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ALCOHOL OXIDASE, A FLAVOPROTEIN FROM SEVERAL BASIDIOMYCETES SPECIES

CRYSTALLIZATION BY FRACTIONAL PRECIPITATION WITH POLYETHYLENE GLYCOL

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SUMMARY

1. A novel enzyme designated 'alcohol oxidase', has been isolated from the mycelium of a Basidiomycete belonging to the family Polyporaceae. In the presence of O_2, this enzyme catalyzes the oxidation of the lower primary alcohols to the corresponding aldehydes and H_2O_2. Unsaturated alcohols are also good substrates, but branched-chain and secondary alcohols are not attacked. Halogenated ethanols are oxidized, but other substituted ethanols are not.

2. The enzyme was purified to the crystalline state by fractional precipitation with polyethylene glycol.

3. Flavin-adenine dinucleotide was identified as the prosthetic group.

4. The pH optimum is 6.5–9. Below pH 6.5, the enzyme is rapidly inactivated.

5. Gel filtration on Sephadex G-200 suggested that the molecular weight of the enzyme is greater than 300 000.

6. The enzyme may be useful for the colorimetric determination of methanol, ethanol, or other lower alcohols.

INTRODUCTION

A preliminary report from this laboratory described the isolation from a Basidiomycete of a novel enzyme which catalyzes the oxidation of methanol and other lower primary alcohols to the corresponding aldehydes and hydrogen peroxide[1]. The present publication describes the crystallization of the enzyme, its characterization as a flavoprotein, and some of its properties. Its production by several species of Basidiomycetes will be described by KERWIN AND RUELIUS[2].

Abbreviation: PEG, polyethylene glycol 6000.

MATERIALS AND METHODS

Polyethylene glycol 6000 (PEG)

Mol. wt. 6000–7500. It was obtained from Baker Chemical. The material was pulverized in a ball mill before use.

Peroxidase

Horseradish peroxidase, 400 units/mg, was purchased from Worthington Biochemical, Freehold, N.J. A 0.1% stock solution in distilled water was prepared and stored at 4° under toluene until needed. This reagent is stable for at least 1 month.

o-Dianisidine dihydrochloride (3,3-dimethoxybenzidine dihydrochloride)

The material was purchased from Eastman Organic Chemicals, Rochester, N.Y. A 1% stock solution was prepared in 0.025 M HCl and stored in the dark at 4°. The solution is stable for at least 1 month.

Peroxidase–dianisidine reagent

To 79 ml distilled water, 10 ml of 0.5 M sodium phosphate (pH 7.5), 10 ml of peroxidase solution and 1.0 ml of *o*-dianisidine solution was added. This reagent was prepared fresh daily. A 2 × concentration was prepared in the same way except that the final vol. was 50 ml.

Methanol–peroxidase–dianisidine reagent

This reagent was prepared fresh daily as described for peroxidase–dianisidine reagent except that 1.0 ml of water was replaced by 1.0 ml of absolute methanol.

Assay of alcohol-oxidase activity

The enzyme was assayed by measuring the H_2O_2 produced in the following system: 1.0-ml aliquots of appropriately diluted enzyme solution were dispensed in duplicate test tubes, which were than placed in a 25° water-bath. At zero time, 4.0 ml methanol–peroxidase–chromogen reagent at 25° was added. The reaction was terminated after 5 min by addition of 0.2 ml of 4.0 M HCl. The color produced was read in a Klett–Summerson photoelectric colorimeter with a No. 42 filter. The reading was corrected for the blank, which was obtained from enzyme and reagent without methanol. H_2O_2 was determined by comparing the color with that obtained from known amounts of H_2O_2. 1 unit is defined as the amount of enzyme that produces 1 μmole of H_2O_2 per min in this system. Specific activity is defined as the number of units per mg nitrogen. Nitrogen was determined by the micro-Kjeldahl method of MINARI AND ZILVERSMIT[3].

Identification of the flavin group of alcohol oxidase.

A pyridine extract of the crystalline alcohol oxidase was examined for flavins by paper chromatography in 2 solvent systems as described by PAZUR AND KLEPPE[4].

