ALCOHOL OXIDASE, A NOVEL ENZYME FROM A BASIDIOMYCETE

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During an investigation of a glucose-oxidizing enzyme from an unidentified Basidiomycete, hydrogen peroxide production was noted in controls which contained no glucose. We subsequently found the hydrogen peroxide to be produced by the enzymic oxidation of methanol, the solvent for the o-dianisidine used in determining glucose oxidase by a peroxidase-chromogen method, cf. Schepartz and Subers (1964). Investigation of the methanol-oxidizing enzyme showed that it catalyzes the oxidation of lower primary aliphatic alcohols to the corresponding aldehydes and hydrogen peroxide: $RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$. For this reason it has been named "alcohol oxidase".

The culture of the organism which produces alcohol oxidase was obtained from the fruiting body of a Basidiomycete belonging to the family Polyporaceae, collected as part of a screening program for antitumor agents from fungi. The enzyme is produced in the mycelium. We obtained it by growing the organism in 2-liter flasks on a rotary shaker at 25°C until enzyme production was optimal. Each flask contained 1 liter of medium consisting of Amber MPH (Amber Laboratories, Milwaukee, Wisconsin) 1% and dextrose 1%. The washed mycelium may be stored frozen with little loss in activity for several months.

Frozen mycelium was homogenized in a Waring Blendor with ice-cold 0.005 M potassium ethyl xanthate in 0.05 M pH 7.5 sodium phosphate buffer (7.5 ml./g. wet mycelium). Centrifugation of the homogenate at 7900g for 15 min. yielded a turbid supernate which contained the alcohol oxidase.

Subsequent operations were carried out at room temperature (ca. 22-25°C). The enzyme was purified by fractional precipitation with polyethylene glycol 6000 (PEG). To 162 ml. of extract, 36.4 g. PEG was added slowly while mixing in a Waring Blendor, yielding a final PEG concentration of 19% (w/v). Thirty min. after addition, the resulting suspension was centrifuged for 15 min. at 7900g. The sediment (Fraction 1) was redissolved in 20 ml. of the xanthate-phosphate buffer, yielding a very turbid suspension. Addition of 0.6 g. PEG (equivalent to 3% w/v) to the suspension, followed by centrifugation, removed particulate material consisting largely of cell debris. The clear supernate (Fraction 2) was then adjusted to a PEG concentration of 19% w/v

Table I

Purification of Alcohol Oxidase

Enzyme Fraction	Volume ml.	Total[1,2] Activity units	Total[3] Nitrogen mg	Specific Activity[4]	Overall Recovery %
Crude extract	162.0	350	125.0	2.8	-
Fraction 2 (3% PEG supernate)	20.0	320	15.5	21.0	91.5
Fraction 3 (Second 19% PEG precipitate)	3.3	306	5.8	53.0	87.5
Fraction 4 (12.5% PEG supernate)	3.1	278	3.3	84.0	79.5

1 The enzyme was assayed by measuring the formaldehyde produced during a 5 min. reaction at 25°C in a 25 ml. Erlenmeyer flask shaken at 80 cycles per min. The system contained 100 μM methanol and alcohol oxidase (0.02 to 0.1 unit) in 1.0 ml. final volume of 0.06 M pH 7.5 sodium phosphate buffer. Formaldehyde was determined as described by Frisell and Mackenzie (1958).

2 A unit is defined as the amount of enzyme that produces 1 μM of HCHO per min. under the above conditions.

3 Nitrogen was determined by a modification of the micro-Kjeldahl method described by Minari and Zilversmit (1963).

4 Specific activity is defined as the number of units per mg. N.

by adding 3.8 g. PEG while mixing well on a magnetic stirrer. One-half hour after the addition the resulting suspension was centrifuged at 11,400g for 15 min., yielding a well-packed sediment (Fraction 3) containing most of the enzyme. The sediment was dissolved in 3.0 ml. of 0.001 M potassium ethyl xanthate in 0.01 M pH 7.5 sodium phosphate buffer, and inactive material was precipitated by addition of 0.44 g. PEG (12.5% w/v). After 30 min., the suspension was centrifuged as above for 15 min., yielding a clear supernate (Fraction 4) which contained most of the activity. The purified enzyme is stable for at least 2 weeks when kept cold and protected from microbial growth. The purification and recovery data are presented in Table I.

