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METHOD FOR THE DETERMINATION OF CATALASE ACTIVITY IN TOBACCO

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A method has been developed whereby small samples of tobacco leaf tissue may be assayed for catalase activity by measurement of initial velocities using an oxygen analyzer. The tissue preparation includes hand-grinding with dry-ice and cell disruption in the presence of buffer in a Virtis micro homogenizer. Centrifugation yields a clear supernate containing essentially all of the activity. Velocity measurements are made on a YSI Model 53 Oxygen Monitor utilizing a Clark-type electrode. A velocity constant is obtained by making several runs at varying enzyme concentrations. Hydrogen peroxide is the substrate. Catalase activity of a given tissue is expressed as the velocity constant per unit weight of sample.

INTRODUCTION

Catalase (EC 1.11.1.6) plays an important role in the metabolism of the leaf because of its function in the recycling of oxygen from the hydrogen peroxide produced in a number of oxidative degradation reactions. Since the discovery of catalase in Connecticut leaf tobacco by Loew in 1900 (12), the determination of this enzyme in tobacco has been the subject of only a limited number of investigations (1,2,5,7,8,10,12,14,15,16,18).

Where quantitative methods have been used, these have been mainly based on manometric (2,12,16) or titrimetric (1,14) techniques. Although valid for comparative purposes, these methods lack the ability to provide the quick response necessary for measurement of the initial velocity which is required for calculation of true catalase activity (13). This type of measurement is possible using an oxygen electrode and a method has been developed whereby small samples of tobacco leaf tissue may be assayed for activity of this enzyme by observation of initial velocities using such an electrode.

EXPERIMENTAL METHODS

The method is applicable to green as well as cured and processed tobaccos. The tissue preparation consists of a series of rather simple steps designed to release the maximum amount of enzyme from the cells. First, two 200 mg samples of laminal tissue are weighed out.[1] One of these is held for moisture determination. The other is placed in a mortar and hand-ground to a powder in the presence of powdered dry-ice. When the dry-ice has evaporated the sample is transferred to the micro flask (5 ml) of a Virtis[2] homogenizer equipped with a micro shaft and blade. After 3.0 ml of 0.05 M sodium phosphate, pH 7.0, are added to the flask (a portion of this volume may be used to facilitate transfer from the mortar) the contents are blended at medium speed for 30 sec. The sample is then transferred as completely as possible to a 15 ml centrifuge tube and allowed to stand at room temperature for 60 min. At the end of this time it is resuspended by use of a vortex-type stirrer for 10 sec. and then centrifuged at 2000 rpm for 5 min. The supernate is poured through a small plug of glass wool to give a clear filtrate containing essentially all of the catalase activity. The pellet contains only trace amounts of activity.

The assay of catalase activity is performed on a YSI (Yellow Springs Instrument Company) Model 53 Oxygen Monitor, using a Clark-type electrode (4). The electrode senses changes in the oxygen content of the reaction solution as reflected in the pO_2. The oxygen concentration is indicated on a meter and displayed on a Honeywell Model 194 potentiometric recorder set at 100 MV.

The YSI system includes a water bath with four sample chambers. For this determination the bath is maintained at 30°C by means of a Lauda Model K-2 circulator and a Brinkmann Thermo-Cool heat exchanger.

All reactions are run in 0.05 M sodium phosphate buffer at pH 7.0 in a total volume of 3.0 ml. The substrate used is hydrogen peroxide prepared by diluting Mallinckrodt 30% Analytical Reagent with distilled water to give a concentration of 0.84 M (usually obtained by a 1:10 dilution). Although this concentration can be determined by titration against $Na_2S_2O_3$, in practice it is found more rapid and convenient to use the spectral method of Beers and Sizer (3) after once calibrating it against the titration. Any necessary adjustment in dilution can then be made to give the required concentration.

The sample which was held for moisture determination is dried in an oven at 95°C for 3 hr, cooled in a desiccator and weighed, the loss in weight being taken as the moisture content. The dry weight of the assay sample is then calculated from this information.

Assay Procedure:

(1) To a sample chamber in the YSI bath at 30°C add an air-saturated solution of 0.05 M sodium phosphate, pH 7.0[3], using 2.8 ml or sufficient volume to give a final total volume of 3.0 ml.