Quantitative estimation of flavin–adenine dinucleotide (FAD) in alcohol oxidase

The FAD content of crystalline enzyme was estimated by the method of SWOBODA AND MASSEY[5]. PEG was first removed from the enzyme by 2 washings with 50%

aq. acetone at −8°. The dry wt. was obtained on separate aliquots of the solution, and FAD was determined spectrophotometrically on other aliquots after trichloroacetic acid extraction. FAD (Calbiochem 90% pure B grade) was used as standard. The impurities in the standard were assumed to be nonabsorbing at 450 mμ, and the readings were corrected to 100% based on this assumption.

Molecular weight by gel filtration

Molecular weight was estimated by gel filtration of crystalline enzyme on Sephadex G-200, as described by LEACH AND O'SHEA[6] except that the temperature was 5°. 8.0 g Sephadex G-200 (Pharmacia) was equilibrated with an 0.05 M sodium phosphate buffer (pH 7.5) containing 0.2 M NaCl. A suspension of the gel was poured into a column (Pharmacia, 2.5 cm × 37 cm) and washed with the same buffer at a flow rate of 20 ml/h. Alcohol oxidase (6.0 units) and beef liver catalase (6000 units) in 2.0 ml buffer containing 5.0 mg dextrose and 4.0 mg Blue Dextran (Pharmacia) were applied under a layer of buffer with a syringe. 2-ml fractions were collected in an automatic fraction collector. Blue Dextran was determined spectrophotometrically. Alcohol oxidase was determined colorimetrically with methanol–peroxidase–dianisidine reagent, and catalase was estimated by the method of BEERS AND SIZER[7].

Carbohydrate determination

The carbohydrate in the crystalline enzyme was measured by the anthrone procedure of SCOTT AND MELVIN[8].

Effect of pH on activity and stability of alcohol oxidase

Alcohol-oxidase activity was determined over a pH range of 5.2 to 9.0, as described in the legend to Fig. 1. Effect of pH on stability of alcohol oxidase was determined as described in the legend to Fig. 2.

Stoichiometry of reaction

Quantitative analyses of formaldehyde, H_2O_2, and residual methanol were carried out on the reaction mixture of methanol and alcohol oxidase. The enzyme used for this experiment was purified as previously described[1]. The PEG-purified enzyme was then freed of catalase and PEG by chromatography on DEAE-cellulose equilibrated with 0.02 M sodium phosphate buffer (pH 7.5). Alcohol oxidase was eluted from the column by a gradient elution with NaCl in the same buffer.

Methanol (4 μmoles) and alcohol oxidase (1.0 unit) were placed in a 25-ml-erlenmeyer flask, and the reaction mixture was brought to a final vol. of 3.0 ml by addition of 0.02 M sodium phosphate buffer (pH 7.5). The reaction was allowed to proceed for 10 min, with shaking in air at 25°, and was then terminated by the addition of acid to pH 3. The H_2O_2 produced during the reaction was determined with the peroxidase–dianisidine reagent. Catalase was added to destroy accumulated H_2O_2 prior to the formaldehyde and methanol determinations. Formaldehyde was determined in 1 aliquot by the method of FRISELL AND MACKENZIE[9]. Other aliquots were heated to 70° for 7 min to destroy catalase, and residual methanol was measured by reacting with 0.5 unit of alcohol oxidase for 10 min in peroxidase–chromogen reagent. The H_2O_2 produced was compared with that obtained by alcohol-oxidase action on standard methanol solutions under the same conditions.

