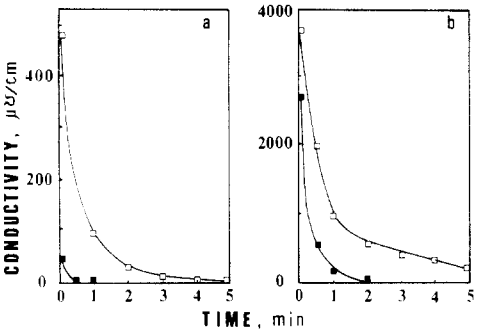

Figure 3. Foaming properties of cross-linked lysozyme and serum albumin. The foaming power and foam stability were determined by measuring the electric conductivity (μv/cm, the reciprocal of resistance) of foam: a, lysozyme; b, serum albumin. Key: □, native protein; ■, cross-linked protein.

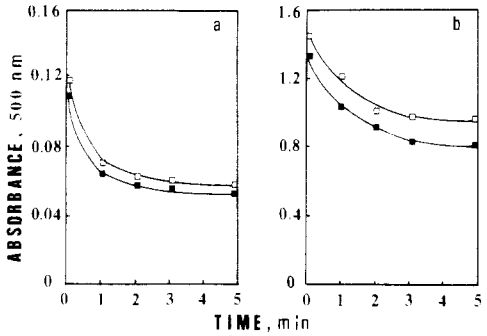

Figure 4. Emulsifying properties of cross-linked lysozyme and serum albumin. The emulsifying activity and emulsion stability were determined by measuring the turbidity at 500 nm of diluted emulsion: a, lysozyme; b, serum albumin. Key: □, native protein; ■, cross-linked protein.

30 s. The foaming power of bovine serum albumin is high (3700 μv/cm) and is significantly lowered (2750 μv/cm) by cross-linking. The foam stability of cross-linked serum albumin was greatly decreased, and the foam was diminished within 2 min.

On the other hand, the emulsifying properties of lysozyme and serum albumin were slightly decreased by cross-linking (Figure 4). The emulsifying properties of lysozyme are originally low. Therefore, the difference in the emulsifying properties between native and cross-linked lysozyme was slightly observed. However, in the case of bovine serum albumin possessing good emulsifying properties, a significant difference between native and cross-linked one was observed, although the difference was smaller than that in foaming properties.

We have proposed in a previous paper (Kato et al., 1985) that the flexibility of proteins detected by protease probe method may play an important role on the foaming and emulsifying properties. This was confirmed by using cross-linked proteins, as shown in this paper. The foaming properties may be more closely affected by the flexibility of proteins than the emulsifying properties. Bovine serum albumin is originally a hydrophobic protein. Therefore, the emulsifying properties of serum albumin may be still good, although it was cross-linked by FNPS.

LITERATURE CITED

Imoto, T.; Fukuda, K.; Yagishita, K. *J. Biochem.* **1976**, *80*, 1313-1318.
Imoto, T.; Hartdegen, F. J.; Rupley, J. A. *J. Mol. Biol.* **1973**, *80*, 637-648.
Kato, A.; Komatsu, K.; Fujimoto, K.; Kobayashi, K. *J. Agric. Food Chem.* **1985**, *33*, 931-934.
Kato, A.; Nakai, S. *Biochim. Biophys. Acta* **1980**, *624*, 13-20.
Kato, A.; Osako, Y.; Matsudomi, N.; Kobayashi, K. *Agric. Biol. Chem.* **1983a**, *47*, 33-37.
Kato, A.; Takahashi, A.; Matsudomi, N.; Kobayashi, K. *J. Food Sci.* **1983b**, *48*, 62-65.
Nakai, S. *J. Agric. Food Chem.* **1983**, *31*, 676-683.
Pearce, K. N.; Kinsella, J. E. *J. Agric. Food Chem.* **1978**, *26*, 716-723.
Shimizu, M.; Takahashi, T.; Kaminogawa, S.; Yamauchi, K. *J. Agric. Food Chem.* **1983**, *31*, 1214-1218.
Ueno, H.; Harrington, W. F. *J. Mol. Biol.* **1984**, *173*, 35-61.
Wold, F. *J. Biol. Chem.* **1961**, *236*, 106-111.

Received for review July 25, 1985. Accepted December 30, 1985.

Segregation of Leptine Glycoalkaloids in *Solanum chacoense* Bitter

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Foliar leptine levels in 60 *Solanum chacoense* accessions were determined in a search for high-leptine plants that might be useful in breeding for resistance to the Colorado potato beetle, *Leptinotarsa decemlineata* Say. New methods were developed for detecting and quantifying leptine glycoalkaloids. A wide range of levels (<2–98 mg % fresh weight) was found among the accessions; most (42 of 60) apparently did not synthesize even traces of leptines. Among six sibs individually sampled from one accession, levels ranged from <2 to 306 mg % in replicated analyses. The results indicate that leptine glycoalkaloids are segregating widely among and within *S. chacoense* accessions. Tubers from high-leptine clones did not synthesize leptines even when greened or wound healed.

INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say, is becoming increasingly difficult to control with available pesticides. New control measures are needed, including the use of resistant varieties in integrated pest management programs. Present potato cvs have little or no resistance and may not be adequate as a germplasm base to improve resistance (Sanford et al., 1984). Resistant interspecific hybrids were developed in Europe in the 1940s (Schwarze, 1963; Torka, 1950), but when pesticides such as DDT became widely available, most of the European breeding research was discontinued.

Resistant wild species and their hybrids with *Solanum tuberosum* in these early European breeding studies generally had high total glycoalkaloid (TGA) levels. The re-

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Table I. Segregation of Foliar Leptines among Sibs of Three *Solanum chacoense* Accessions

	leptine concn, mg % fresh wt[a]							
			foliage from individual sibs					
	bulked foliage		sib no. (cold frame)			sib no. (field)		
accession	3 sibs	12 sibs	1	2	3	4	5	6
PI 320287	38	13	72	0	8	4	3	0
PI 458313[b]	nd[c]	8	260	3	nd	0	5	nd
PI 458310	45	23	131	35	53	60	134	26

[a] Determined by acetylsolanidine concentration (GLC) in hydrolyzed extracts. [b] The six PI 458313 sibs were another sample of the accession grown in the greenhouse. The 3 individual sibs in the cold frame and the 12 in the field were not analyzed. [c] Not detected. Limit for detection is ca. 2 mg %.

sistance in several *Solanum spp.*, particularly *Solanum chacoense*, Bitt., *Solanum demissum* Lindl., and *Solanum polyadenium* Greenm., appears to be associated with the presence of glycoalkaloids not found in *S. tuberosum*, e.g., leptines, demissine, or tomatine, or with high levels of α-solanine (sol) and α-chaconine (chac) [for a review, see Tingey (1984)]. sol and chac are the usual glycoalkaloids found in *S. tuberosum* foliage and tubers, at concentrations of 10–100 mg % fresh weight in foliage (Deahl et al., 1973) and 1–15 mg % fresh weight in tubers (Sinden and Webb, 1972).

Kuhn and Low (1961a) discovered a new class of *Solanum* glycoalkaloids, the leptines, when they investigated the glycoalkaloid composition of a particular *S. chacoense* line that had been inbred by Torka (1950) for a level of resistance approaching immunity (Struckow and Low, 1961). Leptines are acetylated forms of the more common *Solanum* glycoalkaloids [for a review, see Schreiber (1968)]. One of the four leptines Kuhn and Low detected, leptine I, was isolated and shown to have the same structure as chac, except for an acetyl on C_{23} of the steroid aglycone in place of the hydrogen on C_{23} in chac (Kuhn and Low, 1961a, 1961c). Neither chac nor sol inhibit larval development, even at a concentration of 500 mg % (Buhr et al., 1958) and are only weak deterrents of adult feeding at this high concentration (Struckow and Low, 1961). In contrast, when leptine I in crystalline form was infiltrated into potato leaf disks at 100 mg % fresh weight, adults did not feed (Struckow and Low, 1961) and all larvae died (Kuhn and Low, 1961c). When the deterrent activity of leptine I on adult feeding was compared to that of two other feeding deterrents, demissine and tomatine, leptine I proved to be ca. 5-fold more potent (Struckow and Low, 1961). Leptine III, in amorphous form, was also tested and had deterrent activity equivalent to that of leptine I. Apparently acetylation of C_{23} in certain *Solanum* glycoalkaloids can convert weak or impotent glycoalkaloids into potent feeding deterrents.

By transferring to *S. tuberosum* the ability to acetylate solanidine (the aglycone of sol and chac) it might be possible to breed potato cvs that have improved CPB resistance, yet have the normal types and concentrations of glycoalkaloids in their tubers. According to Kuhn and Low (1961a), leptines are not found in *S. chacoense* tubers; therefore, the potential for introducing new and possibly more toxic glycoalkaloids into foods should not be a probelm. In order to use the leptines in resistance breeding it will first be necessary to select or breed high-leptine lines such as the one Kuhn and Low (1961a) studied, which had ca. 160 mg % total of leptines I–IV in the foliage. This high-leptine line is no longer available, and in recent studies of *S. chacocense*, only low leptine levels, or no leptines at all, were found (Gregory et al., 1981; Schwarze, 1963; Sinden et al., 1980). We report here on the rare occurrence of high-leptine genotypes in *S. chacoense* and on the wide segregation of leptine levels among and within accessions. We also identified three high-leptine clones that might be useful in breeding for CPB resistance and determined their complete foliar and tuber glycoalkaloid composition by using a combination of TLC, GLC, and new HPLC analytical methods.

EXPERIMENTAL SECTION

Sampling of Accessions and Individual Sibs. True seeds of 60 *S. chacoense* accessions were obtained from the Potato Introduction Station, Sturgeon Bay, WI. Three seedlings of each accession were transplanted into a sterile potting mixture and grown in pots in a cold frame. Another 12 seedlings of each accession were transplanted into a field plot in June for glycoalkaloid assessments and CPB resistance tests.

