Preparation and Characterization of Acid Hydrolysis Products of the Tomato Glycoalkaloid α-Tomatine

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As part of an effort to define the biological roles of carbohydrate groups of the tomato glycoalkaloid α-tomatine in plants and animals, experiments were carried out to optimize the acid hydrolysis of the tetrasaccharide side chain of α-tomatine to products with three, two, one, and zero sugar groups. This was accomplished by following the time course for hydrolysis in 1 N HCl at 100 °C, isolating the hydrolysis products by chromatography on an aluminum oxide column, and determining the number and nature of hydrolysis products, including free sugars, with the aid of thin-layer chromatography and gas chromatography/mass spectrometry of alditol acetate sugar derivatives. The results show that a 20-min hydrolysis time appears useful for the formation of a mixture of the monosaccharide δ-tomatine, the disaccharide γ-tomatine, and the trisaccharide β_1-tomatine. Efforts to isolate the other possible trisaccharide, β_2-tomatine, were unsuccessful, apparently because xylose is degraded during the hydrolysis. A 70-min exposure achieved complete hydrolysis of α-tomatine to the aglycon tomatidine. α-Tomatine was stable to hydrolysis at 37 °C, suggesting that it may also be stable to comparable acid conditions of the gut of insects, animals, and humans. The approach used should be generally useful for preparing hydrolysis products of glycoalkaloids and other glycosides such as saponins to facilitate assessing the role of the carbohydrate groups of these compounds in host-plant resistance, nutrition, and microbiology.

Keywords: *Acid hydrolysis; aluminum oxide chromatography; carbohydrate analysis; gas chromatography; glycoalkaloids; glycoside; mass spectrometry; thin-layer chromatography; tomatidine; α-tomatine; β_1-tomatine; β_2-tomatine; γ-tomatine; δ-tomatine; tomatoes*

INTRODUCTION

α-Tomatine, a tetraglycoside of the steroidal aglycon tomatidine, occurs naturally in tomatoes. Green tomatoes contain up to ∼500 mg of α-tomatine/kg of fresh fruit weight. The compound is partly degraded as the tomato ripens until, at maturity levels, red tomatoes contain ∼1% of the value found in mature green ones (Friedman and Levin, 1995; Kozukue et al., 1994). However, ripe red fruit grown in the Andean mountains also have a high tomatine content (Rick et al., 1984). It is noteworthy that feeding hamsters mature green tomatoes with an α-tomatine content of 48 mg/kg of fresh weight induced a greater lowering in low-density lipoprotein (LDL) cholesterol than was observed with the same variety of red tomatoes with an α-tomatine content of 0.4 mg/kg of fresh weight (Friedman et al., 1997a).

Although the plant enzymes that have been postulated to degrade α-tomatine during maturation of tomato fruit both during growth and after harvest are not well characterized (Eltayeb and Roddick, 1985; Heftmann and Schwimmer, 1972), several tomatinases (glycosidases that hydrolyze α-tomatine to β_1-, β_2-, γ-, and δ-tomatines and tomatidine) produced by phytopathogenic fungi growing on tomatoes have been isolated and characterized (Larini et al., 1996, 1997; Osbourn et al., 1996; Sandrock and Vanetten, 1998; Sandrock et al., 1996). Although in vivo glucosidase-catalyzed hydrolysis of pyridoxine glucoside occurs in the stomachs of rats and humans (Nakano et al., 1997; Trumbo et al., 1990), it is not known whether analogous glycosidases can hydrolyze tomatines. Since acid hydrolysis products of tomatines appear to be involved in the biosynthesis of the parent compound, in host-plant resistance, in microbiology, and in animal and human nutrition, the ready availability of such compounds should facilitate progress in these areas.

