Purification and Some Properties of Polyvinyl Alcohol-degrading Enzyme Produced by *Pseudomonas* O-3[†]

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Purification was conducted on polyvinyl alcohol (PVA) degrading enzyme produced and secreted into the culture medium by *Pseudomonas* O-3 strain. The enzyme was found to separate into several fractions by ion-exchange chromatography and gel filtration. Among these fractions, a fraction adsorbed to SP-Sephadex C-50 at pH 6.0 was purified to homogeneity by polyacrylamide gel electrophoresis. Some properties of this purified enzyme were examined. Optimum pH and temperature were 9.0 and 40°C, respectively. The enzyme was stable up to 50°C and in a pH range between 5 and 11 at 5°C. The enzyme activity was inhibited by Co^{2+} , Ni^{2+} , EDTA, hydroxylamine and salicylaldehyde. In substrate specificity, this enzyme oxidized several kinds of modified PVA, as well as normal PVA, but did not oxidize other synthetic polymers, such as vinylon, polyacrylamide and polyvinyl acetate. The effect of oxygen on this enzyme was examined, and without oxygen, PVA was not broken down by this enzyme. The molecular weight of this enzyme was estimated by gel filtration on Sephadex G-100 to be approximately 26,000.

In the previous paper,¹⁾ it was reported that a *Pseudomonas* strain isolated from soil utilized polyvinyl alcohol (PVA), a water-soluble synthetic polymer, as the sole source of carbon. This bacterium grew vigorously degrading and assimilating PVA in medium containing PVA. Some aspects of the degradation of PVA by this bacterium were examined, and it was found that this strain produced an enzyme which degraded PVA oxidatively. However, the nature of this enzyme and the mechanism of degradation of PVA by this enzyme are not yet known. In this paper, the purification of PVA-degrading enzyme was carried out. PVA-degrading enzyme was separated into several active fractions by ion-exchange chromatography and gel filtration. Among these fractions, a fraction which was adsorbed by SP-Sephadex, a cation-exchanger, was purified to homogeneity by polyacrylamide gel electrophoresis, and its properties were examined.

MATERIALS AND METHODS

Microorganism and culture. *Pseudomonas* O-3 strain which was isolated from soil was used throughout this study. The composition of the culture medium was as follows: PVA 500, 5.0 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; KH_2PO_4 , 0.4 g; K_2HPO_4 , 3.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; NaCl, 0.1 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; and yeast extract, 1 g in 1 litre of distilled water, pH 7.5. The culture of the organism was carried out on a rotary shaker at 180 rpm at 30°C. The organism was inoculated to 30 ml of culture medium in a 100ml- Erlenmeyer flask and cultured for 7 days. Culture broth of 2.5 ml was seeded in a 500 ml-Erlenmeyer flask containing 200 ml of medium.

Estimation of polyvinyl alcohol. Estimation of PVA was conducted by the method of Finley.²⁾ The method is based on the green color produced by the reaction of PVA with iodine in the presence of boric acid. Colorimetric determination was made by reading the optical density at 660 nm.

Assay of PVA-degrading activity. The reaction mixture consisted of 2 ml of 1 per cent PVA 500 solution, 1 ml of 0.2 M tris-HCl (pH 9.0), 0.5 ml of enzyme solution and 0.5 ml of distilled water. A total volume of 4.0 ml of reaction mixture was incubated on a reciprocal shaker at 120 rpm at 30°C for several hours. After incubation PVA concentration was measured

[†] Degradation of Polyvinyl Alcohol by Microorganisms. Part II.

with an iodometry. PVA-degrading activity was expressed as the difference of $E_{660\text{nm}}^{1\text{cm}}$ ($\Delta E_{660\text{nm}}$) before and after incubation. One unit of enzyme was defined as 0.1 $\Delta E_{660\text{nm}}$ per hr. Specific activity of the enzyme was expressed as PVA-degrading activity per mg protein.