Foliage samples (four midlevel leaves) were taken from each of the three cold-frame sibs when they were ca. 30–50 cm. Leaflets were stripped from the petioles and bulked for each accession, forming a 5–10-g fresh weight sample. A second bulked sample of the accession was formed by taking one midlevel leaf from each of the 12 sibs in the field. Individual sibs (three to eight per accession) were sampled in July and August by harvesting four leaves per plant.

Selected high- and low-leptine plants were transfrerred to the greenhouse for tuberization. Individual clones were propagated by rooting nodal cuttings obtained from tuber-derived plants. These plants (20 of each clone) were transplanted into the field in May of the following year in a paired-plot experiment comparing leptine levels and CPB resistance of high-leptine and low-leptine clones from the same accession. Two midlevel leaves were excised from each of the 20 plants at weekly intervals over 3 consecutive weeks in June for glycoalkaloid analyses.

Leptine Levels and Glycoalkaloid Composition. Leaf samples were boiled for 10 min in ethanol acidified with glacial acetic acid (5%), 20 mL of extraction medium/gram of tissue, 10–30 min after harvest to inactivate enzymes and extract the glycoalkaloids. After filtering, the extracts were evaporated to ca. 1 mL/g tissue in a steam chamber. One milliliter of H_2O was added to each milliliter of concentrated extract and the acidic extract cooled before filtering. For the replicated determinations (Table II) and for HPLC analyses (Table III), the boiled foliage was additionally homogenized in a blender to ensure complete extraction. Aliquots of the extract (1-g tissue equivalents) were evaporated to dryness under an air stream and hydrolyzed for 4 h at 68 °C in 1 N methanolic HCl for the GLC analyses (Gregory et al., 1981; Sinden et al., 1980). Aliquots equivalent to 4 g of tissue were ammonia precipitated (Gregory et al., 1981) for HPLC and TLC analyses. To estimate recoveries of the leptines and other glycoalkaloids in ammonia precipitates, enough concentrated ammonia to raise the pH to 11.0 was added to aqueous extracts of samples of foliage from PI 458313-1 and PI 320287-1. Aliquots (1-g tissue equivalents) of the

Table II. Types of Glycoalkaloids Segregating among Pairs of Sibs from Three *Solanum chacoense* Accessions

accession	sib no.[b]	mg % fresh wt[a] leptines	leptinines	sol + chac	total glycoalkaloid
PI 320287	1	120	29	11	159
	2	0	11	167	178
PI 458313	1	306	100	243	649
	4	0	48	704	752
PI 458310	1	271	141	69	483
	3	51	86	253	390
lsd (0.05)		45	29	79	120

[a] Determined by the concentration (GLC) in hydrolyzed extracts of acetylsolanidine for leptines, hydroxysolanidine for leptinines, and solanidine for sol and chac. Data are averages of three foliage samples. [b] Sib numbers from Table I.

original aqueous extract, the ammonia precipitate, and the ammonia supernatant were analyzed by GLC. To determine the solubility of leptine I in water and ammonia (pH 11.0), 10 mg of the isolated glycoalkaloid was suspended in 2 mL of the solvent. The suspensions were boiled and sonicated for 5 min. After cooling, the suspensions were filtered (Whatman No. 42) and the concentration of leptine I in the filtrate was determined by GLC.

Tubers were exposed to light (16 Einstein s^{-1} m^{-2}) for 14 days prior to analysis for glycoalkaloid composition. Glycoalkaloids were extracted from the greened tubers by using the methods of Bushway et al. (1980). Aliquots (2-g tissue equivalents) of the filtered methanol–chloroform extracts were evaporated to dryness, hydrolyzed, and analyzed by GLC. The remaining extract was ammonia precipitated for HPLC and TLC analyses. Tubers were also wound healed under light, and the tissue was extracted by methods previously described for the induction of solamarine synthesis of *S. tuberosum* (Sinden and Sanford, 1981). Aliquots of the extracts from would-healed tubers were analyzed by GLC and, after ammonia precipitation, by TLC and HPLC.