We previously described an approach designed to establish the structures of monosaccharides comprising the tetrasaccharide carbohydrate side chain of the minor tomato glycoalkaloid dehydrotomatine (Friedman et al., 1997b). The tetrasaccharide of dehydrotomatine was found to be the same as that of α-tomatine. Related studies showed that immunochemical aspects (Sporns et al., 1996; Stanker et al., 1994, 1996) and biological effects of potato and tomato glycoalkaloids on cell membranes (Blankemeyer et al., 1997, 1998; Keukens et al., 1995, 1996), cholesterol (Friedman et al., 1997a; Roddick and Drysdale, 1984), embryos (Friedman and McDonald, 1997; Friedman et al., 1992; Rayburn et al., 1994), and pathogens (Duffey and Stout, 1996; El-Raheem et al., 1995; Lazurevskii et al., 1980; Zhang and Mitchell, 1997) and in the diet (Friedman et al., 1996) are strongly influenced by the nature of the carbohydrate side chain attached to the 3-OH position of the steroidal part of the molecule. The nature, number, and order of attachment of the sugars in the oligosaccharide associated with the glycoalkaloids appear to influence biological potency.

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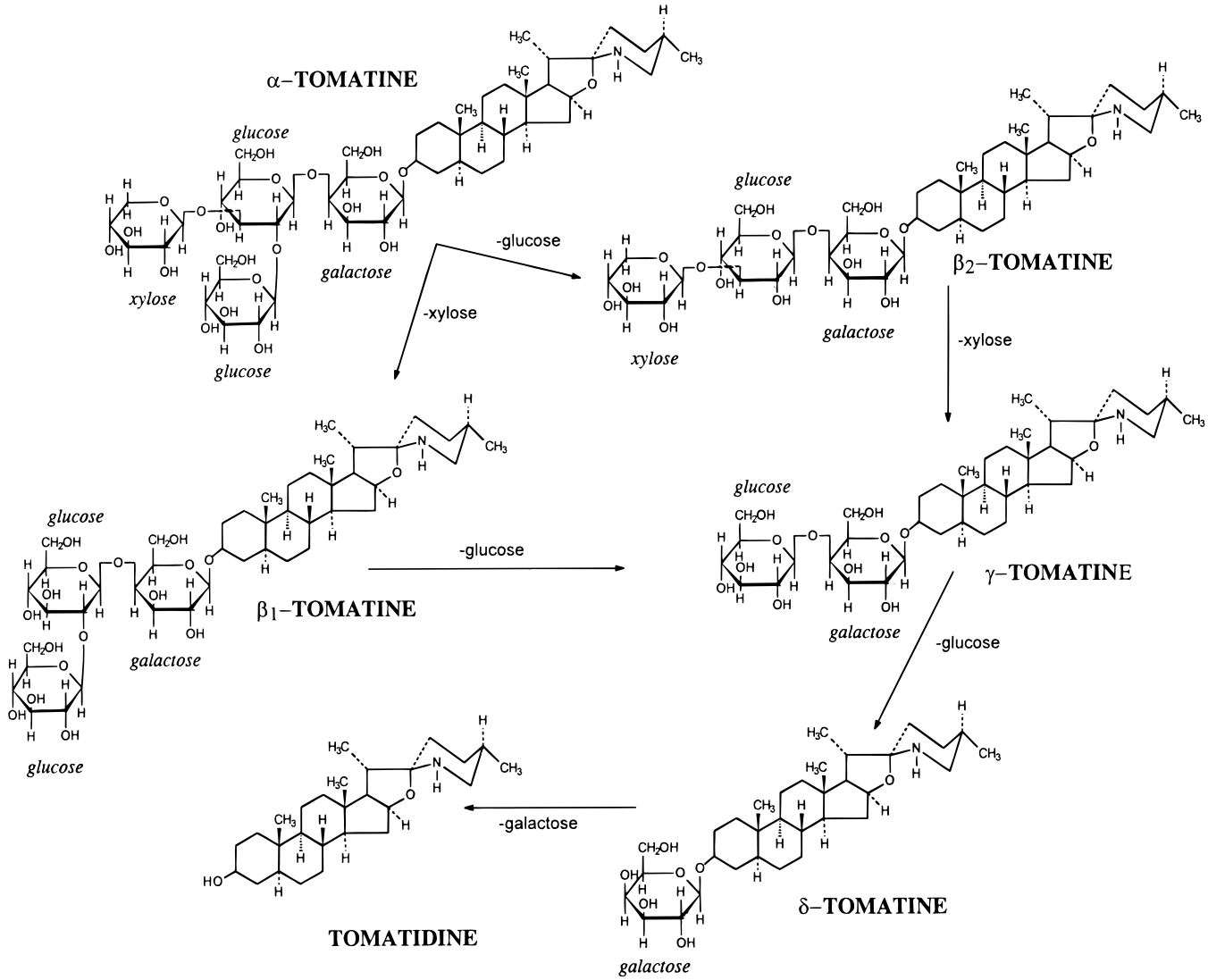