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[1]*If there is a problem in getting a representative sample, the laminal portions of a large enough number of leaves can be ground in a mill, the powder mixed well, and then the necessary 200 mg aliquots removed.*
[2]*Mention of commercial items does not imply their indorsement by the Department over similar products not mentioned.* *Contribution received Oct. 8, 1973. Tob. Sci. XVIII: 52-54. 1974.*

[3]*There is no pH optimum, activity being constant in the range pH 4-8.5 (13).*

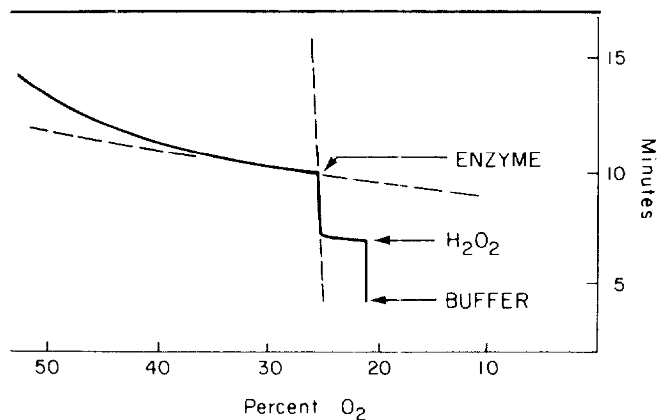
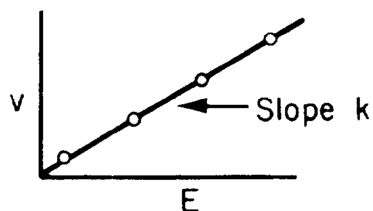


Fig. 1. Recorder trace of catalase reaction. Additions to system are made as indicated. Broken lines are tangents drawn for measurement of initial velocity.

RAW DATA $\% \ O_2 / min = v$

PLOT



ACTIVITY $K_f = \frac{kD}{W}$

Fig. 2. Procedure for handling data. v = initial velocity in % O_2/min, E = enzyme volume in μl, k = velocity constant (slope of plot), K_f = activity of sample, D = dilution and unit correction factor, W = dry weight of sample in gm.

(2) After equilibration for 3 min, insert electrode and adjust instrument to read 100% on the "Air" setting. Then switch to "O_2" setting (instrument generally will read approximately 21-22%). Set chart speed to a convenient rate such as 5 min/in.

(3) Add 100 μl H_2O_2 (0.84 M) and allow to stabilize.

(4) Add 100 μl enzyme solution (tobacco extract) and observe recorder response (**Figure 1**). The change in slope of the trace indicates the initial velocity of the reaction. Repeat the entire procedure at three lower levels of enzyme volume if the slope change is large or repeat at higher levels of enzyme volume if the slope change is small.

(5) When a minimum of four velocity measurements has been made, draw tangent lines (**Figure 1**) and determine the initial velocities as the slope of these lines in terms of % O_2/ min, with corrections being made for any blank activity.

(6) The raw data, that is, the velocities obtained from the recorder traces, are plotted against enzyme volume and a straight line is drawn (**Figure 2**). The slope of this line is k, the velocity constant. The activity of the sample, K_f, is then calculated from the relationship indicated in the figure. In practice the expression for K_f becomes:

$$K_f = \frac{\% \ O_2/\text{min}}{1 \ \mu l} \times \frac{3.0 \times 3.0 \text{ ml}}{\text{gm of tobacco}} \times \frac{1000 \ \mu l}{1 \text{ ml}}$$

RESULTS AND DISCUSSION

The key steps in the preparation of tobacco tissue for catalase determination are the hand-grinding with dry-ice and the cell disruption with the Virtis homogenizer. Other methods of cell disruption such as ultrasonics and the tissue homogenizers or blendors generally used for animal cells were unsuccessful.