Chromatographic standards for leptines I and II and leptinines I and II were isolated from foliage of PI 458310-1 (Table III). Extracts from 800 g of foliage were ammonia precipitated (3×) to remove most nonglycolakaloid impurities. Glycoalkaloids in the extract were separated by column chromatography over acidic alumina (Kuhn and Low, 1961a). Leptine I was chromatographically pure in a few column fractions. Leptine II was further purified from selected fractions by preparative TLC. Leptinines were isolated from an aliquot of the extract that had been treated with base (1 N NaOH in 70% aqueous methanol, 10 min, 60 °C) to cleave the acetyl from the leptine glycoalkaloids (Kuhn and Low, 1961a). Leptinine I was separated from leptinine II by preparative TLC. After isolation of the glycoalkaloid from the alumina column or TLC plates, the glycoalkaloid was dissolved in 0.1% acetic acid, filtered, and then ammonia precipitated. TLC plates (Whatman HP-KF) were usually developed with ethyl acetate–pyridine–water (10:4:1). With 1-h development the glycoalkaloids had the following R_f values: leptine I, 0.73; leptine II, 0.31; chac, 0.55; sol, 0.20; leptinine I, 0.40; leptinine II, 0.13. Isolated glycoalkaloids were acid hydrolyzed (1 N methanolic HCl, 2 h, 68 °C) and the aglycones identified by GC/MS analyses. Sugars in hydrolysates (2 N H_2SO_4, 2 h, 100 °C) were identified by comparing GLC chromatograms of the aldononitrile acetate derivates (Varma et al., 1973) of sugars from the glycoalkaloids with those from hydrolysates of sol and chac (Sigma).

The methods of Morris and Lee (1981), with minor modifications, were used for the HPLC analyses of ammonia-precipitated extracts. The Waters Associates (Milford, MA) HPLC system consisted of a Model 510 pump, a U6K injector, a Model Z radial compression module fitted with a C_{18} 10 cm × 8 mm Radial Pak cartridge, a Lambda-Max 481 spectrophotometer, and a Model 730 data module. The flow rate of the mobile phase, acetonitrile–water–ethanolamine (45:55:0.1 at pH 4.1), was 2 mL/min, and the column effluent was monitored at 210 nm. Sol and chac (Sigma), isolated leptine I, leptine II, leptinine I, leptinine II, and β-chac were individually injected (5 μL of 0.1% acetic acid containing 10 μg of the glycoalkaloid) to determine the retention times of the seven glycoalkaloids. Ammonia-precipitated extracts were dissolved in 0.1% acetic acid and filtered. Aliquots (2–10 μL) of the extracts containing ca. 4–20 μg of total glycoalkaloid were injected. Cochromatography of the isolated standards with ammonia-precipitated extracts showed that impurities in the precipitates did not affect retention times. Leptine I and leptine II peaks were collected during analyses of PI 458310-1 foliar extracts and cochromatographed (TLC) with standards to confirm the identification of these peaks on HPLC chromatograms. sol (Sigma) was used as the standard for quantificationof the glycoalkaloids in extracts by absorbancy at 210 nm.

RESULTS AND DISCUSSION

Leptine Segregation. The average level of foliar leptines in *S. chacoense* accessions varied over a wide range, <2–98 mg %. Of the 60 accessions, 42 did not synthesize leptine levels high enough (ca. 2 mg %) to detect with the GLC method. In the 18 accessions that did have leptines, the levels were usually less than 10 mg %. The highest level found in a bulked foliage sample was 98 mg %, and only 2 of 120 bulked samples of the accessions had levels greater than 50 mg %. None of the 60 accessions synthesized a level as high as the 160 mg % in the very re-

Table III. Glycoalkaloid Compositions of the Foliage and Tubers of Three High-Leptine *Solanum chacoense* Clones by HPLC Analyses of Ammonia-Precipitated Extracts

glycoalkaloid	t_R[a]	mg % fresh wt PI 320287-1 tubers	PI 320287-1 foliage	pI 458313-1 tubers	pI 458313-1 foliage	PI 458310-1 tubers	PI 458310-1 foliage
leptine I	3.36	0	22	0	149	0	114
leptine II	2.76	0	26	0	132	0	76
leptinine I	1.90	2	4	15	35	3	35
leptinine II	1.58	4	5	39	40	6	25
α-chaconine	4.71	63	0.1	85	148	97	4
α-solanine	3.82	61	0.6	131	74	93	7
β-chaconine	6.01	4	0.1	9	72	6	0
total glycoalkaloid		134	57.8	279	650	205	261

[a] Retention time (t_R) in minutes. HPLC conditions: C_{18} 8-mm Radial Pak (Waters Associates); mobile phase of acetonitrile–water–ethanolamine (45:55:0.1); flow of 2 mL/min; detection of 210 nm.

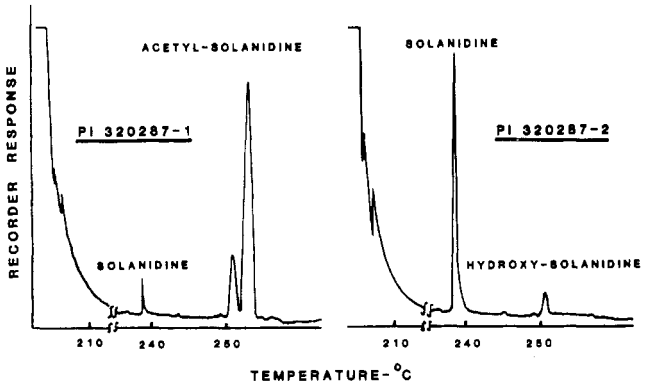

Figure 1. GLC chromatograms of the aglycones in hydrolyzed foliar glycoalkaloid extracts from a high-leptine sib and a no-leptine sib of PI 320287.

sistant, inbred *S. chacoense* line Kuhn and Low (1961a) investigated.