Figure 1. Hydrolytic pathways of the tetrasaccharide side chain of α-tomatine. α-Tomatine = *β*-D-galactopyranoside (3*β*,5α,-22*β*,25S)-spirosolan-3-yl-*O*-*β*-D-glucopyranosyl-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*O*-*β*-D-glucopyranosyl-(1→4). Tomatidine = (3*β*,5α,-22*β*,25S)-spirosolan-3-ol.

To better define the role of the carbohydrate groups of tomatines in nutrition, food safety, microbiology, and plant science, a need exists to develop improved methods for the large-scale preparation of tomatine glycoalkaloids with one, two, and three sugar groups. Our major objective was, therefore, to achieve a maximum yield of each saccharide via a single incomplete acid hydrolysis of α-tomatine to permit isolation and characterization of all possible hydrolysis products. To accomplish this objective, this study (a) defines the kinetic course of the acid hydrolysis, (b) describes conditions for the isolation of hydrolysis products by preparative chromatography, and (c) assigns structures to the isolated compounds with the aid of thin-layer chromatography (TLC) and gas chromatography/mass spectrometry (GC/MS).

Table 1. R_f Values of α-Tomatine and Its Hydrolysis Products on TLC and Yields of Products Isolated after Separation on an Aluminum Oxide Column

compound	R_f with solvent A[a]	B[b]	C[c]	D[d]	yield[e] (%)
commercial α-tomatine	0.09	0.22	0.28	0.59	
commercial tomatidine	0.79	0.85	0.88	0.86	
hydrolysis products					
tomatidine	0.79	0.85	0.88	0.86	27.5
δ-tomatine	0.37	0.53	0.66	0.75	6.6
γ-tomatine	0.19	0.34	0.45	0.67	13.8
β_1-tomatine	0.10	0.22	0.31	0.61	5.7

[a] Chloroform/methanol/1% NH_4OH (2:2:1, v/v/v, bottom layer). [b] Chloroform/methanol/2% NH_4OH (14:6:1, v/v/v). [c] Chloroform/methanol/1% NH_4OH (65:35:5, v/v/v). [d] Chloroform/methanol/1% NH_4OH (5:5:1, v/v/v). [e] Determined from weights of isolated products following chromatography on aluminum oxide.

MATERIALS AND METHODS

Materials. Activated aluminum oxide and anisaldehyde were obtained from Kant Chemical Co., Tokyo, Japan. Silica-coated TLC plates were purchased from Merck, Darmstadt, Germany. All other compounds and reagents came from Sigma Chemical Co., St. Louis, MO.

Time Course of Acid Hydrolysis of Commercial Tomatine. Four vials sealed with Teflon caps, each containing 1 mg of commercial α-tomatine and 1 mL of 1 N HCl, were heated in an oil bath at 100 °C for 15, 30, 45, and 60 min, respectively. The cooled solutions were neutralized with 1 N NH_4OH and partitioned three times with 1 mL of *n*-butanol. The butanol layer was separated and evaporated to dryness. The solid residue was dissolved in 1 mL of methanol. An aliquot (10 *μ*L) of each methanol solution was spotted on a TLC plate.

