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REEXAMINATION OF THE REPORTED OCCURRENCE OF L-CANAVANINE IN *AGARICUS CAMPESTRIS*

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Studies of the amino acid constituents of the mushroom, *Agaricus campestris bisporus* (L.) ex Fr., led to the contention that L-canavanine is a natural constituent of the edible fruiting body [1]. This finding is particularly intriguing since canavanine distribution is otherwise restricted to members of the Lotoideae, a subfamily of the Leguminosae [2–4]. Since the earlier report was based solely on the elution of a ninhydrin-positive substance having the same column retention time as canavanine, the possible occurrence of canavanine in fungi has been reexamined.

Analysis of the free amino acids of *A. campestris bisporus* confirmed the elution of a ninhydrin-positive compound with the same column retention time as L-canavanine (see Experimental). Reaction of this compound with ninhydrin established the presence of approximately 4 mg/kg, assuming a ninhydrin response comparable to canavanine. The paucity of this amino acid necessitated an indirect approach for determining if it was L-canavanine: (1) arginase treatment of the fungal extract and evaluation of the hydrolytic cleavage of canavanine to canaline and urea; (2) catalytic hydrogenolysis of the fungal extract and assessment of the catalytic reduction of canavanine to homoserine and guanidine; and (3) enzymatic and chemical treatment of the fungal extract and determination of canavanine conversion to canaline and eventually *O*-ureidohomoserine.

Arginase-mediated hydrolysis of the fungal extract. Arginase catalyzes a hydrolytic cleavage of L-arginine and L-canavanine forming urea and L-ornithine and L-canaline, respectively. Incubation of 1 ml of fungal extract with 1 ml of commerically-prepared arginase (8 mg/ml) for 3 hr at 37° caused the complete disappearance of the fungal arginine peak, a concomitant increase in the ornithine peak, but only a partial diminution in the peak resulting from the compound possessing the same retention time as canavanine. The fungal extract was augmented with sufficient canavanine to elicit approximately twice the ninhydrin response of the putative canavanine peak. The exogenously supplied canavanine was hydrolyzed completely by arginase under the same experimental conditions.

Catalytic hydrogenolysis of the fungal extract. The fungal extract (2 ml) was incubated with 100 mg Pd black; hydrogen was bubbled gently through the mixture for 6 hr at 22°. Catalytic reduction of the fungal extract did not affect the reputed canavanine peak. Supplementation of the fungal extract with canavanine, prior to catalytic hydrogenolysis, produced a new, ninhydrin-responsive peak with a column retention time of 56 min, corresponding to homoserine.

In addition, catalytic reduction of the canavanine-enriched fungal extract enhanced markedly its response to the Sullivan colorimetric assay for guanidine [5]. These results are consistent with the

successful catalytic cleavage of canavanine to homoserine and guanidine uniquely in the canavanine-supplemented fungal extract.

Conversion of canavanine to canaline and ureidohomoserine. L-Canaline, a naturally occurring γ-oxyamino-containing amino acid, is produced by arginase-mediated hydrolysis of canavanine. This substance, structurally similar to hydroxylamine, completely decomposed in the lithium–citrate buffer employed for amino acid analysis. However, canaline can be chemically carbamylated with carbamyl phosphate to form *O*-ureidohomoserine, a stable amino acid. In an analogous manner, citrulline results from chemical carbamylation of ornithine.

The fungal extract (1 ml) was incubated with 1 ml of arginase (8 mg/ml) and 50 mM carbamyl phosphate (final vol. 3 ml) for 3 hr at 37°. Analysis of such treated fungal extract revealed the complete disappearance of the arginine peak and the concomitant formation of a ninhydrin-reactive species having the retention time of citrulline. Of far greater significance, no ninhydrin-positive species was discernible at the position where reference *o*-ureidohomoserine eluted.