Determination of protein. Protein was determined by the method of sodium carbonate-Folin using bovine serum albumin as a standard or by reading the absorbance at 280 nm.

Measurements of oxygen uptake. Oxygen uptake was measured by a Mitamura 6-30R15 Warburg manometer at 30°C with reciprocal shaking of 100 rpm in 2 cm width. Composition of vessels was as follows: the main room contained 0.5 ml of enzyme solution and 1 ml of 0.2 M tris-HCl (pH 9.0); the side arm contained 0.5 ml of substrates (1 per cent concentration); total volume, 2.0 ml.

Polyacrylamide gel disc electrophoresis. Disc electrophoresis was conducted in β -alanine-acetic acid buffer (pH 4.5) for 60 min with a current of 3 mA per tube according to the method of Reisfeld *et al.*⁽⁸⁾ Protein was stained with amido black.

Materials. PVA 500 and three kinds of modified PVA (Nos. 1, 2 and 3) were purchased from Wako Pure Chemical Industry, Ltd. Modified PVA Nos. 1 and 2 were copolymers of PVA with acrylamide. The content of acrylamide in No. 1 was higher than No. 2 but neither exceeded 15 mole per cent. Modified PVA No. 3 was copolymer of PVA with a vinyl ether compound content of which was below 1 per cent weight. PVA (periodate treated) and X-220 were products of Kurarey Co., Ltd. PVA (periodate treated) was PVA treated chemically with periodic acid which split the 1,2-glycol bonds in the molecules and then hydrogenated with boron hydride. X-220 was on the contrary a sample of PVA with a very high content of 1,2-glycol bonds (about 76 per cent). Contents of 1,2-glycol bonds in PVA were determined by the method of Flory and Leutner.⁽⁴⁾

RESULTS

DEAE-Sephadex chromatography

Pseudomonas O-3 was cultured in the culture medium for 5 days. After completion of culture, the culture broth of 20 flasks each containing 200 ml medium was collected and centrifuged at $20,000 \times g$ for 20 min. The supernatant solution was concentrated in a rotary evaporator under the reduced pressure below 50°C. By this method, about 4000 ml

of solution was concentrated to about 70 to 80 ml. To this concentrated solution, cold acetone (-20°C) was added with stirring to a concentration of 40 per cent. The precipitate was removed by centrifugation at $20,000 \times g$ for 20 min. To the supernatant solution, cold acetone (-20°C) was added in a cold bath (-10 to -20°C) with stirring to a concentration of 80 per cent. The precipitate was recovered by centrifugation at $20,000 \times g$ for 20 min at -5°C . It was then dissolved in 25 ml of 0.05 M tris-HCl (pH 8.5) and dialyzed against the same buffer at 5°C overnight. The dialyzed solution was centrifuged at $20,000 \times g$ for 20 min and applied to a column of DEAE-Sephadex A-50 equilibrated with 0.05 M tris-HCl (pH 8.5). The column was eluted with the same buffer increasing the ionic strength stepwise with sodium chloride. A typical elution profile of PVA-degrading enzyme is shown in Fig. 1. PVA-degrading

FIG. 1. Chromatography of PVA-degrading Enzyme on DEAE-Sephadex.

PVA-degrading enzyme which was precipitated with acetone and dialyzed against 0.05 M tris-HCl (pH 8.5) was applied to a DEAE-Sephadex A-50 column (2.5×20 cm) which had been equilibrated with 0.05 M tris-HCl (pH 8.5). An increasing concentration of tris-HCl (pH 8.5) and sodium chloride was used for the elution buffer. Fractions of 5 ml were collected, and optical density at 280 nm (—) and enzyme activity (-----) were measured.

enzyme was separated into five active fractions: one fraction was not adsorbed to the column, and the other four fractions attached to the column were eluted at different elution volumes. The PVA-degrading enzyme which

was produced by this strain was a mixture of active fractions which differed from each other in electronic property or molecular weight.

