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OKAYAMA, SHIGEKI
Biological Laboratory, General Education Department, Kyushu University

INADA, ASAJI
Biological Laboratory, General Education Department, Kyushu University

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PARTIAL PURIFICATION AND PROPERTIES OF POLYPHENOLASE FROM *AZOTOBACTER* CELLS

SHIGEKI OKAYAMA and ASAJI INADA

(Biological Laboratory, General Education Department, Kyushu University)

In the course of culture of *Azotobacter chroococcum*, color of cells changes from white to brown. The cells from the younger culture did not show any color development with pyrogallol as a substrate, but the older brown cells showed the marked yellow color development. These observations suggest that phenolase presents in the older *Azotobacter* cells. In fact, a certain type of polyphenolase* could be extracted from the acetone dried cells of *Azotobacter chroococcum*. The procedure of partial purification and some properties of the enzyme are described here.

MATERIALS AND METHODS

Azotobacter chroococcum was isolated from soil according to the method of Beijerink (1). The bacteria were cultured in the Burk's liquid medium (2) at 30° with continuous aeration.

Gas uptake was determined with the conventional Warburg manometer at 30°. In most cases, the colorimetric assay procedure was used for the assay of the enzyme activity. The procedure bases on the fact that the oxidation of pyrogallol leads to the formation of yellow colored compounds which have the light absorption maximum at 430 mμ. The reaction mixture consisted of 1 ml of 5 % pyrogallol solution, 1 ml of 0.5 M phosphate buffer (pH 6.0) and 5.0 ml of glass distilled water. At the start of the reaction, 1.0 ml of the enzyme solution was pippeted into the reaction mixture and, after 10 minutes incubation at 30° in a 50 ml Erlenmyer flask, 0.5 ml of 3 N H_2SO_4 was added to stop the reaction. Another reaction was run as a control without the addition of the enzyme solution, until the reaction was stopped. The absorption at 430 mμ was measured with Hitachi electrospectrophotometer type EPU-2, using the control reaction mixture as a blank. One unit of enzyme was defined as the

* Here this term is applied to the enzyme that catalyzes the production of melanine-like pigments through the oxidation of certain polyphenols and has copper as their essential prosthetic group.

difference of 0.01 in optical density at 430 mμ. This assay prosedure well represented the enzyme activity.

In the case of determination of Michaelis constant, the enzyme activity was measured specrophotometrically following the disapperance of 265 mμ peak of ascorbate which reduces the quinones produced as the result of the oxidation of phenols and turns to dehydroascorbic acid having little absorbance at 265 mμ. This method bases on the procedure of Sisler and Evans (3). The reaction mixture consisted of 1 ml of 10^{-4} M ascorbic acid solution, 1 ml of 0.07 M phosphate buffer (pH 6.0), 1 ml of each phenol solution and 0.5 ml of enzyme solution to a final volume of 3.5 ml. All reagents were made up with glass distilled water containing 10^{-3} M ethylendiamine tetraacetic acid, disodium salt. The assay were run at room temperature (20°). The reaction verosity was expressed as the decrease in the optical density at 265 mμ during the first 1 minute.

RESULTS AND DISCUSSION

Procedure of enzyme purification

Bacterial cells were collected when they became brown, usually at 3 or 4 days after the culture had been started. Collected cells were then dried with acetone according to the common method (4). Acetone dried cells were extracted twice with 0.7 M phosphate buffer (pH 7.0) by hand grinding in a chilled mortar and pestle with the aid of powdered glass and the insoluble cell debris were centrifuged off at 14,000 G for 20 minutes. Red brown supernatants were combined and then dialyzed overnight against the same buffer. After pH of the dialyzate (the crude extract fraction) was adjusted with M acetic acid to 6.0, one-twentieth of its volume of M $MnCl_2$ was added dropwisely with mechanical stirring, during this procedure pH was maintained at 6.0 with the occasional dropwise addition of N NaOH. The stirring was continued for additional 20 minutes, and then the heavy white brownish precipitate was filtered off with a filter paper. The filtrate was dialyzed against the buffer for 2 hours with mechanical stirring. The dialyzate was fractionated with the addition of solid ammonium sulfate, and the precipitated fraction between 30 and 50 % saturation was dissolved in the buffer, and then dialyzed aginst the buffer. After centrifuged off the insoluble proteins which appeared during the dialysis, the dialyzate was used as partially purified enzyme preparation. All procedures were carried out at 4°. Summary of purification was shown in Table 1.

Substrate specificity

The crude extract of *Azotobacter* cells could catalyse oxidation of pyrogallol, hydroquinone, catechol and dihydroxyl phenyl alanine (dopa),

but not tyrosine, phenol, resorcinol and phloroglucinol, as shown in Table 2. The oxidation of such phenols was accompanied with the concomitant characteristic color development. Similar color development could be observed also with the oxidation catalyzed by the partially purified enzyme preparation. These color developments were inhibited by dieca as shown later. From these results, it is suggested that the oxidation of such phenols is catalyzed by the phenolase-type enzyme which has no monophenolase activity but has the polyphenolase activity.