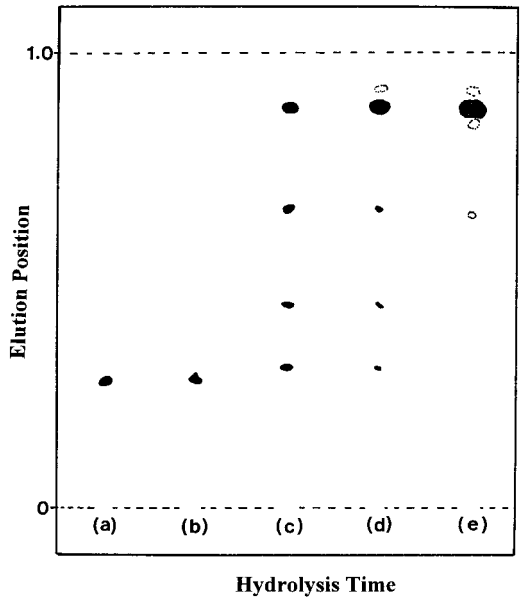

Figure 2. TLC of compounds formed on hydrolysis of α-tomatine in 1 N HCl at 100 °C for various time periods. Hydrolysis times: (a) α-tomatine standard; (b) 15 min; (c) 30 min; (d) 45 min; (e) 60 min. TLC conditions: solvent, chloroform/methanol/1% NH_4OH (65:35:5, v/v/v, bottom layer); detection, anisaldehyde spray followed by heating at 120 °C for 5 min.

An analogous experiment was carried out at 37 °C.

Isolation of Hydrolysis Products. A 10-mL vial with a sealed Teflon cap, containing 100 mg of commercial α-tomatine dissolved in 4 mL of 1 N HCl, was heated at 100 °C for 20 min. The cooled solution was neutralized with 1 N NaOH and partitioned four times with 10 mL of water-saturated butanol. The combined butanol layers were evaporated to dryness on an aspirator at 45 °C, and the residue was dissolved in 6 mL of methanol. The methanol solution was then applied to an aluminum oxide column (30 × 1.5 cm). The compounds were eluted with water-saturated butanol at a flow rate of 0.5 mL/min controlled with a Hitachi L-600 pump. The eluate was collected in 5-mL fractions. The fractions were examined by TLC for detection of hydrolysis products. These were visualized by spraying with the anisaldehyde reagent. Fractions with the same elution position on the TLC plate were combined and evaporated to dryness. These were then characterized further by GC/MS as described below.

Acid Hydrolysis to the Aglycon Tomatidine. The three glycoalkaloids obtained from partial acid hydrolysis of α-tomatine and purified by aluminum oxide chromatography were further hydrolyzed to the aglycon tomatidine as follows. Fractions 13−19 (1.65 mg), fractions 21−28 (2.0 mg), and fractions 36−40 (1.43 mg) were each dissolved in 1 mL of 1 N HCl in a 5 mL vial with a sealed Teflon cap. The vials were heated at 95−100 °C for 70 min. After cooling, the mixture was neutralized with 1 N NH_4OH and partitioned five times with 2 mL of benzene. The combined benzene solutions were washed five times with 2 mL of water. The benzene was then evaporated to dryness and the residue dissolved in 1 mL of benzene. Aliquots of these solutions were used to demonstrate by GC/MS the complete hydrolysis of the glycoalkaloids to the aglycon tomatidine.

Characterization of Carbohydrates as Alditol Acetates. The aqueous phases containing the individual carbohydrates derived from the above-described benzene extractions were desalted by passage through columns of Oregano cation (IR-120, H^+) and anion (CG-400, $HCOO^-$) exchange resins. An internal standard, 203 μg of methyl-β-glucose, was added to the desalted solution. The solution was then evaporated to dryness on a water aspirator at 40 °C.

