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Polyphenol Oxidase in the Roots of *Cucurbita pepo* L.

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Polyfenoloxydasa v kořenech tykve

Z kořenů tykve *Cucurbita pepo* L., sorta „Kabačky", byl získán acetonový preparát, který má polyfenoloxydásovou aktivitu vůči pyrogallolu, podstatně menší vůči pyrokatechinu či DOPA. Je kompetitivně inhibován DIECA, dále KCN, není inhibován NaN_3, což svědčí pro Cu-enzym polyfenoloxydasového typu. Kvantitativní rozdíly mezi variantami „NS" a „-Ca" nebyly zjištěny, kvalitativní rozdíly, dané afinitou vůči substrátu, nejsou průkazné.

Summary

From the roots of *Cucurbita pepo* L. an acetone preparation was obtained with a polyphenol-oxidase activity towards pyrogallol, less marked towards pyrocatechin and DOPA. It is competitively inhibited by DIECA and KCN, but is not indibited by NaN_3, which is evidence that it is a copper enzyme of the polyphenol oxidase type. No quantitative differences between the two variants "NS" and "-Ca" were found nor qualitative differences expressed by the affinity for the substrate.

Introduction

In previous papers (DVOŘÁK 1960, 1962) it was reported that an enzyme is present in acetone preparations obtained from the roots of *Cucurbita pepo* L., which oxidises pyrogallol without the addition of H_2O_2. We tried to elucidate the nature of this enzyme and to compare the qualities of the enzymatic preparation from plants cultivated on a complete and Ca-deficient medium.

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Materials and Methods

The roots of *Cucurbita pepo* L. variety "Kabačky" were grown for 7 days in a complete medium without adding microelements or in a Ca-deficient medium (DVOŘÁK 1960). An acetone preparation was obtained by precipitating the protein extract in a phosphate-buffer solution at pH 8 (DVOŘÁK 1963). The enzyme-activity was tested by the direct Warburg method at 26° C; the basis was three ml. of resuspended preparation in phosphate-buffer $5 \cdot 10^{-2}$ M (30 mg per flask), with other added substances, at pH 6.8; at this pH value the enzyme activity is high and the standard value does not exceed 3 to 5 μl. O_2/10 min./1 flask. If pH rises above 7 the enzyme activity cannot be distinguished from autooxidation. 1 ml. of substrate was placed in the side-arm (0.1 M). This was added to the main contents after T = 26° C was reached. The O_2 consumption was measured during next 60 minutes; the course was linear. Blank values were not subtracted from the results.

Results and Discussion

The enzyme was identified according to its substrate specificity and to its reaction with inhibitors. Pyrogallol was found to be the only satisfactory substrate which at pH = 6.8 had a total O_2 consumption is 18 to 30 μl/10 min./80 mg of preparation. Oxidation of pyrocatechol was 11% and DOPA 7.5%. No oxidation was observed with tyrosine and acetyl-tyrosine. As the final product of the reaction we obtained purpurogallin, which can be extracted with ether.

The sensitivity of the enzyme to some inhibitors is shown on Table 1.

Tab. 1. Comparison of action of some inhibitors to polyphenol oxidase from roots of *Cucurbita pepo* L. pH = 6·8, substrate $2 \cdot 5 \cdot 10^{-2}$M pyrogallol, 30 mg. of the preparation per vessel, temp. 26° C

Inhibitor	Concentration 10^{-3}M	% of activity remaining in var.: "NS"	"-Ca"
DIECA	0.1	105	—
DIECA	2.5	34	70
DIECA KCN	2.5 + 1.0	—	7.5
KCN	1.0	34	39.5
NaN_3	1.0	100	100

With both variants the enzyme-substrate affinity expressed by the constant $K_{(S)}$ (BLADEGROEN 1955) was measured in five cases for pyrogallol concentrations: $12.5 \cdot 10^{-2}$, $2.5 \cdot 10^{-2}$ and $0.25 \cdot 10^{-2}$ M using 30 mg of preparation per vessel. The value for "NS" = $(7.22 \pm 3 \cdot 0.65) \cdot 10^{-3}$ M and for "-Ca" = $(10.3 + 3 \cdot 1.4)\ 10^{-3}$ M. The difference is not statistically significant. DIECA acts as a competitive inhibitor. By variants "NS" and "-Ca" the $2.5 \cdot 10^{-3}$ M DIECA raises $K_{(S)}$ from 8.9 to 28 and from 12.5 to 41 mM, which corresponds to $K_{(I)}$ 0·84 and 2·5 mM. No difference was observed when comparing the acitivity of polyphenol-oxidase in three parallel cultures of the variant "NS" and "-Ca".

