Identification of 5-*O*-Caffeoylquinic Acid in Limpograss and Its Influence on Fiber Digestion

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Chlorogenic acid (5-*O*-caffeoylquinic acid, CHLA) was identified as a major phenolic acid in limpograss. This was based on retention times using high-performance liquid chromatography under isocratic and gradient conditions with several solvent systems, UV spectra, thin-layer chromatography spotting, and identification of caffeic acid and quinic acid in the saponified unknown compound. The 1-, 3-, and 4-*O* isomers of CHLA were produced and separated by HPLC, eliminating them as the possible unknown. Analyses using MS and GC–MS with a derivatized sample were unsuccessful in identifying the compound. Chlorogenic acid was highly soluble in water or methanol and was relatively stable compared to caffeic acid. Concentration of CHLA in herbage was affected by harvest date, morphology, and variety. High concentrations of CHLA were found in herbage sampled early in the growing season. The upper portion of the canopy contained the highest concentrations of CHLA. The tetraploid variety Floralta contained much higher concentrations of CHLA than two diploids or another, less persistent, tetraploid limpograss variety. Chlorogenic acid negatively affected in vitro cell wall digestion at early digestion times, but the mixed bacterial culture appeared to eventually overcome the effect of the CHLA addition. No CHLA was detected in samples undergoing in vitro digestion 6 h after addition of CHLA.

Limpograss [*Hemarthria altissima* (Poir.) Staph. & Hubbard] originated in Africa and is used as a forage crop in the southeastern United States, particularly in Florida. Although conventional laboratory analyses suggest that this perennial grass has acceptable forage quality, animal performance is lower than that predicted by laboratory analyses.

Caffeic acid was previously identified as a major alkali-labile component of limpograss (Cherney et al., 1989b). Low molecular weight phenolic acids, such as caffeic acid, may decrease utilization of forage fiber by limiting microbial digestion of bound structural carbohydrates. Bacterial inhibition of fiber digestion by hydroxycinnamic acids has been observed in several studies (Borneman et al., 1986; Herald and Davidson, 1983). Benzoic, cinnamic, and caffeic acids depressed digestion of cellulose (Jung, 1985).

Caffeic acid is very sensitive to air oxidation and, as a result, is difficult to isolate and quantify in plants (Huang et al., 1986). Although alkali-labile caffeic acid was found in abundance, free caffeic acid was not found in water extracts of limpograss. Our objectives were to (1) identify the chemical form of caffeic acid in limpograss and (2) determine the effect of the identified compound on digestibility.

MATERIALS AND METHODS

Plant Materials. Limpograss (var. Floralta) samples were collected on July 13 and September 7, 1988, near Gainesville, FL. Samples were collected from two different fields and separated into upper and lower canopy (i.e., lower half of plant height was lower canopy). Plants were also separated into leaf blade and stem plus leaf sheath fractions.

In 1989, samples were collected from three replicates of four limpograss varieties: Floralta, Bigalta, Redalta, and Greenalta. Herbage was removed from all plot areas on July 24, 1989, and samples were collected on August 31 and September 19, 1989. Samples also were taken on October 31, 1989, from previously unharvested areas. Roots were sampled to a 15-cm depth on October 31, 1989, and washed to remove soil. Herbage samples were oven-dried (60 °C) and ground to pass a 1-mm screen. For in vitro digestion studies, alfalfa (*Medicago sativa* L.) at first flower and orchardgrass (*Dactylis glomerata* L.) at a heading stage were used as forage substrates.

Extraction of Plant Materials. Herbage samples (0.1 g) were extracted with 10 mL of 100% methanol for 5, 10, 15, and 30 min and 1, 2, 3, 4, 6, 8, 12, 16, 20, and 24 h to determine an optimum extraction time. Subsequent extractions were for 1 h. Plant extracts were filtered and brought up to a 50-mL volume with water, and a portion of this solution was passed through a 0.45-μm nylon 66 membrane filter. In all cases, sample extraction immediately preceded injection.

Analytical Methods. Filtered extract (100 μL) was injected into a high-performance liquid chromatograph (HPLC) under conditions described by Cherney et al. (1989b). Several isocratic and gradient conditions with different solvents were used. Isocratic conditions used included 0.7% aqueous acetic acid (A, 80%) and 50% aqueous acetonitrile (B, 20%) and 0.7% aqueous acetic acid (A, 80%) and 100% methanol (B, 20%). Under gradient conditions, solvent A was either aqueous acetic acid (0.7%) or aqueous phosphoric (1.0%), and solvent B was either aqueous acetonitrile (50%) or methanol (100%). The gradient was 10-20% B over the first 10 min, 20–50% B over the next 15 min, and 50% B for 5 min, followed by reequilibration.