The desalted sugars were dissolved in 3 mL of H_2O to which was added 5 mg of $NaBH_4$. The mixture was kept at room temperature for 2 h. Five drops of acetic acid was then added to stop the reaction, and the solution was evaporated to dryness. The sodium borohydride-derived boric acid was then removed by codistillation with methanol (3 mL added five times). The residue was acetylated by treatment with acetic anhydride/pyridine (1:1.2 mL) for 10 min at 95−100 °C. The reaction mixture was left standing overnight. Methanol (5 mL) was then added and the mixture evaporated to dryness. Methanol addition−evaporation was repeated five times. Chloroform (3 mL) was then added, the mixture was shaken to extract the alditol acetate sugars, and the chloroform was separated from the aqueous layer. This procedure was repeated five times. The combined chloroform extracts were then washed five times with 5 mL of H_2O. The chloroform layers were transferred with a pipet, and the chloroform was evaporated by passing a stream of nitrogen over its surface.

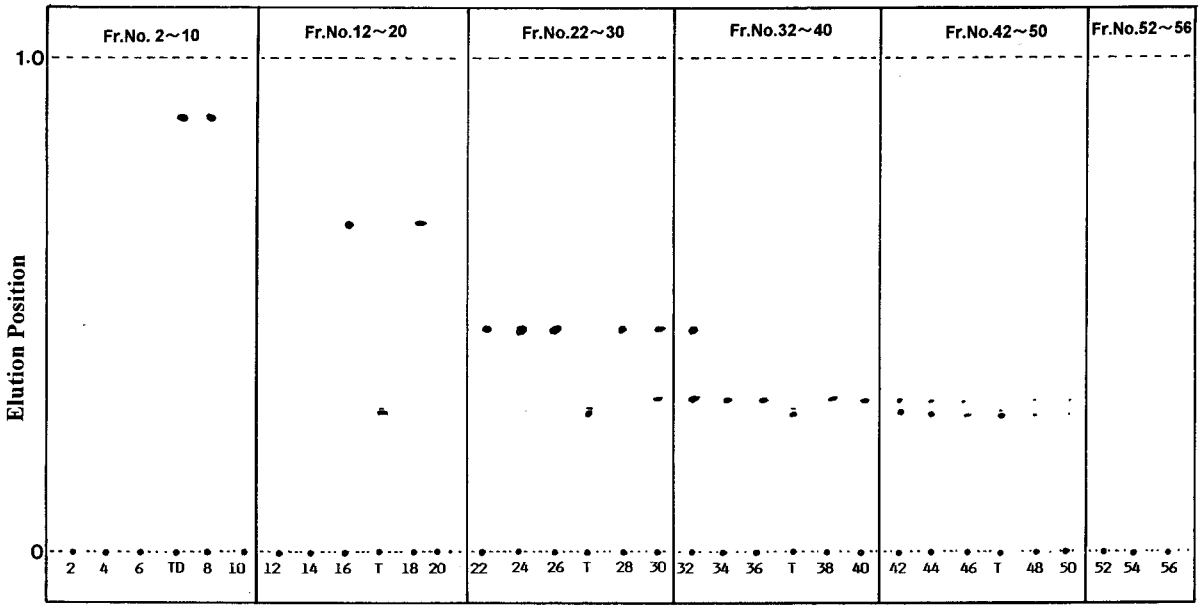

Figure 3. TLC of compounds formed on hydrolysis of α-tomatine in 1 N HCl at 100 °C for 20 min and eluted from the aluminum oxide column. TLC conditions: same as in Figure 2. Abbreviations: Fr., fraction; TD, tomatidine; T, α-tomatine.

