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Biosynthesis of Stizolobinic Acid and Stizolobic Acid in Higher Plants

An Enzyme System(s) Catalyzing the Conversion of Dihydroxyphenylalanine into Stizolobinic Acid and Stizolobic Acid from Etiolated Seedlings of *Stizolobium hassjoo*

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It was demonstrated that an enzyme system(s) extracted from etiolated seedlings of *Stizolobium hassjoo* catalyzed the conversion of L-dihydroxyphenylalanine into stizolobinic acid, α-amino-6-carboxy-2-oxo-2*H*-pyran-3-propionic acid, and stizolobic acid, α-amino-6-carboxy-2-oxo-2*H*-pyran-4-propionic acid, in the presence of $NADP^+$ or NAD^+ under aerobic condition. Enzymically synthesized radioactive stizolobinic acid and stizolobic acid isolated from the reaction mixtures were purified and confirmed to have constant specific radioactivities by cocrystallization with authentic samples.

Maximal activity of the enzyme preparation was obtained by using an insoluble polyphenol adsorbent (Polyclar AT) and a reducing agent (araboascorbic acid) in the extraction medium and by subsequent fractionation of the extract with ammonium sulfate followed by Sephadex G-25 gel filtration. Catalytic activity of the enzyme preparation was more unstable under aerobic condition than anaerobic. Attempts to stabilise the enzyme activity were made by the use of many substances which are known to stabilise other enzymes or expected to arrest the inactivation. Evidence is provided in this paper that the previously proposed biosynthetic pathways of stizolobinic acid and stizolobic acid from dihydroxyphenylalanine proceeded in the cell-free system from etiolated seedlings of *S. hassjoo*.

Stizolobinic acid and stizolobic acid, α-pyrone-bearing non-protein amino acids, were originally isolated from the sap of etiolated epicotyl tips of *Stizolobium hassjoo* (Leguminosae) [1]. These compounds were further detected in other species of the same genus such as *S. pruriens*, *S. utilis* and the synonymous genus such as *Mucuna irukanda* or *M. deeringiana* [1,2]. A recent report concerning the phytochemical investigation of non-fatal mushrooms has revealed that these amino acids were also distributed in some species of Basidiomycetes [3].

On the basis of their structures it has been postulated that these amino acids are derived from 3,4-dihydroxyphenylalanine (dihydroxyphenylalanine) *via* a metapyrocatechase-type of ring cleavage reaction followed by subsequent recyclization and dehydrogenation of the resultant intermediate aliphatic products such as α-hydroxymuconic semialdehyde derivatives [4,5] (Fig. 1). A sequence of the reaction (oxidation, ring opening, dehydrogenation and intramolecular rearrangement) has also been suggested for the biosynthetic pathway of patulin [6].

In a previous paper [7], we have indicated, using a radiotracer feeding technique, that dihydroxyphenylalanine was in fact utilized as a direct substrate for the biosynthesis of these two novel amino acids. In addition, evidence was presented that alanyl side-chain of dihydroxyphenylalanine was incorporated intact into the α-pyrone-6-carboxylic acid moieties [7]. Furthermore, by applying a doubly labelled tracer technique, it was demonstrated that the α-pyrone rings of both amino acids originated directly from dihydroxyphenylalanine *via* extradiol cleavage of the aromatic ring [8]. Similar results appeared in a recent study on *M. deeringiana* [2]. These findings are in harmony with an earlier proposal based on the structural investigation [4].

On the assumption that the proposed routes (Fig. 1) are operative, it should be investigated what kind of enzyme(s) really catalyze the synthetic reaction

Enzymes. Catechol 2,3-dioxygenase or catechol : oxygen 2,3-oxidoreductase (EC 1.13.11.2); monophenol monooxygenase or monophenol, dihydroxyphenylalanine : oxygen oxidoreductase (EC 1.14.18.1).

sequences. Although metapyrocatechase-type enzymes are expected, no evidence has yet been found for these enzymes in any plant material. As a first step of a study on such interesting biological catalysts, the present paper reports the biosynthesis of stizolobinic acid and stizolobic acid by an enzyme system(s) prepared from etiolated seedlings of *S. hassjoo*.

