COMPARISON OF OCTOPINE, HISTOPINE, LYSOPINE, AND OCTOPINIC ACID SYNTHESIZING ACTIVITIES IN SUNFLOWER CROWN GALL TISSUES[1]

Ethan Hack and John D. Kemp*

Department of Plant Pathology, *also Plant Disease Resistance Research Unit

ARS, USDA, University of Wisconsin, Madison, Wisconsin, 53706, U.S.A.

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SUMMARY:

Extracts of sunflower crown gall tissues induced by Agrobacterium tumefaciens strain B_6 catalyze the synthesis of octopine, histopine, lysopine and octopinic acid. These compounds are not synthesized either in extracts of crown gall tissues induced by strains AT1 and C58 or in extracts of habituated sunflower callus. All four synthetic activities require NADPH or NADH, pyruvate, and the appropriate basic amino acid. Incorporation of radioactivity from any one of the four labeled, basic amino acids into its product is inhibited by the other three basic amino acids. All the reactions are inhibited by ε-aminocaproic acid but none are inhibited by the neutral amino acids alanine and phenylalanine.

Virulent strains of Agrobacterium tumefaciens (E. F. Sm. and Town.) Conn, the bacterium inciting crown gall, can be divided into three main groups [1, 2, 3]: 1, those which utilize octopine (N^2-(1-carboxyethyl)-L-arginine) [4] for growth and induce galls containing octopine (such as strain B_6); 2, those which utilize nopaline (N^2-(1,3-dicarboxypropyl)-L-arginine) [5] and induce galls containing nopaline (such as strain C58); and 3, those which do not use either amino acid derivative and induce galls containing neither (such as strain AT1). Galls induced by A. tumefaciens strain B_6 contain, in addition to octopine, N^2-(1-carboxyethyl) derivatives of L-histidine (histopine) [6], L-lysine (lysopine) [7], and L-ornithine (octopinic acid) [5]. Three of these derivatives, octopine, octopinic acid, and lysopine, have been synthesized in crown gall tissue extracts [8, 9]. The fourth derivative, histopine, was recently identified and characterized from crown gall tissue [6] and has not been previously synthesized in vitro.

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In this paper we report tissue-specific in vitro synthesis of octopine, histopine, lysopine and octopinic acid and present results indicating that all four N^2-(1-carboxyethyl)-amino acids may be synthesized by the same enzyme.

MATERIALS AND METHODS

Plant Material - The establishment of tissue culture lines PSCG-B_6 (from a primary crown gall on sunflower, Helianthus annuus, inoculated with A. tumefaciens strain B_6) and HSSS (habituated tissue isolated from a normal H. annuus callus culture) has been described [10; PSCG-B_6 was previously identified as PSCG-4]. The derivation of PSCG-AT1 and PSCG-C58 from H. annuus inoculated with A. tumefaciens strains AT1 and C58, respectively, will be described elsewhere. Tissue cultures were maintained at 27°C in the dark on Linsmaier and Skoog's medium [11] lacking phytohormones. Following a short lag after transfer, tissues grew exponentially for 25-30 days and were routinely harvested for experimentation after 10-24 days.

Tissue extraction - Tissues were homogenized with a Duall[2] homogenizer in an equal volume (w/v) of extraction buffer containing 0.1 M Tris-HCl (pH 8.0), 0.5 M sucrose, 1 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was centrifuged for 20 min at 27,000 X g to remove cell debris. The clear supernatant (crude extract) was layered on a 1.2 X 24 cm column of Sephadex G-25 previously equilibrated with extraction buffer. After elution with extraction buffer, the fraction containing high molecular weight material was retained (gel-filtered extract).

Enzyme assay - The reaction mixture contained 250 µl of crude extract previously diluted 2.5 times with extraction buffer or 250 µl of gel-filtered extract, 16 mM pyruvate, 0.8 mM NADPH, and either 35 µM L-[guanido-^{14}C]-arginine (23 mCi/mmole), 28 µM L-[ring-2-^{14}C]-histidine (55 mCi/mmole), 0.05 µM L-[4,5-^{3}H]-lysine (70 Ci/mmole), or 0.13 µM L-[3-^{3}H]-ornithine (26 Ci/mmole) in a final volume of 300 µl. Other modifications and additions are noted in the results. After an appropriate incubation time at 20°C, the reaction was stopped by addition of 300 µl of 10% (w/v) trichloroacetic acid. The mixture was chilled to 4°C for 15 min and the precipitated material removed by centrifugation. The radioactive material in the supernatant fraction was adsorbed on a 0.9 X 50 cm column of Aminex Q-150S ion exchange resin previously equilibrated with either pH 3.49 [6, 12, 13] buffer for octopine, lysopine, and octopinic acid, or pH 3.12 [12] buffer for histopine. Octopine, lysopine, and octopinic acid were eluted from the resin with pH 3.49 buffer and histopine was eluted with pH 3.12 buffer. The buffer was changed to pH 5.95 for elution of the basic amino acids [6, 13]. The reaction products gave two major peaks of radioactivity when chromatographed as described above. The first peak was the N^2-(1-carboxyethyl)-amino acid and the second peak was the original radioactive amino acid. In each case the radioactive N^2-(1-carboxyethyl)-amino acid synthesized in the enzyme catalyzed reaction co-chromatographed with its authentic counterpart on both the amino acid analyzer and on thin-layer plates [6]. After each run the resin was regenerated with NaOH [12] and re-equilibrated with the desired buffer.