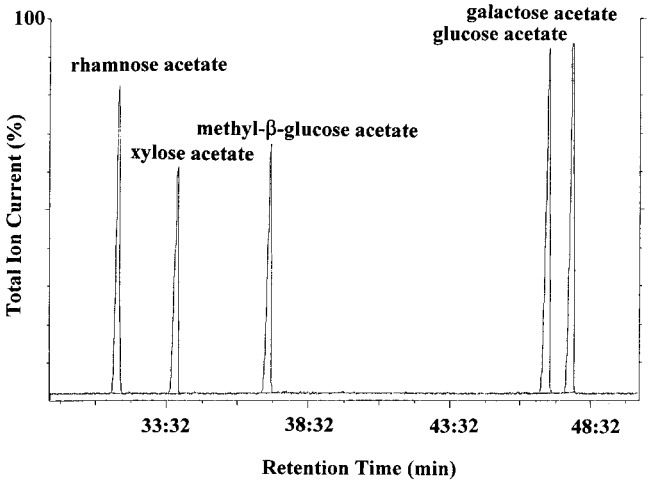

Figure 4. GC of sugar standards determined as alditol acetates.

The residue was dissolved in 2 mL of chloroform. Aliquots of this solution were used for GC/MS.

TLC. TLC was performed on Merck precoated silica gel G plates, 0.25 μm thick. The plates were developed in a chamber with four different solvents shown in Table 1. Spots were visualized by spraying with anisaldehyde reagent and heating for 5 min at 120 °C.

Mass Spectrometry of Tomatidine. Tomatidine was analyzed by liquid secondary ion mass spectrometry (LSIMS) as described previously (Friedman et al., 1993, 1997b).

GC/MS of Carbohydrate Derivatives. GC/MS was performed on a GCQ gas−liquid chromatography ion trap mass spectrometer (Finnigan, San Jose, CA). The split/splitless injector was operated at 240 °C. Injection was made in splitless mode. Chromatography was performed with a 0.25 mm × 30 m, 0.25 μm film, DB-5MS, fused silica column (J&W Scientific, Folsom, CA) with an average helium carrier gas flow set to a constant velocity of 40 cm/s. The column oven temperature was held at 120 °C for 1.5 min and then programmed to 195 °C at 1.5 °C/min. The mass spectrometer was operated in the electron ionization mode with a source temperature of 200 °C. Positive ions were monitored by scanning the analyzer from mass 50 to mass 650 every 0.75 s.

RESULTS AND DISCUSSION

Previously, Keukens et al. (1995) attempted to determine by NMR the structures of all possible hydrolysis products of α-tomatine after hydrolysis in 5% H_2SO_4 at 60 °C using a single hydrolysis time of 16 h and separation on an HPLC column. They could not separate β_1- and β_2-tomatines by HPLC. Biermann (1989) points out that hydrolysis of saccharides with H_2SO_4 has a drawback because of the necessity of its removal using $BaCO_3$ to precipitate sulfate prior to derivatization. Some of the carbohydrate is probably adsorbed during the precipitation step. Formation of sulfated byproducts is also a possibility. For these reasons, we selected HCl as the hydrolysis medium for our kinetic and preparative studies. Thus, our approach complements and extends earlier studies designed to establish conditions for the formation and characterization of a mixture of all possible hydrolysis products of tomatines.

TLC showed that exposure of α-tomatine to 1 N HCl at 37 °C for up to 3 h did not produce any of the hydrolysis products described below. The TLC plate had only the single spot associated with α-tomatine. This observation indicates that α-tomatine is probably not hydrolyzed by similar acid conditions in the digestive tracts of insects and animals, including humans.