Table 1. Purification of polyphenolase from *Azotobacter*

Step	Total protein	Total unit	Specific activity	Yield
	mg		unit per mg protein	%
1. Crude extract	32.80	4,600	140	100
2. $MnCl_2$ supernatant	7.04	4,000	568	87
3. $(NH_4)_2SO_4$ fraction	2.55	2,500	980	54

Table 2. Oxidation of phenols with the crude extract from *Azotobacter* cells

Phenols	O_2 uptake[a]	Color development
	μl	
phenol	0.0	none
tyrosine	0.0	none
catechol	25.8	yellow to brown
hydroquinone	36.6	pink to brown
resorcinol	0.0	none
dopa	7.0	red to brown
pyrogallol	85.2	yellow to brown
phloroglucinol	0.0	none

a) μl O_2 uptake per mg protein per hour. Warburg flasks were charged with 1.0 ml of 0.07 M phosphate buffer (pH 7.0), 1.0 ml of each phenols and 1.0 ml of the crude extract with 0.2 ml of 20 % KOH in center well. Gas phase was air; temperature, 30°.

The enzymatic oxidation of catechol, pyrogallol, hydroquinone and dopa obeyed the Michaelis and Menten's relationship, as shown in Fig. 1. From these relationships, Michaelis constant of the partially purified enzyme for the phenols was determined and presented in Table 3.

The partially purified enzyme preparation did not show any detectable ascorbic acid-oxidase activity.

From these findings, it is concluded that polyphenolase from *Azotobacter* has rather low substrate specificity and has higher affinity for the simpler polyphenols such as catechol, pyrogallol and hydroquinone. These

substrate specificity are similar with plant origin enzymes, and differ from the animal ones (5).

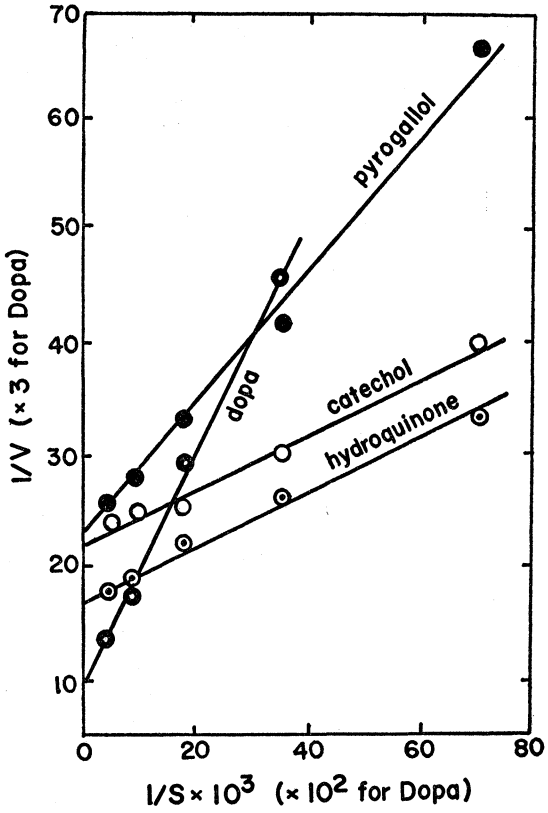

Fig. 1. Effect of substrate concentrations on the reaction velocity, plotted according to the conventional Lineweaver-Burk plot.

Table 3. Michaelis constant of the partially purified enzyme for the phenols

Phenols	K_m
Catechol	1.1×10^{-5} M
Pyrogallol	1.3×10^{-5}
Hydroquinone	2.5×10^{-5}
Dopa	1.3×10^{-3}

Prosthetic group

In order to elucidate the metal prosthetic group, the effects of metal combining reagents were studied. With 2×10^{-3} M potassium cyanide and sodium diethyldithiocarbamate (dieca), the almost complete inhibition was

observed, but not with 2×10^{-3} M $\alpha\alpha'$-dipyridyl and EDTA, as shown in Table 4. $\alpha\alpha'$-Dipyridyl chelates specifically with ferrous iron, coppor and manganese, and is known to inhibit some iron enzymes (6). Dieca is used to distinguish the copper enzyme activity from another metal enzyme activity (6). The color development during the oxidation of catechol, pyrogallol, dopa and hydroquinone were also inhibited with dieca in the same conditions. Slight stimulation observed with $\alpha\alpha'$-dipyridyl and EDTA suggests some metal ions, perhaps ferrous iron, may inhibit this enzyme activity.