The GLC method of glycoalkaloid analysis is particularly suited to the analysis of leptines, which are ammonia soluble, according to Kuhn and Low (1961a). Other method such as titration (Bushway et al., 1980) and HPLC (Morris and Lee, 1981) require purification of the glycoalkaloids in crude extracts by ammonia precipitation prior to quantitative analysis. Even ammonia-precipitable glycoaklaloids such as demissine in *Solanum acaule* are sometimes partially lost in the supernatant when foliar extracts are precipitated (Gregory et al., 1981). The GLC method is rapid and, therefore, suited to the estimation of the glycoalkaloid levels in large numbers of clones because the usual time-consuming precipitation, washing, and collection procedures are not required. King (1980) showed that recoveries of sol added to extracts of *S. tuberosum* foliage were nearly quantitative using similar method of extraction, hydrolysis, and aglycone analysis by GLC. Replicated analyses are necessary, however, to obtain accurate estimates of the foliar glycoalkaloid level in a particular clone or accession. In replicated analyses of a single extract from a sample of PI 458310-1 foliage and an extract of PI 458313-1 foliage, leptine levels ranged from 217 to 243 mg % among three independent analyses of the PI 458310-1 extract and from 323 to 381 mg % among three analyses of the PI 458313-1 extract. Ranges in TGA levels among the three analyses were similar to those for leptines: 412–480 mg % for PI 458310-1 and 618–689 mg % for PI 458313-1. Even greater variation occurred among samples from a single clone. For instance, among three samples of PI 458313-1 foliage taken weekly from the replicated field plot over a 3-week period, leptine levels ranged from 221 to 385 mg %. TGA levels ranged from 484 to 726 mg %.

Hydrolysates of extracts that contained leptines always had a third aglycone, 23-hydroxysolanidine (Kuhn and Low, 1961b), in addition to solanidine and 23-acetylsolanidine. Hydroxysolanidine (leptinidine) is the aglycone of leptinines I and II (Kuhn and Low, 1961a, 1961b) and may be a precursor of acetylsolanidine. Or, it may be formed from acetylsolanidine by esterases in the foliage, and/or by partial cleavage of the acetyl during acid hydrolysis. The presence of three peaks corresponding to solanidine (sol and chac), hydroxysolanidine (leptinines), and acetylsolanidine (leptines) in an elution pattern such as that for PI 320287-1 foliage (Figure 1) is a reliable indicator of the presence of leptines in the foliage even if the leptines are present at low concentration. However, the presence or absence of leptines at concentration below ca. 2 mg % fresh weight cannot be reliably determined by the GLC method because even on chromatograms of hydrolyzed extracts of *S. tuberosum* foliage that does not contain leptines traces of unidentified compounds with retention times (R_t) similar to those of acetylsolanidine and hydroxysolanidine are sometimes present. If chromatograms of *S. chacoense* extracts had no more than a trace peak (<2 mg %) corresponding in R_t to acetylsolanidine, leptines were considered not detected (nd). Chromatograms of most of the *S. chacoense* extracts did not have even traces of acetyl- or hydroxysolanidine. Other no-leptine clones had traces or more of hydroxysolanidine on GLC chromatograms but no acetylsolanidine. No traces of leptines were detected in foliage samples of PI 320387-3, PI 458313-4, and some other no-leptine clones by TLC of aqueous extracts, TLC of ammonia precipitates, or HPLC of ammonia precipitates.

Leptine levels were considerably different in the 3-sib and 12-sib bulked samples of some accessions (Table I), possibly indicating nonuniform distribution of leptines among sibs from certain accessions. To determine whether or not leptine levels might be segregating among sibs, 65 sib plants from 14 accessions were individually screened for their leptine levels. Twenty of the plants were randomly chosen from five accessions that did not have leptines in either of the bulked samples; none of these plants had leptines. Levels ranged from 0 to 260 mg % among the remaining 45 plants from nine leptine-synthesizing accessions. Eleven of the 45 did not have leptines and only five synthesized more than 100 mg %.

In a sample of six sibs from PI 320287, one had a leptine level of 72 mg % (Table I). Two other sibs from this accession, sibs 2 and 6, did not synthesize even traces of leptines. The plant with the highest leptine level in this investigation was identified among six sibs of PI 458313 (Table I). Sib 1 had a level of 260 mg % in the initial screening. In replicated tests (Table II) its leptine level was 306 mg %. Sib 4 from this accession did not have leptines. Levels ranged from 26 to 134 mg % among six sibs of PI 458310. Similar wide differences were also found among the sibs from the other six leptine-synthesizing accessions.

