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PURIFICATION AND CHARACTERIZATION OF DIHYDROGEODIN OXIDASE FROM A FUNGAL STRAIN OF ASPERGILLUS TERREUS PRODUCING (+)-GEODIN

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Dihydrogeodin oxidase (DHGO), an enzyme catalyzing regio- and stereo-specific intramolecular phenol oxidative coupling reaction of dihydrogeodin to give (+)-geodin, was purified up to homogeneity. DHGO was shown to be a blue copper protein with an absorption maximum at 600 nm. The presence of copper atoms was confirmed by atomic absorption analysis. A molecular weight of 153,000 was estimated for the oxidase and it was composed of two equal molecular weight subunits. DHGO had no phenolase activity and showed strict substrate specificity.

KEYWORDS —— phenol oxidative coupling; dihydrogeodin; (+)-geodin; blue copper protein; Aspergillus terreus

The phenol oxidative coupling reaction has been regarded as one of the most important reactions involved in the biosyntheses of complex natural products[1] since Barton and Cohen first proposed that some of the new C-C or C-O bonds could be formed by the pairing of radicals of phenolic substrates generated by oxidation.[1c] Extensive tracer studies have clarified the detailed biosynthetic pathways of a great number of natural products[2] and verified the important role of phenol oxidative coupling reactions in the biosyntheses of many phenolic natural products including alkaloids such

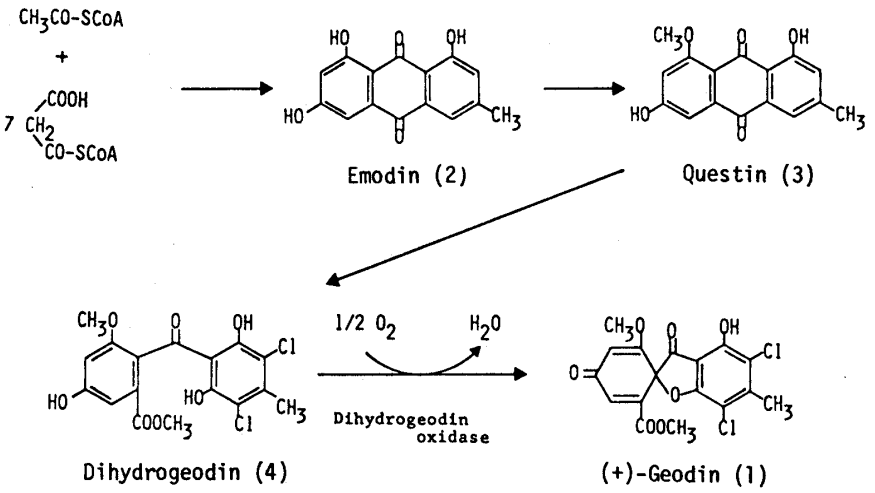


Fig. 1. Biosynthesis of (+)-Geodin in Aspergillus terreus IMI 16043

as morphine. A variety of chemical[3] and biochemical[4] oxidizing systems which are frequently used in *in vitro* phenol oxidation experiments have the common feature that they contain heavy metals as reagents or prosthetic groups. Biosynthetic reactions catalyzed by enzymes undergo strict regio- and stereo-specific control. Model enzyme systems used in the biomimetic experiments, however, lack both regio- and stereo-specificities. Scott[1b] summarized the results of enzymic model oxidations generating new chiral centers. In none of the examples was any optical activity observed in the products. As far as we know, no report has been published on the isolation and characterization of any enzyme catalyzing the specific phenol oxidative coupling reaction involved in the biosynthesis of natural products.

(+)-Geodin (1) is a seco-anthraquinone[5] derived from an octaketide anthraquinone, emodin (2). Emodin (2) is then methylated to give questin (3),[6] which is further converted into a benzophenone by a Baeyer-Villiger type ring cleavage.[7] Dihydrogeodin (4) formed by further methylation and chlorination is the substrate of phenol oxidative coupling reaction to give (+)-geodin (1). The postulated biosynthetic scheme is shown in Fig. 1.

Komatsu[8] showed that a cell free preparation of mycelia of *Penicillium estinogenum*, a (+)-geodin (1) producing strain, was capable of converting dihydrogeodin (4) into (+)-geodin (1). He suggested the participation of the copper ion in this reaction, presumably as a prosthetic group of the coupling enzyme, based on his observation that culture of *P. estinogenum* accumulated dihydrogeodin (4) when it was grown in a medium lacking copper ion. A variety of reactions involved in the biosynthesis of (+)-geodin (1) drew our attention. We have extensively investigated the purification and characterization of enzymes responsible for (+)-geodin (1) biosynthesis in *Aspergillus terreus* IMI 16043 and some of the results have already been reported.[6,9] This communication reports the purification and characterization of dihydrogeodin oxidase (DHGO) which catalyzes the regio- and stereo-specific intramolecular phenol oxidative coupling reaction of dihydrogeodin (4) to yield (+)-geodin (1).

