial or nonmicrobial), the application of pesticides, length of growing season, maturity of the tubers and tuber damage during growth, harvest or transportation all are believed to affect GA synthesis and accumulation (Maga, 1980; Sinden et al., 1984; Sharma and Salunkhe, 1989).

Physiological stress to the harvested tuber may cause significant increases in the GA content. Extremes in the storage temperature, exposure to light, mechanical damage and sprouting can all stimulate GA synthesis (Sharma and Salunkhe, 1989). Methods of GA control are therefore aimed at reducing exposure to stress conditions and slowing down tuber metabolism. Treatments that have been found to slow down GA formation during storage include certain types of packaging, the application of some sprout inhibitors (e.g. isopropyl-N-(3-chlorophenyl)-carbamate), exposure to γ-irradiation and the application of wax, detergents, surfactants or oils (Jadhav and Salunkhe, 1975; Maga, 1980; Jadhav et al., 1981; Mondy et al., 1992). Some of these treatments, however, have practical limitations because of safety considerations and marketing trends.

1.2.2.3 Effect of processing

The oldest known potato processing method is the production of chuño. This process is believed to be about 2000 years old (Salaman, 1949). In this process, still practiced in Peru, potatoes are left out and exposed to the high mountain elements for 2-3 weeks. During this time, the cool/dry/low pressure conditions allow freeze-drying to take place. Potatoes are then stepped on to remove their skins and the remainder is placed in a slow moving riverbed, with straw, and left for another 2 weeks to 2 months and then redried. This process reduces the bitterness and the resulting dry powder, which can be stored for 2 to 3 years, is used in soups, stews and sweet desserts (Rhoades and Rogers, 1982). While this method of processing causes a reduction in the nutrient content, it also reduces the GA content from about 30 mg/100 g in the fresh bitter potatoes to about 4 to 16 mg/100 g in chuño (Christiansen, 1977; Woolfe, 1987).

Large-scale processing of potatoes has become increasingly important in industrialized nations, especially because the appearance and increased consumption of fast foods and snack foods. The percentage of potatoes consumed in the US that were processed by frying, dehydration or canning increased from 2% in 1940 to 51% in 1970 (Woolfe, 1987). In 1980, Americans alone consumed 2 billion kg of french fries and 0.4 billion kg of potato chips (Rhoades and Rogers, 1982).

The production of pre-peeled and/or pre-cut potatoes is a growing industry for supply to the food service sector (Woolfe, 1987). Peeling may remove a significant amount of GA, by removal of the tissue where GA are most concentrated. At the same time, however, tissue damage may cause an increase in GA synthesis in the peeled product through a wound-response mechanism (Sharma and Salunkhe, 1989). The question of GA accumulation also arises in potato-processing plants if pre-cut potatoes are left in conditions of high light intensity and high temperature for a period of time before cooking (Jadhav et al., 1981; Van Gelder, 1991). The use of anoxic water treatment has been shown to be effective for inhibition of light-induced and wound-induced GA formation in potato tissue by inhibiting respiration (Salunkhe and Wu, 1979). Other treatments that are effective in slowing down GA formation during processing include certain types of packaging, exposure to γ-irradiation and the application of wax, detergents,

surfactants or oils (Jadhav and Salunkhe, 1975; Maga, 1980; Jadhav et al., 1981). As mentioned previously, some of these treatments may have practical limitations.

GA are not easily removed or destroyed by cooking due to their heat stability and poor solubility characteristics. Boiling, baking, microwaving, frying or dehydration of potatoes does not cause a reduction in the GA concentration (Bushway et al., 1980a; Bushway and Ponnampalam, 1981). Frying, due to the loss of water, can result in a three- to fourfold increase in the GA concentration (Sizer et al., 1980).

Potato tissue containing GA contents in excess of 14 to 22 mg/100 g have been rated as bitter by taste panelists (Sinden et al., 1976). GA contents over 22 mg/100 g were found to produce a mild to severe burning sensation in the mouth and throat (Sinden et al., 1976). These same sensations have also been correlated with the phenolic content (Mondy et al., 1971). There are differences in the bitterness of different GA and in the perception of bitterness between individuals (Zitnak, 1977). In addition, it has been suggested by some authors that bitterness in processed products can be masked by a high oil content or the addition of flavorings and salt. Taste, therefore, may not be a good indicator of GA content.

1.2.3 Toxicology

Toxicological reviews include those by Morris and Lee (1984), Dalvi and Bowie (1983), Keeler (1983 and 1986) and Slanina (1990).

Potatoes have been responsible for a number of documented episodes of GA poisoning - up to 30 deaths and over 2000 cases of non-fatal poisoning. It has been suggested, however, that this is an underestimation of the actual number of cases. The British Medical Journal (Anon., 1979) reported that many cases, both mild and severe, would be diagnosed as gastroenteritis (a term based on symptoms, rather than cause) and not be recorded as poisoning. If the facilities or expertise are not available to analyze for GA, or if there are no leftovers from a suspected meal, or no tubers of the same lot available for analysis, then confirmation is impossible.

In confirmed cases, where the GA content of the potatoes was reported and the amount of potatoes consumed was estimated, toxic or lethal doses could be calculated. Toxic dose refers to the mg of GA per kg body weight consumed by those who suffered toxic, not necessarily lethal, effects. The symptoms are both gastrointestinal and neurological in nature. They include vomiting, diarrhea (sometimes including blood), severe abdominal pain, drowsiness, apathy (in some cases this alternates with restlessness or shaking attacks), confusion, weakness, depression and in some cases unconsciousness (Willimott, 1933, Reepyah and Keem, 1958; McMillian and Thompson, 1979; Health and Welfare Canada, 1988).

In those cases where people died, and the information is available, a lethal dose can be calculated. This is the mg of GA per kg body weight consumed by those who suffered lethal effects. In these cases, the toxic symptoms, as mentioned before, were severe and death was attributed to strangulation of the bowel (Harris and Cockburn, 1918), respiratory failure (Hansen, 1925), and/or cardiac arrest (Willimott, 1933).

Morris and Lee (1984) calculated toxic and lethal doses based on available and estimated information involving approximately 1400 cases during 15 episodes. These ranged from 2 to 6 mg/kg body weight. From this, the authors concluded that the safety factor between the average GA level occurring in potatoes and a toxic dose is only about four-fold for a 500 g serving. Therefore, it should not be surprising that episodes of GA poisoning, some fatal, have occurred. It has been suggested that potato GA are one of the most serious toxic components in the human diet (International Food Biotechnology Council, 1990; Hall, 1992).

GA levels in potato tubers below 20 mg/100 g, fresh-weight basis (fwb) have often been quoted as "safe" or "acceptable" for human consumption (Osman, 1983; Gregory, 1984; Sinden et al., 1984). The suggestion for 20 mg/100 g as the tolerance limit came from findings, as early as 1924, that potatoes involved in poisoning showed GA contents exceeding 25 mg/100 g (Van Gelder, 1991). It is probably more correct, therefore, to state that a GA content over 20 mg/100 g is potentially unsafe or even hazardous for human consumption.

Gastointestinal symptoms are believed to be due to the saponin-like properties of GA. Membrane destabilization, measured as cell lysis in various cell systems, was shown to occur synergistically when mixtures of GA were present (Roddick and Rijnenberg, 1987; Roddick et al., 1988, 1990 and 1992). This property is believed to be a result of the interactions between GA and membrane sterols (Keukens et al., 1992).

GA also act as cholinesterase-inhibitors, a likely cause of their neurological effects. Susceptibility of cholinesterase to inhibition by GA is determined by the the isozyme(s) present and the type of GA (Alozie et al., 1978; Sharma and Salunkhe, 1989).

The role of GA as teratogenic compounds in humans has been the topic of much controversy in the literature (Renwick, 1972; Chaube et al., 1973; Keeler, 1973; Poswillo et al., 1973; Swinyard and Chaube, 1973; Keeler et al., 1975; Nishie et al., 1975; Chaube and Swinyard, 1976; Keeler et al., 1976; Keeler et al., 1978; Friedman et al., 1992). Much of the debate stems from the complication of maternal toxicosis in the examination of fetal teratogenesis (Baker et al., 1991), the necessity to expose the fetus at the susceptible developmental stage with the required dose (Keeler, 1986) and difficulties with the extrapolation of results from animal models to humans (Mantel and Schneiderman, 1975; Keeler et al., 1991).

α-Solanine and α-chaconine have not been found to be mutagenic in the Ames test, nor is there any evidence to suggest that they are carcinogenic in animals (Slanina, 1990). There is little information available on the subacute or possible chronic toxicity of GA (Van Gelder, 1991).

1.2.4 Analysis

At the retail or processing level, control of GA levels has depended on a secondary indicator, greening of potatoes. This phenomenon is the result of a parallel increase in chlorophyll synthesis, resulting when tubers are exposed to light. In the U.S.A., potato standards state that if more than 5% of the potato weight must be removed to eliminate greened tissue, they are considered damaged, and if the removal exceeds 10%, they are deemed seriously damaged (Maga, 1980). In Canada, potatoes can only be claimed

to be Nc. 1 Grade if no greater than 15% of their surface shows greening or if greening has not penetrated to a depth that would not be removed by ordinary peeling (Consumer and Corporate Affairs Canada, 1981). The use of greening as an indication of GA concentration is unreliable, inaccurate and wasteful since chlorophyll production does not always accompany synthesis of GA, and *vice versa* (Jadhav and Salunkhe, 1975). It is estimated that in the U.S.A., potato losses of between 14 and 27% are due to greening (Morris and Lee, 1984). Extrapolation to the average annual world crop of almost 300 million tons (Rhoades and Rogers, 1982), greening of potatoes may represent a significant and unnecessary economic loss. One might expect that with these kinds of losses and the toxicity risks associated with consuming GA, that GA analysis would be a routine procedure.

Numerous methods of analysis have been reported in the literature, most of which have serious drawbacks in terms of cost, time required and accuracy. These arise because of the diversity of GA and their properties. During the past 25 years there have been 48 reports in the literature with the key words GA and analysis (American Chemical Society, 1993). Methods of GA analysis have been reviewed by Maga (1980), Coxon (1984), Morgan et al., (1985) and Van Gelder (1991). 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.

1.2.4.1 Extraction and purification

In the past, most of the methods for GA analysis have required that the samples to be analysed are relatively pure. This is due to the lack of specificity of the chemical reactions and detection methods traditionally used. Many methods therefore relied on precipitation by ammonia, bisolvent extraction, and/or Soxhlet extraction (Jadhav et al., 1981). Some newer methods for sample clean-up have included the use of hydroxylapatite (Bezbaruah, 1981) and cartridge columns (Crabbe and Fryer, 1980; Carman et al., 1986; Kobayashi et al., 1989; Saito et al., 1990). Although several aqueous or non-aqueous solvent systems have been used for GA isolation (Friedman and Dao, 1992), solubility characteristics can vary between GA

causing incomplete extraction or precipitation. For example, leptine GA are ammonia-soluble (Gregory et al., 1981).

1.2.4.2 Colorimetric, titrimetric and chromatographic analysis

Colorimetric methods that have been reported in the literature for the quantitation of GA include dye-binding methods (Birner, 1969; Coxon et al., 1979), reaction with antimony trichloride (Smittle, 1971) and reaction with paraformaldehyde (Wang et al., 1972). Aglycones, obtained by acid hydrolysis of GA, can be quantitated titrimetrically with phenol (Fitzpatrick and Osman, 1974; Fitzpatrick et al., 1978; Mackenzie and Gregory, 1979; Bushway et al., 1980c; Speroni and Pell, 1980). Methods using antimony trichloride and paraformaldehyde do not quantitate alkaloids that are saturated at C_5 (e.g. demissidine and tomatidine GA) (Coxon, 1984). The disadvantage with all of the colorimetric and titrimetric methods include the requirement for GA extraction and purification prior to assay and the non-specificity of the reagents used.

Thin layer chromatography (TLC) is a useful method, particularly in the qualitative analysis of GA. Solvent systems and detection methods were reviewed by Svendsen and Verpoorte (1983).

High performance liquid chromatography (HPLC) has been reported by several authors in the separation and quantitation of some major GA (Bushway et al., 1980b; Crabbe and Fryer, 1980; Hunter et al., 1980; Morris and Lee, 1981; Bushway et al., 1986; Carmen et al., 1986; Hellenäs, 1986; Kobayashi et al., 1989; Saito et al., 1990; Friedman and Dao, 1992). GA do not have a suitable uv chromophore and therefore absorbance is measured at 208 nm, where many compounds absorb light. This limitation dictates the need for relatively large sample sizes and extensive sample clean-up to overcome background noise (Coxon, 1984; Morgan et al., 1985). In addition, uv absorbance will not detect GA that are saturated at C_5.

The use of gas chromatography (GC) has also been reported for the analysis of many GA (Herb et al., 1975; Coxon et al., 1979; King, 1980). 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). Newer capillary GC methods for GA (Van Gelder, 1985; Lawson et al., 1992) do not require derivitization but GA must first be hydrolyzed to their aglycones.

1.2.4.3 Analysis by immunoassay

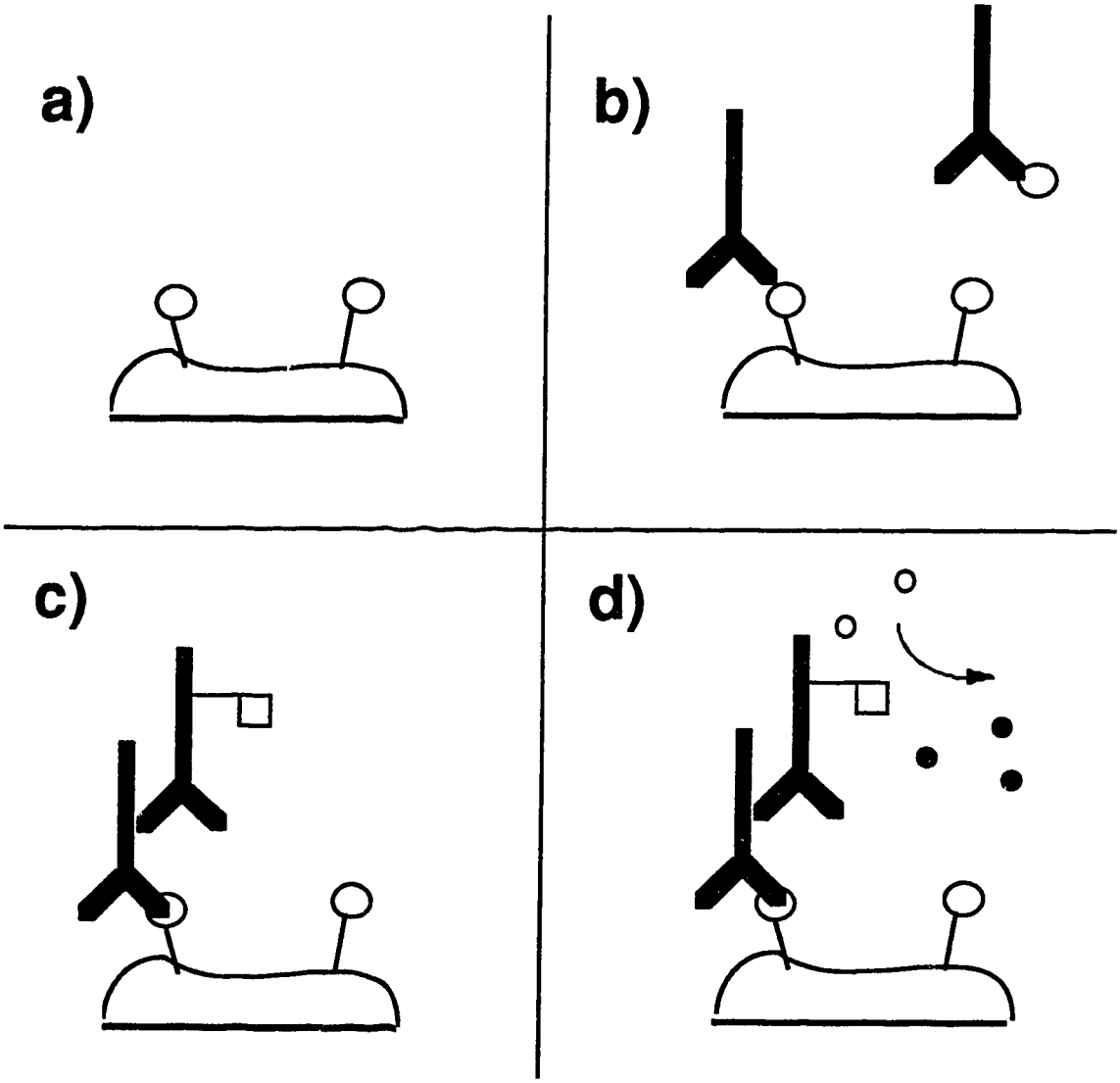
The most promising methods to date for the analysis of GA are the methods based on immunoassay (Vallejo and Ercegovich, 1979; Morgan et al., 1983; Barbour et al., 1991). Immunoassay of GA has been shown to be sensitive, simple and it correlates well with other more expensive methods (Morgan et al., 1985; Hellenäs, 1986). Many samples can be measured simultaneously, increasing the number of samples that can be tested per week, compared with traditional methods (Morgan et al., 1985). Immunoassays, due to their specificity, can eliminate the problem of extraction and purification, which has been a drawback of previous methods.

1.3 Theory of Enzyme Immunoassay

Immunoassays are analytical methods that take advantage of the specificity of antibody (Ab)-antigen (Ag) interaction. There many types of immunoassays including radioimmunoassay, enzyme immunoassay (EI), immunofluorescence assay and luminoimmunoassay, depending on the label used; or competitive, noncompetitive or sandwich, depending on the set-up or whether Ab or Ag are limiting. Interaction between Ab and Ag can occur in solution, at a solid-liquid interphase, can give rise to a soluble product or precipitate. Immunoassay methods and theory was reviewed by Tijssen (1985), Harlow and Lane (1988), Gosling (1990) and Gazzaz et al. (1992). The method that will be used in this study is an Ab capture competitive indirect EI (Harlow and Lane, 1988). The steps involved in this type of assay are presented in Figure 4. EI was reviewed by Maggio (1980), Oellerich (1984) and Gould and Marks (1988).

Ab are immunoglobulins (Ig) composed of four or more protein subunits that exist as pairs of heavy and light polypeptide chains. They can be generated in response to some types of immunologic challenge. An Ab can be classed as IgD, IgM, IgG, IgA, or IgE or subclassed (e.g. IgG_1, IgG_2,

Figure 4. Competitive Indirect EI using Ab capture. a) Ag is immobilized to a solid-phase. b) Competition between immobilized Ag and added sample Ag occurs for the binding sites on added Ab. c) After removing unbound Ab and unbound Ab-Ag complex, Ab bound to immobilized Ag is quantified using a labelled second Ab (e.g. goat anti-rabbit,conjugated to enzyme). d) Bound Ab can be quantitated by measuring enzyme activity. Enzyme activity is inversely proportional to the amount of sample Ag that was added during b).