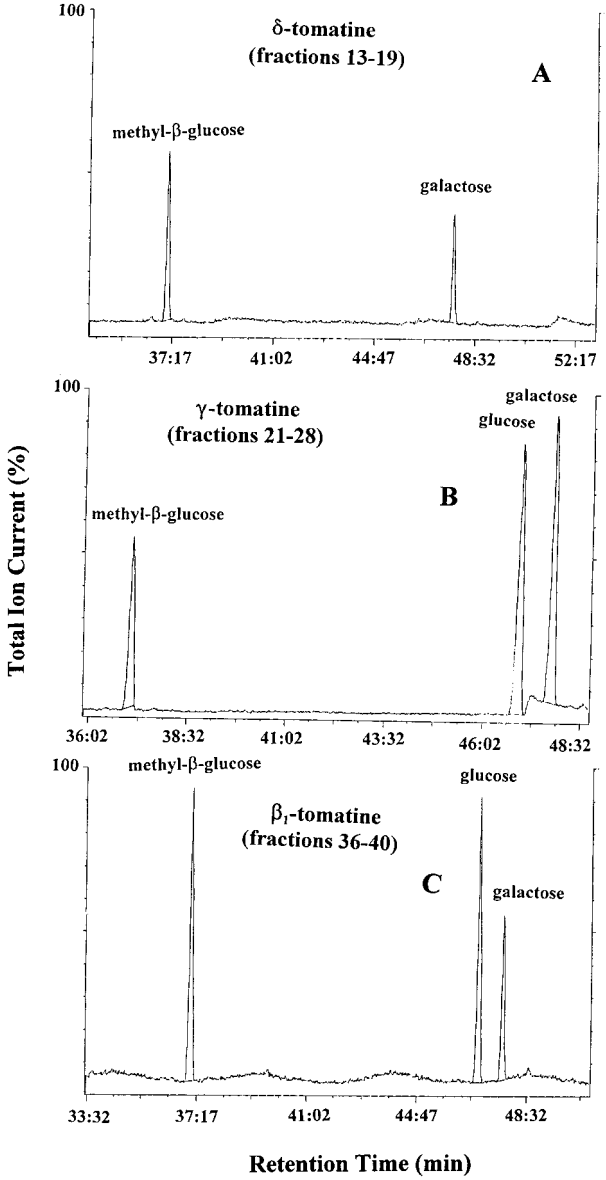

Figure 5. (A) GC of galactose (after derivatization to the acetate) formed on acid hydrolysis of δ-tomatine and the internal standard methyl-β-glucose. (B) GC of a 1:1 ratio of glucose and galactose (as the acetates) formed on acid hydrolysis of γ-tomatine. (C) GC of a 2:1 ratio of glucose and galactose (as the acetates) formed on acid hydrolsyis of β_1-tomatine.

Whether enzyme (glycosidase)-catalyzed hydrolysis occurs in vivo is not known.

Figure 1 shows that partial acid hydrolysis of α-tomatine can in principle produce a total of five compounds: two isomeric trisaccharides, β_1- and β_2-tomatines; the disaccharide γ-tomatine; the monosaccharide δ-tomatine; and the aglycon tomatidine. The time course of hydrolysis of α-tomatine revealed that hydrolysis in 1 N HCl at 100 °C for 20 min generated a mixture of partial hydrolysates suitable for separation by column chromatography on aluminum oxide. Four pure compounds were isolated from fractions 7−9, 13−17, 21−28, and 36−40, respectively, collected from the aluminum oxide column, as determined by TLC (Figures 2 and 3; Table 1).

The compound isolated from fractions 7−9 was identified as tomatidine by comparison on TLC with an authentic sample and by GC/MS. The three compounds