Chemicals - Authentic octopine and octopinic acid were purchased from Sigma Chemical Co. Authentic histopine and lysopine were chemically synthesized as described previously [6].

[2]Mention of companies or commercial products does not imply recommendation or endorsement by the U. S. Department of Agriculture over others not mentioned.

Table 1. Synthetase Activities in Various Tissues.

Tissue	% of CPM in			
	Octopine	Histopine	Lysopine	Octopinic acid
PSCG-B_6	16.5	1.3	12.4	26.8
PSCG-AT1	< 0.05	< 0.05	< 0.1	< 0.2
PSCG-C58	< 0.05	< 0.05	< 0.1	< 0.2
HSSS	< 0.05	< 0.05	< 0.1	< 0.2

Assay conditions were as described in Materials and Methods for crude extracts with the addition of 0.8 mM NADH. Incubation times were 40 min. With the exception of PSCG-B_6 all values were below the limits of detection.

Table 2. Requirements for the Four Synthetase Activities Found in Gel-filtered Extracts of PSCG-B_6.

Assay Conditions	% of CPM in			
	Octopine	Histopine	Lysopine	Octopinic acid
Complete	13.3	2.8	11.7	17.1
+NADH	14.3	3.1	12.1	17.1
+NADH, -NADPH	9.6	2.0	12.7	18.1
-NADPH	0.1	0.3	1.5	3.4
-Pyruvate, +NADH	< 0.05	0.1	0.3	0.4

Assay conditions were as described in Materials and Methods. When added, NADH was present at 0.8 mM. Incubation times were 40 min.

RESULTS

When an aliquot of crude extract from octopine-containing tissue, PSCG-B_6, was incubated for 40 min with pyruvate and [^{14}C]-arginine 16.5% of the total radioactivity was found in the octopine fraction (Table 1). The same extract incubated with pyruvate and [^{14}C]-histidine, [^{3}H]-lysine, or [^{3}H]-ornithine

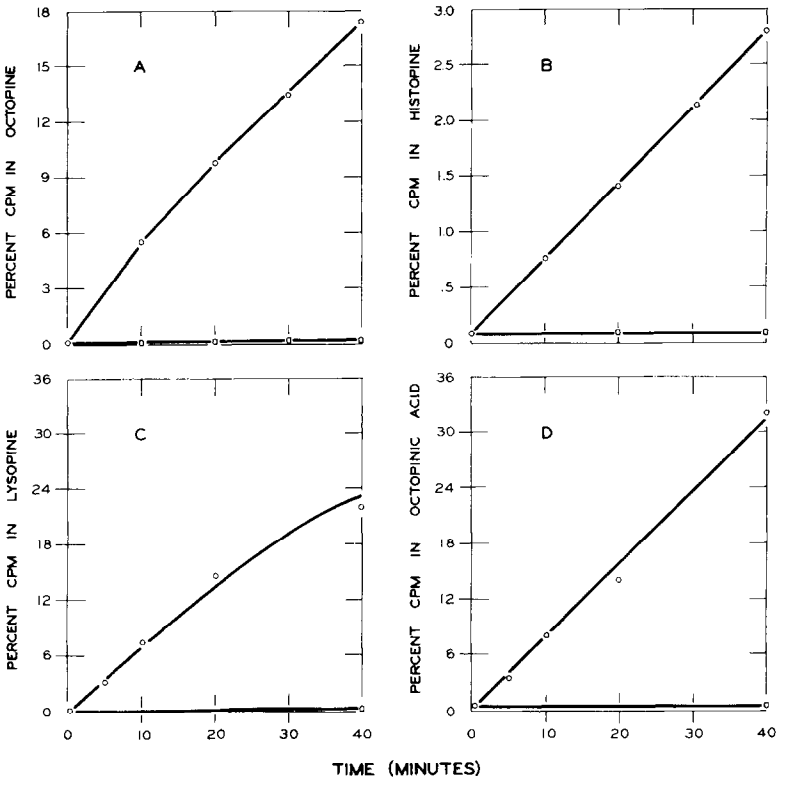

Figure 1. Activity with time in gel-filtered extracts from PSCG-B_6 tissue. A. octopine, B. histopine, C. lysopine, and D. octopinic acid synthesis. (o--o) complete reaction mixture, (□--□) reaction mixture minus pyruvate.

converted 1.3, 12.4, or 26.8% of the radioactivity to histopine, lysopine, or octopinic acid, respectively. The synthesis of all four N^2-(1-carboxyethyl)-amino acids by crude extracts from PSCG-B_6 was dependent on added pyruvate. The enzyme appeared to be saturated by 9 mM pyruvate because there was no increase in the rate of synthesis of octopine when the pyruvate concentration was increased to 18 mM. On the other hand, the amino acid concentrations used were well below saturation. By measuring the reaction rates at increasing concentrations of arginine, the Michaelis constant for arginine was estimated to be 2.4 mM. Because of endogenous NADH and NADPH, the dependence of the reactions on either compound could not be demonstrated in crude extracts.