IgG_3, IgG_4) depending on allotypic determinants in the heavy chain. Variable domains of both heavy and light chains make up the Ab binding site and are therefore responsible for Ab specificity. The general structures of IgG and IgM are presented in stylized form in Figure 5. IgG has two identical binding sites while IgM has ten. Ig structure and function were reviewed by Seiler et al. (1985), Harlow and Lane (1988), Coleman et al. (1989) and Roitt et al. (1989).

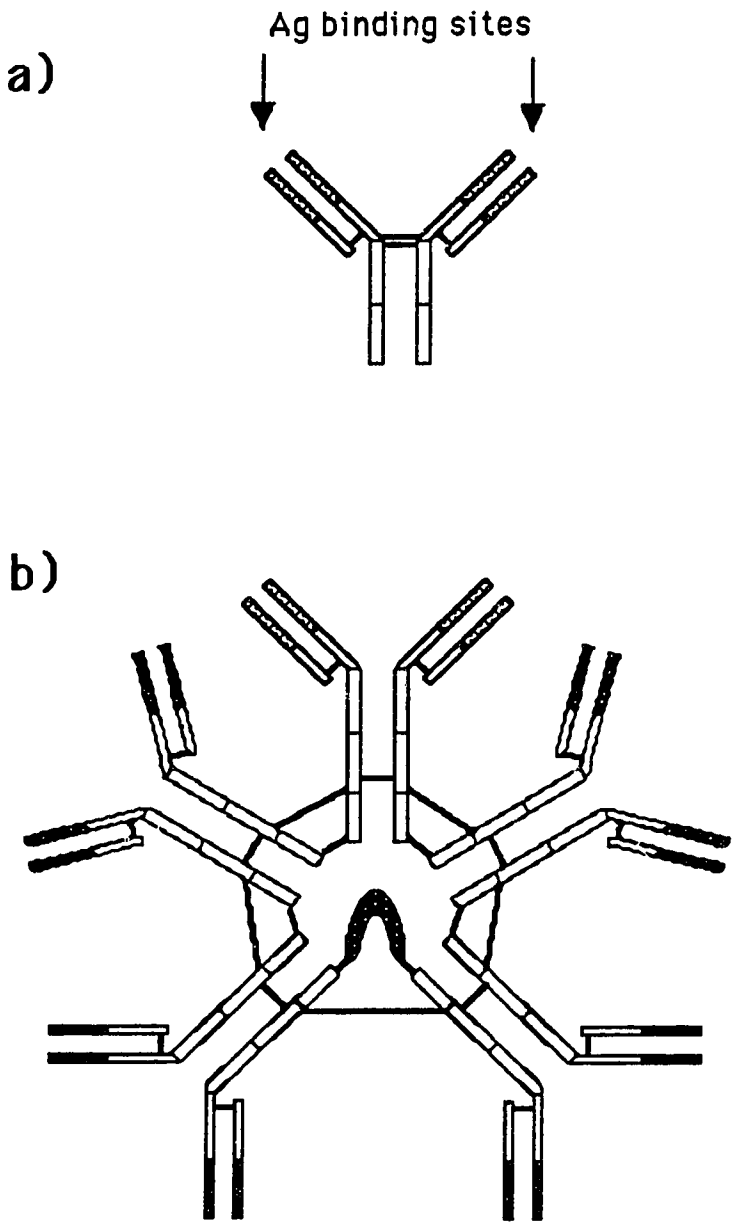
The binding between Ab and Ag is noncovalent and reversible. Attractive forces include hydrophobic, Van der Waals, electrostatic and hydrogen bonding (Roitt et al., 1989). For a bimolecular reversible interaction, the equilibrium constant, K_{eq}, can be defined as the ratio of the forward to the backward rates of the reaction:

$$K_{eq} = k_A/k_B = \frac{[Ab\text{-}Ag]}{[Ab]\ [Ag]}$$

where k_A is the rate of association, k_B is the rate of dissociation, [Ab] is the molar concentration of the unoccupied Ab binding sites, [Ag] is the molar concentration of the unoccupied Ag binding sites and [Ab-Ag] is the molar concentration of the Ab-Ag complex that will be found at equilibrium. The time the reaction takes to reach equilibrium depends on the rate of diffusion. k_A and k_B can vary independently of each other according to the experimental conditions. K_{eq}, also referred to as the affinity constant, determines what proportion of a given solution of Ab and Ag will be found as Ab-Ag complex at equilibrium. Like all reversible equilibrium reactions, the affinity constant is affected by temperature, pH and solvent.

The overall stability of Ab-Ag interaction, also referred to as avidity, is strengthened by the formation of multivalent complexes. Ab can contain two to ten binding sites per Ig. These can give rise to avidity effects that can cause 10^3- to 10^7-fold increases in the effective K_{eq} for IgG and IgM, respectively (Roitt et al., 1989). Ag can be multivalent either because of intrinsic properties (i.e. the Ag contains multiple copies of the same epitope) or when Ag is bound to a solid support providing high local concentrations. Avidity effects arise whenever a complementary pair of Ab and Ag binding

Figure 5. General structures of a) IgG and b) IgM.



sites are held in close proximity to each other by one or more other bound pairs of Ab and Ag binding sites.

An Ab response, after the introduction of an appropriate immunogen to an appropriate animal using an appropriate immunization schedule, involves a series of interactions between Ag presenting cells, T lymphocytes and B lymphocytes (Harlow and Lane, 1988). During the primary response, activated B cells proliferate and differentiate, giving rise to plasma cells. Plasma cells produce large quantities of one type of Ab, initially IgM, a relatively low-affinity Ab. Class-switching can occur during B cell differentiation giving rise to the production of IgG. An increase in the titer of high affinity IgG Ab (affinity maturation) occurs during class-switching as a result of clonal selection and somatic hypermutation (Roitt et al., 1989). The use of multiple immunizations, or boosts, are desirable to induce class-switching. Considerations to be made before immunization (i.e. choice of animal, form of Ag, type of adjuvant, dose, boosts or route(s) of injection) are reviewed in Harlow and Lane (1988) and Tijssen (1985).

Collection of serum from an animal after an appropriate immunization schedule will result in a polyclonal Ab (PAb) preparation. Figure 6 illustrates how PAb can be produced in rabbits. With an ideal immunization protocol the levels of Ab with required specificity will be high but other Ab will always be present. Crude PAb preparations are easy to prepare and may be suitable for some types of analytical-scale experiments, for example, an EI where there is no interference from either other Ab in the serum or contaminants in the sample being analyzed. On the other hand, highly purified Ab is desirable if it is to be coupled to a fluorochrome, biotin, an enzyme or a solid-phase matrix. Pure Ab preparations can be made either by affinity purifying PAb (Assil et al., 1992), or by the production of monoclonal Ab (MAb).

MAb production, usually using mouse cells, involves the generation of a cloned cell line, derived from a B lymphocyte, that secretes a homogeneous supply of Ab (Figure 7). Plasma cells grow poorly or not at all *in vitro* but can proliferate when fused with malignant plasma (myeloma) cells (Pirofski et al., 1992). The resulting hybrid cells are called hybridoma. The production of hybridoma for the immortalization of B lymphocytes was

Figure 6. Diagramatic representation of the production of PAb from rabbits.

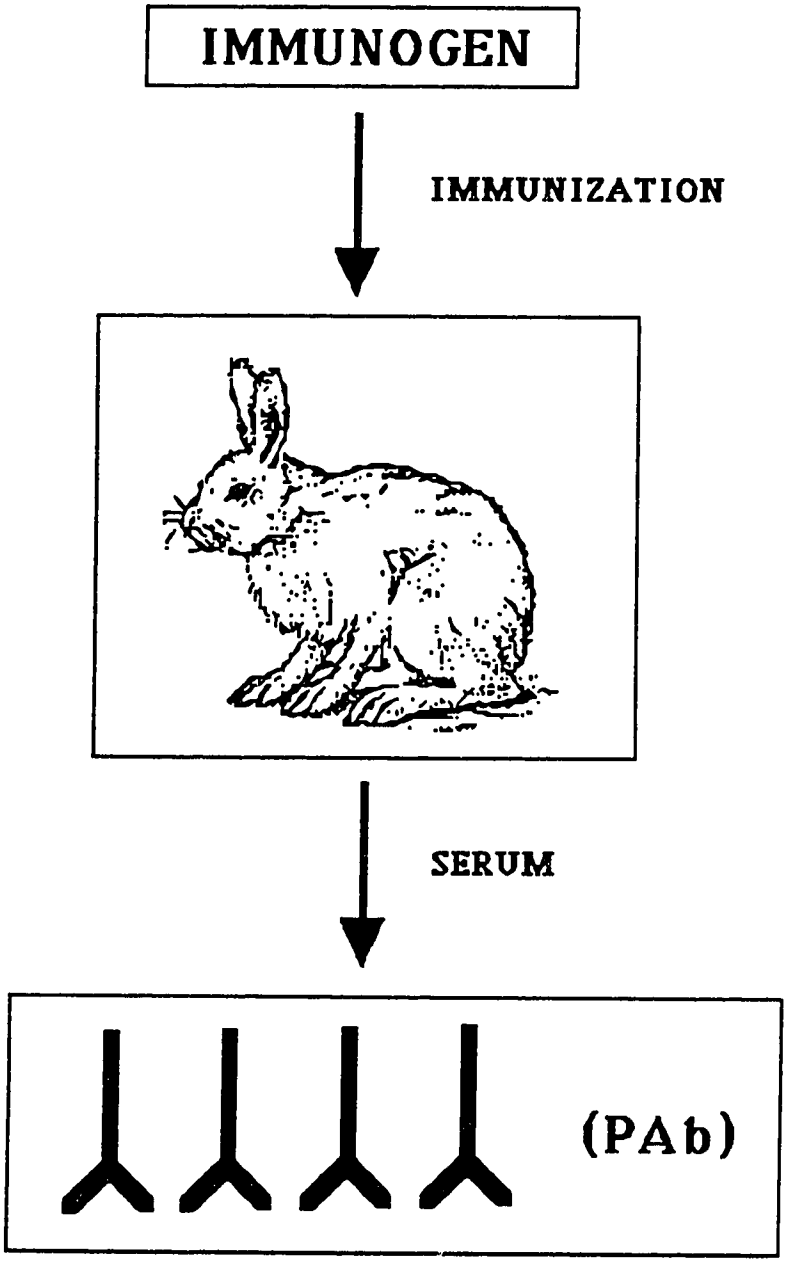
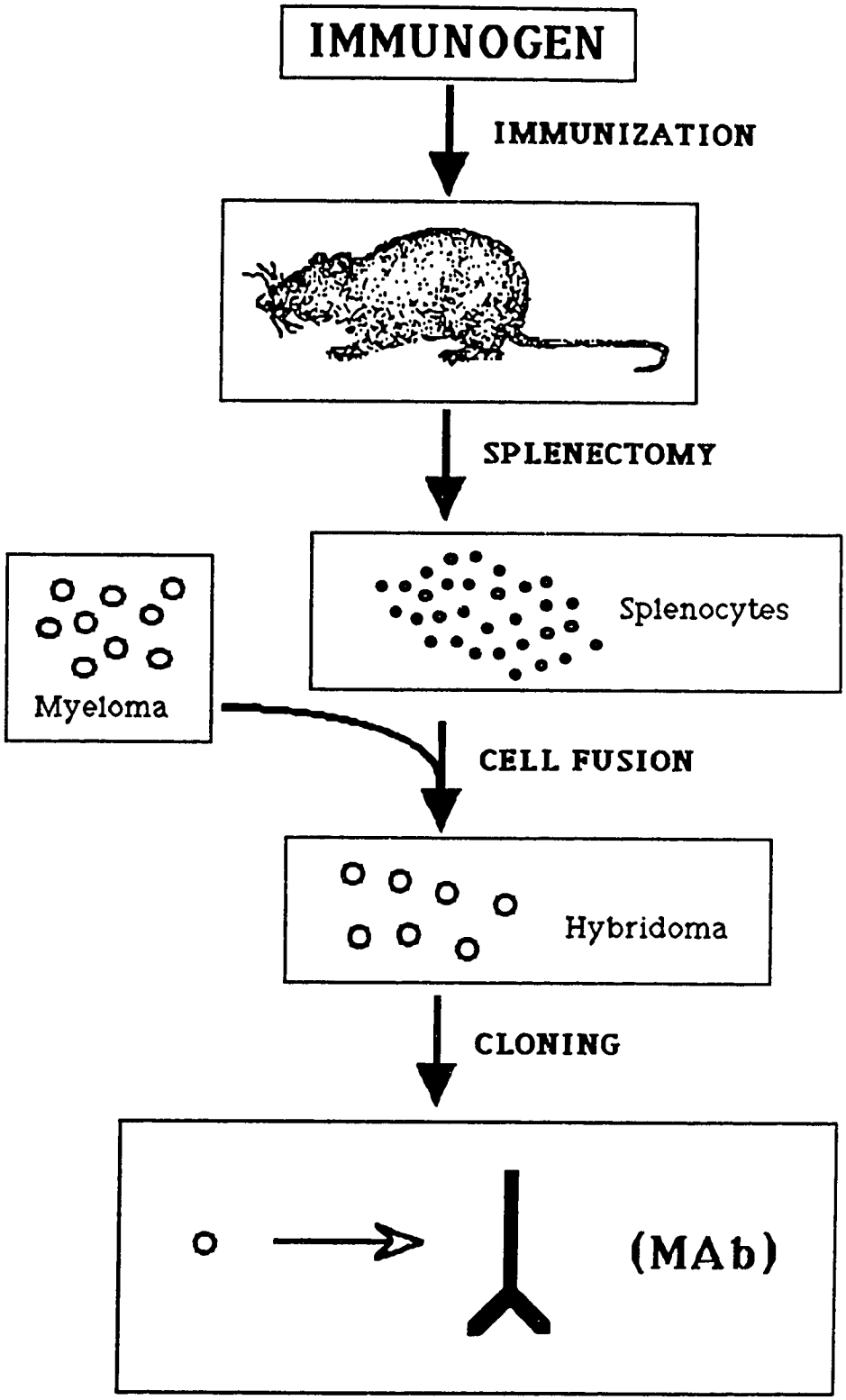


Figure 7. Diagramatic representation of the production of MAb from mice.



first described by Köhler and Milstein (1975). After fusion, parental myelomas can be inhibited using a selective medium, the remaining hybridoma cloned and the desired MAb-producing cells selected. Methodology for the production of MAb was reviewed by Hudson and Hay (1980), Campbell (1984), Westerwoudt (1985), Harlow and Lane (1988) and McCullough and Spier (1990).

Once cells are cloned, the most commonly used method for producing large quantites of MAb involves using rodents to produce ascites fluids after inoculation with hybridoma (i.e. inducing tumor growth *in vivo*). There are several reasons why researchers are tending to move away from this method and towards cell culture for the production of MAb. The production of Ab *in vivo* is costly and contamination of ascites with other Igs, plasma proteins and possibly infectious adventitious agents can be a problem. Other reasons that make this method undesirable include lack of reproducibility between animals, the inability to use rodents for the production of human Ab and there has been a movement towards minimum use of animals in research (McCullough and Spier, 1990).

Production of MAb *in vitro*, on the other hand, has many advantages. Cell culture can be done using relatively well-defined media, allowing for scale-up and continuous culture to produce a virtually endless supply of Ab. Recent advances in cell culture techniques have improved the ability to grow hybridoma in protein-free media or with Ab-free serum supplements, thus enabling easier Ab purification later (Griffiths, 1987; Wilson and Spier, 1987; Hayter et al., 1992).

While many methods of Ig purification abound, the method of choice will depend on the purity and concentration of Ig present in the starting solution (either ascites fluid or cell culture supernatant) and the efficiency of purification required. Ab may be isolated from complex mixtures because of their solubility characteristics, molecular size, electrostatic density and isoelectric point. Methods include ammonium sulfate fractionation, liquid chromatography (affinity, ion-exchange and size-exclusion) and gel electrophoresis. Details of theory and protocol and additional references for Ig purification can be found in Hudson and Hay (1980), Goding (1986),

Menge et al. (1987), Bollag and Edelstein (1991) and Harlow and Lane (1988).

The use of MAb in immunoassay systems has several advantages over the use of a serum containing PAb. MAb give uniform affinity constants, greater reproducibility and continuity of supply, larger linear ranges in immunoassays, better-defined specificity and the ability to combine different MAb and analyze several analytes simultaneously (Seiler et al., 1985; Gosling, 1990). For these reasons, the trend has been towards a greater use of MAb in immunoassays (Gosling, 1990).

1.4 Current Research Objectives

With the obvious need for a simple GA analysis method, such as the immunoassays developed by Vallejo and Ercegovich (1979) and Morgan et al. (1983) for solanidine GA and by Barbour et al. (1991) for tomatidine GA, it is surprising that further improvements of their immunoassay procedures have not appeared in the scientific literature. The radioimmunoassay developed by Vallejo and Ercegovich (1979) yielded a low titer PAb preparation that did not cross-react equally with all solanidine alkaloids. Morgan et al. (1983) obtained suitable cross-reactivities but their method for linking alkaloid to protein resulted in very low hapten to protein ratios. A 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. Barbour et al. (1991) did not successfully determine the hapten to protein ratio of their conjugate. Their method also suffered from cross-reacivity problems because their Ab could bind (not equally) with both solanidine and tomatidine GA.

This thesis reports new methods to prepare GA-protein conjugates in an attempt to improve the EI analysis of GA. Different methods were developed for the preparation of immunogen and solid-phase conjugates to enhance the binding between Ab and free GA during the competitive assay (Vallejo et al., 1982; Wie and Hammock, 1984; Gosling, 1990; Harrison et

al., 1991; Sheth and Sporns, 1991). PAb and MAb preparations were prepared and evaluated in Ab capture competitive indirect EI.

The objective has been to acquire a simple and reliable method to measure GA. In the long term, it would be desirable to have several Ab, each against a different class of GA, and produce an assay that will measure each of these GA simultaneously, rapidly, inexpensively and accurately.

