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Double behaviour of o-diphenol:O_2 oxydoreductase from apricot

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In the research on phenolase complex and, more precisely, on o-diphenol : O_2 oxydoreductase it is not unusual finding two or more substances with enzymic activity in the same tissue and it is actually a matter of discussion whether they are different enzymes or modified forms of the same enzyme.

Along the purification of the enzyme from tea, BENDALL and GREGORY (1) found several active forms that, they assume, are modifications of only one enzyme. SMITH and KRUEGER (5) and BOUCHILLOUX, MCMAHILL and MASON (2) found respectively 4 and 5 different tyrosinases in mushroom and Smith and Krueger support that all of them may occur in the intact tissue. KERTESZ and ZITO (4) also working with enzyme from mushroom found, besides a light component that they consider an inactive depolimerization product, two active forms, whose one is slowly converted into the other one. In the same paper KERTESZ and ZITO consider probable that the different tyrosinases found by SMITH and KRUEGER and by BOUCHILLOUX et al. are modications of the same enzyme as judged, chiefly in the second case, by the similar amino acid compositions.

We want, in the present paper, to report about two types of o-diphenol : O_2 oxydoreductase from apricot and to explain the reasons why we think they are different forms of the same enzyme.

Material and Methods

The enzyme solutions were prepared by grinding in a morter in the cold room 0.5 g of apricot acetone-dried powder and 0.1 M buffer of the desired pH up to 100 ml. The extract was centrifuged at 8.000 × g for 15 min at 0° C and the supernatant used for the experiments.

All the buffers used were prepared according to the tables collected by GOMORI (3).

Enzymic activity : catechol (100 µmoles), ascorbic acid (15 µmoles), phosphate

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or citrate-phosphate buffer of the desired pH (500 μmoles as anions), and enzyme solution (1 ml) in a total volume of 8 ml are allowed to react for 3 minutes at 25° while stirred with magnetic apparatus at such speed (experimentally determined) that oxygen does not limit the rate of the reaction. The last is stopped with 5 ml of 10 % (w/v) HPO_3 and the remaining ascorbic acid titrated with 0.005 N iodine. A blank is run with boiled enzyme. Units of activity are expressed as μmoles of substrate (catechol) oxydized in one minute in the described reaction mixture. The method gives reproducible results and linear with the amount of enzyme, provided the last does not exceed of 3.5 units. In several instances along these experiments, both the volumetric and manometric methods were used and they always gave the same results.

Results and Discussion

When studying the effect of pH of the reaction mixture on the enzymic activity two sharply diferentiated curves were obtained, depending on that the enzyme were extracted with pH 5.0 phosphate-citrate buffer or pH 7.0 phosphate buffer. The alternative use of pH 5.0 citric acid-citrate and acetic acid-acetate, pH 7.0 Tris-maleate and barbital-HCl, and pH 7.5 and 8.0 Tris-HCl buffers showed that the shape of the plot pH-enzymic activity was only dependent on the pH of the extract and that with pH 7.5 or 8.0 the enzyme has the same behaviour as that with pH 7.0. The enzyme extracted at higher pH will be called A and that at lower pH B (Fig. 1). When pH between 5.0 and 7.0 buffers were used, curves of the C type were obtained; the presence of a minimum in C points to a mixture of A and B.

The only found difference between both enzymes is their behaviour versus pH; the type of positive substrate (22 natural and synthetic substrates checked) and the effect of sodium diethyldithiocarbamate, sodium azide and 8-oxyquinoline were quite similar for both enzymes.

A pH 7.0 extract (form A), after centrifuged was changed into pH 5.0 by adding 0.1 M citric acid; in this way the A enzyme was slowly (2-4 days) converted into B at 0° and in 15-20 hr. at

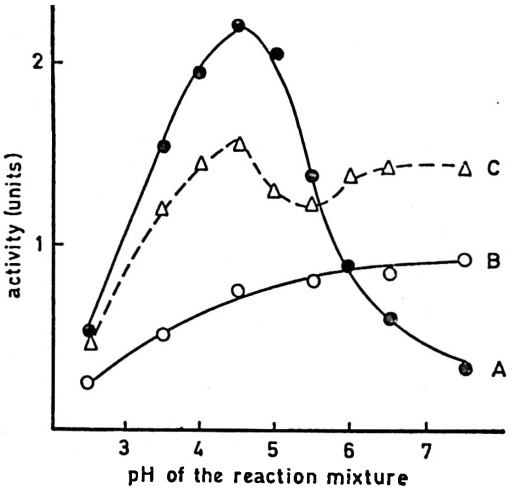

FIG. 1. Relative activity of apricot o-diphenol :O_2 oxydoreductase versus *p*H. The curves correspond to acetone-dried powder extracted with buffer of different *p*H values.
● 0.1 M citrate-phosphate, *p*H 7.0.
○ 0.1 M phosphate, *p*H, 5.0.
△ 0.1 M phosphate, *p*H 6.0.

20° while the enzyme was scarcely destroyed thanks to ist great thermal stability. The same Fig. 1 is useful to illustrate this transformation; as can be seen, the process implies that some activity has disappeared in the low pH region and some appeared in the high pH one. There are, then, powerful reasons to support that they are not two independent enzymes. The B form obtained from A was changed again into pH 7.0 by adding 0.2 M HNa_2PO_4 but no change from B into A could be observed, even after 48 hr at 20°.

When forms A and B were kept in 4 M urea without changing their original pH 7.0 and 5.0 respectively, A enzyme was converted into B in only 4-6 hr. without loss of of activity, measured the last before and after dialysis to eliminate the urea. With 0.4 M urea the change could not be observed. In no case B enzyme was changed into A.

From the urea and change of pH treatments it appears that A can be irreversiblely converted into B and that the conversion may consist of some type of dissociation.

The practical aspect of the problem demands a knowledge about which of forms A or B or both are present in the tissue, but the already demonstrated effect of the extracting solvent (even water extracts a mixture of A and B) demands for the use of not solubilized enzyme. Perhaps some information can be drawn from the following experiment: Two extracts A and B after centrifuged, as usual, yielded two residues «a» and «b». The residues are still active even after washing with their respective buffers and recentrifuged. «a» and «b» were suspended in their buffer and their activity measured at different pH values, taking care that the suspensions were homogeneous when sampling. Both residues acted in the form B.

Two possibilities stand still for A: 1) it do exist in the tissue together with B and is readily solubilized at 7.0 or higher pH, but at low pH it is changed into B; 2) (and more likely) it is not the native enzyme, but an artefact formed by some unknown way when extracted at high pH. In both cases A can be an association product of B with itself or with some other substance; one could distinguish between these kinds of association by analysis of the aminoacid contents but, unfortunately, the enzyme from apricot has resisted attempts of purification by the methods used for mushroom tyrosinases. On the other hand it is doubtful that A enzyme would remain unaltered along the purification.

One fact shows that the suspected association does not take place by the simple presence of the components, but it is in some way related to the natural state of the enzyme: B enzyme is immediately obtained in solution when extracted with pH 5.0 buffer, while transition from A into B by changing pH from 7.0 into 5.0 is slow.

Summary

By extracting o-diphenol :O_2 oxydoreductase from apricot with low or high *p*H buffers two different forms of the enzyme are obtained, as judged by their curves activity-pH.

The enzyme extracted at low *p*H can be irreversiblely converted into the high *p*H one by standing and the conversion is accelerated in the pressence of large amounts of urea, which points to some kind of dissociation.

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