SP-Sephadex chromatography of PVA-degrading enzyme

The organism was cultured, and the supernatant solution of the culture broth was concentrated and fractionated with cold acetone as in the case of DEAE-Sephadex chromatography. The acetone-precipitated protein was dissolved in 0.02 M potassium phosphate buffer (pH 6.0) and dialyzed against the same buffer at 5°C overnight. The dialyzed solution was centrifuged at $20,000 \times g$ for 20 min and applied to a column of SP-Sephadex C-50 equilibrated with 0.02 M phosphate buffer (pH 6.0). The column was eluted with phosphate buffer (pH 6.0) increasing the concentration stepwise. A typical elution profile is shown in Fig. 2. PVA-degrading enzyme was

FIG. 2. Chromatography of PVA-degrading Enzyme on SP-Sephadex.

PVA-degrading enzyme which was precipitated with acetone and dialyzed against 0.02 M phosphate buffer (pH 6.0) was applied to a SP-Sephadex C-50 column (2.5×20 cm) which had been equilibrated with 0.02 M phosphate buffer (pH 6.0). A stepwise increasing concentration of phosphate buffer was used for elution of PVA-degrading enzyme. Fractions of 5 ml were collected, and optical density at 280 nm (—) and enzyme activity (-----) were measured.

separated into two major fractions: one not being adsorbed by the column and the other being adsorbed by it and eluted with 0.05 M

phosphate buffer. The specific activities of each fraction were measured, and it was found that the specific activity of the latter fraction was much higher than the former. The latter fraction (tentatively designated as SP-fraction) was collected and concentrated with Amicon diaflow-filter UM-2 to a volume of less than 10 ml.

Gel filtration of SP-fraction

The concentrated solution was applied to a column of Sephadex G-100 which was equilibrated with 0.05 M tris-HCl (pH 8.5) and gel-filtrated. The elution profile of SP-fraction is shown in Fig. 3. It was shown that SP-fraction

FIG. 3. First Gel Filtration of Sp-fraction on Sephadex G-100.

Fractions from 65 to 85 of SP-Sephadex chromatography were collected and concentrated with Amicon diaflow-filter UM-2 to about 10 ml. The concentrated solution was applied to a Sephadex G-100 column (2.5×85 cm) which had been equilibrated with 0.05 M tris-HCl (pH 8.5) and eluted with the same buffer at a flow rate of 15 ml per hr. Fractions of 5 ml were collected, and optical density at 280 nm (—) and enzyme activity (O---O) were measured.

tion was separated into two fractions: the main peak appeared around tube No. 52 and the minor peak appeared around tube No. 80. The main peak was collected and concentrated with Amicon diaflow-filter UM-2 and once again applied to a column of Sephadex G-100 and gel-filtrated with 0.05 M tris-HCl (pH 8.5). As a result, a single peak was obtained at around tube No. 52 (Fig. 4). Active fractions from tube Nos. 47 to 60 were collected and stored as the purified enzyme. The purified enzyme exhibited a pale pink color.

FIG. 4. Second Gel Filtration of Sp-fraction on Sephadex G-100.

Fractions from 47 to 60 of the first gel filtration were collected and concentrated with Amicon diaflow-filter UM-2 to about 10 ml. The concentrated solution was applied to a Sephadex G-100 column (2.5×85 cm) which had been equilibrated with 0.05 M tris-HCl (pH 8.5) and eluted with the same buffer at a flow rate of 15 ml per hr. Fractions of 5 ml were collected, and optical density at 280 nm (—) and enzyme activity (O---O) were measured.

Criteria of homogeneity of purified enzyme

The homogeneity of the purified enzyme was examined by polyacrylamide gel disc electrophoresis at pH 4.3. As shown in Fig. 5, a single band of protein was obtained.

FIG. 5. Polyacrylamide Gel Electrophoresis of Purified Enzyme.

Electrophoresis was carried out in β -alanine-acetate buffer (pH 4.5) at 3.0 mA per tube for 60 min.