Centrifugation of Fraction 4 removed a small amount of insoluble material which formed during storage in the refrigerator. An aliquot of the supernate was subjected to electrophoresis on cellulose acetate film (Sepraphore III - Gelman Instrument Co., Ann Arbor, Michigan) for 20 min. at 400 volts in a 0.05 M pH 7.5 sodium phosphate buffer. The enzyme activity was detected on the strip by a spray reagent consisting of 10 ml. 0.1% peroxidase (Worthington D grade), 1 ml. of 1% aqueous o-dianisidine dihydrochloride, 1 ml. ethanol, 10 ml. 0.5 M pH 7.5 sodium phosphate buffer and 28 ml. distilled water. The single zone showing enzyme activity was located about 0.8 cm. toward the anode. Procion blue staining (Fazekas de St. Groth et al., 1963) revealed one zone at the same location as the enzyme activity.

Fractionation of blood proteins with PEG was recently described by Polson et al. (1964). Fractional precipitation of alcohol oxidase with this agent is a rapid, convenient procedure for purification. The specific activity is increased about 30-fold with an overall recovery of 80%. The enzyme can be separated from PEG on DEAE-cellulose; adsorbtion of the enzyme occurs from 0.02 M pH 7.5 sodium phosphate and elution is effected with 0.1 M NaCl in the same buffer. The effectiveness of the PEG fractionation appears to be quite dependent upon the concentration of precipitable protein in the various steps. For this reason, reduction in volume is

important as the purification proceeds.

The relative rate of oxidation of several substrates is presented in Table II.

The enzyme has a broad pH optimum from pH 6 to 9. It is unstable in slightly acidic solution. The activity is completely destroyed in 3 hours at pH 5.0.

Table II

Substrate Specificity

Substrate	μM	μM H_2O_2[1]/min.	Relative Activity
Methanol	40	0.6	100
Ethanol	40	0.17	28
n-Propanol	200	0.16	5.3
n-Butanol	500	0.16	2.1
iso-Butanol	1000	0.014	0.1
sec-Butanol	1000	0.00	< 0.01

[1] Hydrogen peroxide production was measured as follows: 2.0 ml. substrate solution (20-500 μM/ml.) and 2.0 ml. peroxidase-o-dianisidine reagent [10 ml. 0.1% peroxidase (Worthington D Grade), 10 ml. 0.5 M pH 7.5 phosphate buffer, 29 ml. H_2O, and 1.0 ml. 1% o-dianisidine dihydrochloride in H_2O] were equilibrated in 100 ml. volumetric flasks under O_2 atmosphere at 25°C in a water-bath shaker set at 80 cycles/min. At zero time, 0.04 units of enzyme in 1.0 ml. of pH 7.5 buffer was added. After 5 min., the reaction was terminated by addition of 0.2 ml. of 4.0 M HCl. The color was read in a Klett-Summerson colorimeter using a No. 42 filter. The relative activity of the substrates was calculated assuming a first order reaction.

Alcohol oxidase appears to be a novel enzyme which oxidizes lower aliphatic primary alcohols to the corresponding aldehydes. Thus, from methanol the enzyme gives a reaction product which yields a positive chromotropic acid reaction, indicating formation of formaldehyde, and from other lower aliphatic primary alcohols the enzyme forms Schiff-positive reaction products. Hydrogen peroxide is formed with each of these substrates, as demonstrated by peroxidase-o-dianisidine.

Methanol is the best substrate tested, being about 3 times as reactive as ethanol, the next best substrate. Reactivity decreases as chain length of the normal primary aliphatic alcohols is increased. Secondary or branched-chain alcohols are not oxidized to any appreciable extent.

One other enzyme that oxidizes methanol preferentially has recently been described. This enzyme was isolated from a Pseudomonas species by Anthony and Zatman (1964). The properties of the two enzymes are quite dissimilar: a mediator (phenazine methosulfate) and ammonium ion are required for the Pseudomonas enzyme, while the Basidiomycete enzyme has no such requirements. The Basidiomycete enzyme appears definitely to be an oxidase in that it requires oxygen and produces hydrogen peroxide.

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