ENZYMES OF THE HOMOGENTISATE RING-CLEAVAGE PATHWAY IN CELL SUSPENSION CULTURES OF HIGHER PLANTS*

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Received 20 November 1973

1. Introduction

It has been known for some years that exogenously supplied tyrosine is extensively metabolized by tissues of higher plants [references in 1], and there is ample of vidence that most, if not all, aromatic constituents of higher plants are subject to catabolic reactions involving cleavage of the aromatic ring [2, 3]. The mechanisms of ring-cleavage, however, have remained obscure.

In the course of an investigation on the biosynthesis of 2-methyl-5-hydroxy-1,4-naphthoquinone (plumbagin) in *Drosophyllum lusitanicum* Link: it was demonstrated by in vivo experiments, that labelled tyrosine, a highly efficient, but indirect, precursor of plumbagin, is degraded in this tissue to acetate most likely via the homogentisate ring-cleavage pathway [4]. Acetate thus formed is reused for biosynthetic purposes by the plant.

We now report the results of studies which demonstrate the presence of the complete catabolic sequence of enzymes from tyrosine to acetoacetate and fumarate in homogenates of cell suspension cultures of *D. lusitanicum*. Furthermore, the key-enzyme of the ring-cleavage reaction, homogentisate oxidase, was unequivocally demonstrated in partially purified extracts of ten other plant species.

2. Experimental

Cultures of *D.lusitanicum* Link. were started from surface-sterilized plant sections. All cultures were maintained on B5 medium [5], which was in some cases supplemented with casein hydrolysate. The origin of other cell cultures and media used have been described elsewhere [6]. Tissues were homogenized in ice-cold 0.02 M potassium phosphate buffer, pH 7.0, and 0.14 M KCl, and cell debris were removed by centrifugation at 20 000 *g*. This crude homogenate was used as enzyme source. For the determination of tyrosine transaminase activity [7], DL-tyrosine-β-^{14}C (specific activity 1 μCi/r ɔ ɛ) seved as substrate. 3-^{14}C-*p*-Hydroxyphenylpyruvate formed during the reaction was transformed into its dinitrophenylhydrazone (m.p. 171–173°C) and crystallixed to constant specific activity. *p*-Hydroxyphenylpyruvate oxidase was assayed in the presence of α, α'-bipyridyl using 3-^{14}C-*p*-hydroxyphenylpyruvate (specific activity 0.45 μCi/μmole) as substrate [8]. The labelled homogentisic acid was isolated by paper chromatography and after methylation identified as 2, 5-dimethoxyphenylacetic acid and the corresponding methyl ester respectively [9]. Homogentisate oxygenase was assayed by either a manometrical or an optical method both described by Knox [10]; both methods agreed within experimental errors. 4-Fumarylacetoacetate hydrolase was assayed by measuring the release of acetoacetate which was determined colorimetrically using diazo-*p*-nitroaniline [11]. Fumarylacetoacetate was prepared by isomerisation of maleylacetoacetate which was generated enzymatically from homogentisic acid [10].

In most cases reactions were linear with time up to 15–30 min. Protein concentrations used ranged from 1.8 to 2.2 mg/ml. Maleic and fumaric acids were separated by TLC on silica gel using diisopropylether–

* Dedicated to Prof. Dr. Maximilian Steiner on the occasion of his 70th birthday.

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formic acid–H_2O = 90:7:3 as solvent system.

3. Results and discussion

The biosynthesis of plumbagin in intact tissues of *D.lusitanicum* as well as in cell suspension cultures of the same plant proceeds via the acetate–polymalonate pathway [4]. In the course of this earlier investigation it had been observed that β-^{14}C-tyrosine was incorporates into plumbagin to an extent of 20% of the label supplied to the plant. The radioactivity of the β-carbon atom of tyrosine was, however, not localized in a single position of the plumbagin molecule but was found in every alternative carbon atom of the molecule. This observation could could only be explained by the assumption of an operative homogentisate ring-cleavage pathway in this plant. Feeding experiments with labelled putative intermediates like homogentisate, maleyl- and fumarylacetoacetate, as well as the observed accumulation of [^{14}C]homogentisate after the application of [β-^{14}C]tyrosine in the presence of α,α'-bipyridyl, an inhibitor of homogentisate oxygenase, supported this assumption. Rigorous proof, however, that the catabolic pathway for the breakdown of tyrosine to acetate via homogentisate is operative in higher plants required demonstration of the individual enzymes involved.