The results of purification of PVA-degrading enzyme (SP-fraction) are summarized in Table

I. The specific activity of the purified enzyme increased about 5-fold compared to the culture filtrate, and the yield of the purified enzyme was about 3 per cent.

Effect of pH and temperature on purified enzyme

The effect of pH on enzyme activity was measured changing the pH of the reaction mixture from 7.8 to 10.2 with 0.2 M ammonium buffer. As shown in Fig. 6, the optimum pH

FIG. 6. Effect of pH on Enzyme Activity.

Reaction mixtures were adjusted at each pH with 0.2 M $\text{NH}_4\text{OH}-\text{NH}_4\text{Cl}$ and incubated shaking at 30°C for 21 hr. After incubation, PVA concentrations were measured with iodometry and compared with the control tube without enzyme solution.

of this enzyme was 9.0. The effect of temperature on the enzyme was measured changing the temperature of the reaction from 30 to 50°C . The result shown in Fig. 7 indicates that the optimum temperature of this enzyme was around 40°C .

Heat and pH stability of purified enzyme

The heat stability of this enzyme was estimated by heating the enzyme solution at temperatures from 30 to 75°C for 15 min and measuring the remaining activities after treatment. As shown in Fig. 8, the enzyme was stable to treatment up to 50°C , but inactivated by higher temperatures and the activity was

TABLE I. SUMMARY OF PURIFICATION PROCEDURES OF PVA-DEGRADING ENZYME (SP-FRACTION)

Procedure	Total volume (ml)	Protein (mg)	Specific activity (unit/mg protein)	Total activity	Yield (%)
Culture filtrate	4000	2400	4.33	10392	100
Acetone ppt. (40~80%)	40	440	13.45	5918	56.9
SP-Sephadex	80	49.6	19.35	959.8	9.2
1st Gel filtration	75	26.7	21.34	569.8	5.5
2nd Gel filtration	75	13.2	22.73	300.0	2.9

FIG. 7. Effect of Temperature on Enzyme Activity.

Reaction mixtures containing 2 ml of 1% PVA solution, 0.5 ml of enzyme solution, 1 ml of 0.2 M tris-HCl (pH 9.0) and 0.5 ml of distilled water were incubated shaking at each temperature for 16 hr. After incubation, PVA concentrations were measured with iodometry and compared with the control tube without enzyme solution.

almost completely lost by treatment at 75°C. The pH stability of this enzyme was examined by treating the enzyme solution at each pH between 2.5 and 12.0 overnight at 5°C and estimating the remaining activities. The results shown in Fig. 9 indicate that the enzyme was stable between pH 5 and 11. At pH below 5, the enzyme activity decreased gradually, but at pH above 11, the activity decreased rapidly.

Effect of metal salts on enzyme activity

The effect of metal salts on enzyme activity was measured by treating the enzyme with 10^{-3} M of various metal salts at 30°C for 20

FIG. 8. Heat Stability of Purified Enzyme.

Reaction mixtures which consist of 2 ml of 0.2 M tris-HCl (pH 9.0), 0.5 ml of enzyme solution and 0.5 ml of distilled water were treated at each temperature for 15 min. After treatment the remaining activities were measured shaking at 30°C for 18 hr.

min and estimating the remaining activities. The results shown in Table II indicate that Ni^{2+} and Co^{2+} inhibited enzyme activity to some extent.

Effect of inhibitors on enzyme activity

Enzyme was treated with 10^{-3} or 10^{-4} M of various inhibitors at 30°C for 20 min, and the remaining activities were measured. Among these inhibitors, EDTA and hydroxylamine inhibited enzyme activity somewhat, suggesting that a certain kind (s) of heavy metal is involved in the enzyme activity (Table III). Among heavy metals, Cu and Fe have been shown so far to be cofactors of oxidases or oxygenases. Chelators of Cu and Fe were, therefore, applied to the enzyme, and their effects were examined. The results shown in Table IV indicate that the

FIG. 9. The pH Stability of Purified Enzyme.