Chapter 2. Materials and Methods

2.1 Materials

Water used in all reactions and EI tests was purified using a Milli-Q system (Millipore Corp., Millford, MA.). α-Chaconine, α-solanine, solanidine, tomatidine, demissidine, solasodine, thimerosal, Tween 20, sodium cyanoborohydride, 4-dimethylaminopyridine, bovine serum albumin (BSA), *Limulus polyphemus* hemocyanin (LPH), ammonium persulfate, sterile dimethyl sulfoxide and Tris-HCl were obtained from Sigma Chemical Co., St. Louis, MO. Antimony trichloride, dioxane, sodium metaperiodate, pyridine, bromocresol green, urea, trypan blue, ammonium chloride and ammonium sulfate (enzyme grade) were purchased from Fisher Scientific, Edmonton, AB. Coomassie Brilliant Blue R-250 was from Bio-Rad Laboratories Canada Ltd., Mississauga, ON. Sodium borohydride was obtained from Terochem Laboratories Ltd., Edmonton, AB. Succinic anhydride and glycerol were obtained from BDH Inc., Edmonton, AB. N-ethyldiethanolamine, N-hydroxysuccinimide, 1,3-dicyclohexyl-carbodiimide and N,N-dimethylformamide were purchased from Aldrich Chemical Co., Milwaukee, WI. 2-Mercaptoethanol was from Serva Feinbiochemica, Heidelberg, Germany. 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 and goat anti-mouse Ab and bovine IgG were obtained from Calbiochem Corp., La Jolla, CA. The Ribi Adjuvant system (for mice) was purchased from Cedarlane Laboratories Ltd., Hornby, ON. Low melting point agarose (electrophoresis grade) and Tris (enzyme grade) were from Gibco BRL, Burlington, ON. Sterile polyethylene glycol (PEG) 1500 in 75 μM HEPES buffer, sodium dodecyl sulfate (SDS) and acrylamide (electrophoresis grade) were purchased from Boehringer-Mannheim Canada Ltd., Laval, PQ.

Dynatech Immulon II microtiter plates and Spectrum dialysis tubing (12,000-14,000 mwco) were purchased from Fisher. Sep Paks were Waters

C-18 cartridges (Millipore). A commercial ELISA kit for solanidine glycoalkaloids (Lot D400 3513A02) was purchased from Holland Biotechnology bv, Leiden, The Netherlands. Syringes, needles and serum separator tubes were from Becton Dickinson and Co., Rutherford, NJ.

Potato samples a to 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 to 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 mo at 5°C in darkness and sample f (extremely green and sprouted) was stored for 6 mo at 5°C in darkness then 6 mo at 25°C exposed to daylight. Sample g was plant material (leaves, stems and flowers) from *S. demissum* obtained from the Department of Plant Science, University of Alberta. After treatments, all samples were cut into 0.5 cm^3 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 thimerosal (0.2 g) in 1.9 L of water, then adjusting the pH to 7.3 with 1 N NaOH. The total volume was 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 thimerosal (PBS-t) was prepared as PBS, but omitting thimerosal.

Rabbits and mice were handled by the Biosciences Animal Services facility at the University of Alberta. The myeloma cell line (NS-1) was a non-secreting clone of P3x63Ag8 (American Type Culture Collection, Rockville, MD.).

Sterile plasticware used for fusion, cloning and tissue culture included 25, 75 or 150 cm^2 polystyrene tissue culture flasks (Fisher), 1, 10 and 25 mL pipets (Fisher), 96 and 24-well covered microtiter plates (Flow Laboratories Inc., Mississauga, ON), reagent reservoirs (Bio-Rad), Millex-GS 0.22 µm filter units (Millipore), 0.2 µm 150 mL Nalgene cellulose acetate bottle top filters (Nalge Co., Rochester, NY), 0.2 µm 500 mL cellulose acetate bottle top filters (Costar Corp., Cambridge, MA), 17 x 100 mm polypropylene

tubes with snap caps (Fisher), 15 and 50 mL conical centrifuge tubes (Corning Glass Works, Corning, NY) and cryovials, canes and sleeves (Nalge).

Several media were used for the culture of myeloma and hybridoma: Serum-free RPMI consisted of Rosewell Park Memorial Institute 1640 (Gibco BRL), with the addition of L-glutamine (2 mM), oxaloacetic acid (0.5 mM), Na pyruvate (1 mM), penicillin (100 units/mL) and streptomycin (100 µg/mL, all purchased sterile from Gibco BRL). Complete RPMI was serum-free RPMI plus 20% (v/v) heat-inactivated calf serum for myeloma, and heat-inactivated fetal calf serum (Gibco BRL) or FetalClone (FC; HyClone, Logan, UT.) for hybridoma. Heat inactivation of sera was performed at 56 °C for 30 min. HT medium consisted of complete RPMI with 20% fetal calf serum, sodium hypoxanthine (100 µM), and thymidine (16 µM). Hypoxanthine and thymidine were purchased sterile as 100x supplements from Gibco BRL. HAT medium consisted of HT medium plus aminopterin (0.4 µM) (sterile as 100x from Gibco BRL). Hybridoma were also cultured using protein-free hybridoma medium (PFHM II, Gibco BRL) and serum-free RPMI plus 1% (v/v) Nutridoma (Boeringer-Mannheim).

2.2 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 Buchi Rotoevaporator R (Fisher) was used for the removal of solvents from samples. 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. Other optical densities were measured using a Hewlett Packard model 8452A diode array spectrophotometer with HP 89532A UV-Vis Software (Hewlett Packard, Waldbronn, Germany). Cells were grown in Forma Scientific water-jacketed controlled atmosphere incubators (Caltec Scientific, Edmonton, AB) and viewed using a PhotoZoom inverted microscope (Bausch and Lomb, Rochester, NY). Cell counting was performed using a Neubauer counting chamber (Fisher). Tissue culture manipulations (subculturing, etc.) were done in a Canadian Cabinets Type IIA model BM6-MM-99-T2 biosafety cabinet (Canadian Cabinets Co. Ltd., Ottawa, ON). Clone selection from soft agar was performed in a Canadian Cabinets model H6-MW-99-C30 laminar flow hood. All autoclaving was performed at 121 °C for 15 min.

Preparative 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% aq. ammonia (2:2:1, v/v/v, lower layer) for the separation of α–chaconine hydrolysis products and ethyl acetate/ methanol/1% aq. 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.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Bio-Rad Mini Protean II separation system and Bio-Rad power supply unit. Gels were 0.75 mm thick. The separation gel was 10% acrylamide in 0.375 M Tris-HCl buffer, pH 8.8, containing 0.1% (w/v) SDS, 0.05% ammonium persulfate and 0.01% TEMED. The stacking

gel was 4% acrylamide in 0.125 M Tris-HCl buffer, pH 6.8, containing 0.1% (w/v) SDS, 0.05% ammonium persulfate and 0.01% TEMED. Standards were used at 1 mg/mL and samples were used at 8X the cell supernatant concentration. Protein samples (20 µL) were mixed with 5 µL denaturing dye solution (25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue in 60 mM Tris-HCl buffer, pH 6.8) and heated at 100 °C for 2 min. Gels were run at 20 mA constant current until the dye front reached the bottom of the gel (approx. 1.5 h), stained for 0.5 h using 0.1% Coomasie Brilliant Blue R-250 in 40% methanol with 10% acetic acid, and destained overnight in 30% methanol with 7% acetic acid solution. Gels were stored in 7% acetic acid.

2.3 Procedures

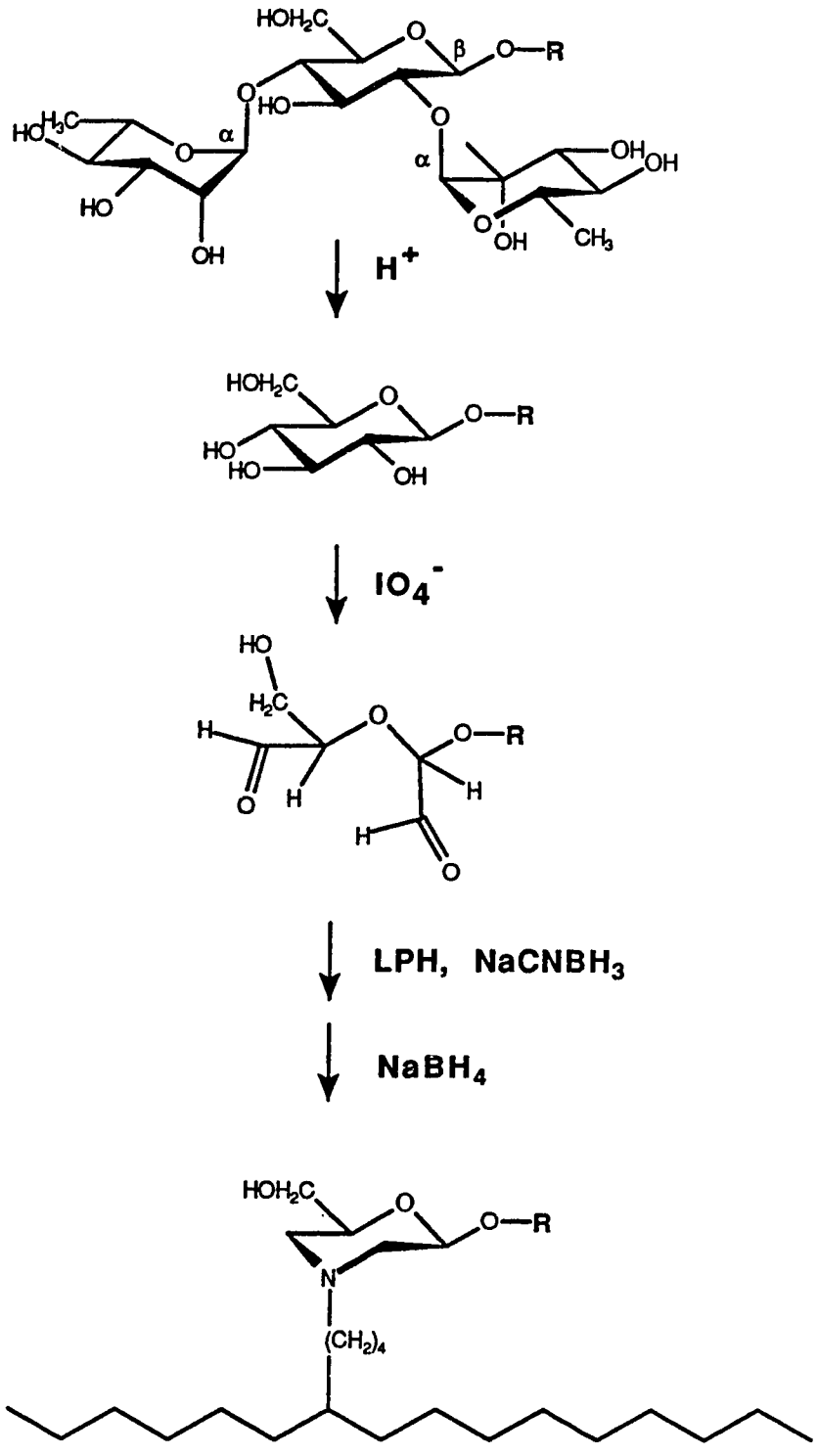
2.3.1 Preparation of Immunogen

A general scheme for the production of immunogen is illustrated in Figure 8.

2.3.1.1 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 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 methanol and 10 mL 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 ethyl acetate followed by 400 mL of ethyl acetate:methanol:aqueous 1% ammonia (375:20:5, v/v/v) and finally 111 mL ethyl acetate:methanol: aqueous 1% aqueous ammonia (100:10:1, v/v/v). The first 190 mL of eluant were discarded and the next 330 mL, containing the desired (identified by TLC) γ–chaconine were collected. Later fractions contained partially

Figure 8. Preparation of Immunogen (R = solanidine).



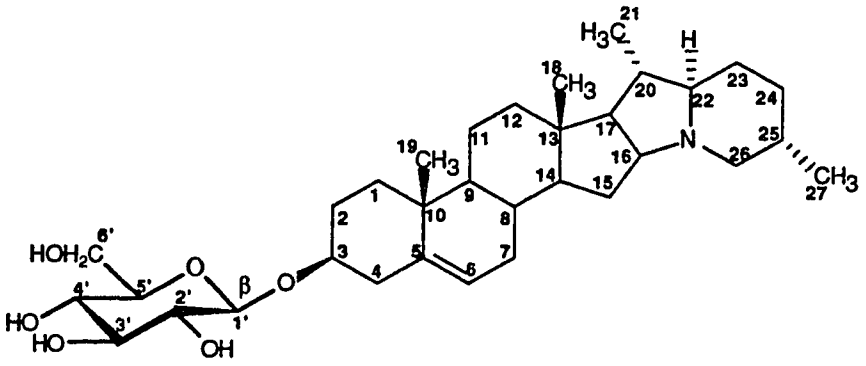
hydrolyzed α –chaconine that could be used in future hydrolyses. The γ –chaconine fraction was evaporated to dryness, dried overnight under vacuum in the presence of phosphorus pentoxide, and weighed. The 60.7 mg of γ–chaconine (MW 560 g/mole, Figure 9) recovered represented a 60% yield for the selective hydrolysis. ^{1}H NMR (200 MHz, solvent CD_3OD, δ 3.35, 4.84) δ 5.38 (broad 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 H, alkaloid), 2.87 (m, 1 H, alkaloid), 2.43 (m, 1 H, alkaloid), 2.25 (m, 1H, alkaloid),1.70 (m, 18 H, alkaloid), 1.24 (m, 9 H, alkaloid), 0.94 (m, 15 H, alkaloid).

2.3.1.2 Conjugation of γ-chaconine to protein

γ –Chaconine (16.5 mg, 0.0295 mM) was added to 2 mL 98% ethanol, 1.5 mL dioxane and 1.5 mL 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 dialysed with water and lyophilized, was added in 3 mL of pH 9.00 N-ethyldiethanolamine buffer (26.6 mL N-ethyldiethanolamine in 1 L water and about 14 mL 6 M HCl to pH 9.00). The protein container was rinsed with 3 x 2 mL portions of pH 9.00 N-ethyldiethanolamine buffer which was added to the reaction mixture along with 100 mg (1.58 mM) sodium cyanoborohydride (Schwartz and Gray, 1977). The reaction mixture was heated, with stirring, for 6 days at 40 °C. At this time 60 mg (1.61 mM) sodium borohydride was added and the reaction mixture stirred overnight at room temperature. The entire reaction mixture was poured into an Amicon ultrafiltration unit and filtered to about 2 mL. The filtration was repeated with 10 mL water, followed by 10 mL 8 M urea, 4X 10 mL water, 10 mL 20% aqueous ethanol and again with 10 mL water. The residue collected was lyophilized. This reaction was also performed using BSA to obtain solid-phase protein conjugates linked in the same manner as the immunogen.

2.3.1.3 Measurement of alkaloid in conjugate

Figure 9. γ-Chaconine.



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 2 M HCl and 4 mL 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 chloroform. The combined organic layers were washed with 5 mL 1% aqueous ammonia solution and evaporated to dryness. The residue was dissolved in 5 mL 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 colorimetric method of Birner (1969). The results were compared with a standard curve made with pure solanidine (Sigma) from 0 to 62 µg/5 mL. For ease of comparison between conjugates, the protein is assumed to have a MW of BSA (66,000 g/mole) 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 molecule of BSA. These three conjugates, linked through a glycosidic bond, will be referred to as LPH-G-14, BSA-G-6 and BSA-G-23, respectively.

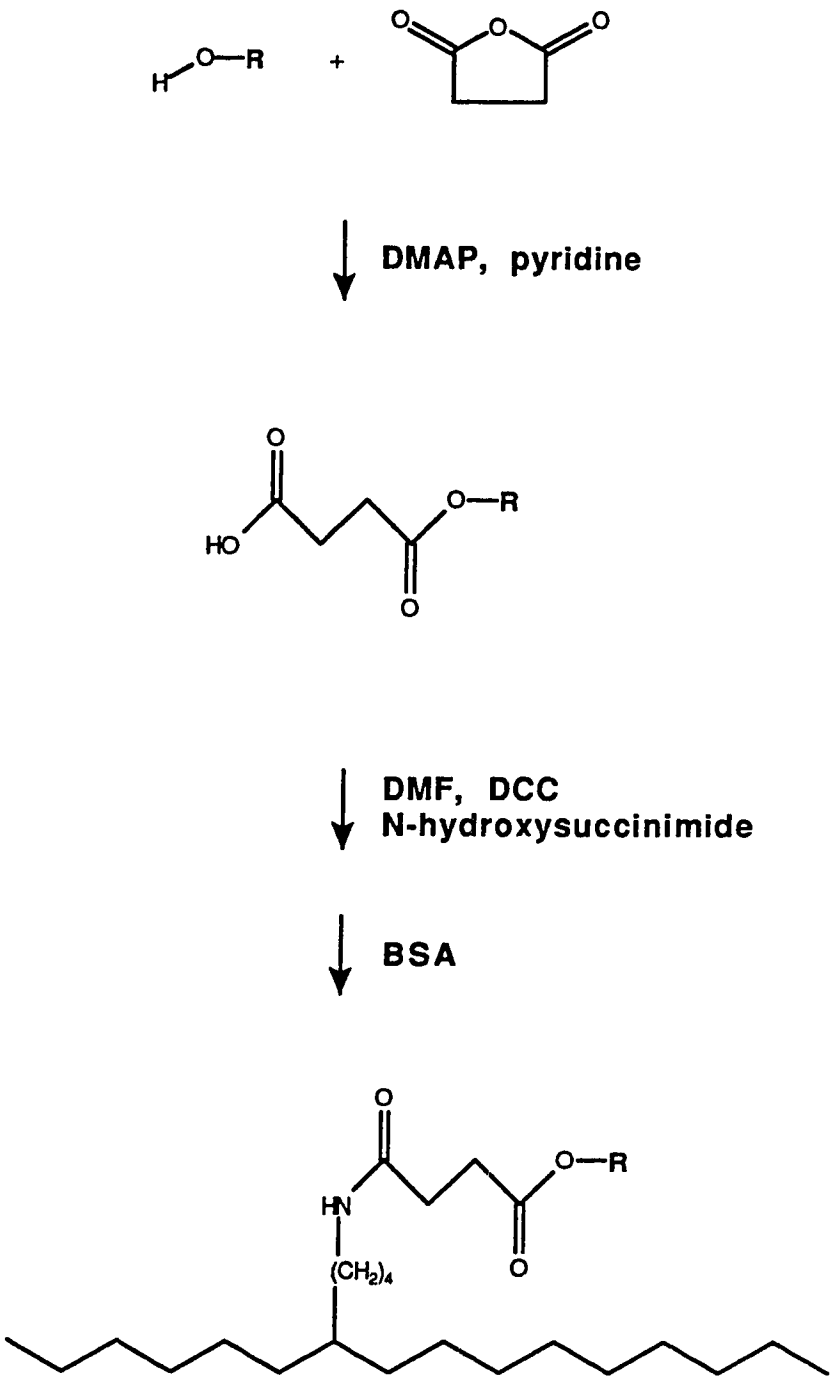
2.3.2 Preparation of Solid-Phase Conjugates

A general scheme for the production of solid-phase conjugates, using a hemisuccinyl linkage, is illustrated in Figure 10.

