AN ENZYME SYSTEM CLEAVING THE AROMATIC RING OF 2,3-DIHYDROXYBENZOIC ACID, FROM LEAVES OF *TECOMA STANS*

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1. Introduction

Evidence for the occurrence of aromatic ring-fission in higher plant tissues has been mounting in recent years ([1, 2] and references therein). However, no report on studies with isolated enzyme systems has appeared so far. In this communication we describe the preparation of an enzyme system from tender leaves of *Tecoma stans* which catalyzes the aerobic oxidation of 2,3-dihydroxybenzoic acid leading to the isolation of an aliphatic product which reacted as a dilactone, tentatively characterized as 2,6-dioxa-3,7-dioxo*bicyclo* (3:3:0) octane-8-carboxylic acid by chemical and physical methods. An intradiol oxygenative cleavage of 2,3-dihydroxybenzoic acid is proposed to account for its formation.

2. Materials and methods

All chemicals used were of the highest grade available commercially. Protein was estimated by the method of Lowry et al. [3]. Ultraviolet spectra were recorded in Unicam SP-700A or Cary-14 recording spectrophotometer. Infrared spectra were recorded in a Carl Zeiss Jena UR10 spectrophotometer using nujol mull or KBr pellet technique.

2.1. *Preparation of enzyme*

All operations were carried out at 0–5° and all centrifugations were at 12,000 *g* and for 10 min unless otherwise stated.

Freshly collected tender leaves of *Tecoma stans* (50 g) were washed thoroughly with 1 mM sodium metabisulfite and crushed to a paste with acid-washed glass powder and 1 mM sodium metabisulfite (92 ml), in a porcelain mortar. The paste was strained through a muslin cloth and centrifuged at 17,000 *g*. The supernatant was stirred with aqueous calcium acetate (10% w/v, 2.5 ml) and centrifuged for 5 min. The supernatant was adjusted to pH 5.2 with 0.1 M citric acid and the precipitate formed in 2 min was removed by centrifugation at 17,000 *g*. The supernatant was stirred for 20 min with a 1% aqueous protamine sulfate (3.5 ml) and the precipitate was removed by centrifugation. The supernatant was stirred for 5 min with 1.2 g of DEAE-cellulose, washed as described by Peterson and Sober [4], and kept suspended in water. The solids were removed by filtration and the filtrate was brought to 0.3 saturation with solid $(NH_4)_2SO_4$. The precipitate formed in 20 min was collected by centrifugation and dissolved in water (40 ml). Any insoluble residue was removed by centrifugation. The clear supernatant was stirred with 7 ml of tricalcium phosphate gel (18 mg solids per ml) for 20 min. The gel was collected by centrifugation and the enzyme eluted from it by stirring for 20 min with 25 ml of 0.1 M citrate-phosphate buffer pH 5.2.

2.2. *Isolation of product*

A reaction mixture consisting of 30 ml of the enzyme preparation (4–5 mg protein) and 20 mg of 2,3-dihydroxybenzoic acid was incubated at 30° for 15 min. The reaction was stopped by lowering the pH to 2 with conc. HCl. The reaction mixture was extracted repeatedly with peroxide-free diethyl ether. The ether layers were combined, dried with anhydrous sodium sulfate and concentrated under vacuum. The

ether solution was applied to a cellulose column (1.8 × 20 cm) packed in benzene. The column was washed successively with petroleum ether (40–60°) and benzene–acetic acid (90:10, v/v). The product was eluted as a single band (located by its green fluorescence under UV light) with methanol–benzene–acetic acid (65:35:15, by vol). The eluate was extracted two times with water and the organic phase was discarded. The aqueous layer was extracted three times with diethyl ether and the combined ether layer was washed repeatedly with 0.01 M HCl to remove acetic acid, dried with anhydrous sodium sulfate and concentrated to about 1 ml under suction. The compound was crystallized by the addition of ethyl acetate.