Thin-Layer Chromatography. Silica plates were washed in chloroform/methanol. Samples and standards were spotted onto the plates and placed in a chamber with 1-butanol/acetic acid/water (4:1:1). When the solvent front approached the top of the plate, plates were removed and checked for phenolics with a UV light source.

Quinic Acid Assay. The presence of quinic acid was determined in samples by reaction with periodate and thiobarbitu-

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rate (Levy and Zucker, 1960) after saponification with 1 M NaOH (Davies et al., 1978). Absorption at 549 nm for the unknown was compared to quinic acid standards.

Mass Spectroscopy. Fractions that were presumed to be CHLA were collected after HPLC separation and evaporated to dryness. Samples were analyzed by using the direct insertion probe (heated to 300 °C) of a Finnigan 4000 mass spectrometer. The compound also was derivatized by adding 0.1 mL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) to the dried compound and heating at 110 °C for 30 min. The derivatized compound was analyzed by using GC–MS with the following column conditions: 30-m DB-1 column (J&W Scientific) held at 100 °C for 2 min and then programmed to 240 °C at 10 °C min^{-1}. Both electron impact (EI) and isobutane chemical ionization (CI) were used in both MS analyses. Fast atom bombardment (FAB) also was used on derivatized compounds.

In Vitro Digestion. Alfalfa (variety Hi-phy) and orchardgrass (variety Pennlate) (0.25 g) were used as substrates to determine the effect of chlorogenic acid (CHLA) on fiber digestion. The alfalfa substrate was harvested at a flowering stage with a neutral detergent fiber (NDF) concentration of 521 g kg^{-1}. The orchardgrass substrate was harvested at a flowering stage with an NDF concentration of 569 g kg^{-1}. A standard in vitro digestion using filtered rumen fluid (Marten and Barnes, 1980) was carried out in sealed serum bottles. After digestion, samples were extracted with neutral detergent solution [Robertson and Van Soest (1980), as modified by Cherney et al. (1989a)], filtered, rinsed, and dried. Chlorogenic acid (0, 5.0, and 10.0 mg) was added to serum bottles at either 2 or 12 h after the start of the incubation with rumen fluid. Standard in vitro digestibility of cell walls was calculated after 0, 12, 24, 36, 48, and 60 h for orchardgrass and after 0, 6, 13, 16, 24, and 30 h for alfalfa.

RESULTS AND DISCUSSION

Identification of Unknown Compound. Limpograss was extracted with methanol, and components in the extract were separated by HPLC. The largest peak detected at 280 or 320 nm had a retention time that did not correspond with any of the common phenolic monomers. This compound was collected as it eluted from the column. The compound would not extract into ether or ethyl acetate without the solution first being acidified to pH 3.

When the collected unknown peak was treated with NaOH, caffeic acid was released and detected by using HPLC. Esters containing caffeic acid occurring in nature are found with tartaric acid, glucose, and quinic acid. The most common ester is 5-*O*-caffeoylquinic acid (CHLA), found in relatively large quantities in coffee beans (Iwahashi et al., 1986) and in other plant storage organs such as potato tubers (Malmberg and Theander, 1985).

Mass spectrometry analysis was performed on the compound in HPLC solvent, ether, and ethyl acetate solutions. No significant response was observed (except for solvent spectra) to allow identification of the compound. It should be noted that the unknown remained in the sample vial and did not volatilize during MS probe analysis. This indicated that the unknown compound may be in some nonvolatile salt form. This collection/analysis procedure was repeated numerous times, changing column temperature and other variables, without success in identifying the unknown. In addition, FAB analysis also proved unsuccessful. Derivatization of the peak of interest with BSTFA followed by GC–MS also gave negative results. Mass spectrometry of authentic CHLA using a probe or GC–MS following derivatization produced the appropriate spectrum identifying this compound. If authentic CHLA was first passed through the HPLC column, however, MS analysis still could not identify the peak as CHLA. The cause of this interference in detection of CHLA is not known. Chlorogenic acid has been isolated and tentatively identified in a number of species by using gas–liquid chromatography, HPLC, or colorimetric procedures (Davies et al., 1978; Lahiry et al., 1977; Malmberg and Theander, 1985); however, none of these studies identified CHLA by using MS.