Dihydrogeodin oxidase (DHGO) activity was assayed photometrically by following the increase of absorbance at 392 nm where the difference spectrum of (+)-geodin (1) to dihydrogeodin (4) gave a maximum. Enzyme preparation was added to a 1 cm light path cuvette which contained 1 m mol of potassium phosphate (pH 6.0), 0.2 μ mol of dihydrogeodin (4) in a total volume of 2.2 ml. All assays were performed at 30° C. The crude mycelial extract which showed DHGO activity was subjected to ammonium sulfate fractionation. The fraction precipitated betweeen 30% and 80% saturation was then treated under an acidic condition (pH 4.5 for 10 min). After removal of the precipitates by centrifugation, the supernatant was dialyzed against 0.05 M Tris-HCl buffer (pH 7.0). This fraction was loaded onto a DEAE-cellulose column, and eluted by using NaCl concentration gradient. An active fraction was then directly applied to a Hydroxyapatite column equilibrated with 0.05 M Tris-HCl buffer (pH 7.0). DHGO was eluted by a continuous gradient of ammonium sulfate concentration. After dialysis, DHGO was again applied to the second DEAE-cellulose column. An active fraction obtained was then loaded onto a chromatofocusing column (PBE 94)[10] and eluted by continuous gradient of pH and NaCl concentration. Further purification with Sepharose 6B and DEAE Sephadex A-25 columns gave the purified DHGO preparation. The results of DHGO purification are summarized in Table I.

The final enzyme preparation showed a single protein band on sodium dodecylsulfate polyacrylamide gel electrophoresis at a position corresponding to a molecular weight of 76,000. The molecular weight of native DHGO was estimated to be 153,000 by TOYO PEARL HW-55S column chromatography, indicating that the oxidase was composed of two equal subunits. The absorption spectrum of DHGO showed maxima at 280 and 600 nm (Fig. 2). The blue colour of the enzyme suggested the presence of

Table I. Summary of Purification Procedures for Dihydrogeodin Oxidase

Step	Volume (ml)	Total Protein (mg)	Total Activity (mU)	Specific Activity (mU/mg)	Recovery (%)
Crude Extract	1120	659	18032	27.4	100
Amm. Sulf. Ppt.	104	360	11440	31.8	63.4
Acid Treatment	86	157	10148	64.6	56.3
1st DEAE-Cellulose	360	40.7	6264	154	34.7
Hydroxyapatite	226	10.4	4723	454	26.2
2nd DEAE-Cellulose	102	5.51	3407	618	18.9
Chromato-focusing	20	2.20	2160	982	12.0
Sepharose 6B	13.5	1.74	1661	955	9.2
DEAE-Sephadex A-25	14.0	0.596	567	951	3.1

One Unit of Activity is defined as the Amount of Enzyme yielding 1 μ mol of Product in a minute at 30° C.

copper as a prosthetic group in the catalytic site of the enzyme molecule. The presence of copper was confirmed by atomic absorption analysis and its content was calculated to be 0.65% by the standard addition method as shown in Fig. 3. This value together with its molecular weight indicates that the enzyme contains 16 copper atoms per molecule. The product formed by this enzyme reaction was identified as (+)-geodin (1) by the comparison of its IR spectrum with that of an authentic sample and the optical rotatory dispersion analysis proved the product to be a 100% pure (+)-isomer. Table II shows the substrate specificity of the DHGO reaction. Among the potential substrates tested, only benzophenones such as sulochrin (5) and griseophenone B (6) reacted to some extent, while no phenolase or tyrosinase activity was observed. Spectrophotometric and oxygen electrode

Fig. 2. Absorption Spectrum of DHGO

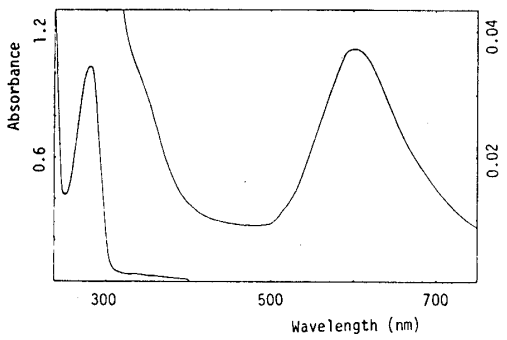
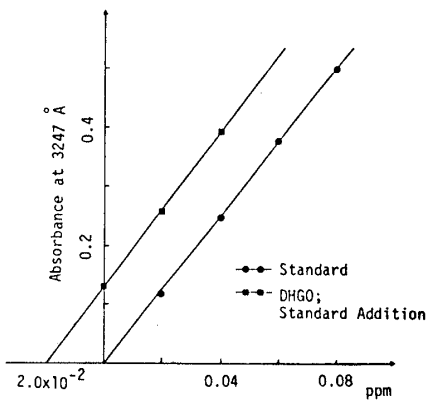
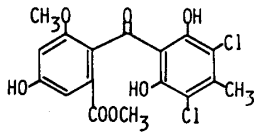