3. Results and discussion

The colorless microcrystals obtained in poor yield (3–4 mg) stick to glass. The material was found to be chromatographically pure. It reacted with hydroxylamine under alkaline and acidic conditions as described by Cain [5], giving red ferric hydroxamate (λ_{max} 500 nm). The formation of the hydroxamate under acidic conditions suggested it to be a dilactone [5]. The UV spectrum of freshly prepared aqueous solutions of the compound showed strong end-absorption with a low intensity broad hump between 240 nm and 280 nm unaffected in 0.02 N HCl (fig. 1), attributable to the n–π^* absorption of the dilactone carbonyls. In 0.02 N NaOH the UV spectrum showed a well-defined peak at 216 nm with enhanced absorbancy while the hump at the longer wavelength region showed a bathochromic shift (fig. 1). These changes are expected in view of the report of Cain et al. [6] that the dilactone, 2,6-dioxa-3,7-dioxo*bicyclo* (3:3:0) octane, which had been isolated by Landa and Eliasek [7] as an artifact from catechol cultures, undergoes rapid hydrolysis in alkaline pH to the α,β-unsaturated monolactone, muconolactone. However, the product of hydrolysis of the dilactone isolated by us is not muconolactone (λ_{max} 207 nm) since its λ_{max} is different. On the other hand its λ_{max} is very close to that reported for β-carboxymuconolactone (215 nm), an intermediate of protocatechuate metabolism by *Neurospora crassa* [8], suggesting structural similarity. It also suggested the presence of a free carboxyl in the dilactone. Con-

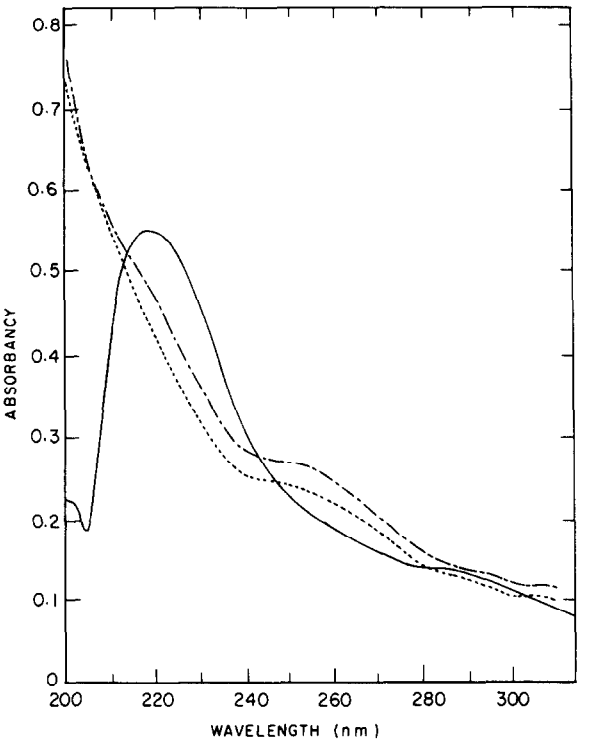

Fig. 1. Ultraviolet absorption spectrum of the isolated product in freshly prepared aqueous solution (· · · · ·), in 0.02 N HCl (– · – · –), and in 0.02 N NaOH (———). The spectra were recorded in a Unicam SP 700A recording spectrophotometer.

firmatory evidence for this was obtained by the fact that it reacted as an acid on chromatograms and its IR spectrum (fig. 2) showed strong broad absorption between 2860 and 2900 cm^{-1} (two bands) characteristic of the carboxylic acid dimer, and a broad hump between 3300 and 3500 cm^{-1} due to the nonbonded OH stretch of the carboxyl. The strong broad band between 1600 and 1800 cm^{-1} indicated multiple CO stretching vibrations. Another strong broad band between 950 and 1200 cm^{-1} indicated the presence of two γ-lactone rings [9]. The strong peak at 1465 cm^{-1} is assigned to the symmetric scissors vibration of the $-CH_2-CO-$ group [10].

The dilactone unexpectedly reacted readily with acidic 2,4-dinitrophenylhydrazine to give a 2,4-dinitrophenylhydrazone (m.p. 146–147°, uncorrected) sparingly soluble in alcohol and highly soluble in $CHCl_3$.

