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DEAMINOCANAVANINE FORMATION FROM CANAVANINE

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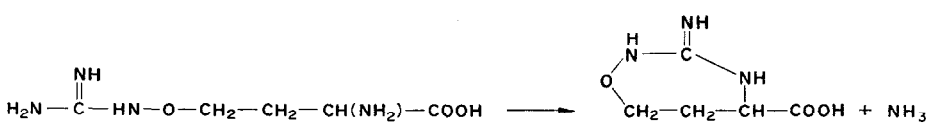
Key Word Index—*Canavalia ensiformis*; Leguminosae; canavanine; deaminocanavanine.

Abstract—The rate of spontaneous canavanine breakdown to deaminocanavanine and NH_3 is affected by temperature, substrate concentration, solvent system, time, and various divalent cations. Ammonium ions may accelerate canavanine decomposition. Canavanine is most stable as a dilute aqueous solution at 4° or in the presence of 0·1 N HCl. When HCl is omitted, deaminocanavanine formation increases as the temperature is elevated from 4 to 37°. In aqueous ethanol or on adding NH_4OH to 100 mM, deaminocanavanine formation is increased. Deaminocanavanine does not appear to play a significant role in the nitrogen metabolism of jack bean.

INTRODUCTION

THE SPONTANEOUS decomposition of canavanine to deaminocanavanine (hexahydro-3-imino-1,2,4-oxadiazepine-5-carboxylic acid) and ammonia was first demonstrated by Kitagawa and Tsukamoto.[1] The structure proposed for this newly discovered compound was subsequently confirmed by catalytic hydrogenolysis.[2] Makisumi[2] also suggested, although no data were presented, that deaminocanavanine was a natural constituent of jack bean, *Canavalia ensiformis* (L.) DC., a leguminous plant known to contain appreciable quantities of canavanine. Töpfer *et al.*[3] found deaminocanavanine in extracts of *Caragana spinosa* (L.) DC., and observed the transfer of radioactivity from labeled canavanine to deaminocanavanine. These studies established the formation of deaminocanavanine from canavanine; however, they were not concerned with the role, if any, of deaminocanavanine in the metabolism of canavanine-containing legumes.



Canavanine instability in solution prompted Hunt and Thompson[4] to take precautions against deaminocanavanine formation during canavanine isolation from jack bean seeds. It is difficult, however, to accurately assess the factors affecting canavanine stability as data on the nature and extent of deaminocanavanine formation are very limited.[1]

[1] A. KITAGAWA and J. TSUKAMOTO, *J. Biochem. Tokyo* **26**, 373 (1937).
[2] S. MAKISUMI, *J. Biochem. Tokyo* **47**, 201 (1959).
[3] V. R. TÖPFER, J. MIERSCH, and H. REINBOTHE, *Biochem. Physiol. Pflanzen.* **161**, 231 (1970).
[4] G. E. HUNT and J. F. THOMPSON, in *Biochemical Preparations*, Vol. 13, p. 41, Wiley, New York (1971).

This paper presents a method for the preparation of canavanine-free deaminocanavanine. In addition, the factors governing canavanine stability have been studied, utilizing the Sakaguchi reagent. A quantitative method for assaying deaminocanavanine has also been developed using the trisodium pentacyanoammonioferrate (PCAF) assay.[5]

RESULTS AND DISCUSSION

Deaminocanavanine Formation

The effect of temperature on deaminocanavanine formation in solution was studied. Both aqueous and ethanolic solvents were used as aqueous ethanol is frequently employed for extracting canavanine from plant tissues.[3,6] The stability of canavanine in 0·1 N NH_4OH was also investigated, as this reagent is employed to elute canavanine in ion-exchange chromatography.[4] The results obtained at 4° revealed little difference between the aqueous and ethanolic solvents (Table 1). Moreover, NH_4OH addition did not affect the breakdown of canavanine. With rise in temperature, increase in deaminocanavanine production was greater in ethanol than in water, and NH_4OH became more efficient in stimulating canavanine decomposition. At 1 mM substrate concentration, little deaminocanavanine was formed under all but the most extreme conditions.