2.3.2.1 Production of solanidine hemisuccinate

Formation of solanidine hemisuccinate was performed using a modified version of Abraham and Grover (1971). 4-Dimethylaminopyridine (26 mg, 21.5 mM) was added to 10 mL pyridine. Succinic anhydride (422 mg, 422 mM) was then added and dissolved, followed by 40.2 mg solanidine (10.1 mM). The mixture was heated under reflux, with stirring, for 22 h at 57 °C. The reaction was followed by TLC and was stopped by the addition of 20 mL H_2O. The mixture (pH 6 to avoid hydrolysis) was extracted with 3 x 25 mL methylene chloride, the organic extracts were combined and rotoevaporated once, then coevaporated with 2 mL toluene to dryness and dried overnight under vacuum in the presence of phosphorus pentoxide.

Figure 10. Preparation of Solid-Phase Conjugate (R = solanidine).



The 48.3 mg of solanidine hemisuccinate (MW 498 g/mole) 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.

2.3.2.2 Conjugation of hemisuccinate to protein

Solanidine hemisuccinate (4.1 mg, 6.2 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 anhydrous dimethylformamide to form an active ester (Hassan, et al., 1988). BSA (215.7 mg) was dissolved in 2 mL PBS. The active ester reaction mixture was then filtered through glass wool into the BSA solution. This reaction mixture was stirred 24 h at 4 °C. The entire reaction mixture was dialyzed with 1 L 8 M urea for 24 h, 4 L 50 mM ammonium carbonate for 24 h and finally, 4 L 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 50 mM ammonium carbonate, centrifuging until the supernatant was clear, and the supernatant was dialyzed against 4 L 25 mM ammonium carbonate for 18 h and lyophilized. The purified product weighed 30.2 mg.

2.3.2.3 Measurement of solanidine in conjugate

The percentage of 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 contained approximately 9 molecules of alkaloid per molecule of BSA (BSA-H-9).

2.3.3 GA Extraction from Potato Samples

GA were extracted from 0.2 g (for potato plant material) and 1.0 g (for potato tuber) freeze-dried powder using 3 x 15 mL methanol and

homogenizing after each addition for 1 min. An additional 1-2 mL methanol was used to rinse the homogenizer probe, and supernatants were pooled and made up to 50 mL. Triplicate potato crude extracts were made and stored at -15 °C until required for analysis.

2.3.4 High Performance Liquid Chromatography

Crude GA 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 GA extract (5 mL) was mixed with 8 mL of water and added to the cartridge, washed with 5 mL 40% aq. methanol, eluted with 15 mL methanol, rotoevaporated at 30°C and taken dissolved in 1 mL methanol. Samples with high GA concentrations (i.e. >20 mg/100 g, fwb) were applied to the Sep-Paks as 1/5 dilutions to reduce the quantities of contaminating substances which otherwise overloaded the cartridges. Purified sample solutions were injected through a 20 µL loop for analysis on a 300 x 3.9 mm µ-Bondapak NH_2 column (Phenomenex, Torrance, CA), operated at 25°C. The mobile phase was acetonitrile/20mM potassium dihydrogen phosphate (75:25, v/v) pumped by a Beckman model 110A/332 pump (Beckman Instruments Inc., Fullerton, CA.) at a flow rate of 1.0 mL/min. The pressure was approximately 1000 p.s.i. Detection was achieved using a Bio-Rad uv monitor, model 1305 at 208 nm. The output was monitored on a Hewlett-Packard 3388A integrator (Hewlett-Packard, Avondale, PE). Sample peak heights were compared with a linear standard curve that was constructed using standards at concentrations from 0-100 µM. Samples were analyzed at least in triplicate.

2.3.5 Polyclonal Ab Production

Preimmunization blood samples (ear, 5 mL each rabbit) were taken from two rabbits (3 month, male, Flemish Giant x Dutch Lop Ear). The rabbits were immunized with 1.0 mg conjugate (LPH-G-14, glycosidic linked LPH conjugate containing 14 GA per BSA equivalent) in 2 mL sterile PBS-t/Freund's Complete Adjuvant (1:1). Injections were performed as 0.5 mL subscapular and 0.5 mL intramuscular, 2 sites each. Four boosts were performed 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 to 2 h, centrifuged at 300 *x g* for 5 min and the serum collected and tested for PAb titer. Three days after the final boost, rabbits were completely bled (cardiac puncture, approximately 120 mL each rabbit). The immune serum obtained, after clotting and centrifugation (see above), was stored at -20 °C until required.

PAb titer for rabbit sera was determined using the following checkerboard EI: Solid-phase BSA conjugate (BSA-H-9) 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. The wells of each row of a microtiter plate were filled with 200 µL/well of one of the above solutions and the final row with 200 µL/well 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 (blocked) with 200 µL 1% BSA in PBS. This solution was allowed to incubate for 1 h at room temperature and then removed as before. The wells were next washed with 3 x 200 µL PBST, for 5 min at 25 °C each wash. Rabbit sera were diluted 1/500 with 0.05% BSA in PBST. Serial dilutions (1/5) to 1/12,500,000 and blanks consisting of 1/500 preimmune sera in 0.05% BSA in PBST were prepared and set aside. Aliquots (100 µL) of methanol were 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 with shaking and humidity for 2 h at room temperature. Wells were emptied and washed as before with 3 x 200 µL PBST. Goat anti-rabbit Ab-peroxidase conjugate was diluted 1/3000, as suggested by the manufacturer, and added to each well in 200 µL quantities, incubated 2 h at room temperature with shaking and humidity, and washed as described before. A solution of peroxidase substrate was prepared by dissolving *o*-phenylenediamine (0.4 mg/mL) and urea peroxide (1 mg/mL) in 0.1 M citrate buffer (pH 4.75) and was added to each well (200 µL/well). The optical density (A_{450nm} - A_{660nm}) was measured after 30 min at room temperature (Sheth and Sporns, 1991). The titer was defined as the minimum serum dilution that gave an absorbance reading that was three times greater than the background levels (no serum).

2.3.6 Enzyme Immunoassay using Polyclonal Ab

For the determination of GA in potato samples, an indirect competitive EI was performed using microtiter plates coated with solanidine-BSA conjugate (0.2 μg/mL BSA-H-9) in PBS and blocked, as described above. All incubations were also performed as described above, however, during incubation with diluted serum (1/100,000), the 100 μL methanol was replaced with 100 μL methanol containing GA (methanol-diluted crude GA 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 serum and sample blanks, where serum blanks contained 1/100,000 dilution of preimmunization serum and 100 μL methanol, and sample blanks contained 1/100,000 immune serum and 100 mL methanol. Results are expressed as:

$$A/A_0 = \frac{(A_{450nm} - A_{660nm})_{sample}}{(A_{450nm} - A_{660nm})_{sample\ blank}}$$

when not corrected for preimmunization serum (i.e. serum blank), or:

$$B/B_0 = \frac{(A_{450nm} - A_{660nm})_{sample} - (A_{450nm} - A_{660nm})_{serum\ blank}}{(A_{450nm} - A_{660nm})_{sample\ blank} - (A_{450nm} - A_{660nm})_{serum\ blank}}$$

when corrected using a preimmunization blank.

Inhibition curves were constructed using 10^{-6} - 10^{2} μM GA in methanol. These were analyzed to determine the four parameters defining the sigmoidal curves (see section 2.4). Each sample GA concentration was determined using the inhibition curve of α-chaconine analyzed on the same plate.

Analysis of HPLC eluate by EI was performed by constructing inhibition curves of α-chaconine in acetonitrile/20 mM potassium dihydrogen phosphate (75:25, v/v).

To compare the sensitivities of the PAb with various GA (cross-reactivity), I_{50} values were calculated. I_{50} is the concentration of GA (μM) giving 50% inhibition (the inflection or midpoint in the sigmoidal curve).

2.3.7 Commercial Solanine ELISA Kit

Potato extracts were analyzed according to the manufacturer's (Holland Biotechnology bv, Leiden, The Netherlands) directions. One kit (containing 12 strips of 8 wells each) allowed analysis of 96 samples, including standards. The number of replicate determinations for each potato sample was therefore limited to three.

2.3.8 Monoclonal Ab Production

2.3.8.1 Immunization of mice

Preimmunization blood samples (tail, 200 μL each mouse) were taken from five mice (3-4 week, female, BALB/c). The mice were then immunized with 1.0 mg conjugate (LPH-G-14) in 2 mL sterile PBS-t/Ribi Adjuvant System. Injections were performed as 0.1 mL subcutaneous and 0.1 mL intraperitoneal, 2 sites each. Boosts were performed in a similar manner on days 21, 56, 91, 196 and 304. Blood samples (tail, 200 μL each) were taken 3 days after each boost, allowed to clot at room temperature for 1 to 2 h, centrifuged in a serum separator tube at 15,000 *x g* and the serum collected and tested for PAb titer (see section 2.3.5).

PAb titers for mouse sera were determined using microtiter plates coated with 1 μg/mL BSA-H-9 in PBS and blocked and washed as described for rabbit sera. Incubation of diluted mouse serum (100 μL/well) and methanol (100 μL/well) was performed at 5 °C, overnight and with humidity. The mouse sera were tested as 1/10 serial dilutions from 1/1000 to 1/1,000,000 and compared with blanks of 1/1000 preimmune sera in 0.05% BSA in PBST. The secondary Ab was goat anti-mouse Ab-peroxidase conjugate diluted to 1/6000, as suggested by the manufacturer.

Otherwise, methods used were performed as for PAb titer (see section 2.3.5).

2.3.8.2 Myeloma cell culture

Myeloma were grown in sterile tissue culture flasks using complete RPMI with 20% calf serum at 37 °C with 7% CO_2 and at 90-100% relative humidity. Cells were subcultured every 2-4 days, using 1/10 to 1/50 dilutions so that cell density remained from 10^5 to 10^6 cells/mL. Viable cells were counted at 1/10 or 1/100 dilution of cell suspension in 0.25% (w/v) trypan blue in PBS, using a Neubauer counting chamber.

Two days prior to fusion, cells were subcultured (1/50 dilution) using complete RPMI with 20% calf serum into eight 75 cm^2 tissue culture flasks so that approximately 10^8 cells would be available for the fusion. On the day of the fusion, cell suspensions were centrifuged at 250 *x g* for 10 min. Supernatants were pooled, cleaned and stored at 4 °C for later use during cell cloning (see 2.3.8.6). Cells were combined into one 50 mL conical centrifuge tube with 50 mL serum-free RPMI. Washing was repeated twice using 50 mL serum-free RPMI each time. After the third washing, centrifuged cells were resuspended in 10 mL serum-free RPMI and the viable cells were counted.

2.3.8.3 Recovery of splenocytes

Three days after the final boost (307 days after initial immunization), one mouse was asphyxiated using CO_2, its spleen removed under sterile conditions, washed in a Petri dish containing 10 mL serum-free RPMI and then transferred to a second Petri dish containing 10 mL serum-free RPMI. Cells were gently teased and flushed from the spleen using two sterile 21-G needles and syringes and transferred to a 15 mL conical centrifuge tube. Cells were centrifuged at 250 *x g* for 5 min and washed twice with 10 mL serum-free RPMI. After the second centrifugation, red blood cells were lysed by treating the cells with 4 mL 0.8% NH_4Cl for 3 min. Cells were centrifuged at 250 *x g* for 5 min, resuspended in 10 mL serum-free RPMI, counted and immediately used for fusion.

2.3.8.4 Fusion of splenocytes and myeloma

Splenocytes (1.8×10^8) and myeloma (1.5×10^7) were combined to give a cell ratio of 12:1, respectively. The mixture was gently mixed for about 1 min and centrifuged at 800 *x g* for 5 min. A 1 mL aliquot of 50% PEG 1500 in HEPES buffer was added to the cells, with gentle mixing over a 1 min period at room temperature and stirred for an additional 1 min. A 1 mL volume of serum-free RPMI was added slowly over the next min and an additional 9 mL over the next 3 min. The cell suspension was centrifuged at 300 *x g* for 5 min and the supernatant decanted. Cells were resuspended in 50 mL HAT medium and plated out as 150 µL aliquots in 8 sterile microtiter plates. Control cells (myeloma) were added to 12 wells.

2.3.8.5 Culture, screening, and selection of hybridoma

Fused cells were incubated at 37 °C with 7% CO_2 and at 90-100% relative humidity. Care was taken not to disturb cell clusters so that an estimation of the number of hybridoma/well could be made. Five days after fusion, 100 µL HAT medium was added to each well. On day six, supernatant from each well was checked for Ab by incubating 20 µL aliquots on microtiter plates that had been previously coated with BSA-H-9 (1 µg/mL) in PBS and blocked and washed as described for mouse sera titer determinations (see section 2.3.3.1). Incubations and all remaining steps of titer determination were also followed but without serial dilutions. The relative Ab activity was expressed as the absorbance (A_{450nm} - A_{660nm}) measured after 30 min at room temperature with peroxidase substrate solution.

On day six, thirty-eight wells that had relative Ab activities of greater than 0.5 were tested for competition in an EI. This was performed as described above for relative Ab activity except 5 µL aliquots of supernatants were incubated with 10 µL 0.1% BSA in PBST and 5 µL of either 0 (blank), 0.02 or 2 µM
α-chaconine in methanol. Results were expressed as A/A_0.

On day nine, 50 µL HAT medium was added to each well. On the tenth day cells from wells that had shown the best competition (absorbance greater than 0.5 when incubated with methanol, but A/A_0 less than 0.15 when incubated with 2 µM α-chaconine) were subcultured (0.02/0.5 dilution)

with HT medium and grown in 24-well microtiter plates. Cells were subcultured (0.5/1 dilution) using HT medium on days 17 and 22.

2.3.8.6 Cell cloning

Beginning twenty-five days after the fusion, cells were cloned using the following method based on limiting dilution in soft agar.

Conditioned medium was prepared by growing myeloma cells in complete RPMI containing 20% calf serum for 2-4 days, centrifuging the cell suspension at 250 *x g* for 10 min and passing the supernatant through a 0.2 µm filter. This 'spent' medium was then fortified with 20% fetal calf serum and 1% 200 mM glutamine.

A 2.4% (w/v) solution of agarose was prepared by suspending agarose (2.4 g) in 100 mL 0.15 M NaCl, heating to 70 °C until dissolved. It was distributed into ten 20 mL scintillation vials, capped, autoclaved and stored at 5 °C until required.

On the day of cloning, serial dilutions of the uncloned cells were made (0.5 mL/1 mL) using conditioned medium in six sterile polypropylene tubes with snap caps and mixed thoroughly after each dilution. The 2.4% agarose solution was melted in a hot water bath at 70 °C and 2.5 mL added to 10 mL conditioned medium to give a 0.48% agarose solution in conditioned medium (soft agar). Aliquots (2 mL) of soft agar were immediately placed into six additional polypropylene tubes and to each of these was added 100 µL from each serial dilution of cells. These were thoroughly mixed on a vortex mixer and dispensed (0.5 mL/well) into a 24-well microtiter plate so that each column in the microtiter plate contained a different serial dilution of cells. Cells in soft agar were incubated at 37 °C with 7% CO_2 and 90-100% humidity.

After 10 days growth clones were selected from the soft agar using an inverted microscope placed in a laminar flow hood. This was done using a mouth-pipeting system that consisted of a sterile 25 µL disposable borosilicate micropipet (Fisher), pipet/hose adapter, sterile tubing (3 mm bore), two 0.22 µm filter units and a mouth piece. Selected cells were immediately placed into 200 µL complete RPMI containing 20% FetalClone

in wells of a sterile 96-well microtiter plate. These were incubated at 37 °C with 7% CO_2 and 90-100% humidity and subcultured every 2-4 days, using 1/10 to 1/20 dilutions so that cell density remained at 10^5 to 10^6 cells/mL. Supernatants were screened and cell lines selected as described for uncloned hybridoma (see section 2.3.8.5).

The cloning procedure was performed twice to ensure stability of the cell line. MAb from cloned cells was isotyped using a Mouse Monoclonal Ab Isotyping Kit (Sigma).

2.3.8.7 Freezing, storage and thawing of cell lines

On the day prior to freezing, cells were subcultured (1/10 dilutions) into fresh complete RPMI containing 20% calf serum. On the day of freezing, approximately 10^7 cells were centrifuged at 250 *x g* for 5 min, resuspended in 1 mL 8% dimethylsulfoxide in calf serum and 0.5 mL aliquots were placed into two freezing vials. These were wrapped in several layers of paper towel and placed at -70 °C overnight. The following day cells were transferred to a liquid N_2 storage tank (-185 °C).

Cells were thawed quickly after removal from liquid N_2 in a 37 °C water bath. Immediately after thawing, cells were asceptically transferred to a centrifuge tube containing 10 mL complete RPMI with 20% calf serum, centrifuged at 250 *x g* for 5 min, the supernatant removed and cells resuspended in complete RPMI with 20% calf serum.

2.3.8.8 *In vitro* Production of MAb

Media used for hybridoma growth included complete RPMI with 20% FC, complete RPMI with 5% FC, serum-free RPMI with 1% Nutridoma and PFHM II in either 10 mL or 100 mL volumes. Prior to growth in either serum-free RPMI with 1% Nutridoma or PFHM II, cells were washed twice using 2 x 10 mL volumes of the new medium and centrifuged at 250 *x g* for 5 min after each wash. Cells were incubated at 37 °C with 7% CO_2 and at 90-100% relative humidity.

Several media were compared for their ability to support cell growth and MAb production in 2 x 100 mL volumes over 3 day periods. Cell growth

was determined by measuring the viable cell densities (see 2.3.8.2) and the results were expressd using the following ratio:

$$\text{Cell Growth} = \frac{(\text{viable cell density})_{final}}{(\text{viable cell density})_{initial}}$$

Relative MAb activity was measured similarly as described for hybridoma screening and selection, however, serial dilutions of tissue culture supernatants from 1/10 to 1/100,000 were made and 100 µL aliquots of each was added to 100 µL aliquots of methanol in the wells of coated microtiter plates (1 µg/mL BSA-H-9).