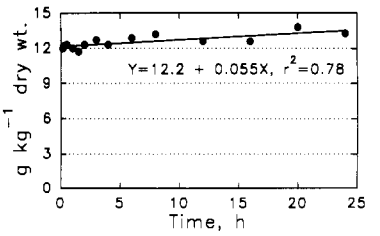

Figure 1. Chlorogenic acid in upper canopy, July harvest limpograss stems extracted with methanol for different time intervals.

Other less direct methods were used to identify the compound, because of the problems with MS analysis. Authentic CHLA produced the same retention times as the unknown of interest when chromatographed by using several different solvents as well as isocratic and gradient conditions. The UV spectra of CHLA and the unknown matched each other well. Results from TLC gave identical results for CHLA and the unknown. Quinic acid was identified by colorimetric means in the unknown compound following saponification.

A caffeoylquinic acid ester can exist in four forms: 1-, 3-, 4-, or 5-*O*-caffeoylquinic acid. The three isomers of CHLA can be produced by heating CHLA in saturated bicarbonate (Nagels et al., 1980). The isomers were separated by HPLC and the 5-*O* isomer corresponded in retention time to the CHLA peak from limpograss. On the basis of the tests mentioned above, the unknown compound was positively identified as CHLA (5-*O*-caffeoylquinic acid).

Chlorogenic acid in limpograss stems was extremely soluble and was released almost immediately after ground tissue was wetted with methanol (Figure 1). There was a small increase in CHLA extracted due to long extraction times, but 1 h was chosen as a convenient standard time of extraction. The release of CHLA after wetting with water was equally as fast (data not shown).

Chlorogenic Acid Content of Tissues. Although samples were oven-dried, previous limpograss samples split in half and subjected to oven-drying or freeze-drying did not differ in caffeic acid concentration (data not shown). Higher concentrations of CHLA ($P < 0.01$) were found in the upper portion of the canopy at both harvests (Table I). Withing a given canopy level, stem plus sheath was higher in CHLA concentration than leaf blades at the first harvest, but lower than leaf blades at the second harvest. Replicate values were very consistent, and all main effects (harvest date, plant component, and canopy level) were significant. Differences in CHLA between harvest dates and morphological components may have significance to a grazing ruminant if CHLA affects digestion or metabolism in the animal.

A previous study (Cherney et al., 1989b) surveyed a range of forage species and found significant quantities of caffeic acid in two species, limpograss and perennial peanut. Although perennial peanut contained substantial quantities of caffeic acid, no CHLA was detected in these samples (data not shown), indicating that caffeic acid is present in a different form in perennial peanut.

Varietal Differences. Cultivars Redalta and Greenalta are diploids, while Bigalta is a tetraploid. Floralta is a relatively new tetraploid cultivar that is more persistent than Bigalta. Floralta had a much higher concentration

Table I. Chlorogenic Acid Concentration (Grams per Kilogram Dry Weight) As Influenced by Harvest Date, Canopy Level, and Plant Part[a]

	July 13	Sept 7
upper canopy		
leaf blade	6.74	5.81
stem plus sheath	11.74	4.98
lower canopy		
leaf blade	4.71	3.06
stem plus sheath	6.44	1.40

	probability (≤)
harvest date	0.04
canopy level	0.01
plant part	0.03
harvest date × canopy level	0.51
harvest date × plant part	0.01
canopy level × plant part	0.03

[a] Means of two replicates. Samples were taken in 1988.

Table II. Chlorogenic Acid Concentration (Grams per Kilogram Dry Weight) As Influenced by Cultivar and Harvest Date[a]

	herbage	roots[b]
cultivar		
Bigalta	2.69	0.31
Floralta	4.08	0.34
Greenalta	2.61	0.25
Redalta	2.22	0.35
BLSD[c]	0.24	NS
harvest date		
Aug 31	4.05	
Sept 19	2.67	
Oct 31	1.98	
BLSD	0.20	

[a] Means of three replicates. Samples were taken in 1989. [b] Roots were harvested only on Oct 31. [c] Bayes least significant difference, $k = 100$ (approximately $P = 0.05$); NS, not significant at 0.05 level of probability.