TABLE 1. EFFECT OF TEMPERATURE AND SOLVENT ON THE FORMATION OF DEAMINOCANAVANINE

Solvent system	Temp.	Canavanine decomposition (%) 2 days	7 days
Water		1·0	1·5
Water + NH_4OH		1·0	1·5
Ethanol		1·0	2·0
Ethanol + NH_4OH	4°	1·0	2·0
Water		1·0	1·5
Water + NH_4OH		1·5	2·0
Ethanol		1·5	2·5
Ethanol + NH_4OH	22°	2·0	4·5
Water		2·0	2·5
Water + NH_4OH		3·0	6·0
Ethanol		4·5	15·0
Ethanol + NH_4OH	37°	9·0	27·0

The canavanine solutions (10 ml) were maintained at the indicated temperature and contained 10 μmol of free base L-canavanine either in water or 75% ethanol; some solutions contained 100 mM NH_4OH. At the indicated intervals, duplicate 2 ml samples were removed and dried. The residue was dissolved in 5 ml of Sakaguchi reagent and analyzed for deaminocanavanine formation. Canavine decomposition values were calculated as deaminocanavanine formation over the initial level of canavanine and expressed to the nearest 0·5%.

The abundance of canavanine in many plant tissues often requires processing canavanine in solutions much more concentrated than 1 mM. Therefore, the canavanine level was augmented systematically to 100 mM and the relationship of substrate concentration to deaminocanavanine formation measured (Table 2). Deaminocanavanine production was accelerated

[5] E. A. BELL, *Biochem. J.* **75**, 618 (1960).
[6] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **6**, 1659 (1967).

as the substrate level rose. Moreover, increasing the temperature from 22 to 37° or substituting aqueous ethanol for water resulted in greater product formation as a function of substrate concentration. As shown in Table 3, canavanine cleavage can be minimized by avoiding an ethanol concentration above 50%. Clearly, addition of NH_4OH augmented the capacity of ethanol to foster canavanine breakdown. Moreover, the higher the ethanol concentration, the greater the effect of NH_4OH.

TABLE 2. CONCENTRATION-DEPENDENT CLEAVAGE OF CANAVANINE

Solvent system	Temp.	Concn (mM)	Canavanine decomposition (%) 2 days	7 days
Water	22°	10	1·0	2·0
		25	1·5	3·0
		50	1·5	4·0
		100	2·0	7·0
Water + NH_4OH	22°	10	1·5	3·5
		25	2·0	5·0
		50	2·5	7·0
		100	3·5	9·0
Water + NH_4OH	37°	10	3·5	11·5
		25	6·0	20·0
		50	8·0	26·0
		100	11·0	34·5
Ethanol + NH_4OH	37°	10	10·5	31·5
		25	14·0	40·0
		50	16·0	41·5
		100	18·0	43·5

Free base L-canavanine solutions were prepared either with water or 75% ethanol at the prescribed concentration and placed at 22 or 37°. When applicable, the NH_4OH concentration was 100 mM. At the indicated intervals, samples were assayed for deaminocanavanine formation as described in Table 1.

Further studies were conducted with NH_4OH to ascertain whether ammonium ion or the associated alkaline pH was responsible for increased canavanine decomposition. Treatment of canavanine with NH_4OH, NH_4Cl, KOH, and NaOH both in water and 50% ethanol revealed that NH_4Cl was just as effective as NH_4OH in fostering synthesis of deaminocanavanine; neither KOH nor NaOH stimulated canavanine breakdown.

A study was also initiated to ascertain if the species of divalent cations employed in enzyme studies of canavanine[7,8] affect the rate of substrate cleavage. Divalent cations such as Co^{2+}, Ni^{2+}, Mn^{2+}, and Mg^{2+} markedly enhanced deaminocanavanine formation. The data obtained for $MgCl_2$ is representative of the entire group and is presented in Table 4. Two distinctive patterns emerge from the data. When NH_4OH is omitted, Mg^{2+} activation of canavanine breakdown in both water and aqueous ethanol peaked at 5 mM $MgCl_2$ while declining at higher concentrations of Mg^{2+}. On the other hand, inclusion of NH_4OH caused increased deaminocanavanine formation throughout the range of Mg^{2+} levels tested.