Reaction mixtures which consist of 0.5 ml of enzyme solution, 2 ml of the buffer solution and 1.5 ml of distilled water were allowed to stand at 5°C overnight. The pH of the reaction mixtures was adjusted with citric acid- Na_2HPO_4 (pH 2.5~8.0), tris-HCl (pH 7.5~9.0) and glycine-NaOH (pH 9.0~12.0). After treatment, the remaining activities were measured shaking at 30°C for 22 hr.

TABLE II. EFFECT OF METAL SALTS ON PURIFIED ENZYME

Test tubes containing 0.5 ml of enzyme solution (with 150 γ protein), 1 ml of 0.2 M tris-HCl (pH 9.0) and 0.5 ml of 4×10^{-3} M metal salts were incubated shaking at 30°C for 20 min. After incubation, 2 ml of 1% PVA solution was added to each tube, and the remaining activities were measured.

Metal salts	Concentration (mol)	$\Delta E_{660\text{nm}}$	Activity (%)
None	—	0.800	100
MgSO_4	10^{-3}	0.750	93.8
FeSO_4	10^{-3}	0.730	91.3
$\text{Pb}(\text{CH}_3\text{COO})_2$	10^{-3}	0.800	100.0
NiSO_4	10^{-3}	0.400	50.0
CoSO_4	10^{-3}	0.480	60.0
SnCl_2	10^{-3}	0.800	100.0
ZnSO_4	10^{-3}	0.850	106.3
$\text{Cd}(\text{CH}_3\text{COO})_2$	10^{-3}	0.800	100.0
CaCl_2	10^{-3}	0.900	112.5
CuSO_4	10^{-3}	0.880	110.0
FeCl_3	10^{-3}	0.800	100.0
AgNO_3	10^{-3}	0.850	106.3
MnSO_4	10^{-3}	0.880	110.0
BaCl_2	10^{-3}	0.880	110.0
HgCl_2	10^{-3}	0.800	100.0

chelators of Cu did not inhibit enzyme activity, while among chelators of Fe, only salicylaldehyde inhibited enzyme activity to some

TABLE III. EFFECT OF INHIBITORS ON PURIFIED ENZYME (1)

Test tubes containing 0.5 ml of enzyme solution (with 250 γ protein), 1 ml of tris-HCl (pH 9.0) and 0.5 ml of 4×10^{-3} M or 4×10^{-4} M inhibitor were incubated shaking at 30°C for 20 min. After incubation, 2 ml of 1% PVA solution was added to each tube, and the remaining activities were measured.

Inhibitors ^{a)}	Concentration (mol)	$\Delta E_{660\text{nm}}$	Activity (%)
None	—	0.510	100
<i>o</i> -Phenanthroline	10^{-3}	0.513	100.6
NH_2OH	10^{-3}	0.355	69.6
PCMB	10^{-4}	0.493	96.7
2,4-DNP	10^{-4}	0.518	101.6
NaF	10^{-3}	0.502	98.4
α, α' -Dipyridyl	10^{-3}	0.520	102.0
EDTA	10^{-3}	0.405	79.4
IAA	10^{-3}	0.523	102.5
NaAsO_3	10^{-3}	0.517	101.4

^{a)} Abbreviations: PCMB, *p*-chloromercuribenzoate; 2,4-DNP, 2,4-dinitrophenol; EDTA, disodium ethylenediaminetetraacetate, dihydrate; IAA, monoiodoacetic acid.

TABLE IV. EFFECT OF INHIBITORS ON PURIFIED ENZYME (2)

Test tubes containing 0.5 ml of enzyme solution (with 50 γ protein), 1 ml of 0.2 M tris-HCl (pH 9.0) and 0.5 ml of 4×10^{-3} M inhibitor were incubated shaking at 30°C for 20 min. After incubation, 2 ml of 1% PVA solution was added to each tube, and the remaining activities were measured.