EXPERIMENTAL PROCEDURE

Plant Material

Seeds of *Stizolobium hassjoo* were harvested in a local field during October – November in 1974 and 1975. Soaked seeds (semisterilized) were germinated in trays with moist vermiculite in the dark at 27 °C. About 95 h after germination, the etiolated seedlings were placed in a cold room for 2 – 3 h at 2 – 4 °C. The excised epicotyls from the seedlings were sterilized with chilled 70% (v/v) ethanol followed by washing with ice-cold sterilized distilled water and used as a source of enzyme preparation.

Preparation of an Enzyme System(s)

The extraction medium was composed (if not mentioned otherwise) of 25.3 mM of araboascorbic acid, 50.0% (w/w) of Polyclar AT and 50.0 mM of Tris-HCl buffer (pH 7.5). The plant material (approx. 200 g fresh wt) was homogenized in the extraction medium (approx. 300 ml) with a Waring blendor and the homogenate was filtered through three layers of nylon cloth and the filtrate was centrifuged at 15000 × *g* for 20 min.

The resulting supernatant was treated with solid ammonium sulfate. The protein fraction precipitating between 55% and 75% ammonium sulfate saturation was collected, dissolved in a minimum amount (3 – 4 ml) of Tris-HCl buffer (50.0 mM, pH 7.5) and then passed through a column of Sephadex G-25. The column was washed with the same buffer. The protein fraction eluted was used as the enzyme preparation. All extraction procedures were performed at 2 – 4 °C as quickly as possible.

Enzyme Assay

The activity of the enzyme system converting dihydroxyphenylalanine to stizolobinic acid or stizolobic acid was assayed by measuring the radioactivity of stizolobinic acid or stizolobic acid formed when L-dihydroxy[β-^{14}C]phenylalanine was added to the reaction mixture as a substrate. The reaction mixture contained the following components, if not indicated otherwise: 0.03 µmol of L-dihydroxy[β-^{14}C]phenylalanine (specific activity 9.2 Ci/mol), 1.0 µmol of L-dihydroxyphenylalanine, 2.5 µmol of $NADP^+$, 2.5 µmol of 2-mercaptoethanol, 15.0 µmol of Tris-HCl buffer (pH 7.5) in a final volume of 3.0 ml.

The reaction mixture was shaken in an incubator at 60 – 120 strokes/min for 2 h at 30 °C. The reaction was stopped by heating on a boiling-water bath for 5 min. Denatured protein was removed by centrifugation and the clear supernatant solution was passed through a column of Amberlite IR-45 resin ($HCOO^-$ form, 1 × 15 cm). The column was then washed with distilled water. Adsorbed radioactive compounds were eluted with 300 ml of formic acid (200 mM). The eluate was concentrated to a small volume with a rotary evaporator at 60 – 65 °C. An aliquot of the concentrated eluate was spotted on Whatman No. 3MM papers and each amino acid was separated by two-dimensional chromatography in butan-1-ol/acetic acid/water (4/1/5, v/v/v, upper layer) and then in phenol saturated with water. The radioactive spot corresponding to stizolobinic acid or stizolobic acid was cut out and the radioactivity in the spot was measured in a liquid scintillation spectrometer (Beckman, model LS 250) after combustion with a sample oxidizer (Packard Tri-Carb, model 305) (counting efficiency 54 – 63%).

Anaerobic experiments were carried out in Thunberg tubes; air in the tubes was evacuated and replaced by nitrogen gas. This procedure was repeated five times before incubation.

The activity of polyphenol oxidase (monophenol monooxygenase) was measured spectrophotometrically by following the initial change in absorbance at 475 nm when L-dihydroxyphenylalanine was used as a substrate [9]. The protein content of the enzyme extract was determined according to the method described by Lowry *et al.* [10].

