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THE NATURAL ABUNDANCE OF L-CANAVANINE, AN ACTIVE ANTICANCER AGENT, IN ALFALFA, *MEDICAGO SATIVA* (L.)

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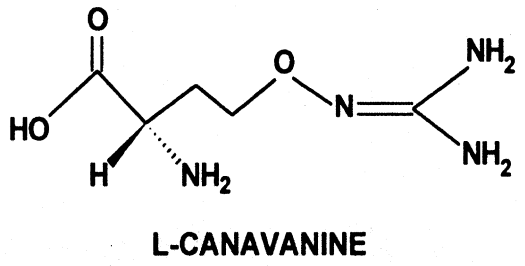
ABSTRACT

L-Canavanine, a potentially toxic antimetabolite of L-arginine that is stored by many leguminous plants, has demonstrative antineoplastic activity against a number of animal-bearing carcinomas and cancer cell lines. This investigation evaluated the natural abundance of this anti-cancer compound in commercially available sprouts, and in ten varieties of the seed of alfalfa, Medicago sativa *(L.). Canavanine abundance in commercially grown sprouts varied according to the source; the young plant stored appreciable canavanine that ranged from 1.3 to 2.4% of the dry matter. Alfalfa seeds were also rich in this nonprotein amino acid as the canavanine content varied from 1.4 to 1.8% of the dry matter. On average, the tested seeds contained 1.54 ± 0.03% canavanine. Alfalfa seed canavanine content was comparable to the levels found in the seeds of representative members of the genus* Canavalia, *which are amongst the more abundance sources of this antimetabolite.*

INTRODUCTION

The nonprotein amino acid, L-canavanine is a potent arginine antimetabolite stored by many leguminous plants (Bell, 1958; Bell et al., 1978) as part of their chemical barrier against predation and disease-causing organisms (Rosenthal, 1977a, 1992).

This arginine antagonist also elicited antineoplastic effects against a number of carcinomas. This observation was made initially by Kruse and McCoy (1958) who reported that canavanine competed with arginine in meeting the growth requirements of Walker carcinosarcoma 256 cells. Kruse et al. (1959) subsequently demonstrated that canavanine was incorporated into the proteins of these cancer cells, and that the diminution in the arginine content of the protein hydrolysate was equal to the amount of canavanine.



L-CANAVANINE

A detailed study of canavanine's anticancer activity was conducted with mice bearing L1210 leukemic cells (Green et al., 1980). These workers reported that DNA synthesis fell to only 9% of the control level, as assessed by [^{3}H]thymidine incorporation, after 12-hourly *i.p.* injections of 20 mg canavanine. In a follow-up study, Green and Ward (1983) reported that canavanine enhanced significantly the efficacy of γ-irradiation of cultured HT-29 cells, a human tumor cell line. The lethal effect of this radiation was augmented both when canavanine was provided prior to as well as after γ-irradiation. These workers provided convincing experimental evidence for their contention that canavanine's lethal effect was manifested preferentially in rapidly proliferating cells – a property essential to chemotherapeutic efficacy (Green & Ward, 1983).

Canavanine disrupted the growth of a colonic carcinoma in male Fischer rats (Thomas et al., 1986). Providing 3.0 g kg^{-1} canavanine by parenteral injection to animals bearing palpable tumors, resulted in a 13% reduction in tumor volume after 5 treatment days (Thomas et al., 1986). Because of the preferential uptake of L-[*guanidinooxy*-1^{4}C]canavanine into

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pancreatic proteins (Thomas & Rosenthal, 1987), canavanine's ability to affect the growth of MIA PaCa-2, a human pancreatic adenocarcinoma cell line, was assessed. Exposing these cells to canavanine, in a minimal arginine-containing media, resulted in significant cell death and an IC_{50} value of 10 μM for canavanine (Swaffar et al., 1994).

The natural abundance of canavanine in alfalfa products has also stimulated considerable interest due to the putative linkage between canavanine consumption and the onset of a systemic lupus erythematosus(SLE)-like syndrome. The first suggestion that canavanine may be deleterious to humans came from studies of the primate, *Macaca fascicularis* (cynomolgus macaque) that were fed alfalfa sprouts (Malinow et al., 1982). These workers reported that canavanine constitutes about 2% of the weight of the fresh sprout. When these animals were maintained on a diet that was supplemented with 40% alfalfa sprouts for 7 months they developed a SLE-like syndrome. In spite of the large amount of consumed legume and the long duration of treatment, this is a noteworthy finding because SLE is a serious disease of humans. It is germane that macaques that developed the disease ceased to exhibit adverse symptoms when alfalfa was removed from their diet. When the animals were fed a diet containing 1% (w/w) L-canavanine sulfate, the SLE-like syndrome was reactivated. Malinow et al. (1984) reported that alfalfa seeds, which were autoclaved for 2 hr, contained no detectable canavanine. There were no symptoms of a syndrome resembling SLE in cynomolgus macaques that were fed autoclaved alfalfa seeds for up to 1 yr. These results supported the contention that canavanine was responsible for the adverse effects of alfalfa seeds and sprouts observed in monkeys.