Inhibitors	Concentration (mol)	$\Delta E_{660\text{nm}}$	Activity (%)
None	—	0.337	100
Cysteine	10^{-3}	0.318	94.4
Thiourea	10^{-3}	0.310	92.0
KCN	10^{-3}	0.337	100.0
Homocysteine	10^{-3}	0.322	95.5
Diethyldithiocarbamate	10^{-3}	0.337	100.0
8-Hydroxyquinoline	10^{-3}	0.343	101.8
α, α' -Dipyridyl	10^{-3}	0.331	98.3
<i>o</i> -Phenanthroline	10^{-3}	0.328	97.5
Salicylaldehyde	10^{-3}	0.247	73.3
EDTA	10^{-3}	0.215	63.8
NH_2OH	10^{-3}	0.138	40.9

extent, suggesting that Fe^{2+} or Fe^{3+} is involved in the activity of this enzyme.

Substrate specificity of the enzyme

The substrate specificity of this enzyme was examined using a Warburg manometer. Oxygen uptakes were measured with various kinds of synthetic polymers as substrates (Table V). Among them, only PVA and modified PVA were oxidized by this enzyme. Therefore, the action of this enzyme is rather specific to PVA. At least the basic structure of PVA seems required for enzyme action development.

TABLE V. SUBSTRATE SPECIFICITY OF PURIFIED ENZYME

O₂ uptakes were measured using a Warburg manometer at 30°C. Main room of vessels contained 0.5 ml of enzyme solution (with 50 γ protein) and 1 ml of 0.2 M tris-HCl (pH 9.0). Side arms contained 0.5 ml of 1% substrate solutions. After preincubation for 15 min, substrate solutions were added to the contents of the main room and incubated for 1 hr.

Substrates ^{a)}	O ₂ uptake (μ l)	Relative activity (%)
PVA 500	36.3	100
Modified PVA No. 1	38.5	106.1
Modified PVA No. 2	36.4	100.3
Modified PVA No. 3	38.9	107.2
PVA (periodate treated)	40.5	111.6
PVA X-220	39.2	108.0
Vinylon	—	0
PVAc	—	0
EVAc	—	0
EVA	—	0
PVP	—	0
PAA	—	0
PEG 200	—	0

^{a)} Abbreviations: PVAc, polyvinyl acetate; EVAc, ethylene vinylacetate copolymer; EVA, ethylene vinylacetate copolymer saponified (content of vinyl acetate, 30.4 mol%; degree of saponification, 76.8 mol%); PVA, polyvinylpyrrolidone; PAA, polyacrylamide; PEG, polyethylene glycol.

Effect of oxygen on enzyme activity

The effect of oxygen on enzyme activity was measured using Thunberg test tubes and changing the conditions of incubation. One tube was incubated shaking, the second one was incubated standing, and the third tube containing nitrogen gas was incubated shaking. These test tubes were incubated at 30°C for

3 hr and after incubation, PVA concentration was measured by iodometry. As a result (Table VI), PVA in the shaking tube with air was degraded most rapidly; PVA in the standing tube was degraded more slowly; and PVA in the N₂-purged tube was not degraded at all. This indicates that the enzyme requires oxygen for the degradation of PVA.

TABLE VI. EFFECT OF OXYGEN ON ENZYME ACTIVITY

The effect of oxygen on enzyme activity was measured using Thunberg tubes. One tube was incubated shaking, the second tube was incubated standing and the third tube air in which was replaced by nitrogen gas was incubated shaking. After incubation at 30°C for 3 hr, PVA concentrations were measured by iodometry.

Condition of incubation	Initial E_{660nm}	E_{660nm} after 3 hr	ΔE_{660nm}
Shaking	0.277	0.140	0.137
Standing	0.277	0.237	0.040
Shaking in N ₂	0.277	0.280	—

Molecular weight of the enzyme

The molecular weight of purified enzyme was estimated by gel filtration on Sephadex G-100. Cytochrome c, α -chymotrypsin, ovalbumin and bovine serum albumin were used as the standards. As shown in Fig. 10, the enzyme was eluted a little earlier than α -chymotrypsin, and its molecular weight was estimated to be approximately 26,000.