Chemicals

The chemicals used in this study were obtained from the following sources: L-dihydroxy[β-^{14}C]phenylalanine (9.2 Ci/mol), The Radiochemical Centre Ltd (Amersham, England); $NADP^+$, C.F. Boehringer and Soehne GmbH (Mannheim, West Germany); NAD^+, Oriental Yeast Co. Ltd (Tokyo, Japan); L-dihydroxyphenylalanine, Nakarai Chemicals, Ltd (Kyoto, Japan); Polyclar AT, General Aniline and Film Corp. (New York, U.S.A.).

RESULTS

Effects of Polyclar AT and Araboascorbic Acid Used in Extraction on the Activity of Enzyme Preparation

The activities of many plant enzymes have been reported to be greatly inhibited by endogenous phenolic compounds [11 – 15]. The seedlings of *S. hassjoo* contain a large quantity of phenolic substances [16]

Table 1. *Effect of araboascorbic acid and Polyclar AT used in extraction on the activity of enzyme preparation*
Extraction was carried out as described in Experimental Procedure except that the amounts of araboascorbic acid and Polyclar AT (as g/g fresh wt of tissue) in the extraction medium were varied as shown. Enzyme activity was measured as radioactivity incorporated in 2 h

Araboascorbic acid	Polyclar AT	Radioactivity incorporated into	
		stizolobinic acid	stizolobic acid
mM	g/g	dis. min^{-1}	
–	–	210	125
2.5	–	1576	1443
5.1	–	3242	4010
25.3	–	4565	4011
50.0	–	2339	3168
–	0.10	1667	1892
–	0.30	3413	3062
–	0.50	2748	4511
5.1	0.30	4101	4340
25.3	0.30	3942	5241
5.1	0.50	4419	5960
25.3	0.50	7641	8108

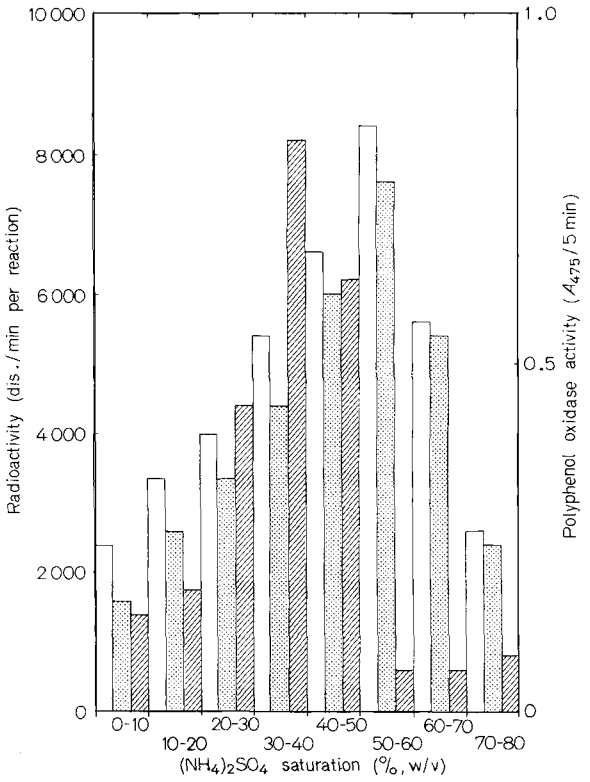

Fig. 1. *Separation of the enzyme system from polyphenol oxidase by ammonium sulfate fractionation.* Etiolated seedlings (approx. 200 g) were homogenized in the extraction medium (25.3 mM araboascorbic acid, 0.5 g Polyclar AT/g, fresh wt of tissue, 50.0 mM Tris-HCl buffer, pH 7.5). The supernatant liquid obtained by centrifugation at $15000 \times g$ for 20 min was fractioned with various concentrations of ammonium sulfate as listed in the figure. Subsequent treatment of the fractioned protein extracts and the enzyme assays were performed as shown in Experimental Procedure. Spotted bars, radioactivity incorporated into stizolobinic acid; empty bars, radioactivity incorporated into stizolobic acid; striped bars, polyphenol oxidase activity

and crude extracts from the etiolated seedlings turned dark brown or black within a few minutes. It was, therefore, necessary to remove these noxious compounds from the enzyme preparation by the addition of a reducing agent and a polyphenol adsorbent.