Pairs of sibs from PI 320287, PI 458313, and PI 458310 (Table I) were selected for replicated field tests of leptine levels and CPB resistance. One sib from each accession was selected for its apparent ability to synthesize a high leptine level. The other sib in the pair was selected because of its low leptine level. PI 320287-2 and PI 458313-3, with no leptines in the initial analyses of single plants (Table I), also did not synthesize leptines in replicated tests of field plants (Table II). Levels in the field plants of PI 458310-3 and the three high-leptine sibs were similar to the levels found in the nonreplicated analyses of single plants. The no-leptine sibs, PI 320287-2 and PI 458313-3, did not synthesize leptines in leaves of young plants (14 days after emergence) in the greenhouse, in 2-month-old plants in the filed, nor in tuberizing (4-month-old) plants in the field. PI 458310-3 had levels ranging from 22 mg % in young plants to 54 mg % in tuberizing plants. The three high-leptine sibs (PI 320387-1, PI 458313-1, PI 458310-1) synthesized 60 mg % or more of leptines in their foliage at all three of these developmental stages. Thus, it appears that either the no-leptine sibs do not have the potential for leptine synthesis, i.e., lack the gene(s) for acetylation, or the gene(s) are repressed during the entire development of the plants.

There were large and significant differences in the total glycoalkaloid (TGA) levels among the three accessions in the replicated tests (Table II). However, the two sibs of

each accession had ca. the same TGA level. A major portion of the total aglycone is acetylated in the high-leptine sibs, resulting in higher levels of leptines and leptinines, and lower levels of sol and chac compared with the low-leptine sibs. This indicates that high- and low-leptine sibs from the same accession do not differ quantitatively in their abilities to synthesize the aglycone and saccharide components of glycoalkaloids. Both sibs synthesize the same saccharide components, solatriose and chacotroise (Schreiber, 1968). Rather, they differ in the types of aglycones they synthesize.

The results suggest the following: (1) Genes for enzymes (esterase synthetases) that acetylate the aglycones of *S. chacoense* glycoalkaloids are not evenly distributed in the species. (2) Most accessions lack the necessary gene(s) for leptine synthesis. (3) In accessions that do synthesize leptines, there usually is a wide segregation for levels of leptines. (4) Clones of *S. chacoense* that synthesize leptine levels of more than 100 mg % such as the inbred line analyzed by Kuhn and Low (1961a) are indeed rare—less than 1% in our *S. chacoense* sample.

Glycoalkaloid Composition. Two of our selections, PI 320287-1 and PI 458310-1, may be especially useful in breeding for CPB resistance because they synthesize ca. 55–88% of their foliar TGA in the form of acetylyated glycoalkaloids (Tables II and III). PI 458313-1 also synthesizes high leptine levels but in addition synthesizes high levels of sol, chac, and leptinines, as did the inbred line that Kuhn and Low (1961a) analyzed. Leptinines are, like sol and chac, only weak deterrents of adult feeding (Struckow and Low, 1961). Ideally, resistant hybrids in a breeding program should synthesize leptine levels high enough to deter larval and adult feeding, yet synthesize relatively low levels of tuber TGA. Excessive TGA level in tubers has been a problem with the use of *S. chacoense* in potato breeding (Schwarze, 1962; Schwarze, 1963; Sinden et al., 1984; Zitnak and Johnston, 1970). Levels of sol + chac greater than 20 mg % in tubers can cause unpleasant bitterness and gastrointestinal distress in some individuals [for a review, see Sinden et al. (1984)]. Inheritance studies have shown that TGA levels are quantitatively inherited in *S. tuberosum* (Sanford and Sinden, 1972). Either high levels of sol + chac or mere presence of certain types of unusual glycoalkaloids in tubers could precipitate questions about a cultivar's food safety.

Although leptines were not detected in tubers from a leptine line by Kuhn and low (1961a), sensitive methods of detection, e.g. HPLC, TLC, and GLC, were not available when Kuhn and Low discovered the leptines. Therefore, before breeding experiments were initiated with these three high-leptine selections, the complete glycoalkaloid composition of their tubers and foliage was determined. We could not detect leptines in greened or check tubers of the three selected high-leptine lines (Table III) using GLC, HPLC, or TLC analytical methods. Nor were leptines detected in wound-healed tuber tissues. The synthesis of high levels of a particular aglycone and its associated glycoalkaloids in foliage, with complete repression of the synthesis in tubers, was first reported for the leptines (Kuhn and Low, 1961a). Repression in tubers was later shown for the solamarine glycoalkaloids, which have tomatidenol as their aglycone, in the cultivar Kennebec (Shih and Kuc, 1974). A major gene inherited from its *S. demissum* ancestor appears to control the synthesis of the foreign tomatidenol aglycone in Kennebec (Sinden and Sanford, 1981). Cutting and wound healing of tubers allows expression of the gene(s) for tomatidenol synthesis in Kennebec (Shih and Kuc, 1974; Sinden and Sanford, 1981). Therefore, we expected expression of the gene(s) for acetylation and resulting leptine synthesis upon cutting and wound-healing tubers from the high-leptine *S. chacoense* clones, but this is not occur.