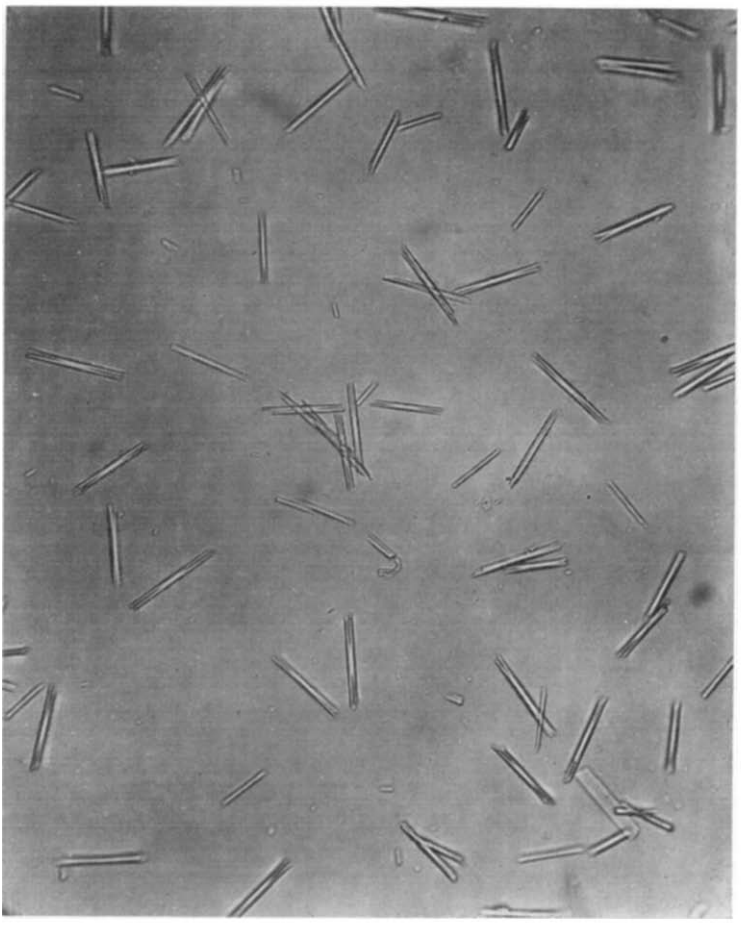

Fig. 1. Crystalline alcohol oxidase (× 100). Crystals were obtained by fractional precipitation with polyethylene glycol as described in Scheme I and Table I.

Kinetics of alcohol-oxidase reaction

The reaction rates for oxidation of methanol, ethanol, *n*-propanol and *n*-butanol by alcohol oxidase were determined at several substrate concentrations. 1 ml of the appropriate substrate solution and 1.0 ml peroxidase–dianisidine reagent (2 × concentration) were added to 16-mm-test tubes. At zero time, 1.0 ml of alcohol-oxidase solution containing 0.07 unit of crystalline enzyme was added. After the reaction had proceeded for 2 min at 20° in an atmosphere of air, the reaction was terminated and the color was read. Michaelis constants (K_m) were derived from curves obtained by plotting the data in accordance with the method of LINEWEAVER AND BURK[10].

Alcohol oxidase in determination of ethanol

For this determination, 4 ml of the peroxidase–dianisidine reagent, followed by 1.0 ml alcohol oxidase (0.2 unit), was added to each 0.1-ml aliquot of ethanol (containing from 0.05 to 0.2 mg). The reaction was allowed to proceed for 15 min at

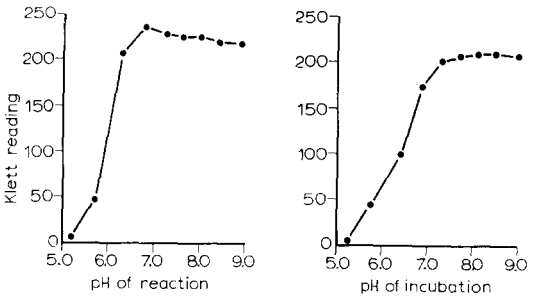

Fig. 2. Effect of pH on activity of alcohol oxidase. 2 ml of 0.5 M buffer of the desired pH and 2.0 ml of 2 × conc. methanol–peroxidase–dianisidine reagent were added to duplicate 16 × 125 mm test tubes. At zero time, 1.0 ml aq.- alcohol-oxidase solution (0.06 unit) was added and alcohol-oxidase activity was measured as described in the text. Buffers were prepared by mixing 0.5 M solutions of the following: (a), acetic acid and sodium acetate (pH 5.2 and 5.7); (b), KH_2PO_4 and K_2HPO_4 (pH 6.3 and 6.8); (c), KH_2PO_4 and Tris (pH 7.25 to 8.9).