Since the homogentisate ring-cleavage pathway is operative in microorganisms [12], extreme care had to be taken to perform these experiments with absolutely sterile plant tissue. For this purpose cell suspension cultures were employed. As summarized in table 1, it was possible to unequivocally demonstrate the presence of four enzymes of the catabolic tyrosine pathway in tissue homogenates of *Drosophyllum*. Special attention was given to the key enzyme of the pathway, homogentisate oxygenase. The product obtained by the action of the plant enzyme on ring-[U-^{14}C] homogentisate [13, 10] was subjected to alkaline hydrolysis (2 H NaOH; 4 hr; 30°C) before and after acid isomerisation [10]. Chromatography of the reaction products yielded labelled maleic (R_f 0.5) and fumaric (R_f 0.8) acid, respectively. Homogentisate oxygenase was purified from *D.lusitanicum* homogenates employing the procedure of Adachi et al. [14] for the bacterial enzyme which was entirely applicable to the plant enzyme. The *Drosophyllum* enzyme was carried through step 5 (DEAE-chromatography) and resulted

Table 1

Activity of enzymes of the homogentisate-ring-cleavage pathway in a cell free preparation from cell suspension cultures of *Drosophyllum lusitanicum* Link.

Enzyme	Total activity nmoles × g^{-1} fr.wt. × min^{-1}
Tyrosine transaminase	17.9
p-Hydroxyphenylpyruvic acid oxidase	5.3
Homogentisate oxygenase	66.8
Flumarylacetoacetic acid hydrolase	21.6

in a 190-fold purification with a yield of 39%. The enzyme was completely inhibited by 5×10^{-3} M α,α'-bipyridyl. The specific activity of *Drosophyllum* homogentisate oxygenase (2.3 U/mg protein) was considerably higher than that reported for rat liver [10] and *Pseudomonas fluorescens* [14] at a comparable step of purification. The only missing enzyme in the entire set of the homogentisate ring-cleavage pathway is maleylacetoacetate isomerase. However, no attempt to detect this enzyme was made since isomerisation of maleyl- to fumarylacetoacetate occurs to a certain extent non enzymatically. Thus, it has been established that tyrosine is catabolized in higher plants according to the same pathway known from animals and microorganisms [12]. The mechanism of breakdown of L-tyrosine to acetate and fumarate via homogentisate seems to be identical in all living organisms so far tested and shows a remarkable phylogenetic constancy.

In order to determine whether capacity to cleave the homogentisate ring is a universal property of higher plants, a range of plant cell culture was examined for the presence of the key enzyme of this pathway: homogentisate oxygenase. It had previously been demonstrated in this laboratory that [U-^{14}C]homogentisic acid-ring, when fed to ten species of plants from 8 different families grown as sterile cell cultures, was rapidly degraded to $^{14}CO_2$ [1]. Homogentisate oxygenase from the same series of cultures was purified through step 4 of the procedure given above [10]. The results are summarized in table 2. All ten different plant cell cultures possess the key enzyme necessary for ring-cleavage of homogentisic acid. These data combined with

Table 2

Occurrence of homogentisate oxygenase in different plant species (values of specific and total activities after the 4th step of purification; protein concentration were determined according to [15].

Species	Family	Specific activity nmoles × mg^{-1} prot × min^{-1}	Total activity nmoles × g^{-1} fr.wt. × min^{-1}
Agave toumeyana Trel.	*Agavaceae*	625	153
Agrostemma githago L.	*Caryopyllaceae*	405	98
Coleus blumei Benth.	*Lamiaceae*	2590	41
Daucus carota L.	*Apiaceae*	250	64
Glycine max (L.) Merr.	*Fabaceae*	495	64
Melilotus alba Medik.	*Fabaceae*	125	13
Nicotiana sylvestris Spagezz, et Comes	*Solanaceae*	1160	18
Phaseolus vulgaris var. nanus (L.) Aschers	*Fabaceae*	515	106
Pimpinella anism L.	*Apiaceae*	170	19
Ruta graveolens L.	*Rutaceae*	390	141

previous results [1] which demonstrated the degradation of ring-[^{14}C]tyrosine and homogentisic acid to $^{14}CO_2$ in vivo allow now the conclusion that the homogentisate ring-cleavage pathway is widespread in the plant kingdom.

In summary, the data shown here unequivocally demonstrate for the first time the presence of the homogentisate ring-cleavage pathway in higher plants. Again, plant cell cultures have proven to be a valuable tool to solve problems of secondary plant metabolism.

Acknowledgements

We are grateful to Miss B. Ries for skilful technical assistance, to Dr. N. Amrhein, Bochum, for reading the manuscript and to the Bundesminister für Forschung und Technologie, Bonn, for financial support.

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