Table 4. Effect of metal combining reagents on enzyme activity

Metal reagent	Final concentration	Enzyme activity[a]	Per cent inhibition
	M	$\times10^{-3}$	%
none	—	128	—
KCN	2×10^{-3}	1	100
	2×10^{-4}	80	38
Dieca	2×10^{-3}	15	88
	2×10^{-4}	60	53
$\alpha\alpha'$-Dipyridyl	2×10^{-3}	172	—
	2×10^{-4}	135	—
EDTA	2×10^{-3}	152	—

a) Increase in optical density at 430 mμ per 20 minutes at 30° with the reaction mixture containing 1 ml of water, 1 ml of the metal reagent solution, 1 ml of 0.5 M phosphate buffer (pH 6.0) and 1 ml of diluted partially purified enzyme.

The inhibition of cyanide was reversible. The enzyme preparation dialyzed against 4×10^{-3} M cyanide was completely lost its activity, but

Table 5. Effect of metal ions on the cyanide-treated enzyme

Metal ion added	Enzyme activity[a]		
	(A) CN-treated[b] enzyme	(B) heated[c] CN-treated enzyme	(A) minus (B)
	$\times10^{-3}$	$\times10^{-3}$	$\times10^{-3}$
none	33	33	0
Cu^{++}	538	440	108
Mg^{++}	57	47	10
Zn^{++}	55	43	12
Co^{++}	128	84	44
Fe^{++}	92	90	2

a) The reaction conditions were the same as Table 3.

b) Dialyzed against 0.7 M phosphate buffer (pH 7.0) containing 4×10^{-3} M KCN for 20 hours in the cold.

c) Heated at 100° for 5 minutes.

the addition of 4×10^{-4} M $CuSO_4$ to this preparation recovered its activity, as shown in Table 5. This recovered activity was almost the same as the activity before the dialysis against cyanide.

In this connection, it is interesting that the culture of *Azotobacter* sometimes failed to give color development, but the addition of 2 mg of copper sulfate to 1,000 ml of Burk's medium ensured the browing.

All these evidences indicate the enzyme which is responsible for the oxidation followed by the color development of phenols is the copper protein, having the rather similar properties with plant origin enzymes and different from animal ones.

Effects of temperature and pH

The logarithm of the verocity was plotted against the reciprocal of absolute temperature in Fig. 2. Drops in the enzymatic verocity was observed above 50° and above 55° it was exceeded by autooxidation verocity. A shift in the configuration of enzyme protein may begin above this temperature.

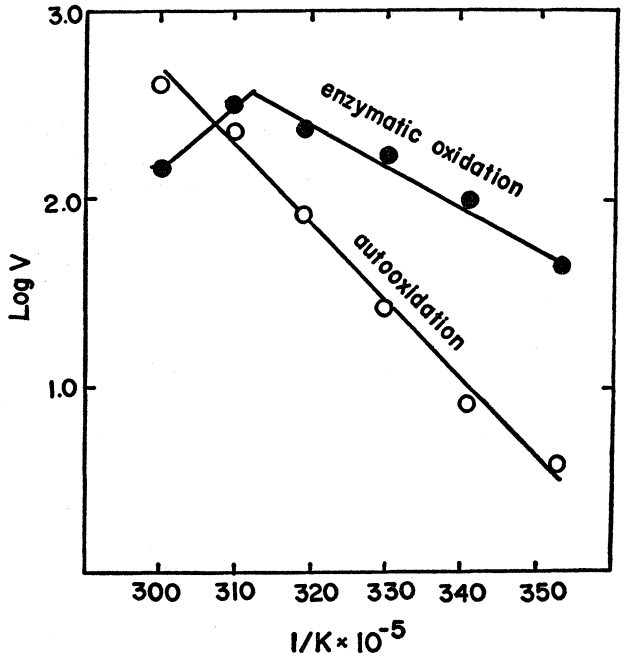

Fig. 2. Effect of temperature on the rate of pyrogallol oxidation

The mean activation energy for pyrogallol oxidation by this enzyme between 10° and 50° was calculated from the slope of the line according to Arrhenius equation as about 9,000 calories per mole, while that for

the autooxidation was much higher values, 19,000 calories per mole. Thus the activation energy for the enzymatic reaction was half of autooxidation.

Whithin the pH range 5.0 to 7.0, the enzyme activity increased with pH with no maximum. Above pH 7.0 the rate of autooxidation exceeded the rate of the enzymatic oxidation.

Stability

Partially purified enzyme preparation could be stored at least 1 week at −15° with little loss in the activity. This preparation was stable at neutral pH; dialysis against the buffer of pH 4.5 and 7.8 for 30 hours at 0° causes the loss of 32 and 24% of the activity respectively, compared to the dialyzate against the buffer of pH 7.0.

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SUMMARY

From *Azotobacter chroococcum* cells a polyphenolase was partially purified and characterized in some properties. This enzyme did not catalyze the oxidation of tyrosine, phenol, resorcinol and phloroglucinol, but could catalyze the oxidation of pyrogallol, hydroquinone, catechol and dopa with the concomitant color development. Michaelis constants of the enzyme for these phenols and the mean activation energy of pyrogallol oxidation were estimated. Analysis of metal prosthetic group revealed that the enzyme has the copper as its essential prosthetic group.

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