Foliage of the three high-leptine clones contained seven glycoalkaloids (Table III); all were unsaturated forms, i.e. solanidine vs. demissidine glycoalkaloids (Osman and Sinden, 1977). Leptines I and II were the primary foliar glycoalkaloids, and the concentration of leptine I was similar to that of leptine II. The foliar TGA levels of PI 320287-1 and PI 458310-1 were considerably lower when measured by the HPLC method (Table III) compared with the more direct GLC analysis (Table II). This was probably caused by incomplete precipitation of the glycoalkaloids in aqueous extracts of these two clones (Gregory et al., 1981).

Recovery experiments with an extract from a foliage sample of PI 320287-1 showed that less than 40% of the TGA in the aqueous extract was recovered in the ammonia precipitate from this foliage sample. When the aqueous extract, ammonia precipitate, and ammonia supernatant were analyzed by GLC, only 34% of the leptines, 37% of the leptinines, and 36% of the sol + chac in the aqueous extract were detected in the ammonia precipitate. With a sample of PI 458313-1 foliage, 91% of the sol + chac, 78% of the leptines, and 62% of the leptinines were recovered in the ammonia precipitate, indicating that recoveries of leptines and other glycoalkaloids in ammonia precipitates vary with the clone. Most (84–96%) of the unrecovered amounts of the glycoalkaloids were found in the ammonia supernantants. Ammonia solubility of the leptines (Kuhn and Low, 1961a) does not completely explain the very poor recoveries of glycoalkaloids in ammonia precipitates from certain *S. chacoense* accessions (Gregory et al., 1981) and clones because large amounts of ammonia-precipitable glycoalkaloids such as sol and chac also remain in the ammonia supernatant. We found that, whereas leptine I is quite soluble in water (ca. 1.5 mg/mL), compared with solanine (ca. 0.01 mg/mL), leptine I is only slightly soluble (ca. 0.18 mg/mL) in ammonium hydroxide at pH 11.0. Heftman (1967) has suggested that nonnitrogenous sterols in foliage of certain *Solanum spp.* can interfere with the precipitation process or the collection of the precipitated glycoalkaloids by centrifugation. With extracts of *S. chacoense* foliage containing large amounts of leptines, the solubility of leptines in ammonia could be an additional factor affecting recovery.

Knowledge of a plant's glycoalkaloid composition can be important in studying the role of these compounds in insect resistance (Schwarze 1963, Sinden et al., 1980; Struckow and Low, 1961; Tingey, 1984). To determine the complete glycoalkaloid composition of an extract the individual glycoalkaloids must first be separated. This can be difficult with extracts containing complex mixtures of seven or more glycoalkaloids. Kuhn and Low (1961a) were not able to separate all 10 major glycoalkaloids in their extracts by either column or paper chromatography. Schwarze (1963) used TLC in an attempt to demonstrate the presence of leptine I in some *S. tuberosum* × *S. chacoense* hybrids, but the results were inconclusive because of the poor separations of leptine I from the partial hydrolysis products of sol and chac. We were able to separate all seven glycoalkaloids in extracts from our high-leptine plants using TLC. However, the separations varied with the purity of the extracts, the concentration of the individual glycoalkaloids, and the time of development. With reversed-phase HPLC (Figure 2; Table III) we consistently separated and identified the seven glycoalkaloids. In ad-

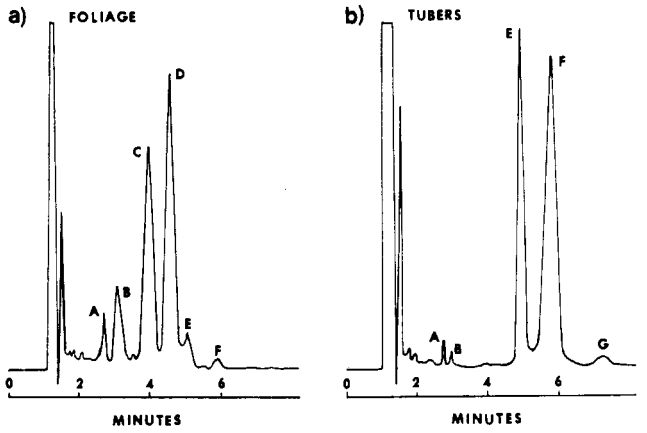

Figure 2. HPLC separation of glycoalkaloids in the foliage and tubers of high-leptine PI 458310-1: (a) foliar glycoalkaloids; (b) tuber glycoalkaloids (greened tubers). Peaks: A = leptinine II; B = leptinine I; C = leptine II; D = leptine I; E = α-solanine; F = α-chaconine; G = β-chaconine.

dition, with the HPLC method of analysis, the relative concentration of each glycoalkaloid present in the ammonia-precipitated extract can be estimated.