Fig. 3. Effect of pH on stability of alcohol oxidase 1 ml of enzyme solution (0.06 unit) was incubated for 3 h at 25° with 1.0 ml of 0.1 M buffer of the desired pH. The solutions were then adjusted to pH 7.7–8.0 by addition of 2.0 ml of 0.5 M phosphate buffer (pH 7.7). 1 ml of 4 × methanol–peroxidase–dianisidine reagent was added and the enzyme assayed as described in the text. Buffers were prepared by mixing 0.1 M solutions of the following: (a), acetic acid and sodium acetate (pH 5.25 and 5.75); (b), KH_2PO_4 and K_2HPO_4 (pH 6.4 and 6.8); (c), KH_2PO_4 and Tris (pH 7.3 to 9.0).

25° and was then terminated by the addition of 0.2 ml 4.0 M HCl. The color was read on a Klett–Summerson colorimeter equipped with a No. 42 filter.

RESULTS

Extraction, purification, and crystallization of alcohol oxidase

The fermentation, extraction, and preliminary purification were carried out essentially as described previously[1], but on a larger scale. For the extraction, 250 g of frozen mycelium was thawed in 1870 ml of freshly prepared 5 mM potassium ethyl xanthate in 0.05 M sodium phosphate buffer (pH 7.7). The thawed mycelium was homogenized in a 1-gallon Waring Blendor for 3 periods of 4 min each. The homogenate was cooled in an ice-bath for 5 min between each period. Centrifugation of the homogenate at 7250 × *g* for 15 min yielded a turbid supernatant which contained the alcohol oxidase.

First PEG precipitation

To 1915 ml of extract at room temperature (22–25°), 431 g PEG powder (19% w/v) was added slowly while the extract was mixed on a magnetic stirrer. 30 min after the addition, the resulting suspension was centrifuged for 15 min at 7250 × *g*. The sediment (Fraction 1) was redissolved in 160 ml of the xanthate–phosphate buffer to yield a final vol. of 200 ml of very turbid suspension.

Second PEG precipitation

To the suspension was added 6.0 g PEG powder (3% w/v). Centrifugation at 12 900 × *g* yielded a clear supernatant (Fraction 2) which contained the enzyme and a voluminous precipitate consisting largely of cell fragments.

Third PEG precipitation

To 186 ml of Fraction 2, 35.4 g of PEG powder (19% w/v) was added slowly, with stirring. 0.5 h after the addition, the resulting suspension was centrifuged at 12 900 × *g* for 15 min, yielding a well packed sediment which contained most of the enzyme. The sediment was dissolved in xanthate–phosphate buffer, yielding 44 ml of a clear greenish-amber solution (Fraction 3).

Fourth PEG precipitation

To 43.5 ml of Fraction 3, 2.18 g PEG (5% w/v) was added during continuous stirring. The clear solutions became turbid when cooled to 0° for 1 h. Insoluble material was removed by centrifugation at 4° for 15 min at 12 900 × *g*. The clear supernatant (Fraction 4) contained most of the alcohol oxidase. Subsequent operations were carried out in the cold room (4–5°).

First crystallization

Fraction 4 (44.5 ml) was placed in a 100-ml-graduated cylinder illuminated from the side by a microscope lamp. A solution of PEG (40 g made up to 100 ml with 0.05 M sodium phosphate buffer (pH 7.7)) was added dropwise during continuous stirring. The solution gradually became faintly turbid. When the PEG had reached 6.7% (w/v) the turbidity suddenly increased, and at this point no more PEG was added. The suspension was stored overnight in an ice-bath. Examination of the precipitate under a microscope revealed a mixture of crystals and amorphous material. Centrifugation of the suspension at 1250 × *g* for 20 min at 4° yielded a yellow sediment which was covered with a thin purple layer. The sediment was dissolved in 40 ml fresh xanthate–phosphate buffer (Fraction 5).

