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Cacao Oxidase

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The isolation and properties of cacao oxidase are presented. The oxidizing enzyme in cacao is a polyphenol oxidase similar to the oxidases in mushrooms and sweet potatoes. It has a high activity in the oxidation of 4-substituted catechol compounds. The oxidase is inactivated rapidly by wet heat and by cyanides and sulfides.

Fermentation is the most important step in the tropical processing of cacao beans. This processing produces a stable product and aids in the development of desirable flavor and color. Many of the changes which the bean undergoes during the fermentation process are obscure. Some changes are due to yeasts, molds and bacteria which decompose the mucilaginous substances or pulp surrounding the bean, while others are due to the natural enzymes of the bean which develop color and flavor during fermentation. These changes resemble those which occur in the browning of many fruits and vegetables due to the enzymatic oxidation of phenolic substances.

Fresh unfermented cacao beans are purple or white and have a harsh, astringent flavor attributed to the phenolic constituents. Cacao oxidase apparently initiates and hastens oxidative reactions involving the phenolic compounds, which in cacao are predominantly catechins, anthocyanins and related tannins. These oxidative reactions appear to be responsible for the decrease in astringent flavor and purple color during fermentation and, therefore, are important in the production of high-quality cacao products.

The enzymatic oxidation processes in cacao have been discussed by Brill,[1] Ciferri[2] and Knapp,[3] but no precise description of the oxidase is available. This paper describes the isolation and properties of cacao oxidase.

Measurement of Oxidase Activity.—Several techniques for the estimation of oxidase activity are available. Colorimetric methods using reagents such as pyrogallol,[4] guaiacol,[5] indophenol[6] and *o*-phenylenediamine have been used. With pyrogallol, the cacao enzyme used in the present work formed purpurogallin, a colored product with a maximum absorption at 305 millimicrons. A crude cacao enzyme had a purpurogallin number of 9 compared to a figure of 0.26 for whole mushroom and the value of 96 reported by Keilin[4] for the purest mushroom oxidase. The cacao oxidase also formed colored products with guaiacol, 2,6-dichlorobenzenoneindophenol and *o*-phenylenediamine. The colorimetric methods were found valuable for qualitative tests or semiquantitative estimations, but they lack reliability for quantitative measurements.

Manometric methods have been used to measure enzyme activity.[7] In the present work, oxygen uptake measurements were made with a Barcroft–Warburg apparatus on a system including cacao enzyme, *p*H 6 buffer, and different substrates. Under these conditions, the enzyme had greater activity with *p*-cresol than with catechol or cacao tannin.

The ascorbic acid–catechol method of Sreerangachar[8] was found to be the most reproducible method for measuring cacao oxidase. The test system contains the enzyme material, a *p*H 5 buffer, catechol or other phenol as substrate and ascorbic acid. Air is drawn through the mixture at a constant rate for a fixed period of time at 40°. Rate studies indicated that a 10-minute reaction gave reproducible results. As the catechol is oxidized, it in turn oxidizes the ascorbic acid, loss of which is followed analytically by indophenol titration. The loss of ascorbic acid is a measure of the enzyme activity of the sample for the oxidation of the substrate. An average deviation of 5% was observed between duplicate measurements using the following procedure.

Oxidase Method.—Pipet 5 ml. of *p*H 5 buffer (McIlvain 0.1 *M* citric acid and 0.2 *M* disodium phosphate) and 5 ml. of ascorbic acid–catechol solution (0.1% ascorbic acid plus 0.5% catechol) into a 200 mm. × 30 mm. test-tube. Weigh cacao or enzyme sample in a small glass cup (10 × 15 mm.) and transfer to the test-tube.

Insert a rubber stopper having a 5-mm. glass tube inlet extending to the bottom of the test-tube and a glass tube outlet at the top. Transfer the reaction vessel to a water-bath held at 40°. Connect the inlet tube to another tube containing water at 40° and the outlet tube through a flowmeter to a vacuum line. Pull air through the reaction vessel at the rate of 8 l. per hour. At 10 minutes reaction time pipet 5 ml. of the mixture into a 125-ml. erlenmeyer flask containing 1 ml. of 20% H_3PO_4. This drop in *p*H stops the oxidation reaction. Measure the excess ascorbic acid which should be not less than 50% of the total by titrating with indophenol solution (0.1% sodium 2,6-dichlorobenzenone-indophenol standardized with 0.01 *N* sodium thiosulfate in the presence of H_3PO_4).

Run a control reaction with each series of tests using 5 ml. of both the ascorbic acid–catechol and buffer solutions, titrating exactly as described above. Subtract the titration of the sample from the control titration, multiply by 2 and calculate the milligrams of ascorbic acid oxidized by 1 g. of sample on the basis of 1 ml. 0.01 *N* thiosulfate solution = 0.88 mg. ascorbic acid.

Cacao samples were prepared for oxidase measurement by drying beans from fresh pods under vacuum at 45°, hand

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shelling, milling, and defatting with hexane. The oxidase activity was found to be fairly stable when the material was kept dry and cold.