[7] M. DAMODARAN and K. G. A. NARAYANAN, *Biochem. J.* **34**, 1449 (1940).
[8] G. A. ROSENTHAL, *Plant Physiol.* **46**, 273 (1970).

Several investigators have employed 0·1 N HCl for extracting canavanine from plant material.[9,10] The ability of this reagent to stabilize canavanine in solution was assessed with 75% ethanol at 37° after a 7 day incubation period employing the canavanine concentrations shown in Table 2. In addition, 100 mM L-canavanine and 5 mM $MgCl_2$ were incubated with and without 0·1 N HCl in 75% ethanol as described in Table 4. These incubation conditions represented the most adverse situations for maintaining canavanine stability. Yet, in all cases, inclusion of 0·1 N HCl effectively prevented canavanine decomposition.

TABLE 3. THE EFFECT OF ETHANOL CONCENTRATION ON CANAVANINE STABILITY

Solvent system	Canavanine decomposition (%)
Water	1·5
Ethanol-25%	1·5
Ethanol-50%	2·0
Ethanol-75%	2·5
Ethanol-90%	5·0
Water + NH_4OH	2·0
Ethanol-25% + NH_4OH	2·0
Ethanol-50% + NH_4OH	2·5
Ethanol-75% + NH_4OH	4·5
Ethanol-90% + NH_4OH	10·0

Utilizing the solvent systems indicated, 10 mM free base L-canavanine solutions were prepared and placed at 22°. Some solutions possessed 100 mM NH_4OH. 7 days later, product assays were conducted as described in Table 1.

Deaminocanavanine Assay with PCAF

Utilizing the PCAF reaction, the extinction of the deaminocanavanine and canavanine chromogen complexes were determined as a function of substrate concentration. Both compounds obey Lambert–Beer's law to 0·7 mM substrate concentration. The deaminocanavanine-PCAF chromogen possessed a violet-red color (E_{max} = 535 nm) as opposed to the characteristic magenta (E_{max} = 530 nm) obtained with canavanine. For equimolar quantities, canavanine gave a 12% greater extinction than deaminocanavanine. Maximum extinction was achieved after 40 min for both deaminocanavanine and canavanine color formation; essentially the same rate of color development was observed for both compounds in the dark.

Natural Occurrence of Deaminocanavanine

It is not known if deaminocanavanine is a natural constituent of canavanine-containing legumes. The possible occurrence of deaminocanavanine was evaluated in jack bean, utilizing the three plant samples possessing the greatest canavanine concentration: 6-week-old pods, 12-day-old leaves, and hydrated seeds.[8,11] Deproteinized extracts of the above

[9] E. A. BELL, *Biochem. J.* **70**, 617 (1958).
[10] S. NAKATSU, S. HARATAKE, Z. SAKURAI, N. ZYO, Z. NISHIHARA and M. HAYASIDA, *Seikagaku* **36**, 467 (1964).
[11] G. A. ROSENTHAL, *Plant Physiol.* **47**, 209 (1971).

samples were subjected to ion-exchange chromatography with Dowex-50 (NH_4^+) to remove canavanine, arginine, and other basic compounds, and the effluent was concentrated. All operations were conducted at 4° and were completed in 2 hr.