2.3.8.9 MAb purification

Tissue culture cell suspensions (2 x 100 mL) were collected and centrifuged at 300 *x g* for 10 min to remove hybridoma. The supernatants were centrifuged at 3000 *x g* at 5 °C for 30 min to remove any remaining cells and insoluble protein. Ammonium sulfate (62 g/200 mL liquid) was then added slowly (over 15 min) to obtain 50% saturation and left to stir at 5 °C overnight. The mixture was centrifuged at 3000 *x g* at 5 °C for 30 min to collect protein precipitated by 0-50% saturated ammonium sulfate. Further fractions (i.e. 50-60% and 60-80%) were also collected to check for MAb that were not precipitated by the 0-50% saturated ammonium sulfate fraction. Each fraction was dialyzed against 3 x 1000 mL PBS at 5 °C for 18 h. Dialyzed protein samples were made up to 25 or 50 mL with PBS and tested for protein concentration and relative MAb activity. Protein concentration was determined by measuring the absorbance at 280 nm (A_{280nm}) against a blank (PBS) and using the following equation (Harlow and Lane, 1988):

$$[\text{protein}], (\text{mg/mL}) = A_{280\,nm} \times (1\ \text{mg/mL} / 1.35)$$

The purities of fractions collected from cell supernatants were checked using SDS-PAGE. These fractions were applied to the gels at 8X the concentration of cell culture supernatants. Further reference to "purified MAb preparations" specify the ammonium sulfate precipitated fraction (0-50%) from 1G9-4-5 supernatant after 3 days growth in serum-free RPMI with 1% Nutridoma.

2.3.8.10 Effect of freeze-drying on MAb activity

Purified MAb preparation, at approximately 1 mg/mL in PBS, was dispensed in 100 µL aliquots into 6 microcentrifuge tubes, covered using filter paper and frozen to -70 °C. Three samples were freeze-dried and then rehydrated with the addition of 100 mL PBS to each sample. The remaining three frozen samples were thawed at room temperature for 1 h. Serial dilutions of all six samples were made and the relative MAb activities were determined as described above (see section 2.3.8.8).

2.3.8.11 Effect of pH during competition

The effect of pH during the competition was studied by adjusting the pH of the PBST used to dilute MAb. Solutions of HCl or NaOH of the same ionic strength as the PBST (0.16 M) were used to achieve several PBST buffers having pH values of 6, 7, 8, 9 and 10. Inhibition curves using 100 µL α-chaconine in methanol (0-100 µM) were determined using plates coated with 0.2 µg/mL BSA-H-9 and purified MAb preparation applied at 1 µg/mL in 0.05% BSA in PBST (pH 6, 7, 8, 9 or 10). Washing and peroxidase activity were done as described for previous assays (see section 2.3.8.8).

2.3.8.12 Effect of single Ab incubation

Microtiter plates were coated using BSA-H-9 (0.2 µg/mL), blocked and washed as described above (see section 2.3.5). GA standards in methanol (100 µL/well) were added, followed immediately by 100 µL of a solution containing 1 µg/mL purified MAb and a 1/3000 dilution of anti-mouse Ab-peroxidase conjugate in 0.05% BSA in pH 7.3 PBST. These were incubated together with shaking and humidity for 2 h at room temperature. Washing and peroxidase activity was done as described previously (see section 2.3.5).

2.4 Modelling and Statistical Analysis

The equations for inhibition curves were determined using the Excel/Solver computer package (Microsoft Corp., Redmond, WA). The data collected for standard solutions were used to determine the four-parameters defining the following equation for a sigmoidal or four-parameter curve:

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

where **y** is the response measurement (i.e. B/B_0, A or A/A_0), **x** is the GA concentration, **a** is the **y**-intercept, **b** is the curvature parameter (i.e. slope at the inflection point), **c** is the concentration giving 50% reduction in **y** (also referred to as the I_{50} value) and **d** is the value of **y** at infinite (saturating) **x** (also referred to as background absorbance). The four parameters **a**, **b**, **c**, and **d** were estimated by minimizing the sum of squares of the observed data. Graphs were prepared by plotting the $\log_{10}$ of the GAconcentrations.

Statistics were performed using the Statview computer program (Abacus Concepts Inc., Berkley, CA). I_{50} values were analyzed (as log I_{50} values) using a single factor factorial ANOVA with significance levels of 95%. Comparisons of means were made using Fisher's Protected Least Significant Difference.

Chapter 3. Results and Discussion

3.1 Production of Protein-Solanidine Conjugates

The molecular weight of GA (<1,000) 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 a protein carrier. 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 crosslinking by the two oxidized sugars on solanine. Also, we believe that excess ethylene glycol 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. The method used by Vallejo and Ercegovich (1979) involved producing a hemisuccinate of solanidine and conjugating to BSA using a mixed anhydride method. These authors reported alkaloid to protein ratios of 4 to 1, determined using acid hydrolysis and titrimetric analysis. Conjugates having 8 to 25 groups per BSA equivalent are considered optimal for obtaining a good immune response (Erlanger, 1980).

Our approach was to start with α-chaconine rather than α-solanine for the production of immunogen through a glycosidic linkage. 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, 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. BSA and LPH conjugates containing up to 23 and 14

molecules of alkaloid per BSA equivalent, respectively, were obtained (Appendix I).

The production of conjugates through a hemisuccinyl group was also accomplished, yielding BSA-solanidine conjugates containing 9 molecules of alkaloid per BSA. This method, used here for the production of the solid-phase conjugates, is relatively rapid and simple to perform.

3.2 Polyclonal Serum in the Quantitation of Solanidine Alkaloids

In order to use Ab effectively in competitive EI, the Ab should have similar affinity for the solid-phase GA as it does for the soluble GA originating from the sample. Bridge heterology (the use of 2 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 an ester linkage, using BSA as protein. Ester linked conjugates were prepared by producing solanidine hemisuccinate from solanidine, and through an active ester condensation, linking to BSA.

The PAb 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) compared with using the glycosidic linking arm (approximately 1/2,000,000). Because the PAb was raised against a conjugate containing a glycosidic linking arm, LPH-G-14, one might expect the PAb to have a greater affinity for the same type of solid-phase. Surprisingly, the PAb showed a greater affinity for the alkaloid linked

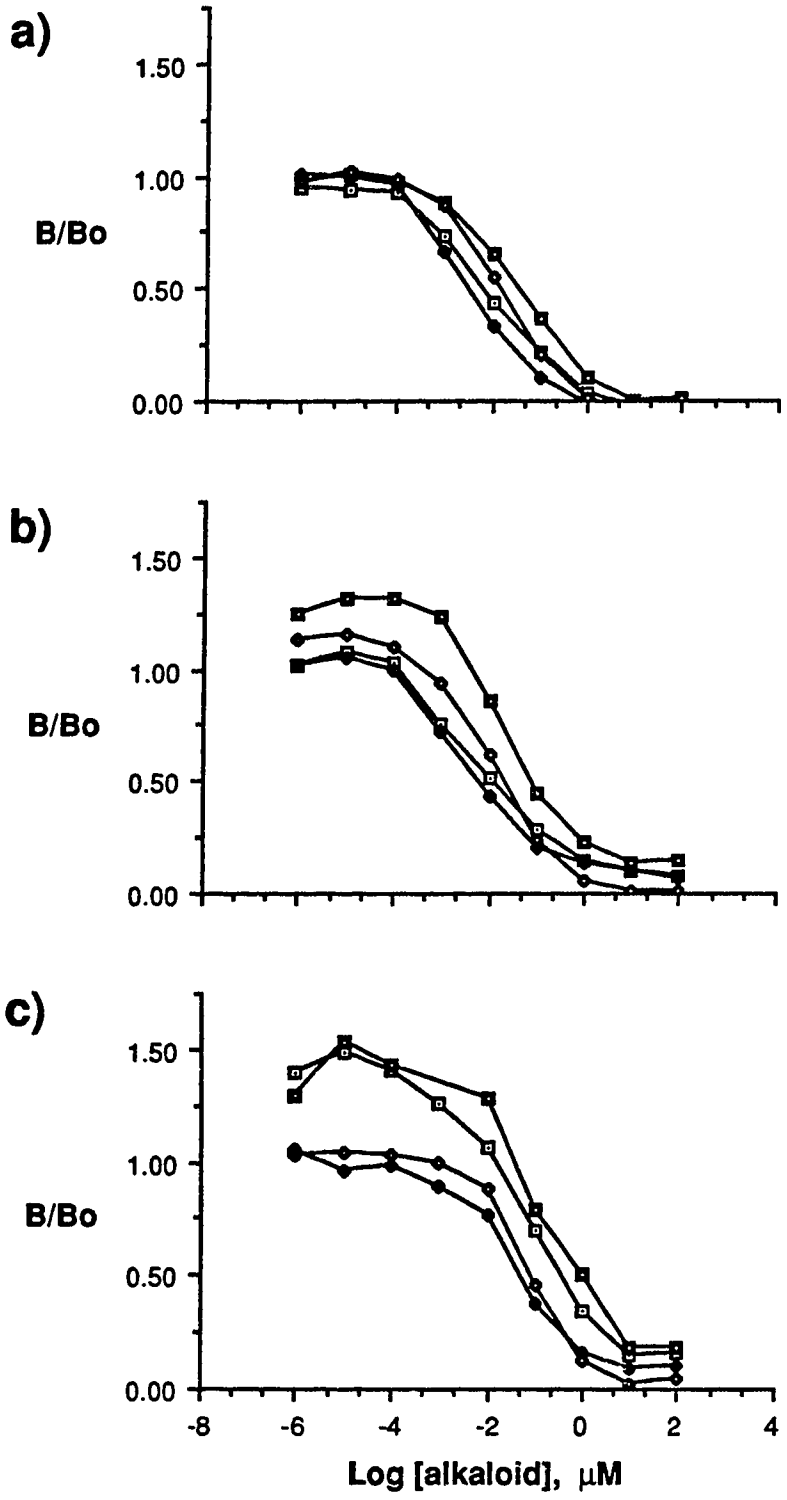
in a different way than the immunization conjugate, regardless of the ratio of GA to BSA. One explanation may be that the hemisuccinyl group contains a flexible methylene chain, allowing favorable orientation of the GA within the Ab binding sites. Another reason may relate to differences in the ability of the conjugates to bind to the microtiter plates.

Figure 11 compares the inhibition curves when the three solid-phase conjugates were used in competitive EI with PAb using α-chaconine or its aglycone, solanidine. Conjugates were applied to the microtiter plates at a concentration, determined by the checkerboard to give a value of B_0 of approximately 0.2 to 0.3 absorbance units when the serum dilution was 1/100,000. This was achieved when plate coating was performed using 0.08 µg/mL, 2.0 µg/mL and 0.40 µg/mL for BSA-H-9, BSA-G-6 and BSA-G-23, respectively. The use of B/B_0 (corrected using a pre-immunization blank) rather than A/A_0 (uncorrected) reduced background absorbance somewhat for all conjugates. Only the BSA-H-9 conjugate gave zero background absorbance when corrected using a pre-immunization blank. 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 or BSA-G-23 were used as solid-phase conjugates. This may indicate that some of the PAb had an affinity for the linking arm. Using a linking arm during the assay that is different from that used during the immunization therefore allows the PAb to be more sensitive to soluble GA (containing glycosidic groups) in the analyte. It may also be possible that avidity effects account for the differences between solid-phase conjugates because comparisons were made using different coating concentrations.

The ester-linked conjugate was found to be easier to make, 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 conjugates. For these reasons, further experimentation was performed using the ester linked conjugate, BSA-H-9.

The final step of the EI system used in this study is the determination of peroxidase activity. Enzyme concentration is proportional to the initial rate of

Figure 11. Competitive EI using PAb for the analysis of α-chaconine (squares) and solanidine (circles) in solution. Inhibition curves were performed using a) 0.08 µg/ml BSA-H-9; b) 2.0 µg/ml BSA-G-6; and c) 0.4 µg/ml BSA-G-23. Sera were diluted 1/100,000. Solid or empty symbols represent the two rabbit sera used. Adapted from Plhak and Sporns, (1992).



the reaction in the presence of excess substrate (Cornish-Bowden, 1979). Measurement of the reaction end-point, however, rather than initial rate, is easier to perform and can be valid in some circumstances. In order to make sure that the incubation time of 30 min (recommended by the manufacturer) was optimum and/or crucial, optical densities were measured every 5 min after the addition of substate solution and inhibition curves compared. The results (Figure 12) indicate that after at least 10 min and up to 30 min, the A/A_0 values were not greatly affected by enzyme reaction time even though the rate was no longer the initial or maximal rate (Figure 13). The incubation time of 30 min was maintained to maximize absorbance readings and therefore maximize the signal to noise ratio. Measurement at this time (i.e. when the reaction rate has decreased) also reduces the variation within each microtiter plate due to the reading time of the instrument (i.e. it takes about 120 sec to read an entire microtiter plate, column by column).

The effect of solid-phase concentration and PAb concentration in the EI is illustrated in Figure 14. It appears from these data that both sensitivity and the amount of background absorbance is affected by both the solid-phase conjugate concentration and PAb concentration. Because the type of EI used here is an indirect competitive assay, it would be expected that non-saturating levels of both of these components would increase sensitivity. The use of a low PAb concentration would decrease interference from low affinity Ab present in the serum, thus decreasing background absorbance. Limiting Ab concentrations would also allow competition between solid-phase and soluble GA for the Ab binding sites to occur at lower concentrations of soluble GA. The use of lower concentrations of solid-phase conjugate would be expected to decrease avidity effects, thus allowing soluble GA to compete more effectively and therefore at lower concentrations. Sensitivity and accuracy also depends on the signal to noise ratio, and for this reason there is a practical lower limit of solid-phase conjugate or PAb concentration. The use of BSA-H-9 at 0.2 µg/mL and PAb diluted to 1/100,000 is considered to be optimum for this EI because the A_0 was approximately 0.5, background absorbance was minimized and sen[illegible] maximized.

Inhibition curves, constructed [illegible] to is GA and PAb, are illustrated in Figure 15. Four-parameter sig[illegible] [illegible]ons were computed and I_{50}

Figure 12. Effect of peroxidase reaction time on inhibition curves for α-chaconine. Plates were coated using 1 µg/mL BSA-H-9 and serum was diluted 1/50,000. Each point is the average of 6 determinations.

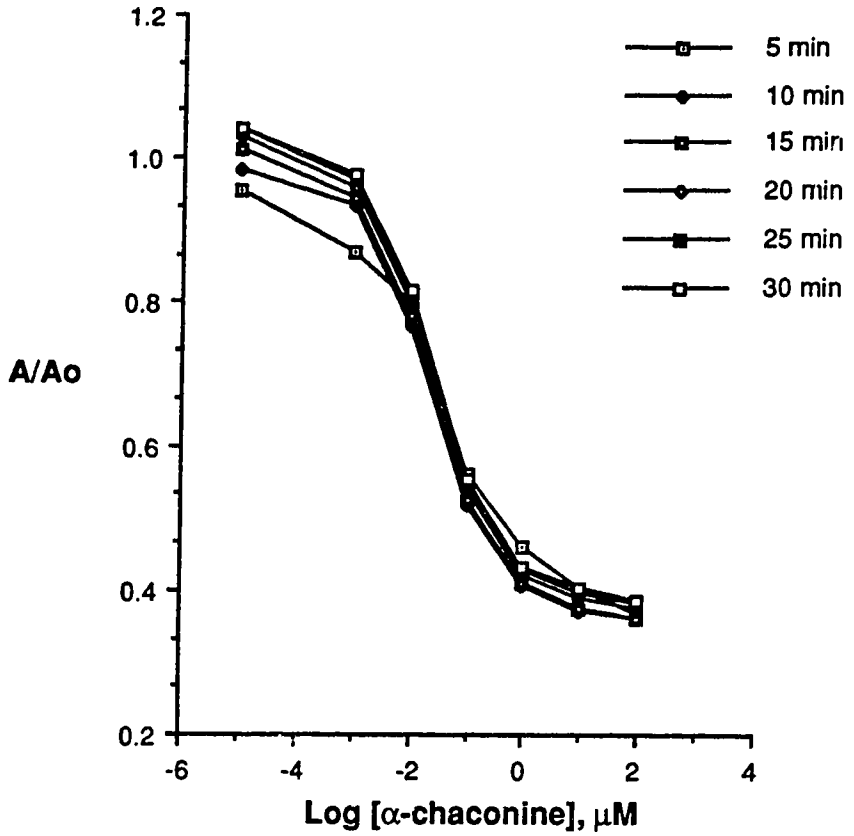


Figure 13. Reaction profile for bound peroxidase in EI. Conditions are the same as those specified in Figure 12. Each point is the average of 6 determinations.

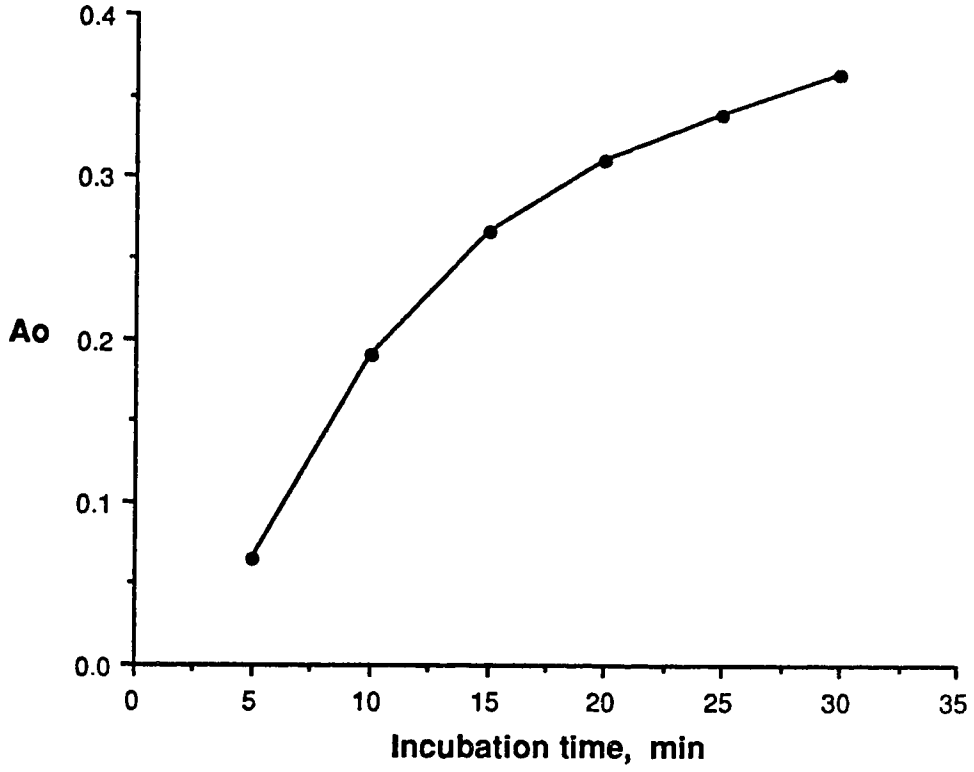


Figure 14. Effect of solid-phase coating concentration and serum dilution on inhibition curves used for EI. Each point is the average of 6 determinations. The legend indicates the concentration of BSA-H-9 (μg/mL) used, and the serum dilution used (in parentheses).

