[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Reversible Inactivation of Polyphenol Oxidase

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From both colorimetric and manometric activity measurements on polyphenol oxidase preparations from apples, prunes and mushrooms, acting on catechol, it has been found that, besides the "reaction inactivation" occurring in the presence of excess ascorbic acid, another type of enzyme inactivation (or inhibition) occurs which is reversible by ascorbic acid. The inhibiting substance reducible by ascorbic acid is not *o*-quinone or its polymerization products. Inactivation of either type can be reversed to a substantial degree by precipitating the enzyme from the reaction mixture with acetone and redissolving it.

Miller, *et al.*,[2a] studied the action of polyphenol oxidase of mushroom origin on catechol by a chronometric method[2b] in which ascorbic acid, present during the course of the reaction, prevents the accumulation of *o*-quinone. They found an inactivation which seemed to occur concurrently with the enzyme reaction, but was not due to the instability of the enzyme, *per se*, nor the reaction product, *o*-quinone. This type of inactivation therefore has been called "reaction inactivation."[3] Ludwig and Nelson,[4] in the same laboratory, had observed earlier that inactivation of the mushroom enzyme occurred in the absence of ascorbic acid, and was not reversible by ascorbic acid. It is the purpose of this paper to describe a type of polyphenol oxidase inactivation which is reversible by ascorbic acid and thus different from that previously described. Both types of inactivation are shown to be at least partially reversible by recovering the enzyme from the reaction mixture through acetone precipitation.

Experimental

Enzyme activity measurements were made both by the manometric method (oxygen uptake) and the colorimetric method described by Ponting and Joslyn.[5] The latter was modified as follows: catechol concentration was increased to 0.1 *M*, the final volume was reduced to 10 ml. from 23 ml. and readings were made in an Evelyn colorimeter with the 400 mμ filter instead of the 420 mμ filter, since the absorption maximum of *o*-quinone, the colored reaction product, has been shown by Mason[6] to be near 400 mμ. In the manometric method a total volume of 3–5 ml. of liquid was used in 50-ml. flasks on Summerson-type differential manometers. In order to avoid enzyme inactivation due to dilution during temperature equilibration, as encountered by Ludwig and Nelson,[4] the concentrated enzyme solution was added to the side-arm and tipped in after equilibration. The usual procedure was to tip approximately 0.5 ml. of enzyme solution into 3 ml. of 0.1 *M* acetate buffer, *p*H 5.0, containing 0.1 *M* catechol and 20 mg. of ascorbic acid if the latter was used. The center cups contained 0.2 ml. of 20% KOH on filter paper to absorb CO_2. Temperature was maintained at 25°. In certain experiments, polyphenol oxidase was precipitated from the reaction mixture by adding 2 volumes of acetone precooled to −20°, then centrifuged out and redissolved in buffer.

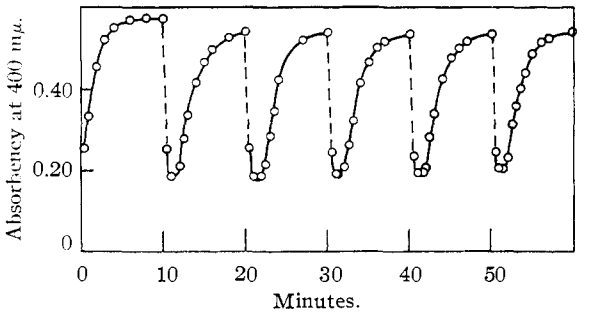

Fig. 1.—Product inhibition of color formation in a polyphenol oxidase–catechol mixture and its reversibility by ascorbic acid: 0.1 ml. of partially purified apple enzyme preparation + 10 ml. of 0.1 *M* catechol in 0.1 *M* acetate buffer, *p*H 5.0; 0.05 mg. of ascorbic acid added in 0.05 ml. of water at 10, 20, 30, 40 and 50 minutes.

Results and Discussion

Figure 1 shows the normal rapid falling off of the reaction rate as measured by the colorimetric method, using a partially purified apple polyphenol oxidase preparation. It also shows that the inhibition can be reversed completely by adding sufficient ascorbic acid to remove most of the *o*-quinone. This does not imply that *o*-quinone is responsible for the inhibition, however, because it was found that the addition of synthetic *o*-quinone does not affect the rate of quinone formation in an enzyme–catechol mixture. Evidently a reaction product (possibly a semiquinone) is formed which inhibits the enzymic reaction and which is reducible by ascorbic acid. With this enzyme preparation "reaction inactivation" did not occur as is evident from the constancy of the slopes after addition of ascorbic acid. It is believed that failure of "reaction inactivation" to occur was due to the large amount of impurities in the enzyme preparation. Calculations showed that in this experiment oxygen was not a limiting factor.

In Fig. 2 are shown colorimetric curves for purified prune and mushroom[7] enzymes. With these enzyme preparations there was apparently both inhibition reversible by ascorbic acid and also reaction inactivation, since the slopes of the color formation curves fell off rapidly with succeeding additions of ascorbic acid. A possible alternative explanation is that there is only one type of inactivation which is partially reversible by ascorbic acid. Dehydroascorbic acid has been found previously[5] to cause no inhibition of polyphenol oxidase in the concentration which might be present.