It remains to be determined whether the gene(s) for acetylating solanidine will be expressed in *S. chacoense* × *S. tuberosum* hybrids. The low frequency of high-leptine sibs among the 720 in our *S. chacoense* sample possibly indicates that recessive gene(s) control the high levels of leptine synthesis. Even if the ability to synthesize high leptine levels such as the 100 mg % that causes adults and larvae to cease feeding (Kuhn and Low, 1961c; Struckow and Low, 1961) cannot be transferred to *S. tuberosum*, the reported potency of leptine I is so high that considerably lower levels might still partially deter larval and adult feeding. A report on the CPB resistance of the six *S. chacoense* clones in Table II will be published subsequently.

ACKNOWLEDGMENT

We express our appreciation to S. F. Osman, ERRC, Philadelphia, PA, for advice on the chemistry of the leptines and the GC/MS analyses.

LITERATURE CITED

Buhr, H.; Toball, R.; Schreiber, K. *Entomol. Exptl. Appl.* **1958**, *1*, 209.
Bushway, R. J.; Wilson, A. M.; Bushway, A. A. *Am. Potato J.* **1980**, *57*, 561.
Deahl, K. L.; Young, R. J.; Sinden, S. L. *Am. Potato J.* **1973**, *50*, 248.
Gregory, P.; Sinden, S. L.; Osman, S. F.; Tingey, W. M.; Chessin, D. A. *J. Agric. Food Chem.* **1981**, *29*, 1212.
Heftman, E. *Lloydia* **1967**, *30*, 209.
King, R. R. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 1226.
Kuhn, R.; Low, I. *Chem. Ber.* **1961a**, *94*, 1088.
Kuhn, R.; Low, I. *Chem. Ber.* **1961b**, *94*, 1096.
Kuhn, R.; Low, I. *Tagungsber. Deut. Akad. Landwirtschaftswiss.* **1961c**, *27*, 7.
Morris, S. C.; Lee, T. H. *J. Chromatogr.* **1981**, *219*, 403.
Osman, S. F.; Sinden, S. L. *J. Agric. Food Chem.* **1977**, *25*, 955.
Sanford, L. L.; Sinden, S. L. *Am. Potato J.* **1972**, *49*, 209.
Sanford, L. L.; Ladd, T. L.; Sinden, S. L.; Cantelo, W. W. *Am. Potato J.* **1984**, *61*, 405.
Schreiber, K. In "The Alkaloids"; Manske, R. H. F., Ed.; Academic Press: New York, 1968; Vol. X, Chapter 1.
Schwarze, P. *Zuchter* **1962**, *32*, 155.
Schwarze, P. *Zuchter* **1963**, *33*, 275.
Shih, M.-J.; Kuc, J. *Phytochemistry* **1974**, *13*, 997.
Sinden. S. L.; Webb, R. E. *Am. Potato J.* **1972**, *49*, 334.
Sinden, S. L.; Sanford, L. L.; Osman, S. F. *Am. Potato J.* **1980**, *57*, 331.
Sinden, S. L.; Sanford, L. L. *Am. Potato J.* **1981**, *58*, 305.
Sinden, S. L.; Sanford, L. L.; Webb, R. E. *Am. Potato J.* **1984**, *61*, 141.
Struckow, B.; Low, I. *Entomol.* Expt. *Appl.* **1961**, *4*, 133.
Tingey, W. M. *Am. Potato J.* **1984**, *61*, 157.
Torka, M. *Am. Potato J.* **1950**, *27*, 263.
Varma, R.; Varma R. S.; Wardi, A. H. *J. Chromatogr.* **1973**, *77*, 222.
Zitnak, A.; Johnston, G. R. *Am. Potato J.* **1970**, *47*, 256.

Received for review July 22, 1985. Revised manuscript received November 4, 1985. Accepted January 21, 1986. Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Synthesis of Alkoxy-, (Alkylthio)-, Phenoxy-, and (Phenylthio)pyrazines and Their Olfactive Properties

Hideki Masuda and Satoru Mihara*

The following series of 22 new pyrazines have been synthesized: two 5-alkyl-2-methoxy-3-methylpyrazines, four 5-alkyl-2-ethoxy-3-methylpyrazines, four 5-alkyl-3-methyl-2-phenoxypyrazines, three 5-alkyl-3-methyl-2-(methylthio)pyrazines, five 5-alkyl-2-(ethylthio)-3-methylpyrazines, and four 5-alkyl-3-methyl-2-(phenylthio)pyrazines. The IR, NMR, and mass spectra of these pyrazines were measured. $M^+ - SH$ peaks were observed for most of the mass spectra of (methylthio)- and (ethylthio)pyrazines. Odor descriptions and odor threshold values of a number of these pyrazines are presented and compared with each other.

Because of the characteristic flavors that possess roasted or smoky odors and low threshold values, alkoxy- and (alkylthio)pyrazines have been widely used as flavor ingredients (Maga, 1982). In 1969 (a,b) Buttery et al. reported the isolation of 2-isobutyl-3-methoxypyrazine from green peppers. The extremely potent odor was shown to be a character-impact compound of bell peppers with an

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0021-8561/86/1434-0377$01.50/0