The oxidase activities of different cacaos are shown in Table I. The oxidase activity of uncured beans ranged from 224 to 665. These variations may be due to maturity, variety, or the conditions under which the beans were held between harvesting and analysis. Oxidase activity probably decreases during the storage of beans in the harvested pod, particularly when temperature and humidity are high. Commercially fermented beans contained little if any oxidase activity. Sanchez beans which are subjected to very mild fermentation, showed a low oxidase content.

Preparation of Crude Oxidase.—For high stability, cacao oxidase preparations should be completely free of tannin substrate. Fresh Panama Forastero cacao pods were obtained by air express about two to seven days after harvesting. They were opened in a cold room at 0°; the beans were shelled by hand and immediately placed in a 50% acetone solution held at about −20°. Twenty-five beans were comminuted in the Waring Blendor and extracted four successive times at 0°, using a total of 1600 ml. of solvent. The solids were separated by centrifuging at 0° after each extraction. The residue was filtered with suction, washed thoroughly with 50% acetone, dried with pure acetone, and defatted with petroleum ether (20 to 40°, b.p.). The dry fat-free and tannin-free product, designated as crude enzyme concentrate, was stored at −20°. A loss of about 10% in oxidase activity was observed during storage for 1 month.

Purification of Oxidase.—Plant oxidases are generally insoluble in all common organic solvents but are soluble in water, particularly in the *p*H range of 7 to 10. Keilin and Mann[4] extracted polyphenol oxidase from mushrooms and latex with water. Rudkin and Nelson[9] used 0.2 *M* disodium phosphate (*p*H 8.3) to dissolve sweet potato tyrosinase, a phenolase which catalyzes the oxidation of tyrosine. Sreerangachar[3] found optimum extraction of tea oxidase at *p*H 10. In the present work, 0.1 *M* citric acid adjusted to *p*H 7 with 0.2 *M* disodium phosphate was the best solvent for cacao oxidase. This buffer extracted 77% of the total activity compared to 32% at *p*H 5.0, 64% at *p*H 6.0 and 56% at *p*H 8.0.

TABLE I

OXIDASE ACTIVITY OF VARIOUS CACAOS

Sample description	Drying Time, hours	Drying Temp., °C.	Oxidase activity, mg. ascorbic acid per g.[a]
White Criollo (fresh)	64	45	336
Purple Criollo (fresh)	64	45	330
Trinidad (fresh)	64	45	224
Forastero (fresh)	64	45	358
Bahia (fresh)	24	45	620
Puerto Rico (fresh)	24	45	665
Panama (fresh)	None		179
Panama (fresh)	64	45	95
Panama (fresh)	6	82	3
Panama 50% acetone extracted	Acetone at R.T.		482[b]
Panama, 50% acetone extracted	Acetone at R.T.		880[b]
Costa Rica White Baranca (fresh)	Acetone at R.T.		308[b]
Sanchez (sun-dried)	Sun		18
Sanchez (sun-dried)	Sun		17
Sanchez (sun-dried)	Sun		6
Accra (fermented)	Sun		6

[a] Activity data are calculated on dry, fat-free basis. [b] Data calculated on weight of 50% acetone extracted residue, which amounts to about 60% of dry, fat-free cacao.

Attempts to improve extractability of the relatively coarse crude enzyme concentrate by grinding with and without water in a chocolate mill and with sand in a mortar, resulted only in a decrease of activity. This decrease could be due to the effect of heat or of metal contamination.

Several methods have been proposed for the purification of plant oxidases. Keilin and Mann[4] precipitated mushroom oxidase from a water extract with ammonium sulfate. This was dialyzed, fractionally precipitated with lead acetate, adsorbed on calcium phosphate, eluted with potassium phosphate solution and precipitated with acetone. When Keilin's technique was applied to cacao oxidase, large losses were encountered during the adsorption or elution. The oxidase was also adsorbed readily on activated alumina, but recovery of the adsorbed material was poor.

Ammonium sulfate was found to be a good precipitant for cacao oxidase. At 80% saturation only 12% of the oxidase activity remained in solution and 60% of the oxidase was found in the precipitate, indicating some inactivation during the salt precipitation. The product obtained from a *p*H 7 buffer extract by precipitation with ammonium sulfate (80% saturation) and dialysis in cellophane had an ascorbic acid activity of 2400 compared to 600 for the crude enzyme. The precipitated product had a nitrogen content of 13.6% and had a copper content of 0.034%. This is much less than the 0.30% reported by Keilin[4] for mushroom oxidase. However, the best preparation of tea polyphenolase contained only 0.08% copper and no iron.[8] Spectroscopic examination of the cacao oxidase indicated that copper and phosphorus were the only inorganic constituents, and that iron is not a prosthetic group in cacao oxidase as in many other plant oxidases.