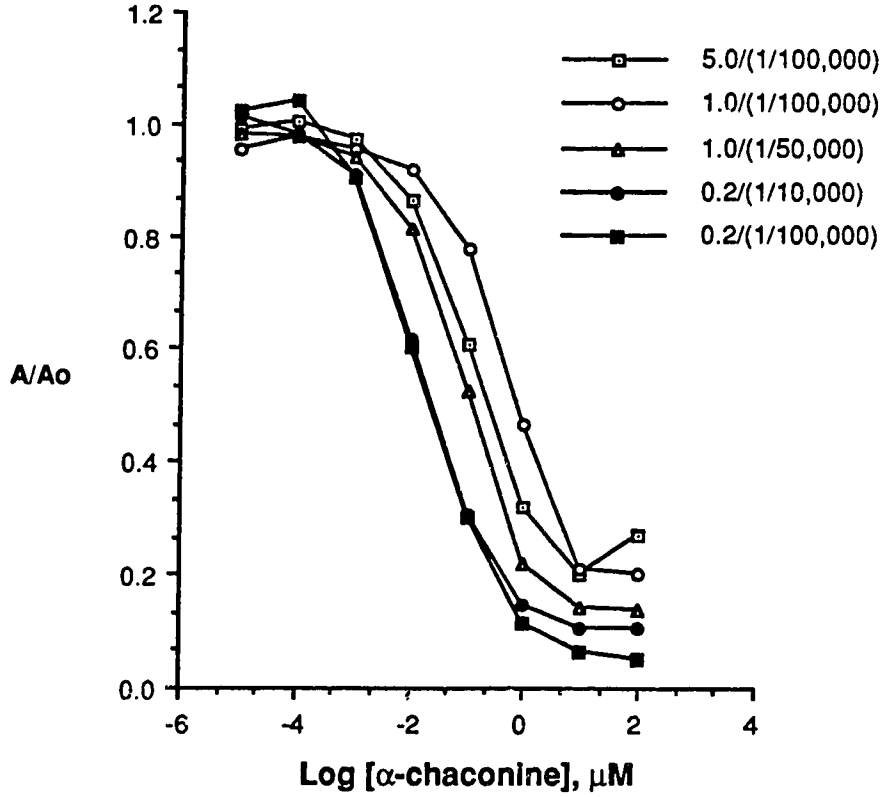
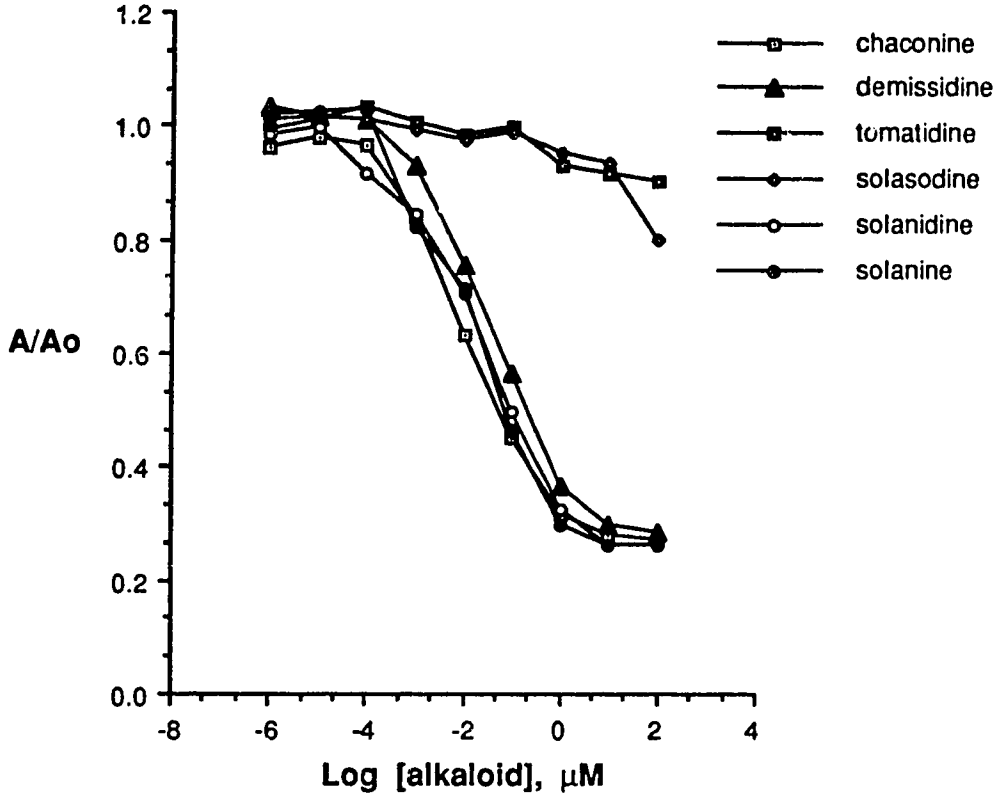


Figure 15. Inhibition curves using various GA and PAb. Plates were coated using 0.2 µg/mL BSA-H-9 and serum was diluted 1/100,000. Each point is the average of 5 determinations.



values calculated. PAb cross-reactivity data is shown in Table III. The PAb showed very little affinity for the two spiral alkaloids tested, tomatidine and solasodine, demonstrated 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. The solanidine GA, α-solanine and α-chaconine showed similar ability to compete (I_{50} values were not significantly different, $P>0.05$). These two GA make up the majority of GA in commercially grown potatoes. The PAb showed a slightly lower affinity for solanidine a... demissidine (significant, $P<0.05$). Demissidine is saturated at C_5 and solanidine does not have a glycosidic group at C_5. These portions of the molecules would be expected to be somewhat distant from the Ab binding site and this may explain why the I_{50} values are only slightly higher than those of α-solanine and α-chaconine.

The analyte coefficient of variation (CV) in EI is very high for extremely low and extremely high values of concentration (Bunch et al., 1990). In order to determine which part of the curve has the lowest error, EI analysis of standard solutions was performed repeatedly and the sigmoidal calibration curves calculated. The solid-phase conjugate, BSA-H-9, was used at 0.2 µg/mL and PAb was used as a 1/100,000 dilution. The predicted concentration values of the standard solutions were calculated (i.e. using the curve equations generated) and the % CV for predicted values was plotted against the $\log_{10}$ of the concentration (Figure 16). This revealed that the EI was most accurate (10 to 20% CV) between 0.01 to 1 µM GA. All determinations of sample extracts were therefore made using sample dilutions that gave at least 2 results in this range. Depending on the sample, total sample dilution ranged from 1/100 to 1/25,000.

Table IV compares the results when potato samples were analysed by our EI (UofA EI), HPLC and the Holland Biotechnology (HB ELISA) EI. These samples were chosen to give a wide variation in GA concentration and are arranged in ascending order in the table. Correlation between the two immunological methods, UofA EI and HB ELISA, was good ($r=0.971$). Correlation between the immunological methods and HPLC, when only commercial potato tubers were used, was also good ($r=0.978$). The leaf extract from *S. demissum* (sample g) was the only sample analysed that

Table III. I_{50} values for various steroidal alkaloids using PAb in EI.
The I_{50} values are the concentration of GA (μM) giving 50% reduction in A/A_o (curves are illustrated in Figure 15).

Steroidal Alkaloid*	I_{50} , (μM)
tomatidine	> 100
solasodine	> 100
demissidine [ab]	0.044
solanidine [ab]	0.040
α-solanine [bc]	0.026
α-chaconine [c]	0.019

* letters in superscript indicate groupings based on statistical comparisons using a 95% level of significance (P = 0.05). Comparisons were made of $\log_{10}$ [GA], (mM).

Figure 16. Inhibition curve for α-chaconine in solution and the coefficient of variation (CV) associated with each average point. Each A_o point was the averageof 6 determinations. Adapted from Plhak and Sporns, (1992).

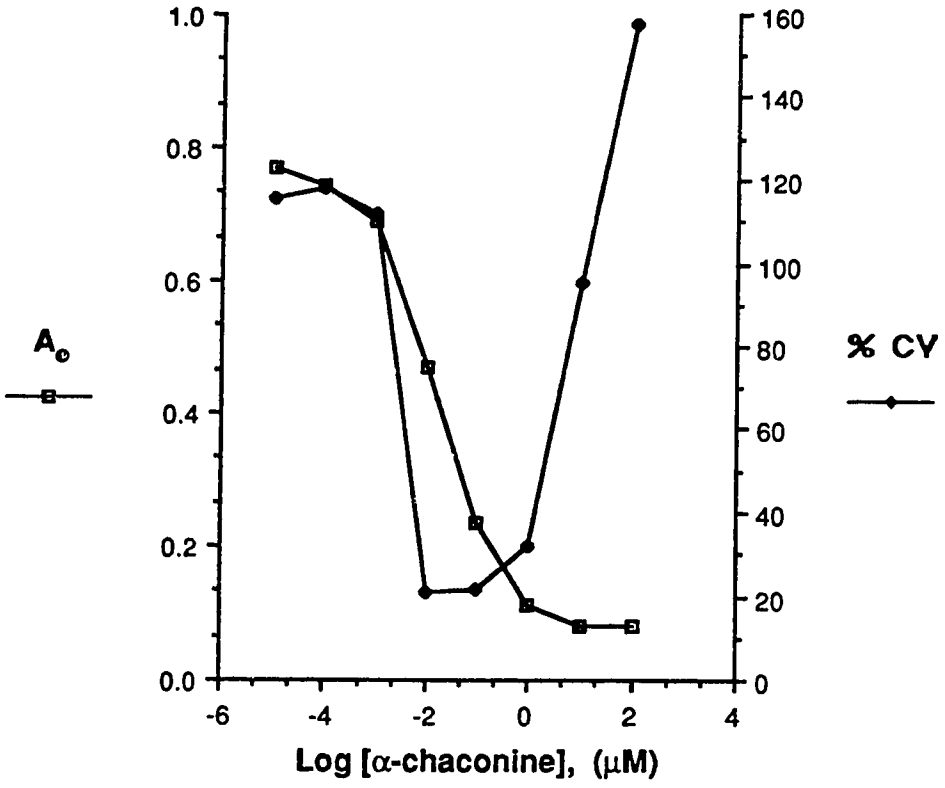


Table IV. Comparison of [GA] obtained by three methods. Adapted from Plhak and Sporns, (1992).

Sample *	[GA], (mg/100 g, fwb**)		
	UofA EI***	HB ELISA	HPLC
a	1.2 (47)	0.67(258)	0.97(175)
b	4.7 (30)	2.7 (8.5)	2.7 (24)
c	8.0 (24)	7.1 (27)	6.0 (14)
d	8.0 (21)	11 (29)	5.4 (4.6)
e	9.9 (33)	12 (15)	8.3 (10)
f	56 (18)	55 (5.8)	57 (13)
g	123 (11)	148 (21)	9.7 (79)

*See materials.

**Assumed 20% moisture for all samples. Values in parentheses are % CV. The number of determinations performed for the three methods used were 6, 3, and 6, respectively.

***UofA EI was performed using 0.2 μg/mL BSA-H-9, 1/100,000 serum dilution and α-chaconine as standard.

gave significantly less GA when analyzed by HPLC when compared with 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 analysed by EI. This revealed the presence of two non-uv-absorbing GA at 13 and 18 minutes (Figure 17). Although the identity of these two compounds was not confirmed, *S. demissum* has been reported to contain demissine or commersonine, demissidine substituted with lycotetraose and commertetraose, respectively (Schreiber, 1968; Van Gelder, 1991).

The % CV varied considerably between methods. For all methods, the variation was the greatest when GA concentration was low. The HPLC analysis of sample g, however, also exhibited a high % CV when demissidine GA were present. The average % CV for the three methods, UofA EI, HB ELISA and HPLC, were 26, 52 and 46 %, respectively.

3.3 Monoclonal Ab Production and Purification

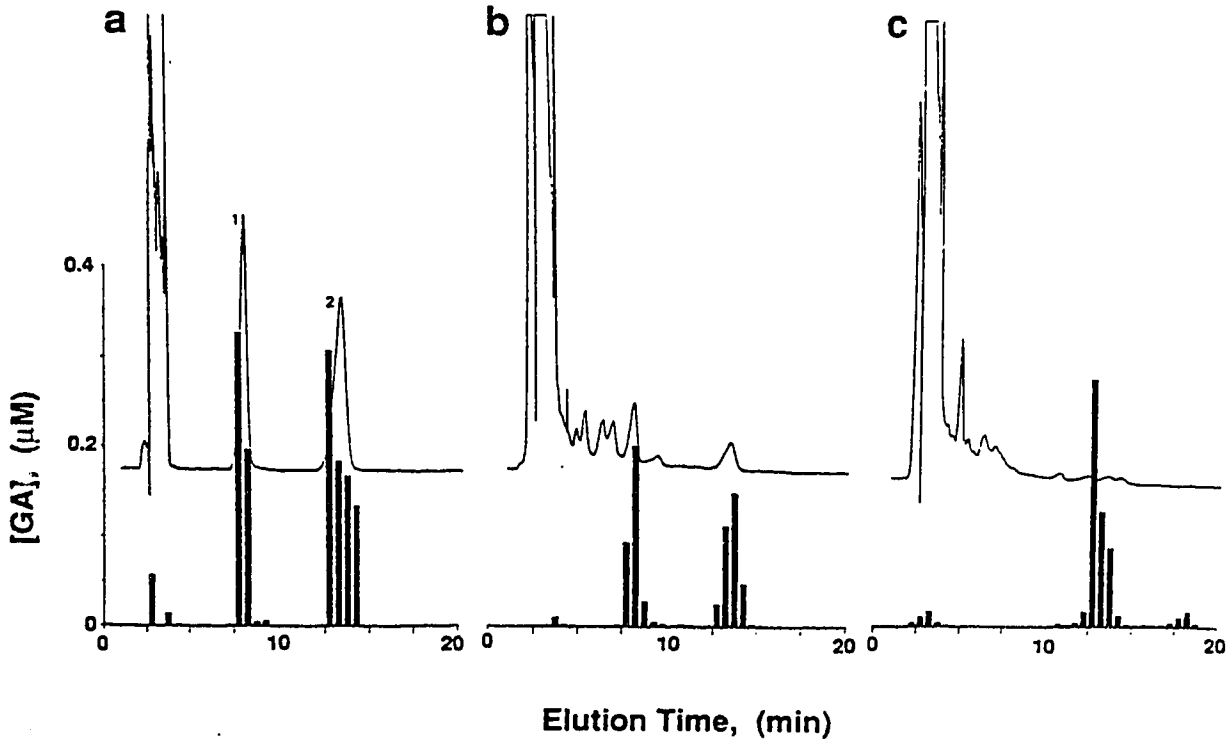
Fusion of splenocytes with myeloma gave rise to at least 10 hybridoma per well, therefore a total of approximately 5000 hybridoma. These actively grew in the presence of aminopterin while control cells (myeloma) did not. Six days after fusion, screening of supernatants revealed that all wells, except 35 of the 480 wells, contained cells that were producing Ab specific for solanidine alkaloids. Cells from 38 wells that produced an absorbance greater than 0.7 were tested for their ability to be competitively removed from the solid-phase in the presence of 0, 0.02 or 2 μM α-chaconine. Competitive inhibition results, expressed as A/A_0, are given in Appendix II. Of these, cells from wells 1G9, 2D11, 4D5, 5F3, 7F5, 8C10, 8D6 and 8G9 retained their ability to make Ab, as revealed by their relatively high values for A_0 and the Ab produced gave low A/A_0 values in the presence of 2 μM α-chaconine. They were therefore selected for further studies. Selected cell suspensions were subcultured into 24-well microtiter plates. Two days after subculturing, supernatants were tested and compared for Ab in the presence of α-solanine, α-chaconine, solanidine, demissidine or tomatidine (Appendix III). The best

Figure 17. HPLC chromatograms of steroidal alkaloids. Superimposed bar graph illustrates EI detection of aliquots collected. Adapted from Plhak and Sporns, (1992).

a) Standard solution containing 100 µM each of solanidine (eluting at solvent front), α-chaconine (peak 1) and α-solanine (peak 2).

b) *Solanum tuberosum* (commercial, sample b) tuber extract.

c) *S. demissum* leaf extract.



growth rate, measured as the percentage of cells covering the bottoms of the wells, was observed for cells originally in wells 1G9, 7F5, 8C10, 8D6 and 8G9. Ab production from cells in well 8C10, however, had declined, as indicated by the relatively low value for A_0. Ab produced from the actively-growing cells did not seem to differ greatly in their ability to recognize the various alkaloids tested. Further screening, up to 21 days later, revealed that the only Ab-producing cells were in wells 1G9, 7F5, 8D6 and 8G9 (Appendix IV).

During cloning, only cells from 1G9, 7F5 and 8G9 survived in the soft agar. Only cells from 1G9 and 8G9, however, continued to produce MAb after cloning. Each of these cell lines were cloned twice and several cell lines were obtained. Of the clones that produced MAb, one clone, 1G9-4-5, was found to be the most vigorous grower and produced a relatively high titer of MAb. Isotyping revealed that 1G9-4-5 cells produced IgG1. This cell line was used in the following studies.

In the large-scale production of MAb *in vitro*, it is desirable to use a growth medium that not only supports growth and MAb production, but also makes downstream MAb purification simple. The use of protein-free or Ig-free products was therefore of interest. FetalClone (FC) is an ultrafiltered bovine serum product that does not contain Ig. Nutridoma is a serum replacement that contains insulin, transferrin, BSA, low density lipoproteins from bovine plasma, vitamins, amino acids and other small organic molecules. The working concentration recommended by the manufacturer is 1% Nutridoma. The effect of 5% or 20% FC and 1% Nutridoma in serum-free RPMI on cell growth and MAb production was evaluated when cells were grown in 10 mL volumes. The results (Table V) showed that complete RPMI with 20% FC gave a slightly higher growth rate and MAb production than complete RPMI with 5% FC. 1% Nutridoma did not support cell growth, however, MAb production was not reduced to any great extent during the two day period of culture.

In a later study (Table VI), cells were grown in 100 mL volumes, and cell growth and MAb production was evaluated. Cell growth was greatest when grown in complete RPMI with FC. MAb production, however, was greatest when growth was limited, in the protein-free used (PFHM II) or in 1%

Table V. Cell Growth and MAb production in serum-containing and serum-free culture media using 10 mL volumes during 2 days of culture.

Measurement	Media Used*		
	Complete RPMI with 5% FC	Complete RPMI with 20% FC	Serum-Free RPMI with 1% Nutridoma
initial cell density (cells/mL)	1.6×10^5	1.3×10^5	1.5×10^5
cell density, 2 days (cells/mL)	3.6×10^5	3.8×10^5	1.6×10^5
cell growth**	2.25	2.92	1.07
relative Ab activity of culture supernatant, 3 days, 1/100 dilution (A_o)	1.34	1.75	1.48

* All media also contained added oxaloacetic acid, sodium pyruvate, sodium glutamate and penicillin/streptomycin (see materials).