Table 1 shows the effects of araboascorbic acid and/or Polyclar AT used in extraction on the activities of enzyme preparations. It is evident in Table 1 that homogenation of the tissue in the presence of araboascorbic acid and/or Polyclar AT gave a higher activity of the enzyme preparation than in the absence of them. The highest activity was obtained by homogenization in 25.3 mM of araboascorbic acid and 50.0% of Polyclar AT.

Separation of the Enzyme System Converting Dihydroxyphenylalanine to Stizolobinic Acid and Stizolobic Acid from Polyphenol Oxidase

The tissues of *S. hassjoo* seedlings contain polyphenol oxidase, which also uses dihydroxyphenylalanine as a substrate. We tried to separate the enzyme system converting dihydroxyphenylalanine to stizolobinic acid and stizolobic acid from the polyphenol oxidase by ammonium sulfate precipitation. As shown in Fig. 1, the highest activity of the enzyme system and the lowest one of polyphenol oxidase were found in the precipitate formed between 50% and 60% ammonium sulfate saturation. The fraction between 55–75% ammonium sulfate saturation was used after gel filtration with Sephadex G-25 as the enzyme preparation in further experiments.

Evidence for the Formation of Stizolobinic Acid and Stizolobic Acid from L-Dihydroxyphenylalanine in the Enzyme System

The reaction mixture, consisting of the enzyme preparation and L-dihydroxy[β-^{14}C]phenylalanine, was incubated for 2 h at 30 °C in the presence of $NADP^+$ under aerobic conditions. The reaction was stopped by heating and the resulting denatured protein was centrifuged off. The supernatant liquid was passed through an ion-exchange column and eluted with formic acid. The eluate, containing radioactive compounds and carrier amino acids, was brought to a small volume and then spotted on strips of Whatman No. 1 papers. Ascending one-dimensional chromatography was carried out in phenol saturated with water

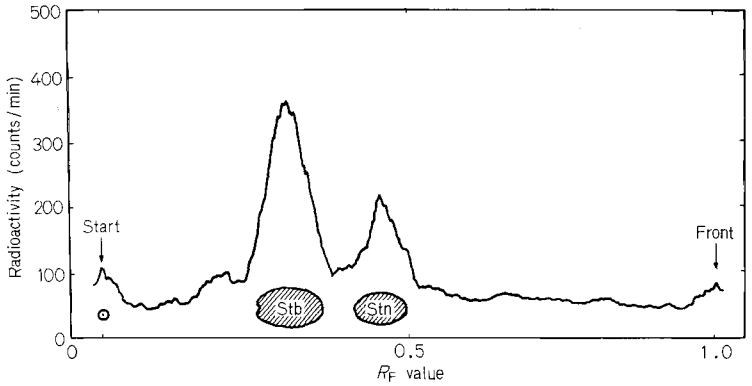

Fig. 2. *Scanning of radioactivities on a paper chromatogram of the amino acids separated from a reaction mixture.* The shaded spots illustrate the positions of authentic stizolobinic acid (Stn) and stizolobic acid (Stb) which were added as carriers. For details of the procedure see Experimental Procedure

Table 2. *Incorporation of L-dihydroxy[β-^{14}C]phenylalanine into stizolobinic acid and stizolobic acid by the enzyme preparation*
Enzyme assays were carried out as described in Experimental Procedure and Table 1

Incubation system	Expt	Radioactivity incorporated into	
		stizolobinic acid	stizolobic acid
		dis. min^{-1}	
Complete	1	4430	5928
	2	5693	9124
	3	5568	9011
Extract boiled	1	134	262
	2	162	73
	3	122	111

as a developing solvent. Relative location of the radioactivities and the ninhydrin-positive areas on a strip are shown in Fig. 2. Radioactivities were found to coincide with the positions of stizolobinic acid and stizolobic acid. To further confirm the chromatographic evidence that these amino acids were formed enzymically from dihydroxyphenylalanine, radioactive spots corresponding to stizolobinic acid and stizolobic acid were cut out from paper chromatograms and eluted with water and the eluates were evaporated to a small volume.