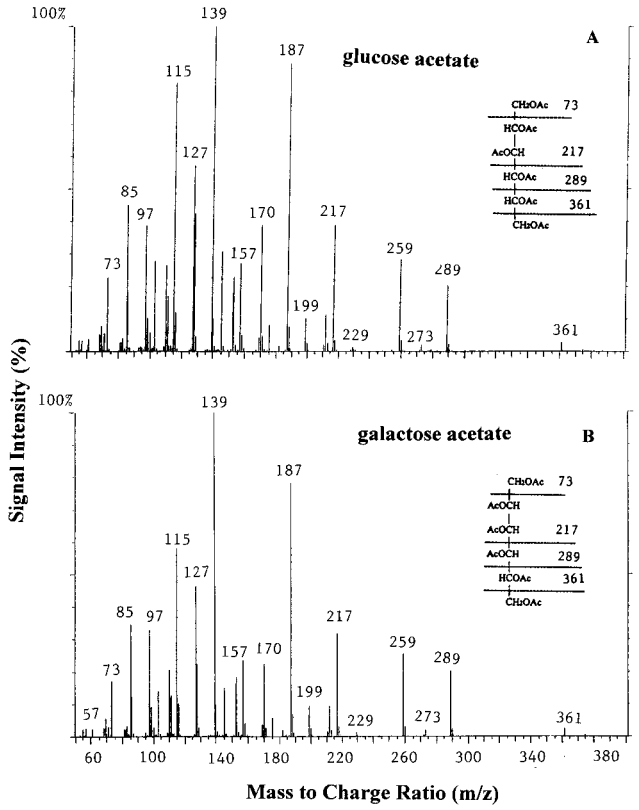

Figure 6. (A) Mass spectrum of glucose (as acetate) derived from hydrolysis of β_1- and γ-tomatines. (B) Mass spectrum of galactose (as acetate) derived from hydrolysis of δ-tomatine.

from fractions 13−17, 21−28, and 36−40, respectively, were further acid hydrolyzed with 1 N HCl for 70 min at 100 °C to form the aglycon and individual sugars. The aglycon was identified as tomatidine by GC/MS, suggesting that these compounds are glycoalkaloids. Analysis of the sugars associated with these glycoalkaloids permits assignment of structures to these compounds. This was accomplished by converting the free sugars to alditol acetate derivatives and determining the nature and molar ratios of the sugars by GC/MS. Since fractions 13−17 contained only galactose (Figures 4 and 5A), this compound was identified as the monoglycoside δ-tomatine. Fractions 21−28 contained glucose and galactose in a molar ratio of 1.00:1.053 (Figure 5B). These sugars are therefore associated with the diglycoside γ-tomatine. Fractions 36−40 also contained glucose and galactose but in a molar ratio of 1.94:1.00 (Figure 5C). These sugars are identified with the triglycoside β_1-tomatine.

Figure 6A shows the mass spectrum of glucose (as the acetate) derived from the hydrolysis of both γ-tomatine and β_1-tomatine. The corresponding spectrum for galactose formed on hydrolysis of δ-tomatine is shown in Figure 6B. The mass spectra of these sugar acetates are identical to those observed with standard sugar acetates determined separately.

The formation of the second possible trisaccharide β_2-tomatine (Figure 1) was not observed. Because this compound would contain xylose and because this sugar was not found in any of the hydrolysis solutions, the pentose is probably degraded in the acid conditions used for hydrolysis. This compound can be obtained by fungal enzyme-catalyzed hydrolysis of the xylose−glucose glycosidic bond of tomatine (Larini et al., 1996; Sandrock et al., 1996) and possibly also by enzyme-catalyzed xylosylation of UDP-xylose and γ-tomatine, in analogy to reported glycosylations by other UDP-sugars (Moehs et al., 1997; Stapleton et al., 1991; Zimowski, 1994). It is relevant to note that previously we could not find the hydrolysis product β_1-solanine in a hydrolysis mixture of the potato glycoalkaloid α-solanine (Friedman et al., 1993).

The mass spectra of glucose and galactose acetates formed on acid hydrolysis of tri-, di-, and monoglycosides, β_1-, γ-, and δ-tomatines (Figures 5 and 6), were analogous to those described previously for the monosaccharides derived from hydrolysis of the tetrasaccharide side chain of α-tomatine and dehydrotomatine (Friedman et al., 1997b). These observations support the structures assigned to β_1 -, γ-, and δ-tomatines formed on incomplete acid hydrolysis of α-tomatine. They also demonstrate that it is possible to isolate these compounds on a preparative scale (Table 1, last column) for further studies of their roles in the plant, in the diet, and as antimicrobial compounds.

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