**Cell growth is the ratio of the final cell density/initial cell density.

Table VI. Cell growth and MAb production in various culture media using 100 mL volumes during 3 days of culture.

Measurement	Media Used*		
	Complete RPMI with 20% FC	PFHM II	Serum-Free RPMI with 1% Nutridoma
initial cell density (cells/mL)	2.8×10^5	1.4×10^5	2.8×10^5
cell density, 3 days (cells/mL)	6.5×10^5	1.5×10^5	2.6×10^5
cell growth**	2.32	1.07	0.93
relative MAb activity of culture supernatant, 3 days, 1/100 dilution (A_{450nm} - A_{660nm})	1.35	2.66	2.75
relative MAb activity of $(NH_4)_2SO_4$) purified sample, (0-50% fraction/1000 mL) (A_o)	1.08	1.14	1.22
total protein purified from 200 mL supernatant (mg)	98	25	15
specific MAb activity*** (A_o/mg protein)	0.011	0.046	0.081
MAb production (µg/mL/day)	327 (impure)	50	83

* All media also contained added oxaloacetic acid, sodium pyruvate, sodium glutamate and penicillin/streptomycin (see materials).

**Cell growth is the ratio of the final cell density/initial cell density.

***Specific MAb activity is the relative MAb activity (0-50% fraction/1000 mL) / mg protein collected.

Nutridoma in serum-free RPMI. Hayter et al. (1992) also found that serum-reduced media resulted in reduced growth rate and increased MAb production and related this to the percentage of cells in the G1 and G2 phases of the cell cycle.

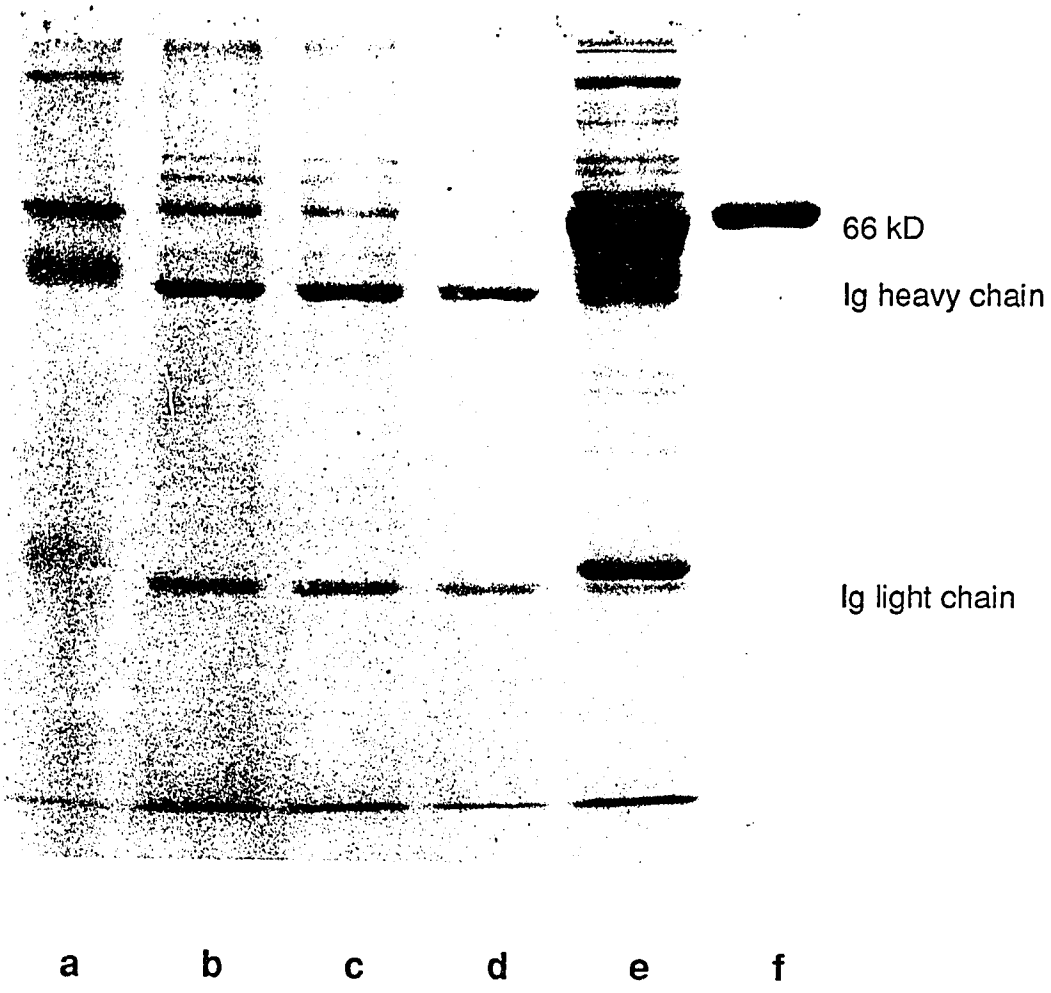
The supernatants from the above experiments were collected and purified using ammonium sulfate precipitation and dialysis. Fractions were collected using 0-50%, 50-60% and 60-80% saturated ammonium sulfate. Gel electrophoresis (Figure 18) revealed that the MAb preparations (0-50% or 50-60% fractions) obtained from cell growth in either PFHM II or in serum-free RPMI with 1% Nutridoma were relatively free from contaminating proteins while the 0-50% fraction obtained from growth in complete RPMI with 20% FC contained many of the serum proteins. Contamination with non Ig protein is presumably the cause for the low specific MAb activity that was found for this sample in Table VI. A commercially obtained bovine IgG standard showed less defined bands for the heavy and light chains than our murine MAb preparations purified from serum-free media. This bovine standard is from plasma and therefore would be expected to be a PAb preparation.

The 0-50% saturated ammonium sulfate fractions contained the majority of relative MAb activity, however, the 50-60% fractions also contained MAb. The relative MAb activities of these 50-60% fractions were approximately half of those for the 0-50% fractions. The relative MAb activities in the 60-80% fractions were very low, indicating that the Ig can be precipitated easily by 0-60% ammonium sulfate. The advantage of using serum-free media to grow hybridoma is to be able to use this simple purification procedure and obtain a relatively pure MAb preparation.

Freeze-drying was not found to significantly ($P>0.05$) decrease the relative MAb activity of purified samples. Samples appeared to rehydrate very quickly after drying. While freeze-drying in these small aliquots (0.1 mL) and at this protein concentration (1 mg/mL) had no effect, further studies should be made to test the effect of volume and concentration on the ability to freeze-dry purified MAb.

Figure 18. SDS-PAGE of MAb fractions collected after ammonium sulfate precipitation.

a) Bovine IgG (Calbiochem Corp.)
b) MAb from cells grown in PFHM II, 0-50% saturated $(NH_4)_2SO_4$.
c) MAb from cells grown in serum-free RPMI with 1% Nutridoma, 0-50% saturated $(NH_4)_2SO_4$.
d) MAb from cells grown in serum-free RPMI with 1% Nutridoma, 50-60% saturated $(NH_4)_2SO_4$.
e) MAb from cells grown in complete RPMI with 20% FC, 0-50% saturated $(NH_4)_2SO_4$.
f) BSA.



3.4 Monoclonal Ab in the Quantitation of Solanidine Alkaloids

Supernatant from 1G9-4-5 cells after 3 days growth in PFHM II was collected and used to compare the three solid-phase conjugates in competitive EI (Figure 19). Conjugates were applied to the microtiter plates at a concentration, determined by a checkerboard EI, to give a value of A_0 of approximately 0.3 when the supernatant dilution was 1/5. This was achieved when plate coating was done with 0.05 µg/mL, 2.0 µg/mL and 0.20 µg/mL for BSA-H-9, BSA-G-6 and BSA-G-23, respectively. The background absorbances were not found to be significantly different when the different solid-phase conjugates were used. Unlike the results obtained using PAb, there appears to be no affinity for the linking arm, as evidenced by relatively low background absorbances. Similar to the results obtained for PAb, however, high absorbances (higher than A_0) at very low concentrations seemed to be a problem for the conjugates linked through glycosidic bonds. For this reason, further experimentation was done using the ester linked conjugate, BSA-H-9.

MAb titer was determined using purified MAb preparation (see section 2.3.8.9) and microtiter plates coated with BSA-H-9 (1 µg/mL) . The titer was found to be 10 ng/mL.

The EI was optimized for solid-phase and MAb concentrations by constructing inhibition curves using combinations of BSA-H-9 at 0.04, 0.2 and 1 µg/mL and purified MAb preparation at 0.01, 0.1 and 1 µg/mL. The inhibition curves in Figure 20 illustrate the effect of solid-phase concentration in the EI when MAb was used at a concentration of 1 µg/mL. Lowering the amount of coating conjugate from 1 µg/mL to 0.2 µg/mL improves the sensitivity of the assay, apparent from the shifting of the curve to lower concentrations of α-chaconine. This would be expected to reduce any avidity effects resulting from Ag multivalency on the solid-phase.

High absorbance values (higher than A_0) at low concentrations of α-chaconine was always apparent for inhibition curves constructed using 0.04 µg/mL BSA-H-9, regardless of the MAb concentration, or using 1 µg/mL BSA-H-9 unless MAb was at 0.01 µg/ml. This phenomenon is therefore not only

Figure 19. Competitive EIA using MAb for the analysis of α-chaconine in solution. Inhibition curves were performed using 0.05 µg/mL BSA-H-9 (squares), 2 µg/mL BSA-G-6 (triangles) and 0.2 µg/mL BSA-G-23 (circles) as solid-phase conjugates. MAb was a 1/5 dilution of 1G9-4-5 supernatant. Each point is the average of 3 determinations.

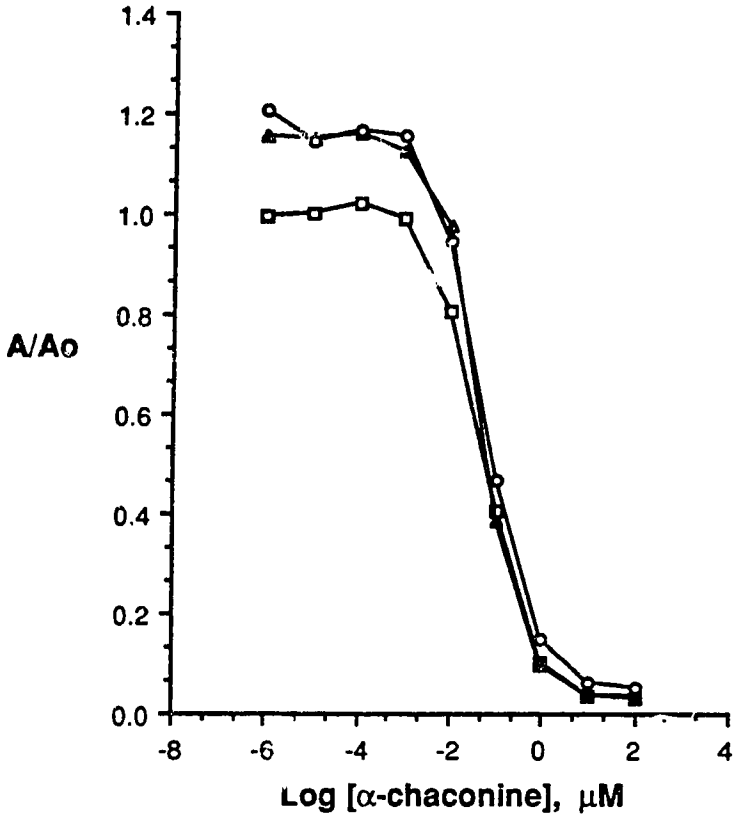
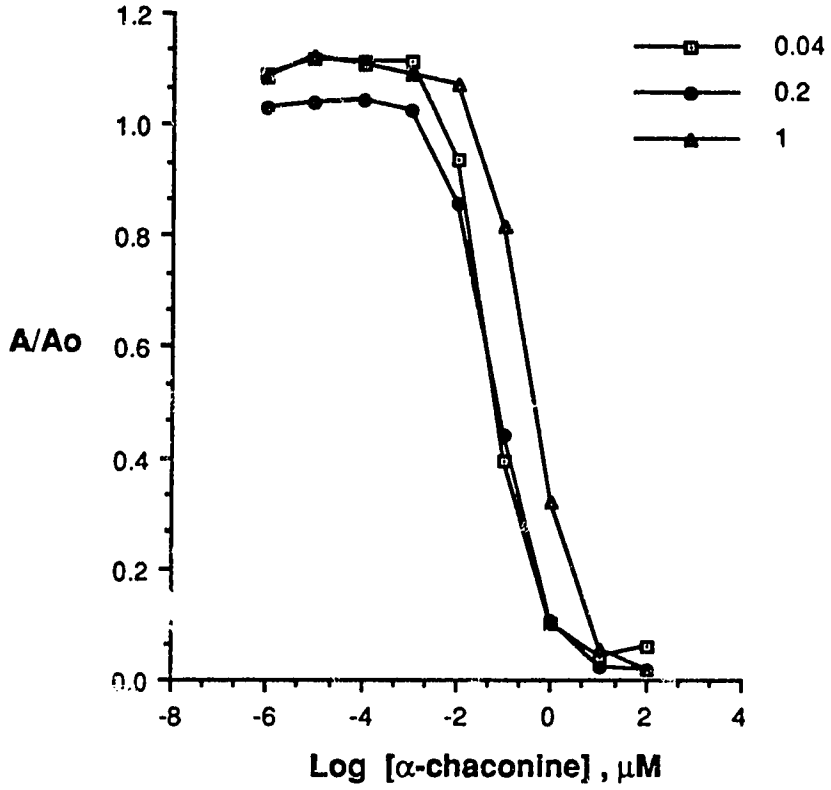


Figure 20. Effect of solid-phase conjugate concentration on inhibition curves. MAb was used at 1 µg/mL. Legend indicates concentration of solid-phase conjugate in µg/mL. Each point is the average of 2 determinations.



related to the type of solid-phase conjugate (i.e. linking arm) used in the assay but also to its concentration and the amount of Ab present.

MAb concentration, when tested from 0.01 to 1 µg/mL, did not affect the sensitivity of the assay, at least when using 0.04 to 1 µg/mL BSA-H-9 for the solid-phase (Figure 21). It did, however, affect the background absorbance when used at limiting concentrations (i.e. 0.01 µg/mL). This can be explained by the low absorbance values that would contribute to a low signal to noise ratio.

Inhibition curves, constructed using various GA and MAb, are illustrated in Figure 22. Four-parameter sigmoidal equations were computed and I_{50} values calculated. MAb cross-reactivity data are shown in Table VII. Similar to the results using PAb, the MAb showed very little affinity for the two spiral alkaloids tested, tomatidine and solasodine, determined by very high I_{50} values. Also, the affinity of MAb is greater for α-chaconine and α-solanine than for solanidine or demissidine alkaloids.

Figure 23 illustrates the effect of pH on inhibition using α-chaconine with MAb. The best sensitivity was obtained using PBST at pH 7. Changing the pH of the PBST from 6 to 10 caused an increase in the values for A_0 from 0.48 to 0.65 absorbance units, indicating that more MAb was binding to the solid-phase. The pH affects the dissociation state of both the GA in solution and the Ab and therefore affects the affinity constant. While a PAb preparation usually reacts over a wide range of pH, a MAb preparation may have a very defined and discrete pH optimum (Campbell, 1984). An increase in affinity between MAb and solid-phase GA may be one explanation for the reduced sensitivity of the assay at higher pH.

Solanidine GA contain secondary nitrogens that act as weak bases in solution. A change in pH will therefore change the relative amounts of unprotonated and protonated GA in solution. The pK of α-solanine at 15 °C is 6.66 (Merck and Co. Inc., 19[illegible]). Assuming that the pK of α-chaconine is similar to α-solanine, the relative amounts of the unprotonated and protonated forms of α-chaconine in a given solution can be calculated as a function of pH. Figure 24 illustrates this effect, calculated for a 1 µM aqueous solution. From this figure, it can be seen that at pH 6 or lower a greater

Figure 21. Effect of MAb concentration on inhibition curves. Plates were coated using 0.2 µg/mL BSA-H-9. Legend indicates concentration of MAb in µg/mL. Each point is the average of 2 determinations.

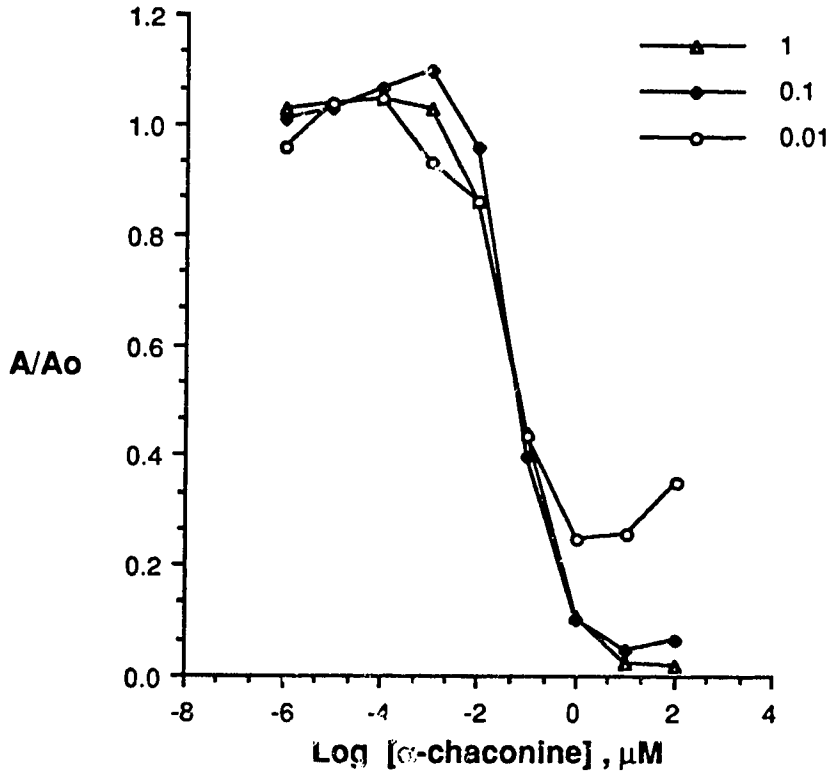


Figure 22. Inhibition curves using various GA and MAb. Plates were coated using 0.2 µg/mL BSA-H-9 and MAb was used at 1 µg/mL. Each point is the average of 6 determinations.