An authentic sample of each amino acid was added to the concentrated water eluate and the amino acids were recrystallized several times from propan-1-ol/water (6/4, v/v). The recrystallized stizolobinic acid or stizolobic acid showed a constant specific radioactivity (290 and 340 dis. min^{-1} μmol^{-1}, average specific radioactivity during the last four times of recrystallization, respectively). In control experiments with boiled enzyme preparation, only negligible amount of radioactivity was detected in the areas corresponding to the amino acids (Table 2).

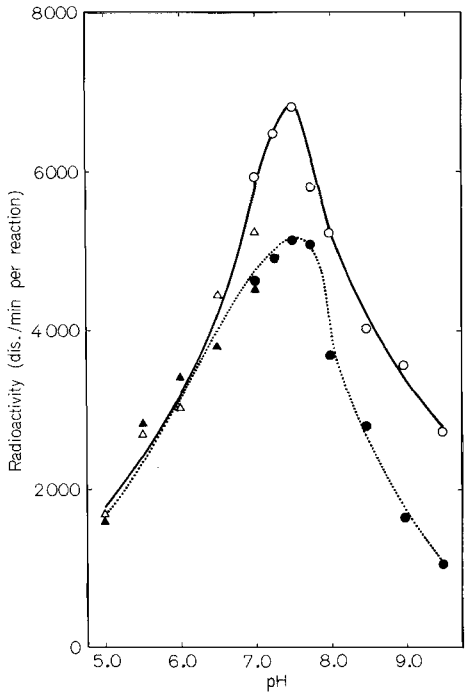

Fig. 3. *Effect of pH value on the enzymic synthesis of stizolobinic acid and stizolobic acid from dihydroxyphenylalanine.* Enzyme assays were carried out as described in Experimental Procedure except that the buffer used in the reaction mixture was varied as shown: (△, ▲) 5.0 mM Tris-maleate buffers; (○, ●) 5.0 mM Tris-HCl buffers. (·····) Stizolobinic acid; (——) stizolobic acid

Effect of pH on the Enzyme Reaction

The standard assay was modified by substituting 5.0 mM Tris-maleate or 5.0 mM Tris-HCl buffer at various pH values for the 5.0 mM Tris-HCl buffer, pH 7.5. The pH at the start of the incubation ranged from 5.0 to 9.5. The pH optimum for tracer incorporation into both stizolobinic acid and stizolobic acid appeared to be about 7.5 in Tris-HCl buffer (Fig. 3).

Table 3. *Effect of aerobic or anaerobic conditions on the conversion of L-dihydroxy[β-^{14}C]phenylalanine into stizolobinic acid and stizolobic acid by the enzyme system*
Assays were carried out as described in Experimental Procedure and Table 1

Expt	Gas phase	Radioactivity incorporated into	
		stizolobinic acid	stizolobic acid
		dis. min^{-1}	
1	air	3327	5207
	N_2	237	510
2	air	5796	6329
	N_2	654	565
3	air	5548	6390
	N_2	569	496

Time Course of the Enzyme Reaction and Effect of Enzyme Concentration on the Reaction

The reaction velocity of conversion of exogenously applied radioactive-labelled dihydroxyphenylalanine into both heterocyclic amino acids by the enzyme preparation was constant for 120 min and then rapid decrease of the rate of incorporation of radioactivity occurred. For this reason, a 2-h incubation period was adopted in all subsequent experiments. A linear relationship was obtained between enzyme activity and amount of enzyme protein used up to 0.23 mg/ml of reaction mixture.