The results of manometric measurements of oxygen uptake with prune enzyme both in the presence and absence of ascorbic acid are shown in Fig. 3. It is evident that the presence of excess ascorbic acid

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

(2) (a) W. H. Miller, M. J. Mallette, L. J. Roth and C. R. Dawson, THIS JOURNAL, **66**, 514 (1944); (b) W. H. Miller and C. R. Dawson, *ibid.*, **63**, 3375 (1941).

(3) I. Asimov and C. R. Dawson, *ibid.*, **72**, 820 (1950).

(4) B. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).

(5) J. D. Ponting and M. A. Joslyn, *Arch. Biochem.*, **19**, 47 (1948).

(6) H. S. Mason, *J. Biol. Chem.*, **181**, 803 (1949).

(7) A commercial mushroom tyrosinase preparation obtained from Mann Biochemical Co.

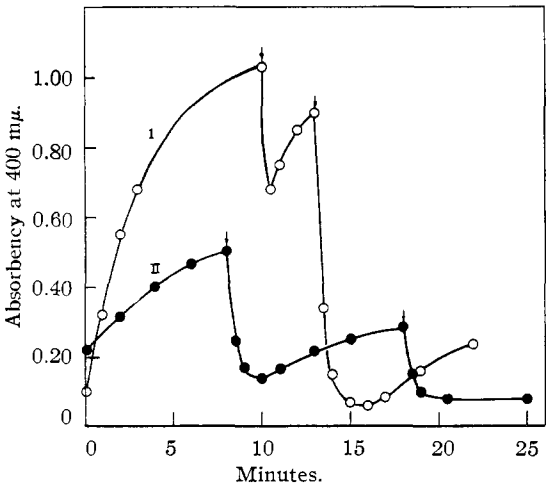

Fig. 2.—Showing both reversible inhibition and reaction inactivation: curve I, purified prune polyphenol oxidase; curve II, commercial mushroom polyphenol oxidase (tyrosinase). Arrows indicate addition of ascorbic acid solution containing 1 mg./ml.; 0.05 ml. added at 10 min. (curve I) and at 8 and 18 min. (curve II), 0.10 ml. added at 13 min. (curve I). Other conditions as in legend for Fig. 1.

delays enzyme inactivation and allows a greater amount of oxygen uptake before inactivation (compare curves I and V). This indicates that the type of inhibition portrayed in Fig. 1 has been prevented by the ascorbic acid and only "reaction inactivation" is occurring. In the absence of catechol the rate of oxygen uptake of ascorbic acid plus enzyme was the same as that of ascorbic acid alone.

It also may be seen in Fig. 3 that, regardless of which type of inactivation occurs, inactive enzyme may be restored to activity by precipitating it from the reaction mixture with cold acetone, redissolving it in buffer and adding catechol (curves I and III). Complete regeneration of the enzyme activity is not to be expected, of course, due to the unavoidable loss accompanying precipitation. However, recoveries are usually 50–70% of the original activity, which compares favorably with recoveries in ordinary precipitations for purification purposes. This is in contrast to the report of Nelson and Dawson[8] that inactivation is irreversible, in the case of mushroom enzyme. These workers, however, did not attempt recovery of their enzyme from the reaction mixture.

(8) J. M. Nelson and C. R. Dawson, *Advances in Enzymol.*, **4**, 99 (1944).

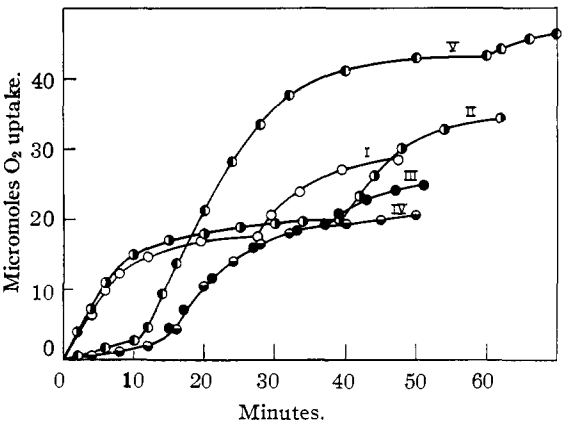

Fig. 3.—Effect of products on polyphenol oxidase–catechol reaction, measured manometrically, in the presence and absence of ascorbic acid: curve I, 0.6 ml. of prune polyphenol oxidase added to catechol at zero time, precipitated from reaction mixture at 27.5 min. with acetone, redissolved in original volume and added to fresh catechol; curve II, control for curve I; 0.6 ml. of enzyme added to catechol at zero time, not precipitated from reaction mixture; additional enzyme (0.6 ml.) added at 39 min.; curve III, 0.2 ml. of enzyme, excess ascorbic acid (20 mg.); enzyme added at 12 min., precipitated from reaction mixture with acetone at 37 min., redissolved in 0.5 ml. of buffer and added to fresh catechol and ascorbic acid; curve IV, control for curve III; 0.2 ml. of enzyme, not precipitated. Curve V, 0.5 ml. of enzyme added to catechol + excess ascorbic acid at 10 min., precipitated from reaction mixture with acetone at 60 min., redissolved in 0.5 ml. of buffer and added to fresh catechol and ascorbic acid.

Ludwig and Nelson[4] are confirmed in their finding that *o*-quinone or its polymerization products do not enter into the inactivation, because addition of fresh enzyme to the quinone-containing reaction mixture after inactivation is complete (Fig. 3, curve II) causes oxygen uptake to resume at the original rate. Addition of fresh catechol to such a reaction mixture causes no increase in oxygen uptake; therefore the reaction has stopped only for lack of active enzyme.

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