QUERCETINASE, A DIOXYGENASE CONTAINING COPPER[1]

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SUMMARY

Quercetinase, a dioxygenase that cleaves the heterocyclic ring of quercetin to yield carbon monoxide and 2-protocatechuoyl phloroglucinol carboxylic acid, was found by the emission spectrograph to contain copper. Iron was not detected. Compounds such as diethyldithiocarbamate and toluene-3,4-dithiol that chelate copper quite specifically were potent inhibitors (10^{-7} M) whereas 1,2-dihydroxybenzene-3,5-disulfonate (10^{-2} M) which is quite specific for ferric iron, was not an effective inhibitor. The native enzyme is believed to contain the cupric ion for a color complex with 2,2'-biquinoline was not obtained unless reducing agents such as ascorbate or sodium dithionite were added.

Many studies on the reaction mechanisms of mono-oxygenases (mixed function oxygenases) and dioxygenases have emphasized the role of metals (1). Mixed function or mono-oxygenases that incorporate one atom of oxygen in the substrate while reducing the other may contain either copper or iron, but some are reported to lack a metal (2,3). Dioxygenases, enzymes that add both atoms of oxygen to the substrate (2,3,4), have been found to contain iron. We have found that a dioxygenase, quercetinase, produced by Aspergillus flavus contains copper and not iron.

Quercetinase is an induced extracellular dioxygenase produced by Aspergillus flavus when grown on rutin, a flavonoid

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glycoside (5). The enzyme oxidatively cleaves the heterocyclic ring of quercetin to yield carbon monoxide and the phenolic ester, 2-protocatechuoylphloroglucinol carboxylic acid. Purification of the enzyme yielded a preparation (25.5% recovery) that by centrifugal studies, disc electrophoresis, isoelectric focussing and gel filtration appeared to contain a single protein with a molecular weight of 111,400 ± 4000 (6). One mole of enzyme is capable of oxidizing 20.3×10^3 moles of quercetin per minute and based on the weight of mycelium (dried to constant weight at 25°C on millipore filters) recovered at the time of harvesting, one mg of mycelium (dry basis) excreted 7.5 units (μ moles quercetin oxidized per hour) or 6.16×10^{-6} μ moles of enzyme. This is a rough estimate, because autolysis and sporulation was occurring at the time of harvesting. The enzyme is colorless except in highly concentrated solutions containing about 30% protein. Such solutions have a very pale greenish color. The absorption spectrum (pH 6.0 in air) had a single peak with a maximum at 280. No significant absorption was observed with a sample containing 26 mg of protein per ml between 350 and 800 mμ as compared to laccase which has a distinct peak at 615 mμ (7,8), or the pink copper protein of erythrocytes which has absorption peaks at 372, 503 and 760 (9). Higher concentrations of quercetinase, if this had been possible, may have revealed absorptions other than that for protein.

Two different preparations of purified quercetinase, 0.03 M with respect to MES [2-(N-morpholino)ethanesulfonic acid] buffer, pH 6.0, were prepared for metal analyses. The first preparation (10 mg protein, 5 ml) was applied at 10°C to a column of Sephadex G-50 (25 × 45 cm) that had been previously washed successively with 300 ml of 1 mM EDTA (ethylenediaminetetraacetic acid), 300 ml

of 1N HCl, then with glass distilled water until acid free. The enzyme was eluted with glass distilled water and collected in test tubes previously washed with 1N HCl and rinsed with glass distilled water. Those fractions containing enzyme were pooled and concentrated to 1.0 ml in a Sartorius Membranfilter (British Drug Houses). The concentrate was quantitatively transferred to a 5 ml volumetric flask, and made up to volume with glass distilled water. The Membranfilter and the volumetric flask had been washed with 1 mM EDTA and rinsed with glass distilled water. The second preparation of purified quercetinase (6.67 mg protein, 7 ml) was incubated at 10°C in 1 mM EDTA, pH 6.0, for 15 minutes. Then the enzyme was absorbed onto and eluted from the column of Sephadex G-50, and concentrated in the same manner as the first preparation. These treated preparations and the water controls were analyzed for metal content (Table 1). The data indicated that copper was the only metal present in significant and consistent concentrations, and was equal to about 2 atoms per molecule of enzyme.