Requirements of Aerobic Condition and Adenine Nucleotides for the Reaction

The effect of aerobic or anaerobic conditions on the incorporation of labelled dihydroxyphenylalanine into both amino acids was examined. When the enzyme reaction was carried out in an atmosphere of nitrogen gas, the rate of radioactivity incorporation was greatly reduced (Table 3). Table 4 shows the requirements of adenine nucleotides for the reaction. $NADP^+$ was required for conversion of dihydroxyphenylalanine to both stizolobinic acid and stizolobic acid, and the nucleotide could be replaced by NAD^+.

Enzyme Stability

The enzyme preparation was unstable, especially in aerobic conditions. The stability of the enzyme system under various storage conditions is shown in Table 5 and 6. Storage for 24 h at 4 °C in the air reduced the enzyme activity to 11% or 8% of the initial with stizolobinic acid or stizolobic acid formation, respectively.

Table 4. *Effect of $NADP^+$ or NAD^+ on the conversion of L-dihydroxy-[β-^{14}C]phenylalanine into stizolobinic acid and stizolobic acid by the enzyme system*
Assays were carried out as described in Experimental Procedure and Table 1

Adenine nucleotides	Concentration	Radioactivity incorporated into	
		stizolobinic acid	stizolobic acid
	mM	dis. min^{-1}	
$NADP^+$	0	821	713
	0.33	2857	3291
	0.83	5592	6912
	1.67	3421	3841
	3.33	1856	2433
NAD^+	0	626	815
	0.33	3113	3927
	0.83	4464	4803
	1.67	3322	4013
	3.33	1768	2030

Table 5. *Effect of various compounds on enzyme stability during storage*
The enzyme preparation was kept with each compound tested during storage for 24 h at 4 °C. Each compound was added to the enzyme preparation at a final concentration of 1 mM, except 20% (v/v) glycerol. Enzyme assays were then carried out as described in Experimental Procedure and Table 1

Compounds	Radioactivity incorporated into	
	stizolobinic acid	stizolobic acid
	dis. min^{-1}	
Glycerol	1021	595
L-Ascorbic acid	516	461
Glutathione (reduced form)	452	671
Dithiothreitol	419	431
Cysteine	622	631
2-Mercaptoethanol	495	428

In an attempt to stabilise the enzyme activity, the effects of a number of substances known to stabilise other enzyme activities or to prevent the inactivation were tested. The addition of a given concentration of glycerol, L-ascorbic acid, glutathione (reduced form), dithiothreitol, cysteine and 2-mercaptoethanol to the enzyme preparation did not prevent inactivation during storage (Table 5). Alteration of ionic strength or enzyme concentration was also ineffective, but considerable stabilisation could be obtained by replacement of air in the enzyme storage vessel by nitrogen gas. After 24 h of storage in a Thunberg tube evacuated and filled with nitrogen gas at 4 °C,

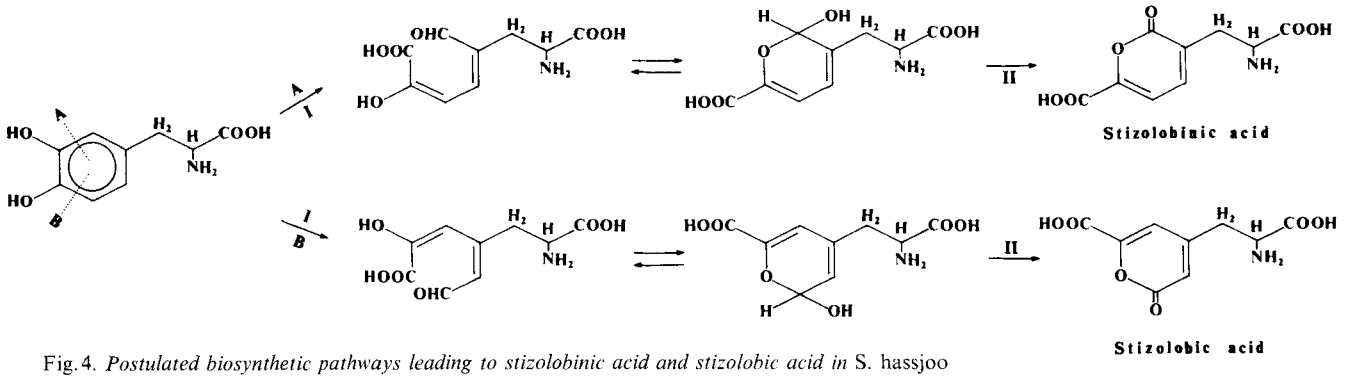

Fig. 4. *Postulated biosynthetic pathways leading to stizolobinic acid and stizolobic acid in* S. hassjoo