TABLE 4. THE ACTIVATION OF DEAMINOCANAVANINE FORMATION BY Mg^{2+}

Solvent system	Mg^{2+} Concn (mM)	Canavanine decomposition (%)
Water	1·0	3·0
	2·5	4·0
	5·0	4·5
	10·0	4·0
	25·0	2·0
Ethanol	1·0	16·0
	2·5	18·0
	5·0	20·0
	10·0	14·0
Water + NH_4OH	1·0	7·5
	2·5	11·0
	5·0	16·0
	10·0	20·0
	25·0	23·0
Ethanol + NH_4OH	1·0	28·0
	2·5	32·0
	5·0	36·0
	10·0	38·5

Solutions of free base L-canavanine (10 mM), prepared with water or 75% ethanol, contained the indicated level of $MgCl_2$ in a final volume of 10 ml. When applicable, 100 mM NH_4OH was also included. The substrate solutions, placed at 37° for 7 days, were assayed for deaminocanavanine according to the protocol of Table 1.

The leaf sample, consisting of five pair of primary leaves weighing 11 g and containing 386 μmol canavanine, exhibited a Sakaguchi response equivalent to 0·11 μmol of deaminocanavanine. Thus, even assuming that all of the Sakaguchi response is correctly attributed to deaminocanavanine, an average of only 22 nmol deaminocanavanine occurred in the primary leaves of jack bean. Admittedly, this minute amount may have resulted from canavanine decomposition during the extraction and isolation of deaminocanavanine. Assays of the pods and seeds revealed even less Sakaguchi response. Addition of known amounts of deaminocanavanine to the jack bean leaf extracts established that at least 94% of the deaminocanavanine was consistently recovered.

Metabolism of Deaminocanavanine

No evidence could be found for deaminocanavanine utilization in extracts of roots, leaves, pods, seeds, and cotyledons of jack bean, tested over a range of pH values from 7·2 to 9·5. Little, if any, deaminocanavanine occurs naturally in the canavanine-rich tissues of jack bean and this compound probably represents an artifact of canavanine isolation.

EXPERIMENTAL

Preparation. Free base L-canavanine (2 g) was dissolved in 15 ml of 67 mM NH_4OH before addition of 85 ml EtOH. After refluxing for 12 hr, the solution was evaporated to dryness and the residue dissolved in hot H_2O. Most of the coloration was removed with activated charcoal. The solution was then concentrated and the deaminocanavanine precipitated by the slow addition of 3 vol. EtOH. After standing for 18 hr at 4° the crystals were washed with EtOH and ether. Yield 915 mg (51%). The known compound (deaminocanavanine) had m.p. 257–258° (decomp.) $[\alpha]_D^{22} + 25 \pm 1°$. (Found: C, 37·7; H, 5·8; N, 26·4 Calc. for $C_5H_9O_3N_3$: C, 37·7; H, 5·7; N, 26·4%.) The compound was unreactive to ninhydrin.

Assay. The Sakaguchi reaction[12] was conducted by a modification of the procedure of van Pilsum *et al.*[13] The assay solution (final vol. 5 ml) contained the sample and 1 ml of a solution prepared by mixing equal vol. 10% NaOH + 2% thymine and 0·04% 1-naphthol in 95% (v/v) EtOH. Color formation was initiated with 0·5 ml of 16% (v/v) commercial Clorox. 1 min later, color development was terminated with 0·5 ml of 3·0% sodium thiosulfate. Assay of deaminocanavanine with the Sakaguchi reagent revealed that up to 0·2 mM, E_{510} was proportional to substrate concentration. At a canavanine level of less than 0·4 mM in the 5 ml assay mixture, no significant canavanine interference of the deaminocanavanine assay occurred. Consequently, all samples were diluted appropriately prior to evaporation and assay.

The PCAF assay[14] was performed by mixing 1·0 ml of sample, 1·0 ml of 300 mM potassium phosphate buffer, pH 7·0, and 0·2 ml of 1% potassium persulfate. The addition of 0·1 ml of 1% PCAF, which had been photoactivated by standing in the light for 30 min, commenced color production. E_{535} was determined after 40 min.

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[12] S. SAKAGUCHI, *J. Biochem. Tokyo* **5**, 133 (1925).
[13] J. F. VAN PILSUM, R. P. MARTIN, E. KITO and J. HESS, *J. Biol. Chem.* **222**, 225 (1956).
[14] W. R. FEARON and E. A. BELL, *Biochem. J.* **59**, 221 (1955).