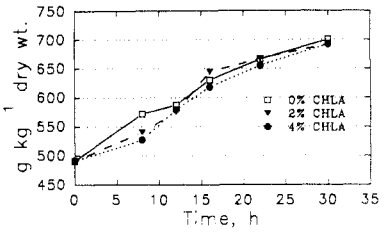

Figure 2. Influence of chlorogenic acid on in vitro digestibility of alfalfa. Chlorogenic acid was added (2 h after addition of rumen fluid) to incubation bottles at 0, 2, or 4% of the alfalfa dry weight.

of CHLA than the other three cultivars, particularly at the first harvest data (Table II). The cultivar × harvest date interaction was highly significant, because of the very high CHLA concentration (6.1 g kg^{-1}) in Floralta at the first harvest date.

Phenolic glucosides may play a role in pest resistance (Lyons et al., 1988). It is possible that CHLA contributes to pest resistance and may help explain why Floralta is more persistent than Bigalta, which is significantly lower in CHLA concentration. Roots contained relatively low levels of CHLA, with no apparent differences between cultivars (Table II). These results may have been negatively influenced, however, by washing of roots, because CHLA is highly soluble in water.

Digestibility. Chlorogenic acid was added to three replicates of sealed serum bottles containing a buffer, rumen fluid, and either alfalfa (Figure 2) or orchardgrass (Figure 3) 2 h after addition of rumen fluid. These two

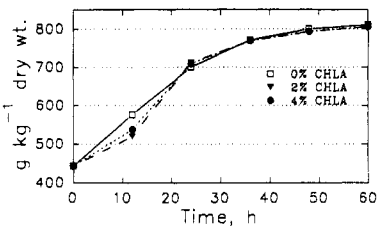

Figure 3. Influence of chlorogenic acid on in vitro digestibility of orchardgrass. Chlorogenic acid was added (2 h after addition of rumen fluid) to incubation bottles at 0, 2, or 4% of the alfalfa dry weight.

species had no detectable endogenous CHLA. Serum bottles were removed from the incubator at intervals to determine the progress of cell wall digestion. Removal times were different for alfalfa and orchardgrass because legume cell walls digest much faster than grasses. In both species CHLA negatively affected digestion at the earlier times, with a 7.8% drop in digestibility for alfalfa (at 8 h) and a 9.8% drop in digestibility of orchardgrass (at 12 h). The mixed bacteria culture appeared to eventually overcome the effect of the CHLA addition. A standard 48-h in vitro digestion, therefore, probably would not show a significant effect due to CHLA.

One other possible action of CHLA would be to directly affect animal performance if it was absorbed into an animal's bloodstream. Besides the potential effects of CHLA and/or caffeic acid on bacterial growth, CHLA has been shown to reduce the availability of lysine in vitro (Davies et al., 1978), and both chlorogenic and caffeic acids inhibited retinoic acid epoxidation (Iwahashi et al., 1986).

Survival of CHLA. Chlorogenic acid (5 mg, or 2% of sample weight) was added to incubating samples of orchardgrass at 2 and 14 h and liquid removed 2, 6, and 10 h after addition of CHLA. The liquid was filtered and analyzed for CHLA by using HPLC. No CHLA was detected at any time in incubating control samples that had no CHLA added. When added at 2 h, 27.5% (SD = 5.6) of the CHLA remained 2 h after it was added to the incubating sample. When added at 14 h, 10.5% (SD = 0.6) of the CHLA remained 2 h after it was added to the vessel. Six hours after addition of CHLA, there was no detectable CHLA in the incubation vessels.

It appears that CHLA may have a bactericidal effect and is broken down as it exerts this effect. Caffeic acid was detected in samples 2 h after addition of CHLA, but in relatively small quantities (ca. 0.5% of original CHLA added at either 2 or 14 h). The presence of caffeic acid 2 h after addition of CHLA indicates that breakdown of CHLA may start with the breaking of the ester bond. One mole of CHLA should release 1 mol of caffeic acid. Caffeic acid has been shown to depress digestion (Jung, 1985), but it is not very stable (Cherney et al., 1989b) and is broken down quickly. After CHLA and caffeic acid are broken down, their negative effect on digestion is removed, such that extent of the in vitro digestion is not adversely affected.

Results indicate that CHLA is a major soluble phenolic in limpograss. Concentration of CHLA is affected by harvest date, morphology, and variety. Chlorogenic acid may be a source of pest resistance in limpograss, but its effect on ruminants cannot be easily determined through in vitro studies.

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