Table 6. *Storage effect on enzyme activity under various conditions*
The enzyme preparations were stored for 24 h in Thunberg tubes filled with nitrogen gas or in open test tubes under various conditions. The initial activity was 7294 dis. min^{-1} over 2 h with stizolobinic acid and 8198 dis. min^{-1} over 2 h with stizolobic acid. Enzyme assays were carried out as described in Experimental Procedure and Table 1

Storage conditions		Radioactivity incorporated into	
temperature	gas phase	stizolobinic acid	stizolobic acid
°C		dis. min^{-1}	
−20	N_2	5252	5247
	air	955	1232
4	N_2	2610	3669
	air	791	651
27	N_2	318	174
	air	193	134

the enzyme activity was about 64% or 55% of the initial activity for stizolobinic acid or stizolobic acid formation, respectively, while after 24 h of storage under a nitrogen atmosphere at −20 °C, 72% of the initial activity for stizolobinic acid formation and 64% for stizolobic acid formation was retained (Table 6).

DISCUSSION

An enzyme system(s) prepared from the etiolated seedlings of *S. hassjoo* has been shown to be able to catalyze the conversion of isotopically labelled dihydroxyphenylalanine into stizolobinic acid and stizolobic acid. In previous studies *in vivo* we have shown that these amino acids may originate directly from dihydroxyphenylalanine by extradiol cleavage of the aromatic ring [7,8]. The findings described in this paper provide evidence that the biosynthetic reactions of these amino acids proceed in a cell-free preparation from a higher plant.

Hydroxylation and oxidative disruption of aromatic rings have been suggested for some steps in the catabolic processes of phenols, aromatic amino acids and other substances [5,17−23], but many of these suggestions remain speculative and tentative. For a cleavage reaction to proceed readily, the aromatic ring of the substrate must be raised at the initial step to a sufficient oxidation level by introducing two hydroxyl groups. Molecular oxygen is then introduced across an aromatic carbon-carbon double bond between two hydroxylated carbon atoms or adjacent to one of the hydroxyls with simultaneous cleavage of that bond to form primary reaction products such as *cis-cis* muconic acid derivatives [24]. The oxidative ring cleavage reaction of aromatic compounds could be adopted for the biosynthesis of stizolobinic acid and stizolobic acid from dihydroxyphenylalanine.

In conversion of dihydroxyphenylalanine to these amino acids by the enzyme preparation from *S. hassjoo* seedlings, aerobic conditions were absolutely required. Although no experiments with isotopically labelled oxygen have yet been performed, it is possible to predict from the results that molecular oxygen may be consumed in the oxidative cleavage of the carbon-carbon linkage between the 2 and 3 position or 4 and 5 position in the aromatic ring of dihydroxyphenylalanine at step I in the proposed pathways for the biosynthesis of stizolobinic acid and stizolobic acid (Fig. 4). On the other hand, a hydrogen acceptor such as $NADP^+$ or NAD^+ was inevitably needed for the overall reaction, indicating that dehydrogenation should occur in the course of the reaction sequence, presumably at step II in the pathways.

The enzyme preparation was unstable, due to rapid inactivation under aerobic conditions. Inactivation of the enzyme preparation, however, was decreased by replacing air with nitrogen gas. Oxygenases such as metapyrocatechase (catechol 2,3-dioxygenase) which acts on catechol are extremely sensitive to oxygen and the air-inactivation is thought to be mainly due to oxidation of the ferrous ion to ferric form in the active centre [25]. Though the properties of the enzyme from *S. hassjoo* still remain to be investigated, it may

possess divalent iron as an integral part of the enzyme molecule.