Second crystallization

PEG solution was added to Fraction 5 to a level of 7.6 g per 100 ml. Cooling the solution for 1 h in an ice-bath made it slightly turbid. Centrifugation for 15 min at 16 900 × *g* yielded a clear amber supernatant (Fraction 5a) and a purple black sediment. Further dropwise addition of PEG solution to the supernatant to a final concentration of 10.8 g per 100 ml increased the turbidity. Storage of the suspension for 3 days at 0° yielded a heavy precipitate consisting of a thin purple upper layer and a yellow lower layer. Examination of this material under the microscope revealed that the solid was mostly crystalline. Centrifugation of the suspension at approx. 160 × *g* yielded a turbid supernatant (which contained mostly amorphous material) and a yellow, mostly crystalline precipitate (Fraction 6). The crystals were washed by resuspending them in PEG solution (12.5 g per 100 ml 0.05 M phosphate buffer (pH 7.7)). Centrifugation of this suspension at 160 × *g* yielded a yellow crystalline precipitate and a turbid supernatant which contained much of the amorphous material.

Third crystallization

The crystals were taken up in 30 ml of fresh xanthate–phosphate buffer, affording a clear yellow solution. PEG solution was added to the point of incipient turbidity, and the solution was then stored overnight at 0°. Centrifugation at 160 × *g* yielded a sediment consisting of uniform crystals (Fraction 7).

The complete purification procedure is outlined as a flow diagram in Scheme I,

SCHEME I

FLOW DIAGRAM FOR THE PEG FRACTIONATION OF ALCOHOL OXIDASE

The separation procedure is described in the text.

Mycelium
↓
Mycelial extract
↓
PEG (19 g/100 ml)
↓
Centrifuge

Precipitate (Fraction 1)	*Supernatant*
Dissolve in buffer	(discard)
Add PEG (3.0 g/100 ml)	
↓	
Centrifuge	
Supernatant (Fraction 2)	*Precipitate*
Increase PEG to 19 g/100 ml	(discard)
↓	
Centrifuge	
Precipitate (Fraction 3)	*Supernatant*
Dissolve in buffer	(discard)
Add PEG (5.0 g/100 ml)	
Cool to 0°	
↓	
Centrifuge	
Supernatant (Fraction 4)	*Precipitate*
Add PEG solution until turbid	(discard)
Hold overnight at 0°	
↓	
Centrifuge	
Precipitate (crystals, Fraction 5)	*Supernatant*
Dissolve in buffer	(discard)
Add PEG (7.6 g/100 ml)	
Hold 1 h at 0°	
↓	
Centrifuge	
Supernatant (Fraction 5a)	*Precipitate*
Add PEG solution until turbid	(discard)
Hold 3 days at 0°	
↓	
Centrifuge	
Precipitate (crystals, Fraction 6)	*Supernatant*
Wash with PEG solution	(discard)
Dissolve in buffer	
Add PEG solution until turbid	
↓	
Centrifuge	
Precipitate (crystals, Fraction 7)	*Supernatant*
Suspend in PEG solution for st orage	(discard)

TABLE I

CRYSTALLIZATION OF ALCOHOL OXIDASE

Fractionation was carried out as described in the text and in Scheme I. Methods for determination of enzyme activity, nitrogen, and specific activity are given in the text.

Enzyme fraction	*Vol. (ml)*	*Total activity (units)*	*Total nitrogen (mg)*	*Specific activity*	*Overall recovery (%)*
Crude extract					
Fraction 1 (19% PEG ppt.)					
Fraction 2 (3% PEG supernatant)	186	6850	227	30.2	86
Fraction 3 (second 19% PEG ppt.)	44	5950	99	60.1	75
Fraction 4 (5% PEG supernatant)	44.8	5300	101	52.5	66
Fraction 5 (first crystallization)	40.9	4590	47.5	96.5	57
Fraction 6 (second crystallization)	30	3940	31.8	124	49
Fraction 7 (third crystallization)	30	3900	23.8	157	49

and the quantitative data are summarized in Table I. The crystals were stored as a suspension in 0.05 M phosphate buffer (pH 7.7) containing PEG (12.5% w/v). The enzyme is stable for at least 5 months under these conditions. A photomicrograph of the crystals is shown in Fig. 1.