The fungal extract was treated as above after addition of 3 μmol of canavanine. Amino acid analysis of the canavanine-enriched fungal extract established the production of a ninhydrin-positive peak coinciding exactly with the elution position of *O*-ureidohomoserine.

Without direct isolation and characterization of the fungal compound, it is impossible to state unequivocably whether or not canavanine is a natural constituent of *A. campestris bisporus*. However, the ability of arginase to hydrolyze the fungal amino acid, and the overlap in the column retention time of the fungal compound and canavanine support the structural similarity of this compound to canavanine. In contrast, the fungal compound is not cleaved by catalytic hydrogenolysis nor does it produce an arginase-mediated hydrolysis product reactable with carbamyl phosphate to form *O*-ureidohomoserine. These findings suggest that the fungal amino acid is not canavanine. It is, therefore, suggested that the edible fungus, *Agaricus campestris bisporus*, contains an amino acid structurally related to, but not identical to L-canavanine.

This study illustrates the inherent danger in establishing chemotaxonomic groups based on inadequate data such as retention time or R_f value.

EXPERIMENTAL

Preparation of the fungal extract. Approximately 4 kg fr. fruiting bodies of *A. campestris bisporus* were thoroughly washed with dist. H_2O and air dried. The fruiting bodies (1·0 kg) were ground exhaustively in a Waring blender with 1 litre of 50% aq. EtOH containing 0·25 N H_2SO_4. Fungal slurries were clarified by vacuum filtration and the resulting cake reground with 1 litre of the above solvent. The pooled filtrates were centrifuged at 18000 *g* for 15 min, adjusted to pH 7·0–7·2, and placed at 4° overnight. The pptd material was removed by centrifugation as above. The clarified fungal extract was stirred mechanically for 24 hr at 4° with approx. 2 l. of Dowex 50-X8 (NH_4^+, 100–200 mesh). The resin, collected by vacuum filtration, was washed with approximately 30 l. of deionized H_2O prior to eluting the basic fungal amino acids by mechanical stirring with 1 litre of 0·1 N NH_4OH for 4 hr at 4°. The resin was separated from the solvent by vacuum filtration and the filtrate concentrated to 50 ml *in vacuo*. During the concentration procedure, the condenser was cooled to 4° while maintaining the fungal extract at no more than 25°. This precaution was taken as canavanine undergoes a temperature-dependent decomposition to deaminocanavanine and NH_3 [6]. Dissolved protein was removed from the concentrated fungal extract by the addition of 8 g TCA. The TCA was subsequently removed by 3 successive extraction with an equal vol. Et_2O. Finally, the fungal extract was adjusted to pH 7·6, concd to 20 ml *in vacuo*, and stored at −30°.

Amino acid analysis. Fungal amino acids were separated and quantitatively determined by colorimetry using a completely automated Technicon TSM HPLC Amino Acid Analyzer. The conc. fungal extract was evaporated to dryness and the residue dissolved in 0·3 N lithium citrate equilibrium buffer (pH 2·0). The buffered amino acid soln, containing norleucine as an internal standard, was injected into 2 blank sample cartridges and placed on the amino acid analyzer. The amino acids were eluted sequentially from ion-exchange resin column with lithium citrate buffers and reacted with hydrazine sulfate and ninhydrin to form a colored complex with an extinction maxima at 570 nm. The following buffer sequence was employed: (1) *acid–neutral amino acids*; 0·3 N lithium citrate buffer with a pH sequence of 2·70, 2·60, 3·30, 4·15; (2) *basic amino acids*; 0·5 N lithium hydroxide buffer with a pH sequence of 3·80, 5·25 and 0·8 N lithium hydroxide buffer (pH 6·00). The columns were washed with a strongly alkaline 0·3 N lithium hydroxide buffer. Retention times (in min) were: *O*-ureidohomoserine 52, homoserine 56, citrulline 95, norleucine 152, ornithine 238, canavanine 282 and arginine 299.

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