Extracts from the nopaline-containing tissue (PSCG-C58), from tissue containing neither octopine nor nopaline (PSCG-AT1), and from habituated callus

Table 3. Inhibition of Radioactive Octopine, Histopine, Lysopine, and Octopinic Acid Synthesis When Various Amino Acids Were Present.

Amino Acid	% Inhibition			
	Octopine	Histopine	Lysopine	Octopinic acid
No Addition	0	0	0	0
Arginine	--	96	95	97
Histidine	85	--	92	89
Lysine	98	96	--	97
Ornithine	92	96	97	--
ε-aminocaproate	93	94	97	94
Alanine	5	14	9	12
Phenylalanine	12	0	6	0

Assay conditions were as described in Materials and Methods with the exception that the listed non-radioactive compounds were present at a concentration of 16 mM. Incubation times were 40 min. Percent inhibition was calculated as (1-amino acid/no addition)X100.

tissue (HSSS) contained no octopine, histopine, lysopine, or octopinic acid synthesizing activity even when both NADPH and NADH were added (Table 1).

After gel filtration the activities in PSCG-B_6 tissue extracts were dependent on both added pyruvate and NADPH (Table 2). The rates of all four reactions were constant for at least 40 min (Figure 1). NADH substituted for NADPH in all reactions (Table 2). However, the rates were not significantly increased by the addition of NADH and NADPH together.

The similar requirements for the four activities suggested that they were all catalyzed by the same enzyme. The results of a study of competition between amino acid substrates supported this hypothesis (Table 3). Non-radioactive amino acids were added to the reaction mixture at 16 mM. This

concentration was such that a competitive inhibitor with a Ki similar to the Km of arginine should give about 90% inhibition of octopine synthesis. Each of the activities was inhibited by the addition of any one of the other three basic amino acids. For example, incorporation of radioactivity into octopine was inhibited by histidine, lysine, or ornithine and incorporation into histopine was inhibited by arginine, lysine, or ornithine (Table 3). If the reaction site on the enzyme requires a basic amino acid then neither alanine, which lacks a side chain, nor phenylalanine, which does not have a basic side chain, should inhibit the reactions. This was indeed the case (Table 3). Finally, -aminocaproic acid, which has a basic side chain but no α-amino group to react with pyruvate, inhibited all four synthetic reactions (Table 3).

DISCUSSION

Extracts of PSCG-B_6 tissue synthesize N^2-(1-carboxyethyl)-derivatives of arginine, histidine, lysine, and ornithine. The inhibition experiment (Table 3) suggests that the same enzyme is responsible for the synthesis of all four derivatives. The apparent specificity of this enzyme for the basic amino acids makes it unlikely that there are N^2-(1-carboxyethyl)-derivatives of other common amino acids in PSCG-B_6.

Bomhoff [8] found that octopine synthesis in crown gall tissues of Nicotiana tabacum, Scorsonera hispanica, and Parthenocissus tricuspidata used pyruvate, arginine, and NADH. Lejeune [9] reported that lysopine and octopinic acid synthesis in the same tissues had analogous requirements. In contrast, Birnberg et al. [14] proposed that crown gall tissues of Vinca rosea contain two different octopine synthesizing enzymes, one specific for NADH and one for NADPH. We find that the four activities in sunflower crown gall tissue are dependent on pyruvate, a basic amino acid, and either NADH or NADPH, but NADH and NADPH activities are not additive. Our results suggest that there is one enzyme that uses either NADH or NADPH. However, we cannot rule out the possibility of an NADH enzyme and an NADPH enzyme, either of which can be inhibited by the other coenzyme. The NADPH-dependent reaction is likely

to be the more important *in vivo* because the NADP(H) pool in plant tissues is maintained largely in the reduced form while the NAD(H) pool is largely oxidized [15].

The presence of N^2-(1-carboxyethyl)-amino acid synthesizing activity in PSCG-B_6 tissue but not in PSCG-AT1, PSCG-C58, or HSSS supports the hypothesis [2] that this enzyme may be controlled by a gene(s) on the tumor-inducing plasmid of *A. tumefaciens*. The plasmid apparently transfers about 3.5×10^6 daltons of DNA to host cells [16]. This could code for no more than four polypeptides of molecular weight 50,000 and thus is probably not sufficient for four separate N^2-(1-carboxyethyl)-amino acid synthesizing enzymes together with other functions necessary for tumor induction. Therefore, our conclusion that one enzyme catalyzes all four reactions is consistent with the amount of genetic information transferred.

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