Identification and quantitative analysis of FAD in alcohol oxidase

The flavin extracted from alcohol oxidase migrated at the same rate as FAD in both chromatographic systems. These results are shown in Table II. The FAD content of acetone-washed alcohol oxidase is 0.97%. The minimum molecular weight based on this figure is 85 000.

TABLE II

PAPER CHROMATOGRAPHY OF FLAVIN FROM ALCOHOL OXIDASE

Solvent 1, *n*-butanol–acetone–acetic acid–water (5:2:1:3, by vol.); Solvent 2, *n*-butanol–methanol–0.5% disodium phosphate (4:1:2, by vol.).

Component	*Migration in Solvent 1 (cm)*	*Migration in Solvent 2 (cm)*
Extract of alcohol oxidase	1.6	4.0
FAD	1.5	3.8
FMN	5.5	8.8
Riboflavin		17.5

Molecular weight

Gel filtration of alcohol oxidase on Sephadex G-200 gave the results shown in Table III. The calculated molecular weight for catalase closely approximates the values obtained with gel filtration by LEACH AND O'SHEA as well as literature values obtained with other methods[6]. It appears, therefore that our chromatographic procedure at 5° yields reliable results for proteins with molecular weights at least as high as 230 000. The V_e/V_o ratio of 1.16 for alcohol oxidase suggests that its molecular

TABLE III

GEL FILTRATION OF ALCOHOL OXIDASE

Mol. wt. calculated from Eqn. 1 of LEACH AND O'SHEA[6]: log mol. wt. = − 0.959 (V_e/V_0 − 1) + 5700, where V_e is the protein elution volume and V_0 is the column void volume.

Component	*Elution vol. (ml)*	V_e/V_0 *ratio*	*Mol. wt.*
Blue Dextran (V_0)	62		
Alcohol oxidase	72	1.16	>300 000
Catalase	84	1.35	232 000

weight is above 300 000. It appears likely, therefore that the enzyme contains several FAD residues per mole.

Carbohydrate analysis

No carbohydrate was detected in crystalline alcohol oxidase by the anthrone method[8]. It appears, therefore, that the enzyme is not a glycoprotein.

Effect of pH on activity and stability

Fig. 2 shows that the enzyme has a broad pH optimum from 6.5 to 9.0, but that the activity falls off rapidly below pH 6.5. The enzyme is stable from pH 7 to 9, as shown in Fig. 3. Below pH 7.0 the stability decreases. At pH 5.0 activity is lost completely and irreversibly after 3 h incubation at 25°.

TABLE IV

STOICHIOMETRY OF METHANOL OXIDATION BY ALCOHOL OXIDASE

Methanol was oxidized at pH 7.5 for 10 min. Residual methanol, H_2O_2, and formaldehyde were determined as described in the text.

Methanol at start (μmole)	*Methanol used (μmole)*	*H_2O_2 produced (μmole)*	*Formaldehyde produced (μmole)*
1.0	0.68	0.68	0.66

Stoichiometry of the alcohol-oxidase reaction

The stoichiometry of the methanol oxidation catalyzed by alcohol oxidase is demonstrated by the data in Table IV. Thus the reaction can be described by the following equation:

$$\text{methanol} + O_2 \rightleftarrows \text{formaldehyde} + H_2O_2$$

Substrate specificity of alcohol oxidase

Table V shows that methanol, ethanol, *n*-propanol, *n*-butanol, *n*-amyl alcohol, allyl alcohol, propynyl alcohol, chloroethanol and bromoethanol are substrates.

The Michaelis constants for several alcohols (Table VI) indicate that methanol

TABLE V

SUBSTRATE SPECIFICITY OF ALCOHOL OXIDASE

2 ml of the substrate solution at the concentration shown in the table was pipetted into test tubes. 3 ml of peroxidase–dianisidine reagent containing 0.08 unit of PEG purified alcohol oxidase was added. The reaction was terminated after 5 min by addition of 0.2 ml of 4.0 M HCl and the H_2O_2 was measured as described above.