Given the growing evidence of the anti-cancer efficacy of canavanine, and the evidence linking its consumption to a SLE-like syndrome, we re-opened the important question of how effectively alfalfa, *Medicago sativa* (L.), provided as commercially-available sprouts, could serve as a dietary source of canavanine. We also assessed the variation in seed canavanine content by determining its natural abundance in 20 varieties.

Prior study of canavanine abundance in this plant was limited to the simple observation of Bell (1958) that alfalfa seeds available in the United Kingdom contained 1.46% canavanine by dry weight. Data were not provided on how this value was determined nor were common articles of everyday diet evaluated.

METHODS AND MATERIALS

Materials

A local Kroger, Inc. supermarket provided fresh alfalfa sprouts on three occasions from late June through mid-July of 1996. Multiple fresh sprout samples were also obtained at this time from New Natives, a grower in Aptos, CA, and the Good Food Cooperative of Lexington, KY. Sprouts were also grown from seed, available from the Lexington, KY seed stores, in the greenhouse of the University of Kentucky under ambient conditions in July through August. Seeds of the following varieties of *M. sativa* were obtained from Sphar & Co. (a division of Scott Seed Co.), Winchester, KY: Pioneer Hi-Bred 5454L and 5373L, ABT 405, ABT Supercuts, Aggressor, Alfagraze, Scott Brand ProBlend, Affinity + Z. Amerigraze, and Total + Z. Pioneer Hi-Bred International, Inc., Johnston, Iowa provided ten seed varieties. Chicago's Indoor Gardens provided two seed varieties.

Chemicals and Biochemicals

L-Canavanine was isolated from jack bean seeds, *Canavalia ensiformis*, and purified by re-crystallization as described elsewhere (Bass et al., 1995). Automated amino acid analysis established the absence of contaminant amino acids in the canavanine. NMR, elemental analysis, melting point, and optical rotation values were within established parameters (Bass et al., 1995). All reagents were obtained from Fisher/Agros Corp. or Sigma/Aldrich Chemical Co.

Preparation of the Plant Extract

Twenty g of alfalfa sprouts, either fresh or frozen at –20°C, were ground with a Servall Omni-mixer at full power for 3 min with 60% aqueous ethanol containing 1% (v/v) HCl. The slurry was decanted into a centrifuge bottle along with two washes of the mixing chamber. After centrifugation at 12,200 × g for 25 min, the supernatant solution was decanted into a volumetric cylinder. This extraction procedure removed 95 ± 1% of the canavanine of the plant sample as determined by stirring the pellet in the above solvent overnight at 3°C.

Canavanine Verification

About 20 mL of the final extract was taken to pH 7.6 with N NaOH and filtered over Whatman no. 1 paper. The filtrate was concentrated by rotary evaporation *in vacuo*, and the residue suspended in deionized water

and evaporated as above. The final residue was taken up in 10 mL of 50 mM tricine buffer (pH 7.6) containing 1 mM $MnCl_2$ and 0.1% (v/v) 2-mercaptoethanol. After filtering the suspension as above, 0.4 mL of the filtrate was reacted overnight at 37°C with 100 µL of purified arginase, prepared as described elsewhere (Rosenthal, 1977b). The purified arginase contained sufficient enzyme to degrade at least a 5-fold excess of the amount of canavanine thought to be in the sample. Deionized water (0.5 mL) was added to the reaction mixture, which was then evaluated colorimetrically for canavanine. These assessments, insuring that canavanine and only canavanine was responsible for the magenta, PCAF-canavanine chromogen, always resulted in a complete loss of chromogen.

A 25 mL sample of the plant extract was dispensed into a centrifuge tube and treated with 0.85 mL of 4N NaOH. After mixing thoroughly, the turbid solution was clarified by centrifugation at 27,000 × g for 15 min. Colorimetric analysis, in triplicate, was conducted on an appropriate sample of the final supernatant solution (Rosenthal, 1977b). All reported values are the mean ± SEM of 4 separate determinations for the sprouts and 3 determinations for the seeds.

Colorimetric Analysis

The plant extract (1.0 mL) was treated with an equal vol of 200 mM potassium phosphate buffer (pH 7.0) and 0.2 mL of 1.0% (w/v) potassium persulfate. After vortexing vigorously, 100 µl of 1.0% (w/v) pentacyanoammonioferrate (PCAF) solution was added, the tube agitated vigorously once again, and color development allowed to proceed for at least 30 min. The PCAF-canavanine chromogen was read at 530 nm, where chromogen formation obeys Lambert-Beer's law to 0.8 mM. PCAF was prepared from recrystallized commercial sodium nitroprusside dihydrate after the method of Fearon (1946).