DISCUSSION

In the previous paper, it was shown that PVA was degraded oxidatively by the enzyme produced by *Pseudomonas* O-3 strain. Purification of this enzyme was carried out, and it was found that the enzyme consisted of several fractions which were separated from each other by ion-exchange chromatography and gel filtration. Whether these fractions are isozymes or enzymes of different kinds cannot be decided until each fraction is purified, and the properties are defined. In this paper, a fraction which was adsorbed by SP-Sephadex, a cation exchanger was purified by ion-exchange chromatography and gel filtration to homo-

FIG. 10. Estimation of Molecular Weight by Gel Filtration.

Molecular weight of purified enzyme was estimated by gel filtration on Sephadex G-100 (2.5 × 85 cm). The column was eluted with 0.05 M tris-HCl (pH 8.5) at a flow rate of 15 ml per hr. 1, cytochrome c (M.W., 12,400); 2, α -chymotrypsin (M.W., 25,000); 3, purified enzyme; 4, ovalbumin (M.W., 45,000); 5, bovine serum albumin (M.W., 67,000).

geneity as exhibited by polyacrylamide gel electrophoresis, and some properties of the purified enzyme were examined. The optimum pH of this enzyme was in the alkaline region, pH 9.0, and its optimum temperature was around 40°C. It was stable under 50°C and at a wide pH range from 5 to 11. Among metal salts, Ni^{2+} and Co^{2+} inhibited enzyme activity. Among enzyme inhibitors, EDTA and hydroxylamine inhibited enzyme activity, suggesting heavy metal (s) is involved in the enzyme activity. Several metals have so far been known to be involved in oxidoreductases. Among them, Cu and Fe are known to be metal components of oxygenases. Of those chelators which interact with Cu or Fe, salicylaldehyde inhibited enzyme activity somewhat, suggesting Fe^{2+} or Fe^{3+} is involved in the enzyme activity. On the contrary, sulfhydryl reagents such as mercuric chloride or PCMB did not inhibit enzyme activity, indicating that the sulfhydryl group did not play an important role in the expression of enzyme activity.

The substrate specificity of this enzyme was examined with various kinds of synthetic

polymers including genuine and modified PVA. Three kinds of modified PVA were oxidized by the enzyme as well as the original PVA. They were copolymers of PVA with acrylamide or vinyl ether compound, and their contents in the polymer were below 15 mole per cent being water-soluble. It can be said that modification of PVA to this extent does not interfere with degradative activity of the enzyme. On the other hand, these polymers in which the hydroxyl groups of PVA are blocked by formaldehyde or acetyl groups being water-insoluble, such as vinylon and polyvinyl acetate, were not oxidized by this enzyme, suggesting that the hydroxyl groups in PVA are essential for enzyme activity. Water-soluble synthetic polymers with vinyl linkages, such as poly acrylamide and polyvinylpyrrolidone, were not oxidized either. PVA which was treated with periodate and has no 1,2-glycol bond in the molecule was oxidized by the enzyme, as well as PVA 500 which contains 1.4 mole per cent of 1,2-glycol bonds, indicating that the enzyme does not specifically split the 1,2-glycol bonds in PVA. From the fact that PVA X-220 which has a very high content of 1,2-glycol bond was oxidized by the enzyme, it was assumed that the enzyme split the PVA molecule without discrimination of the 1,2-glycol bonds (head-to-head bonds) and normal 1,3-glycol bonds (head-to-tail bonds).

Effect of oxygen on the enzyme action was examined, and it was found that oxygen was required for enzyme action, and without oxygen the enzyme did not exhibit activity. However, the classification of this enzyme to oxidases or oxygenases could not be determined until the breakdown mechanism of PVA by this enzyme is clarified.

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