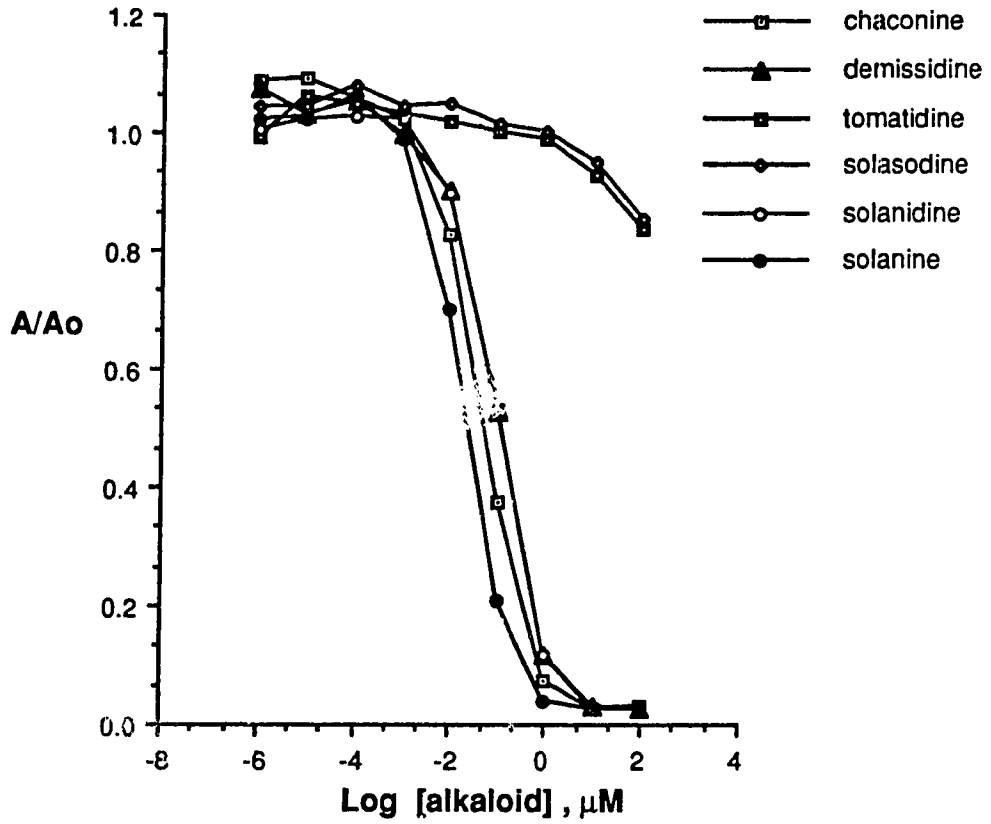


Table VII. I_{50} values for various steroidal alkaloids using MAb in EI.
The I_{50} values are the concentration of GA (μM) giving 50% reduction in A/A_o (curves are illustrated in Figure 22).

Steroidal Alkaloid*	I_{50}, (μM)
tomatidine	> 100
solasodine	> 100
solanidine [a]	0.103
demissidine [a]	0.092
α-chaconine [b]	0.043
α-solanine [c]	0.021

* letters in superscript indicate groupings based on statistical comparisons using a 95% level of significance ($P = 0.05$). Comparisons were made of the $\log_{10}$ [GA], (μM).

Figure 23. Effect of pH on competitive inhibition. Plates were coated using 0.2 µg/mL BSA-H-9 and MAb was used at 1 µg/mL with α-chaconine as standard. Each point is the average of 4 determinations.

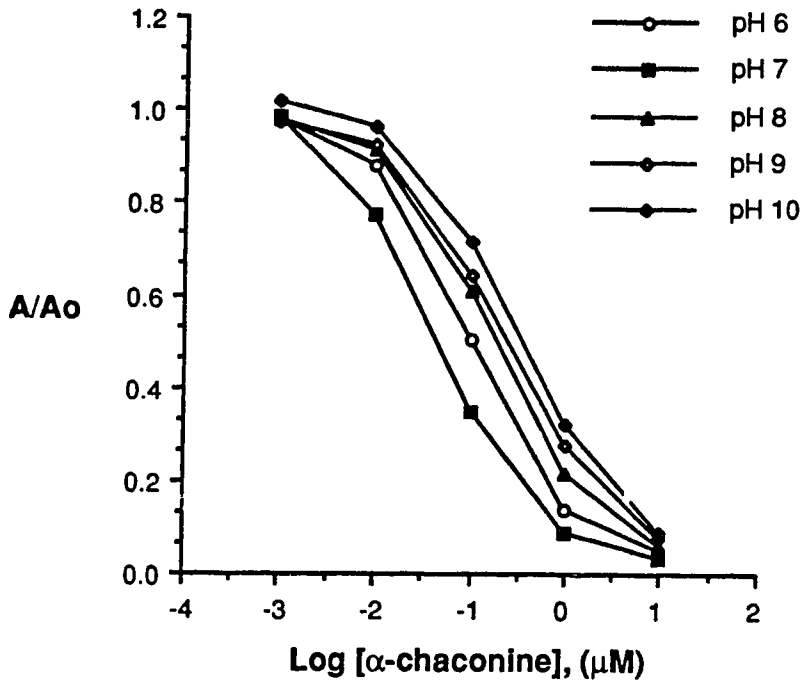
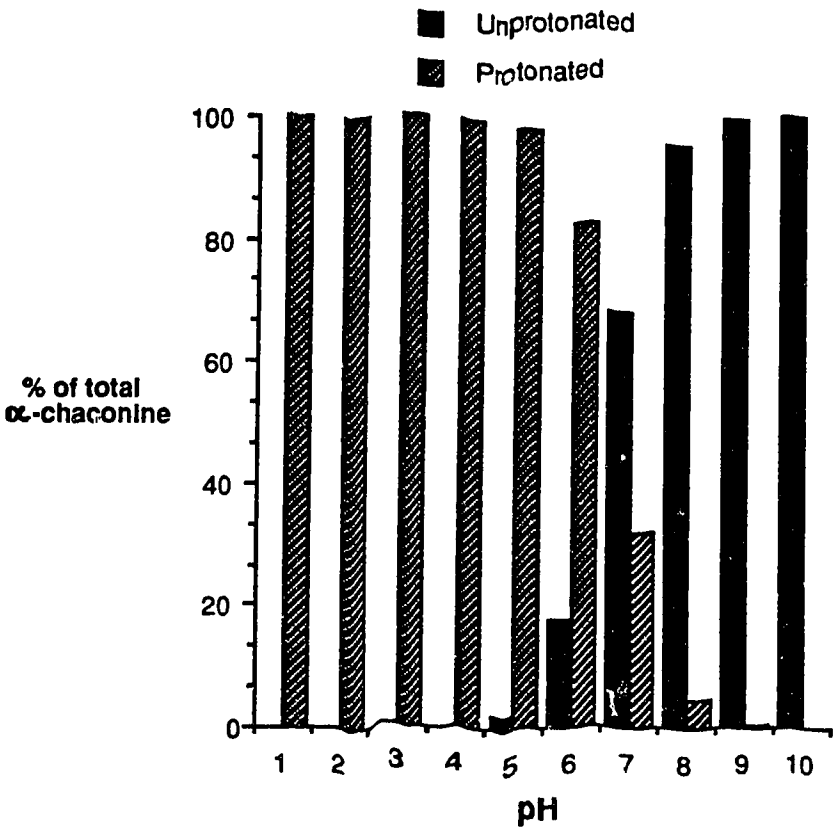


Figure 24. Calculated effect of pH on α-chaconine in a 1 μM solution.

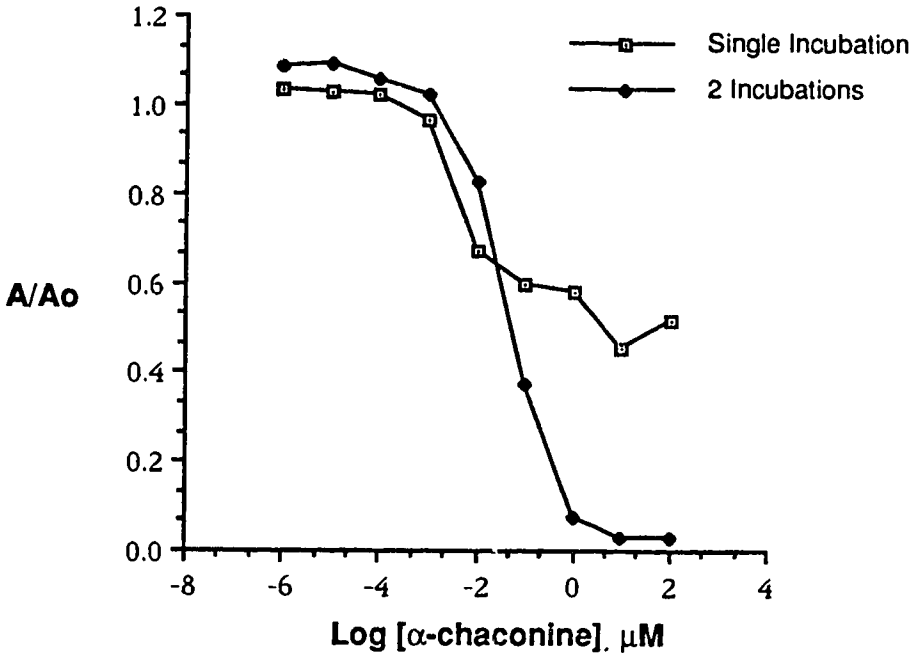


proportion of the α-chaconine in solution exists in the protonated form. Because the immunization was performed at physiological pH and clonal selection was performed using PBST at pH 7.3, it is likely that the MAb will be directed against (i.e. will have greater affinity for) the unprotonated form. The effect of pH on the protonation states of α-chaconine in solution or at the solid-phase seem to explain the effects of low pH on the sensitivity of the EI.

The MAb would also be expected to be greatly affected by pH because it is probable that several pK values within or around the Ab binding site determine its conformation. The conformation and ionic state of the Ab determine its specificity, affinity and ultimately the sensitivity of the EI in which it is used.

In a final study, an attempt was made to shorten the assay time by combining two incubation steps into a single incubation. While it is conventional to do the two Ab incubations (i.e. anti-GA Ab and anti-mouse Ab conjugate) separately, with a washing step in between, theoretically it is not necessary. A version of this method was reported by Naser (1990) where co-incubation of Ab with anti-Ab improved sensitivity by about 10-20 fold. Figure 25 compares the inhibition curves for α-chaconine obtained using the conventional two incubations or a single incubation. When both Ab binding reactions occur together, the background absorbance and variability is higher. This likely occurred from the formation of multimeric complexes between the immunoreactants and the fact that some entrapment or non-specific binding occurred. It may be possible to decrease background absorbance by reoptimizing the concentrations of the solid-phase GA conjugate or the MAb.

Figure 25. EI Using a single Ab incubation. Plates were coated using 0.2 µg/mL BSA-H-9. The two Ab incubations, the MAb (1 µg/mL) competition with α-chaconine and the secondary Ab, anti-mouse Ab-peroxidase conjugate (1/6000 working dilution) were either incubated together in a single incubation, or in two incubations with washing in between. Each point is the average of 6 determinations.



Conclusions

Improved methods for the synthesis of glycoalkaloid 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 moles of alkaloid per mole of bovine albumin) than earlier methods. *Limulus polyphemus* hemocyanin (LPH)-solanidine conjugates, used for immunization, produced high serum titers (>10^6) in rabbits and high MAb titers (10 ng/mL). It is believed that the novel methodology used to produce immunogen (partial acid hydrolysis of α-chaconine and selective reductive condensation using sodium cyanoborohydride) resulted in a homogeneous preparation with optimum substitution of alkaloid groups per protein, leading to a good antibody response. The use of a different protein and linking arm for the preparation of solid-phase conjugates is also believed to improve the sensitivity of the competitive assay.

Competitive EI using PAb or MAb 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 (using PAb) and HPLC. EI was found to be more comprehensive than HPLC because demissidine glycoalkaloids from *Solanum demissum* were also quantified. The %CV was also found to be better for EI methods, compared to HPLC. High %CV in HPLC is believed to be due to the losses during sample purification, prior to analysis. EI, due to its specificity, is the first method for GA analysis that does not require a purified extract for analysis. It also quantitates both glycosides and aglycones simultaneously, without a requirement for acid hydrolysis. These advantages should help to eliminate the problems of sample work-up and incomplete recovery of GA that make traditional methods difficult to perform.

The sensitivity of the EI systems developed here allow for the analysis of highly diluted sample extracts, thereby reducing the possibility of interference from other substances present. Several variables were found to

have an effect on EI sensitivity using α-chaconine, including the concentrations of solid-phase conjugate and MAb and the pH of the PBST used during the competition.

Fusion of murine splenocytes and myeloma resulted in a high number of hybridoma producing MAb that could be used in competitive EI. Serum-free or protein-free media were useful for MAb production in stationary tissue culture. Hybridoma growth rate was not found to be related to MAb production, therefore, even though growth in serum-free media was found to be restricted, MAb production during a 3 day culture period was not. Cloned cells were shown to produce MAb in tissue culture at a rate of 83 µg/mL/day. Supernatants could be easily purified using ammonia sulfate precipitation and purified MAb remained stable during freeze-drying and rehydration.

The EI developed here can be used to quantitate GA common in commercial cultivars of potatoes. The current trend in breeding practices, however, will require that other, less common GA also be measured. Greater use of "wild" species of potatoes in breeding may mean that other (eg. spiral and leptine) alkaloids will become more prevalent in commercial potatoes (Gregory, 1984; van Gelder and Schefter, 1991). There is no convenient method available at this time to screen for all types of alkaloids simultaneously. Further research efforts are therefore required to obtain additional MAb-producing cell lines for analysis of these groups of potato alkaloids. It may be feasible to design an EI that uses several MAb simultaneously, each directed towards a different group of GA and each using a different marker (e.g. different fluorochromes). In such a test system, it would be possible to measure either specific or total GA in a sample.

The toxicological significance of *Solanum* GA dictates the need for more routine analysis of GA by breeders, producers, processors and/or regulators. The methodology described in this thesis has begum the process of developing a truly comprehensive assay system for these compounds. This enzyme immunoassay system has the potential of surpassing traditional chemical and chromatographic methods because of improved specificity, the ability to obtain high titer Ab preparations, a low background absorbance and the ability to measure a greater number of samples simultaneously, factors that should contribute to a lower cost of sample analysis.

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Appendix I

Summary of protein conjugates produced

Aglycone	Protein	Linkage	# of Groups*
solanidine	LPH	glycosidic	14
solanidine	BSA	hemisuccinate	9
solanidine	BSA	glycosidic	6
solanidine	BSA	glycosidic	23
solasodine**	LPH	hemisuccinate	11
solasodine**	BSA	hemisuccinate	8

* Determined using hydrolysis/colorimetry or microanalysis; results expressed as moles of alkaloid per BSA equivalent.

**Solasodine conjugates were prepared for future studies.

Appendix II

Ability to competitively remove Ab from solid-phase using 0.02 μM or 2 μM α-chaconine (initial screening results, 6 days after fusion).

Sample	A_o	A/A_o, 0.02 μM α-chaconine	A/A_o, 2 μM α-chaconine
8G9	0.592	0.42	0.03
8D6	0.495	0.93	0.04
2D11	0.703	0.67	0.06
3F3	0.585	0.77	0.07
1G9	0.867	0.62	0.08
3D9	0.396	0.86	0.08
4D5	0.774	0.93	0.08
8C10	0.542	0.75	0.10
5F3	0.619	0.72	0.10
2E2	1.175	0.91	0.10
4B5	0.573	0.86	0.12
1C4	0.764	0.89	0.13
7F5	0.968	0.57	0.14
4B9	0.751	0.88	0.16
5G6	0.732	0.97	0.17
7E2	0.843	0.91	0.18
2D8	0.691	0.88	0.18
2E7	0.672	0.95	0.19
7B6	0.826	0.93	0.19
3D6	0.582	0.97	0.21
6C5	0.605	0.97	0.22
6D11	0.749	0.99	0.23
6B11	0.754	0.96	0.25
6D9	0.769	0.95	0.25
3G8	0.911	0.99	0.27
2G5	0.711	0.92	0.28
2C11	0.806	0.98	0.31
3C11	0.753	0.94	0.34
2G9	0.712	0.94	0.34
5D5	1.104	0.96	0.35
1B8	0.797	1.28	0.36
2C2	0.822	0.96	0.37
5B6	0.745	0.97	0.45
4C9	0.724	1.06	0.48
2F7	0.926	1.20	0.49
5G5	0.832	0.90	0.50
3D8	0.295	1.04	0.6[1]
5E2	0.609	1.00	0.64

Appendix III

Competition using various steroidal alkaloids (9 days after fusion).

Sample	A_0	Alkaloid	A/A_0, 0.02 µM alkaloid	A/A_0, 2 µM alkaloid
1G9	0.369	α-chaconine	0.33	0.03
		α-solanine	0.47	0.03
		solanidine	0.87	0.15
		demissidine	1.05	0.16
		tomatidine	1.27	1.31
7F5	0.251	α-chaconine	0.27	0.04
		α-solanine	0.56	0.05
		solanidine	1.04	0.19
		demissidine	1.05	0.22
		tomatidine	1.01	1.20
8D6	0.653	α-chaconine	0.70	0.02
		α-solanine	0.84	0.03
		solanidine	0.95	0.18
		demissidine	0.90	0.10
		tomatidine	0.91	0.93
8G9	0.175	α-chaconine	0.22	0.05
		α-solanine	0.80	0.06
		solanidine	1.15	0.17
		demissidine	1.12	0.21
		tomatidine	1.22	1.14
2D11	0.043	α-chaconine	0.35	0.21
		α-solanine	1.07	0.30
		solanidine	0.88	0.28
		demissidine	0.88	0.30
		tomatidine	1.21	1.23
4D5	0.031	α-chaconine	0.39	0.29
		α-solanine	0.74	0.36
		solanidine	0.81	0.32
		demissidine	0.81	0.39
		tomatidine	0.74	0.94
5F3	0.073	α-chaconine	0.93	0.16
		α-solanine	0.90	0.22
		solanidine	0.90	0.36
		demissidine	0.88	0.25
		tomatidine	0.62	0.75
8C10	0.023	α-chaconine	0.78	0.44
		α-solanine	0.91	0.52
		solanidine	0.78	0.57
		demissidine	0.87	0.61
		tomatidine	0.70	1.00

Appendix IV

Ability to competitively remove Ab from solid-phase using 0.02 μM or 2 μM chaconine (21 days after fusion).

Sample	A_o	A/A_o, 0.02 μM chaconine	A/A_o, 2 μM chaconine
1G9	0.600	0.92	0.07
7F5	0.309	0.55	0.04
8D6	0.518	0.73	0.06
8G9	0.604	0.87	0.03
2D11	0.043	0.75	0.57
4D5	0.014	1.11	1.03
5F3	0.040	1.06	0.68
8C10	0.037	1.07	0.71