Dry Weight Determination

The sprout or seed dry weight for *M. sativa* was determined by drying at 60°C in a convection drying oven (Precision Scientific, Inc., Model 18) for 2 d. The *Canavalia* seeds were pulverized mechanically prior to dry weight evaluation.

Recovery

The recovery of canavanine by the standard extraction procedure was established by the addition of 5 µCi of L-[*guanidinooxy*-^{14}C]-canavanine to the plant material. Evaluation by liquid scintillation spectroscopy revealed

Table 1. Canavanine abundance in fresh alfalfa sprouts.

Source	Canavanine content (% dry matter)	Dry matter	Relative abundance*
Supermarket			1.78
Sample 1	1.44 ± 0.02	10.5	
Sample 2	1.51 ± 0.01	9.0	
Sample 3	1.49 ± 0.03	9.6	
Commercial grower-1			1.00
Sample 1	1.31 ± 0.02	5.9	
Sample 2	1.41 ± 0.03	5.9	
Food Cooperative			
Sample 1	2.35 ± 0.03	5.8	1.75
Sample 2	2.38 ± 0.04	6.1	
Commercial grower-2			2.00
Sample 1	2.40 ± 0.01	6.7	
Sample 2	2.43 ± 0.02	6.6	
Greenhouse grown			1.95
Sample 1	2.37 ± 0.02	6.8	
Sample 2	2.33 ± 0.03	6.5	
Greenhouse grown			2.04
Sample 1	2.65 ± 0.02	5.9	
Sample 2	2.71 ± 0.03	6.3	

* Average canavanine content relative to the average dry matter, comparison made to commercial grower-1.

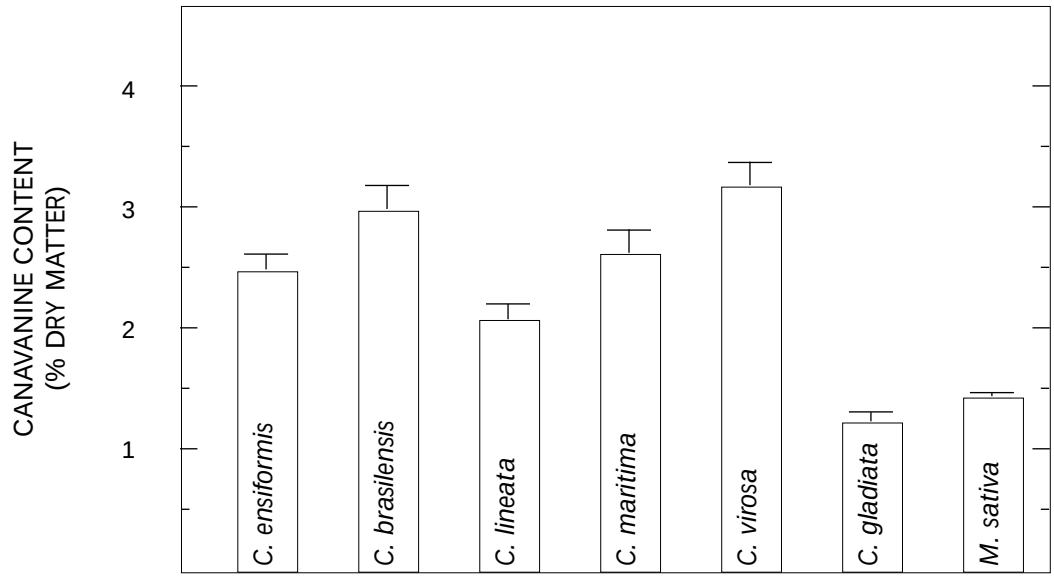

Fig. 1. The relative abundance of seed canavanine in *M. sativa* and several members of *Canavalia*. The seeds were processed as described in the text. Each value is the mean ± SEM for 3 independent determinations.

Table 2. Canavanine abundance in Pioneer Hi-Bred alfalfa seeds.

Variety	Canavanine content (% dry matter)*
5246L	1.78 ± 0.02
5312L	1.58 ± 0.01
7373L	1.52 ± 0.01
5396L	1.48 ± 0.03
5454L	1.56 ± 0.02
5472L	1.46 ± 0.03
5683L	1.48 ± 0.02
5715L	1.42 ± 0.02
5929L	1.62 ± 0.02
5939L	1.53 ± 0.03

Average seed dry matter = 4.9%

that 97% of the radiolabeled canavanine was recovered in the final extract taken for colorimetric analysis.