Substrate	*Concentration (μmoles/2 ml)*	*Relative activity*
Methanol	10	100
Ethanol	40	28
n-Propanol	200	5.3
n-Butanol	500	2.1
iso-Butanol	1000	0.1
sec.-Butanol	1000	0.01
Benzyl alcohol	40	0
Anisyl alcohol	40	0
n-Amyl alcohol	100	1
2-Bromoethanol	50	4
2-Chloroethanol	50	6
Phenethyl alcohol	100	0
Hydracrylonitrile	100	<1
Ethylene glycol	100	0
Methyl cellosolve	100	0
Glycolic acid	100	0
Ethanolamine	100	<0.5
2-Mercaptoethanol	100	0
Choline	100	<0.5
1,3-Propanediol	100	0
1,2-Propanediol	100	<0.5
1,4-Butanediol	100	<0.5
Allyl alcohol	10	17
2-Propyn-1-ol	10	45
Glycerine	100	0
DL-Serine	200	<0.5

is the best substrate and that reactivity decreases as the chain length of the normal primary aliphatic alcohols is increased.

Determination of ethanol with alcohol oxidase

The enzyme is useful for the determination of ethanol. Oxidation of ethanol at concentrations from 0.05 to 0.2 mg per 0.1 ml is shown in Table VII.

TABLE VI

ACTIVITY OF ALCOHOL OXIDASE WITH SEVERAL SUBSTRATES

The Michaelis constants (K_m) of alcohol-oxidase oxidation of the listed substrates were determined as described in the text.

Substrate	K_m *(mM)*
Methanol	1.52
Ethanol	10.0
n-Propanol	54.6
n-Butanol	133

TABLE VII

DETERMINATION OF ETHANOL WITH ALCOHOL OXIDASE

The H_2O_2 produced by oxidation of ethanol was determined as described in the text.

Ethanol (mg/0.1 ml)	*Klett reading*
0	0
0.05	46
0.1	96
0.15	144
0.2	184

DISCUSSION

Fractional precipitation of proteins with PEG was first described by POLSON *et al.*[11], who used the method to obtain electrophoretically pure γ-globulin and fibrinogen from plasma. We have found PEG to be an effective and convenient agent for the fractionation of alcohol oxidase. Initial PEG fractionation to concentrate the alcohol oxidase and remove inactive material purified the enzyme to the degree that it could then be crystallized from a solution to which PEG had been added to the point of turbidity. Although POLSON *et al.*[11] isolated highly purified proteins by PEG precipitation, the present publication appears to describe the first use of PEG for the crystallization of a protein. Thus, only the one agent was necessary to purify the enzyme from the crude extract to the crystalline state. Because of the sensitivity of alcohol oxidase to solutions below pH 7, we avoided PEG precipitation at lower pH values, a procedure which POLSON and his group had utilized with blood proteins. This demonstrates that PEG fractionation is valuable even where pH manipulation is not feasible.

Our experience indicates that the concentration of precipitable protein is an important factor in the mechanism of PEG fractionation. Precipitation of the relativily dilute enzyme from crude extracts is probably aided by the presence of other precipitable proteins and possibly cellular debris. Thus, in our initial attempts to remove extraneous protein and cell debris by a preliminary precipitation at low levels of PEG (6 g/100 ml), subsequent recovery of enzyme was poor when additional PEG was added to precipitate the enzyme from the supernatant. We attribute this result to the low concentration of total precipitable protein after the preliminary PEG precipitation. As the purification of the enzyme progressed, we found it important to concentrate the enzyme to the point where it could be precipitated efficiently by PEG.

The crude mycelial extracts of Basidiomycete also contain a very high level of polyphenol oxidase. This enzyme catalyzes the oxidation of phenolic substances present in the extracts to colored oxidation products. Since polyphenol oxidase is a copper protein, it is inhibited by several copper-complexing reagents[12]. Some amber pigment formed in spite of the inclusion of inhibitor in the buffer.

No amber pigment remained after the first crystallization, but at this stage of purification a purple pigment precipitated along with the enzyme. This pigment was removed from the crystals by slow-speed centrifugation, which left it in suspension,

while the yellow crystals appeared in the sediment. The origin of the purple pigment is uncertain. It may arise from the oxidation of phenolic materials by polyphenol oxidase and be present during the initial stages of purification but obscured by the presence of brown pigments. Alternatively, it may result from the inadvertent production of a semi-quinoid form of flavoprotein. The semi-quinoid form of the enzyme–substrate complex of the flavoprotein D-amino-acid oxidase is a purple pigment[13].