Specificity and Properties of Cacao Oxidase — The relative substrate specificity of cacao oxidase compared to mushroom and sweet potato oxidases was determined by substituting various phenolic compounds for catechol in the ascorbic acid method. The mushroom enzyme was prepared by the method of Keilin and Mann[4]; the sweet potato enzyme by the Rudkin and Nelson procedure.[9]

The cacao oxidase had about four times as much activity with certain catechol compounds, such as caffeic, hydrocaffeic and chlorogenic acids, as with catechol. The sweet potato oxidase also had about four times the activity with these compounds as with catechol, but the mushroom oxidase had only slightly higher activities with these para-substituted compounds than with catechol (Table II). The cacao oxidase had little activity with either phenol or triphenols. Keilin[4] reported that mushroom oxidase had a higher activity with *p*-cresol than with *o*- or *m*-cresol. In the present work all three oxidases showed a higher activity with *p*-cresol than with *o*- or *m*-cresol. With α-methylcaffeic acid the cacao oxidase was only one-third as active as with hydrocaffeic acid, but the activity of the mushroom preparation was the same. Substituent groups on phenols other than OH may affect the activity of cacao oxidase. Cushing[10] reported that the oxidation of a monophenol by tyrosinase fails when there is any group other than OH in the 2-position.

The differences in activities of the three enzyme preparations (Table II) do not necessarily mean that the products contained more than one type of enzyme. Variations in activity may be due to the presence of activators of inhibitors. It has been observed by Adams[11] and Nelson[12] that the degree and method of purification affect the relative activity with different substrates. The data

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in Table II on the more highly purified preparations from cacao and mushrooms show no large change in activity ratios, except that the ratio of *p*-cresol to catechol activity decreases on purification.

TABLE II

ACTIVITIES OF CRUDE AND PURIFIED OXIDASES FROM CACAO, MUSHROOM AND SWEET POTATO WITH DIFFERENT SUBSTRATES[a]

Substrate	Crude cacao	Purified cacao	Crude mushroom	Purified mushroom	Crude sweet potato
Catechol	367	1650	340	3550	55
Caffeic acid	1496	..	...	..	...
Hydrocaffeic acid	1404	..	400	..	168
Chlorogenic acid	1440	7000	364	2540	320
α-Methylcaffeic acid	545	..	340	..	...
4-Butyrylcatechol	304	..	115	..	...
(−)-Epicatechol (from cacao)	680	3870	172	2220	...
Quercitrin	..	300	...	360	...
Rutin	..	280	...	100	...
Phenol	12	..	...	..	...
Hydroquinone	68	..	110	..	40
Resorcinol	23	..	6	..	...
Pyrogallol	20	..	...	..	...
Phloroglucinol	6	..	...	..	...
Gallic acid	19	..	...	..	...
Tyrosine	6	..	...	..	...
p-Cresol	244	740	100	740	7
m-Cresol	82	..	50	..	0
o-Cresol	0	160	0	130	0

[a] Results expressed as mg. ascorbic acid oxidized per gram of substrate.

Cacao oxidase causes reversible changes in chlorogenic acid that affect its light absorption. Spectrophotometric curves show a pronounced increase in absorption in the range of 200 to 320 millimicrons resulting from a five-minute reaction of 1 part of crude cacao oxidase on 200 parts of chlorogenic acid in water solution. The absorption remained nearly constant after the initial five-minute reaction period. The oxidation reaction is apparently reversible, because acidification of the oxidized solution with sulfuric acid reduced the absorption to that of the original solution. The changes in absorption of chlorogenic acid solutions are different from those reported by Mason[13] for the enzymatic oxidation of catechol. He found that the characteristic absorption of catechol at 276 mμ was replaced rapidly by *o*-benzoquinone absorption at 390 mμ, the *o*-benzoquinone later being polymerized to polyphenols. The present absorption data do not indicate any benzoquinone formation in the oxidation of chlorogenic acid. The chlorogenic acid may reach an equilibrium state and not go through the benzoquinone stage and subsequent polymerization to polyphenols.

Oparin[14] found that the oxidase of sunflower seeds catalyzed the oxidation of chlorogenic acid, a diphenol, to a respiratory pigment. This reaction was reversed by amino acids. A similar reversible process may exist in fresh viable cacao beans. This equilibrium could maintain the purple or white colors of the living beans and prevent the formation of dark brown or black colors of oxidized tannins.

Both Fickendey[3] and Loew[3] have reported that cacao oxidase is destroyed by heating at 75°. Shacter[15] found complete inactivation of serum catecholase in 15 minutes at 80°. Cacao oxidase is rapidly inactivated by heat, particularly in the presence of water. A loss of 67% in activity was found after the crude oxidase was held 20 minutes at 90° in a dry state. With 25 to 50% water present there was a complete loss of activity in 20 minutes at 90°. With 50% water, 87% of the activity was lost in 4 hours at 60°, and at 44° complete inactivation was observed in 64 hours.

Effect of Sulfides, Cyanides and Metals on Activity.—Inactivation of plant oxidases by reagents such as sulfides and cyanides is caused by removal of the metal from the enzyme.[8,10] Cacao oxidase resembles the other plant oxidases, in that it is almost completely inactivated by hydrogen sulfide and sodium cyanide.

Iron and manganese do not affect the ascorbic acid activity of cacao enzyme. Copper alone will catalyze the oxidation of ascorbic acid in the presence of air and catechol. However, the combined catalytic effect of added copper and enzyme on the catechol–ascorbic acid system is considerably less than the sum of their individual effects.

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