The enzyme preparation used in this work catalyzed both reaction sequence A leading to stizolobinic acid and sequence B to stizolobic acid in Fig. 4. Therefore, the preparation must contain two enzyme systems although the same pH optima were found for both stizolobinic acid and stizolobic acid formation and fractionation by ammonium sulfate precipitation did not succeed in separating the two enzyme systems. The isolation of each enzyme system is in progress.

From the results presented here, it may presumably be expected that metapyrocatechase-type enzymes also operate in higher plants as in mammalian and bacterial systems [26, 27].

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REFERENCES

1. Hattori, S. & Komamine, A. (1959) *Nature (Lond.) 183*, 1116–1117.
2. Ellis, B. E. (1976) *Phytochemistry, 15*, 489–491.
3. Chilton, W. S., Hsu, C. P. & Zdybak, W. T. (1974) *Phytochemistry, 13*, 1179–1181.
4. Senoh, S., Imamoto, S., Maeno, Y., Tokuyama, T., Sakan, T., Komamine, A. & Hattori, S. (1964) *Tetrahedron Lett. 46*, 3431–3438.
5. Senoh, S. & Sakan, T. (1966) in *Biological and Chemical Aspects of Oxygenases* (Bloch, K. & Hayaishi, O., eds) pp. 93–99, Maruzen, Tokyo.
6. Tanenbaum, S. W.,& Bassett, E. W. (1959) *J. Biol. Chem. 234*, 1861–1866.
7. Saito, K., Komamine, A. & Senoh, S. (1975) *Z. Naturforsch. 30c*, 659–662.
8. Saito, K., Komamine, A. & Senoh, S. (1976) *Z. Naturforsch. 31c*, 15–17.
9. Horowitz, N. H., Fling, M. & Horn, G. (1970) *Methods Enzymol. 17A*, 615–620.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
11. Mason, H. S. (1955) *Adv. Enzymol. 16*, 105–184.
12. Chapon, L., Chollot, B. & Urion, E. (1961) *Bull. Soc. Chim. Biol. 43*, 429–442.
13. Harel, E., Mayer, A. M. & Shain, Y. (1964) *Physiol. Plant. 17*, 921–930.
14. Pierpoint, W. S. (1966) *Biochem. J. 98*, 567–580.
15. Loomis, W. D. & Battaile, J. (1966) *Phytochemistry, 5*, 423–438.
16. Hattori, S. & Shiroya, M. (1955) *Physiol. Plant. 8*, 63–70.
17. Hayaishi, O. & Hashimoto, K. (1950) *J. Biochem. (Tokyo) 37*, 371–374.
18. Evans, W. C., Smith, B. S. W., Linstead, R. P. & Elvidge, J. A. (1951) *Nature (Lond.) 168*, 772–775.
19. Knox, W. E. & Edwards, S. W. (1955) *J. Biol. Chem. 216*, 479–487.
20. Chapman, P. J. & Dagley, S. (1960) *Biochem. J. 75*, 6p–7p.
21. Leete, E. (1967) *Annu. Rev. Plant Physiol. 18*, 179–196.
22. Fischer, N. & Dreiding, A. S. (1972) *Helv. Chim. Acta, 55*, 649–658.
23. Impellizzeri, G. & Piattelli, M. (1972) *Phytochemistry, 11*, 2499–2502.
24. Dagley, S., Evans, W. C. & Ribbons, D. W. (1960) *Nature (Lond.) 188*, 560–566.
25. Nozaki, M., Ono, K., Nakazawa, T., Kotani, S. & Hyaishi, O. (1968) *J. Biol. Chem. 243*, 2682–2690.
26. Decker, R. H., Kang, H. H., Leach, F. R. & Henderson, L. M. (1961) *J. Biol. Chem. 236*, 3076–3082.
27. Kojima, Y., Itada, N. & Hayaishi, O. (1961) *J. Biol. Chem. 236*, 2223–2228.

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