The tobacco catalase solutions, prepared as above,

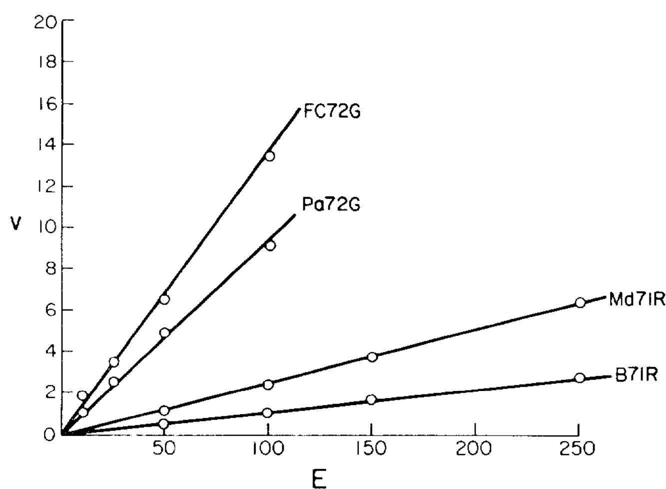


Fig. 3. Effect of enzyme concentration on initial velocity. v = initial velocity in % O_2/min, E = enzyme volume in μl. Samples used as examples: FC72G, flue-cured 1972, green; Pa72G, Penna. filler 1972, green; Md71R, Maryland 1971, redried; B71R, Burley 1971, redried.

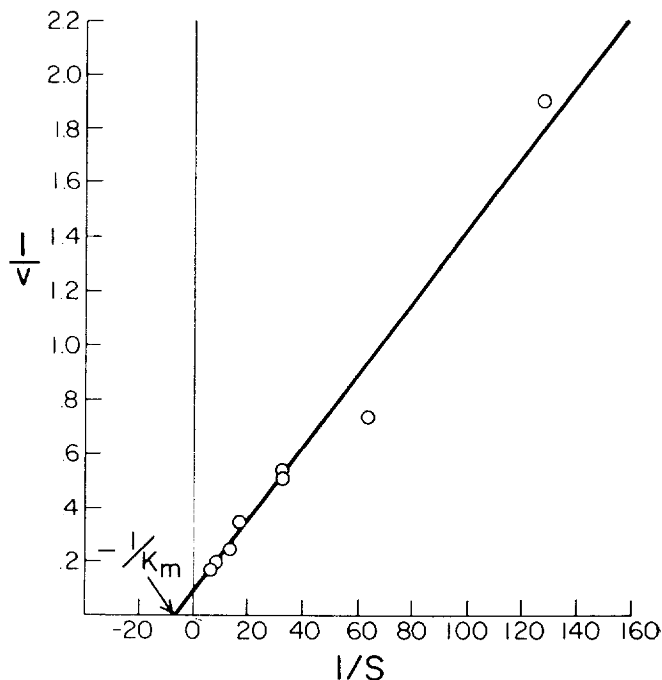


Fig. 4. Effect of substrate concentration on initial velocity. Lineweaver-Burk plot where v = initial velocity, S = substrate concentration and K_m = Michaelis constant.

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proved to be relatively stable when stored in the refrigerator. The activity decreases slowly over a period of time with 90% still remaining after seven days and 80% after 18 days.

Hydrogen peroxide is stated as the substrate of choice. This is in contrast to work by Goldstein (9) in which an oxygen electrode was used to measure catalase activity in animal tissues. Although Goldstein stated that sodium perborate or hydrogen peroxide could be used, with the former being preferred, in the present case perborate was found entirely unsuitable due to its instability in the reaction medium. Also, Goldstein recommends the addition to Triton X-100 to the reaction mixture to maximize the activity. This detergent was without effect on the tobacco enzyme.

Figure 3 shows four typical plots used to derive the velocity constant, k. It also indicates that the initial velocity of the reaction is directly related to the enzyme concentration.

The effect of substrate concentration was observed by measuring initial velocities over a range of hydrogen peroxide concentrations from 7.88 to 157 mM at constant enzyme concentration. The results (**Figure 4**) were plotted according to the kinetic treatment of Lineweaver and Burk (11). This plot shows that the concentration of substrate used in the above assay method is not at the maximum of the range and therefore the reaction being measured is not inhibited by any excess substrate. Also, the plot indicates that the Michaelis constant, K_m, for tobacco catalase is 0.145 M. This value is about five times higher than that reported in the older literature (6,17), but is comparable to values determined by the newer techniques. [Goldstein (9) reports a K_m of 0.100 M for bovine liver catalase.]

The method is applicable to an extremely wide range of catalase concentrations with K values measurable over a span of better than 1500-fold (<100 to >150,000, due to the high sensitivity of the oxygen electrode and the ability of the system to react to very dilute enzyme solutions.

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