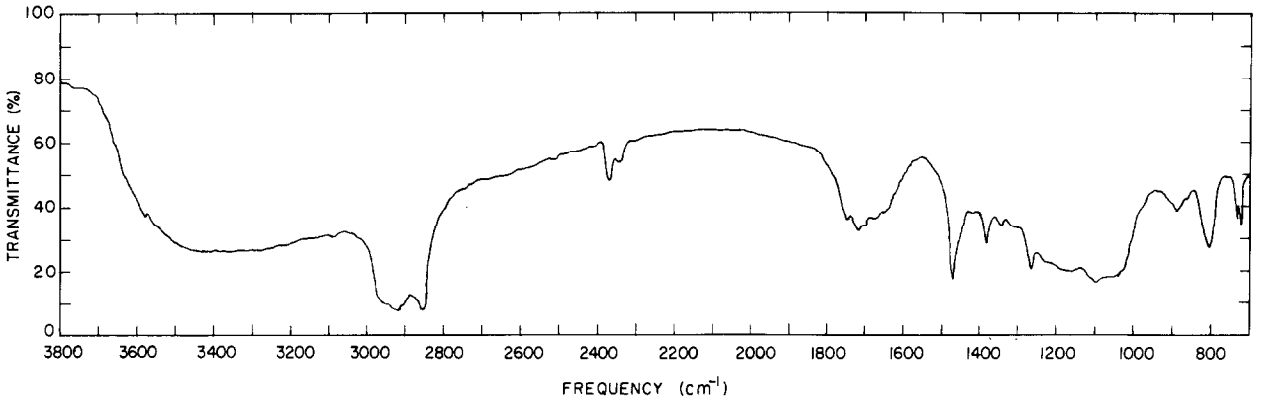

Fig. 2. Infrared spectrum of the isolated product in nujol mull.

Its alcoholic solution showed λ_{max} at 357 nm showing nonconjugation of the carbonyl [11]. The IR spectrum of the 2,4-dinitrophenylhydrazone in a KBr pellet showed a peak at 3440 cm^{-1} on a broad band (nonbonded NH stretch) and a sharp strong peak at 3295 cm^{-1} (bonded NH stretch). The strong intensity of this peak suggested it to be a bisdinitrophenylhydrazone. The peak assigned to the carboxyl group in the IR spectrum of the parent compound was missing from the IR spectrum of the 2,4-dinitrophenylhydrazone. Possibly the preparation of the derivative is accompanied by decarboxylation.

The data presented here strongly suggest that the isolated product of oxidation of 2,3-dihydroxybenzoic acid by the enzyme preparation from *Tecoma stans* is a γ-dilactone carboxylic acid. An initial intradiol oxygenative cleavage of 2,3-dihydroxybenzoic acid could lead to the formation of such a compound by the sequence of reactions depicted in fig. 3. On this basis the compound may be designated as 2,6-dioxa-3,7-dioxo-*bicyclo* (3:3:0) octane-8-carboxylic acid (IV, in fig. 3). This is the first report on aromatic ring fission by an isolated enzyme system from a plant source. This is also the first report on direct intradiol cleavage of 2,3-dihydroxybenzoic acid in any system. In bacterial systems it undergoes extradiol cleavage [12]. In fungi it is enzymatically converted into catechol which undergoes intradiol cleavage [13].

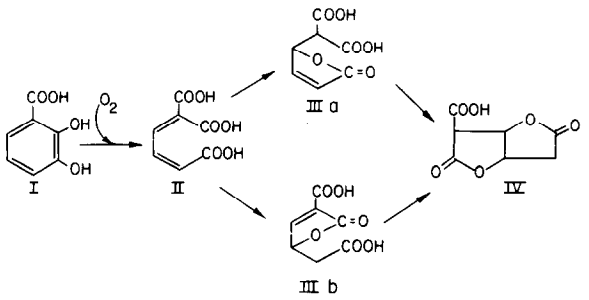

Fig. 3. Proposed scheme for the formation of the dilactone carboxylic acid from the oxidation of 2,3-dihydroxybenzoic acid by the enzyme system from the leaves of *Tecoma stans*. I. 2,3-dihydroxybenzoic acid. II. α-Carboxy *cis, cis*-muconic acid. IIIa. γ-Dicarboxymethyl Δ^{α}-butenolide. IIIb. α-Carboxy-γ-carboxymethyl Δ^{α}-butenolide (α-carboxymuconolactone). IV. 2,6-Dioxa-3,7-dioxo*bicyclo* (3:3:0) octane-8-carboxylic acid.

Investigations are under way to ascertain whether the dilactone is a true enzymic product formed from α-carboxymuconic acid, which apparently is the first product of intradiol cleavage of 2,3-dihydroxybenzoic acid.

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