To distinguish polyphenol oxidase from ascorbinoxidase, which also appeared active in the preparation, 10^{-4} M of DIECA was used as a selective inhibitor. (DVOŘÁK 1963). By using two substrates (ascorbic acid and pyrogallol) not only independently but also in combination it was shown that with a non-inhibited preparation no additive effect occurs when we use both substrates together. Pyrogallol was oxidised in the presence of 10^{-4} M DIECA only if ascorbic acid was not present (Fig. 1). Likewise no additive effect was observed when using hydroquinone although this substance does not prevent the oxidation of pyrogallol.

From the results obtained we can assume that the most effective inhibitor, also at a relatively high pH value, was $2.5 \cdot 10^{-4}$M DIECA, which chiefly forms Cu-complexes. On the other hand, the same inhibitor at the concentration 10^{-4} M acts selectively only towards ascorbate oxidase. JAMES (1953a, b) gives

DIECA as the most acceptable selective inhibitor for copper-oxidases. Azide, which inhibits Fe-enzymes and is less active towards polyphenol oxidase (KEILIN 1936, JAMES 1953a) does not influence the oxidase at all under our experimental conditions. The enzyme can therefore be considered as a Cu-protein type of polyphenol oxidase, but with a relatively small affinity towards the substrate, $K_{(s)}$ being approximately 10^{-2} M. In the case of tyrosinase which oxidises pyrocatechol C. WARNER (1951) gives $K_{(s)}$ for the enzyme prepared from *Psaliotta* as 2.8 . 10^{-4}M, but for the preparation obtained by the same author from potatoes 5 . 10^{-3}M, the latter value being nearer to that obtained in our experiments. We fully agree also with previous authors, that the pH is extremely important. The activity rises together with the pH value as it is possible to distinguish it from autooxidation of pyrogallol. WARNER (1951) gives an optimal pH of 7.5 for the enzyme from potatoes and *Psaliotta* and JAMES et al. (1948) 7.8 for *Atropa belladona*; but in both cases cited above the authors used pyrocatechol as the substrate.

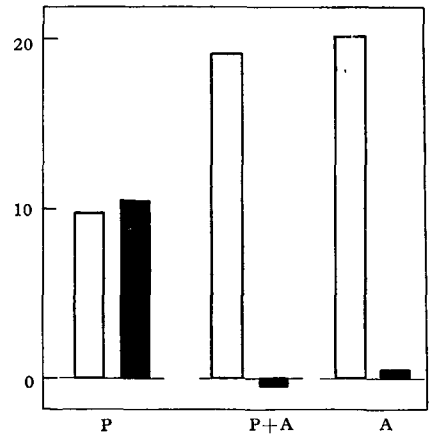

Fig. 1.

Activity of preparation when using two different substrates and 10^{-4} MD IECA as inhibitor. y = μl. O_2/10 min./20 mg. acetone preparation. Empty columns = unblocked, full columns = blocked preparations. P = pyrogallol, A = ascorbic acid. Numbers are average values from two experiments.

We made two particular observations in studying polyphenol oxidase. The first was the small ability to oxidase pyrocatechol, which is the typical substrate for this type of oxidase. When using pyrogallol as a substrate, the interference of peroxidase which is also present in the preparation, is out of question; neither peroxide nor any other preparation where oxidation could occur were added to the preparation. It is further interesting to point out the impossibility to oxidase ascorbic acid indirectly by means of polyphenol: our type of oxidase obviously does not work because of the low redox potential of the ascorbic acid.

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Полифенолоксидаза в корнях тыквы

Из корней тыквы *Cucurbita pepo* L., сорт «Кабачки» получен ацетоновый препарат, отличающийся полифенолоксидазной активностью по отношению к пирокатехину или к *DOPA*. Препарат компетитивно торможен *DIECA*, дальше KCN, не торможен NaN_3, что говорит в пользу его идентификации как Cu-энзима полифенолоксидазного типа. Количественных различий между вариантами „NS" и «—Ca» не установлено, качественные различия, определяемые сродством по отношению к субстрату недостоверны.