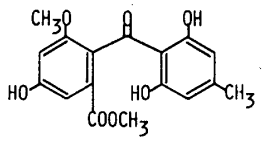
Fig. 3. Quantitative Analysis of Copper in DHGO by Atomic Absorption Spectrometry



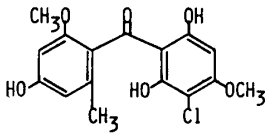
analysis to determine the stoichiometry of DHGO reaction revealed that one half mol of oxygen is consumed for the formation of one mol of (+)-geodin (1). From the characteristics of the enzyme mentioned above, it is concluded that DHGO is a new type of blue copper protein, specific for (+)-geodin (1) biosynthesis, and definitely differs from enzymes so far studied in enzymic model reactions. As far as we know this is the first report that the enzyme catalyzing phenol oxidative coupling reaction specific for natural product biosynthesis is purified and characterized.



Dihydrogeodin (4) 100%



Sulochrin (5) 25%



Griseophenone B (6) 19%

Table II. Substrate Specificity of DHGO

REFERENCES

1) a) W.I.Taylor and A.R.Battersby, "Oxidative Coupling of Phenols," Marcel Dekker, New York, 1967; b) A.I.Scott, Quart. Rev. Chem. Soc., 19, 1 (1965); c) D.H.R.Barton and T.Cohen, "Festschrift A. Stoll," Birkhäuser, Basel, 1957; d) D.H.R.Barton, Pedler Lecture, Chem. Brit., 3, 330 (1967).

2) T.A.Geissman and J.D.Bu'Lock (ed.), "Specialist Periodical Report; Biosynthesis," Vol. I-VI, The Chemical Society, London, 1972-1980.

3) H.Musso, Angew. Chem., 75, 965 (1963); T.Kametani and K.Fukumoto, Synthesis, 657 (1972); P.D.McDonald and G.A.Hamilton, "Oxidation in Organic Chemistry," Part B, Chapter II, ed. by W.S.Trahanovsky, Academic Press, New York, 1973; T.Kametani, K.Fukumoto and F.Satoh, Bioorg. Chem., 3, 430 (1974); S.Tobinaga, Bioorg. Chem., 4, 110 (1975).

4) B.R.Brown, in Ref. 1a, p.167.

5) R.F.Curtis, C.H.Hassall and D.R.Parry, J. Chem. Soc. Perkin I, 1972, 240; H.Fujimoto, H.Flash and B.Franck, Chem. Ber., 108, 1224 (1975); S.Gatenbeck and L.Malmström, Acta Chem. Scand., 23, 3493 (1969).

6) Isao Fujii, Yutaka Ebizuka and Ushio Sankawa, Chem. Pharm. Bull., 30, 2283 (1982).

7) B.Franck, "The Biosynthesis of Mycotoxins," ed. by P.S.Steyn, Academic Press, New York, 1980, p.157.

8) E.Komatsu, Nippon Nogei-Kagaku Kaishi, 31, 698 (1957); Idem, ibid., 31, 909 (1957).

9) Isao Fujii, Yutaka Ebizuka and Ushio Sankawa, Abstracts of Papers, The 99th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, August, 1979, p.207; Hiroshi Iijima, Sachiko Tsukita, Yutaka Ebizuka and Ushio Sankawa, Abstracts of Papers, The 99th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, August, 1979, p.207; Isao Fujii, Yutaka Ebizuka and Ushio Sankawa, Abstracts of Papers, The 100th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1980, p.226; Isao Fujii, Hiroshi Iijima, Yutaka Ebizuka and Ushio Sankawa, Abstracts of Papers, The 102nd Annual Meeting of Pharmaceutical Society of Japan, Osaka, April, 1982, p.546; Isao Fujii, Yutaka Ebizuka and Ushio Sankawa, Abstracts of Papers, The 102nd Annual Meeting of Pharmaceutical society of Japan, Osaka, 1982, April, p.547; Isao Fujii, Hiroshi Iijima, Yutaka Ebizuka and Ushio Sankawa, Abstracts of Papers, 25th Symposium on the Chemistry of Natural Products, Tokyo, Japan, 1982, p.218.

10) L.A.AE.Sluyterman and O.Elgersma, J. Chromatogr., 150, 17 (1978); L.A.AE.Sluyterman and J.Wijdenes, J. Chromatogr., 150, 31 (1978).

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