PAZUR, KLEPPE AND CEPURE[14] have shown recently that glucose oxidase, a flavoprotein obtained from *Aspergillus niger*, is a glycoprotein. Since alcohol oxidase is also a flavoprotein oxidase, it was investigated for the presence of a carbohydrate moiety. None was found with the anthrone test. Moreover, the absence of inhibition by chelating agents (sodium ethylenediamine tetraacetate, sodium diethyldithiocarbamate, or potassium ethyl xanthate) may suggest that alcohol oxidase is a not metalloprotein. However, VALLEE[15] has pointed out that failure to observe enzyme inhibition by metal-complexing agents should not be considered absolute proof of the absence of metal in the enzyme reaction.

Methanol was the best substrate tested. Although ethanol and higher homologues are also substrates, the activity declines rapidly as the chain length of the alcohol is increased; *e.g.*, *n*-amyl alcohol was only about 1% as active as methanol. Unsaturation in the chain appears to enhance the activity. An example of this may be seen in allyl and propynyl alcohols, which are both more active than the saturated alcohol propanol. The alcohol dehydrogenase of the pea has a similar preference for unsaturated alcohols[16]. 2-Bromo- and 2-chloroethanols are substrates, but the activity of both halogenated alcohols is less than that of ethanol. Branched-chain alcohols and secondary alcohols were inactive. Alcohols having C_6H_5–, CN–, HO–, CH_3O–, –COOH, $-NH_2$ or –SH as substituents on the second carbon of ethanol were inactive. Both propanediols tested were also inactive.

Although the enzyme is not specific for ethanol, it is potentially useful for the detection and quantitative analysis of ethanol in the absence of other alcohols. For example, the colorimetric method described in this paper could be readily adapted to analysis of ethanol in serum. For maximum sensitivity, a method utilizing a fluorogenic substrate for peroxidase, *e.g.* homovanillic acid such as in the method of GUILBAULT, KRAMER AND HACKLEY[17], could be readily developed for alcohols. Methanol could be determined specifically by detection of formaldehyde[9] after oxidation of methanol by the enzyme. In the absence of ethanol or other lower alcohols, it could also be determined using the coupled peroxidase–dianisidine reagent for the detection of the H_2O_2 produced in the reaction.

The metabolic role of alcohol oxidase in the Basidiomycete is not known. It seems likely, however, that the enzyme plays a significant role in the metabolism of at least some Basidiomycetes, since appreciable amounts have been detected in the mycelia of 6 species out of 8 tested from this class of organisms[2].

Alcohol oxidase is a novel enzyme which differs from known alcohol-oxidizing enzymes in several respects. The enzyme found in Basidiomycetes by FARMER, HENDERSON AND RUSSEL[18] oxidizes benzyl alcohol and anisyl alcohol but does not oxidize ethanol and *n*-butanol. Alcohol oxidase, on the other hand, oxidizes the latter two substrates but not the aromatic alcohols. The properties of alcohol oxidase also distinguish it from other previously reported methanol-oxidizing enzymes. The Pseudomonas enzyme discovered by ANTHONY AND ZATMAN[19] is a methanol dehydro-

genase which requires the presence of ammonia and a mediator, phenazine methosulfate. The methanol-oxidizing enzyme described by ORME-JOHNSON AND ZIEGLER[20] is a particulate enzyme found in liver microsomes; it catalyzes the oxidation of methanol and ethanol to the corresponding aldehydes, but requires the presence of NADPH as well as O_2. The alcohol oxidase of Basidiomycetes, however, is a flavoprotein requiring O_2 and producing H_2O_2. It catalyzes the following reaction:

$$ROH + O_2 \rightleftarrows RCHO + H_2O_2$$

where R can be methyl, ethyl, propyl, butyl, allyl, propynyl, chloroethyl, or bromoethyl.

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