RESULTS

Sprouts

Analysis of alfalfa sprouts revealed that while the concentration of canavanine varied in this table legume, the sprouts were a significant source of canavanine. (Table 1). The sprouts obtained directly from commercial grower-2 contained the most canavanine having an average value of 2.4% canavanine by dry weight. These alfalfa sprouts were heavily laden with seed coats, and they possessed on average 6.7% dry matter. In contrast, the sprouts, provided by commercial grower-1 had a canavanine content of 1.36% of the dry matter. Adjusted for the difference in dry matter between the two samples, the sprouts provided by commercial grower-2 had more than twice the canavanine content as compared to the samples of commercial grower-1. This analysis points out the variation in canavanine content that can occur in these samples. This variation reflects both differences in the natural abundance of canavanine within the seed, but undoubtedly it also results from differences in the cultural practices used in growing the sprouts from seed. The alfalfa sprouts, purchased from the supermarket, were heavily laden with testas, which increased the dry weight of the sprout samples. These samples only contained 1.47% canavanine, but this is predicated upon an average dry matter of 9.7%. Once again, taking the dry matter into account, the supermarket samples contained levels of canavanine comparable to sprouts obtained from the other sources (Table 1). Alfalfa sprouts, grown under ambient greenhouse conditions and a supplemental nitrogen fertilization in a rich, loamy soil, produced large amounts of stored canavanine. This finding lends credence to the stated importance of cultural practices in rearing canavanine-rich alfalfa sprouts (Table 1).

Seeds

In a preliminary screening of 10 varieties of alfalfa seed provided by Sphar Seed Co. and listed elsewhere, Pioneer Hi-Bred 5454L and 5373L were the richest in canavanine. Pioneer Hi-Bred International developed their

Pioneer Hi-Bred lines for their resistance to insect infestation, hardiness, high yield, and overall robustness. Subsequent analysis of 10 variants of the Pioneer Hi-Bred seed line revealed a canavanine content on a dry weight basis that equaled 1.54 ± 0.03%, and ranged from 1.4 to 1.8% (Table 2). The preliminary screening of the seeds other than Pioneer Hi-Bred varieties revealed a canavanine content that ranged from 0.7 to 1.2%.

Canavanine in *Canavalia*

In order to compare the natural abundance of canavanine in *M. sativa* to some other canavanine-storing legumes, the canavanine content of alfalfa seeds was compared to a number of species of *Canavalia*, which are rich sources of canavanine (Fig. 1). This comparative evaluation disclosed that while *M. sativa* contained less seed canavanine than the studied members of *Canavalia* did, its natural abundance is comparable to these efficient canavanine-storing legumes.

DISCUSSION

The studies of Malinow et al. (1982) followed established scientific protocols and had the appropriate and necessary controls-overall the evidence is substantial and convincing that a SLE-like syndrome can be manifested in *M. facicularis* when they are provided the indicated levels of canavanine, under conditions of chronic exposure. Malinow et al. (1982) reported that they had to provide 1% (w/w) of canavanine sulfate to elicit the SLE-like symptoms. Given a 6% dry matter content and a 2% canavanine content for alfalfa sprouts, a human would have to maintain an alfalfa sprout consumption of nearly 5.5 times the fresh weight of the food consumed by the experimental animal. Taking into account the far greater mass of a human, one could not reasonably eat sufficient alfalfa sprouts, even if one consumed nothing else, to achieve equivalent canavanine consumption.

Most importantly, information is not available on the relative effectiveness of hepatic detoxification of canavanine in this macaque as compared to a human and other mammals. Canavanine moves quickly to the liver where hepatic arginase efficiently catalyzes the hydrolysis of this arginine antagonist. In the adult rat, such metabolic catabolism coupled with urinary excretion resulted in a rapid drop in blood serum canavanine to an innocuous level before detrimental effects were manifested. Serum elimination curves following intravenous or subcutaneous administration of canavanine established that canavanine was rapidly cleared from the serum of the animal. Orally administered canavanine showed a bioavailability of 43%. Therefore, most of the administered canavanine did not find its way into the rat bloodstream.

Extensive research in our laboratory has demonstrated that the rat is only somewhat sensitive to the adverse effects of canavanine. In contrast, the mouse and hamster are extremely resistant to orally-administered canavanine. The question then is which of these mammals is the most appropriate test system for human comparison?

On the other hand, Thomas et al. (1986) demonstrated that chronic canavanine exposure was detrimental to both the neonatal and adult rat. It is germane that in order to achieve this pernicious effect, the rats were administered 3 g/kg canavanine daily for 6 days. This is a high dose that would require the consumption of absurd amounts of alfalfa to provide comparable exposure. Finally, nearly 25 years of detailed study of canavanine in the tobacco hornworm, *Manduca sexta* has demonstrated unequivocally that chronic, long-term exposure to this arginine antagonist produces the most deleterious effects from canavanine exposure.

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