Table 1. Metal Content of Preparations of Purified Quercetinase (Moles per mole of quercetinase*)

	Cu	Fe	Si	Mg	B
Preparation 1	1.86	0.34	5.64	0.434	4.85
Preparation 2	1.52	<0.2	1.62	0.234	0.31

*The aqueous samples were qualitatively analyzed in the emission spectrograph to determine what metals were present. The copper content was confirmed by atomic absorption measurements with a possible error of ±0.05 µg. We are indebted to Dr. D. S. Russell of the Division of Chemistry, National Research Council of Canada, Ottawa, who kindly made these analyses. The large differences in the amounts of silicon and boron present in the preparations are attributed to different treatments given the samples before analysis.

Table 2. Effect of Inhibitors on Quercetinase Activity

Inhibitor	Concentration, M	Inhibition, %
EDTA	2×10^{-3}	0
Tiron	1×10^{-2}	3.5
KCN	5×10^{-3}	7.0
8-Hydroxyquinoline	1×10^{-3}	75.9
	3.8×10^{-4}	50.0
Ethylxanthate	1×10^{-5}	78.5
	4.3×10^{-6}	50.0
Diphenylthiocarbazone	5×10^{-6}	79.9
	2×10^{-6}	50.0
Toluene-3,4-dithiol	1.6×10^{-6}	90.8
	6.7×10^{-7}	50.0
Diethyldithiocarbamate	5×10^{-7}	94.7
	3.8×10^{-7}	50.0

*Activity determined spectrophotometrically (6). The reaction mixture (1.0 ml) contained 0.5 ml of 0.1 M MES buffer pH 6.0, inhibitor, 0.3 units quercetinase, and water. The reaction was begun after preincubation of enzyme and inhibitor for 3 min. at 25°C by the addition of 0.05 ml of 1.2 mM quercetin in dimethylsulfoxide. Water for reagents was glass distilled and the MES buffer was extracted with 0.01% diphenylthiocarbazone in CCl_4.

Confirmation that copper was present in quercetinase and related to enzymatic activity was obtained by means of tests with inhibitors (Table 2). Compounds such as ethylxanthate, diphenylthiocarbazone, toluene-3,4-dithiol and diethyldithiocarbamate that are highly specific for copper inhibited the dioxygenase at low concentrations. 8-Hydroxyquinoline, although less specific for copper, also inhibited. Tiron (1,2-dihydroxybenzene-3,5-disulfonate) a chelating agent specific for ferric iron (10,11) was not an effective inhibitor even though the enzyme and inhibitor were preincubated for periods up to 6 hr. In addition α,α'-dipyridyl (10^{-3} M) and o-phenanthroline (10^{-4} M)

were previously found to be ineffective as inhibitors of quercetinase (5).

A colorimetric method employing cuproine (2,2'-biquinoline) was used to determine whether the enzyme contained cuprous or cupric ion (12). The method of Nakamura et al. (13) was employed except that ascorbate (4 mg/ml) and Na dithionite (3.52 mg/ml) were used as reducing agents for the determination of total copper. The enzyme preparation contained 1×10^{-3} μg of cuprous ion per mg, a negligible amount indicating that cuprous ion is not present. On addition of the reducing agents, the color of the cuprous ion-cuproine complex appeared. The total copper content with this procedure was 1.2 μg per mg of quercetinase, somewhat higher than that obtained by atomic absorption. Quercetinase, in the absence of substrate, thus appears to contain the cupric ion, two moles per mole of enzyme.

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