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BRIEF COMMUNICATION

A Suitable Method for Localization of IAA Oxidase

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Abstract. The method for histochemical detection of IAA oxidase proposed by Sagi was tested, based on colour reaction of natural products of IAA oxidation with Ehrlich-like reagent.

As IAA oxidase is called an enzyme system or complex with oxidase and peroxidase activity (Pilet and Gaspar 1968, Macháčková and Zmrhal 1974, Schneider and Wightman 1974). Theoretically, locations where oxidase and peroxidase activities are detected — especially if simultaneously — may be considered as sites where IAA oxidase is present. The IAA oxidase system can be localized either by ordinary methods for detection of oxidases and peroxidases using selective inhibition or by methods based on substrate specificity. The latter is much more convenient. Aasheim and Iversen (1972) tried to exploit the decarboxylation activity of IAA oxidase for its visualization by reaction of released CO_2 with $SrCl_2$. More promising are methods based on colour reactions which are frequently used for detection of IAA isozymes in gels. The azocoupling method of Endo (1968) seems not to be applicable for sections because of its positive reaction with proteins. Sagi (1972a) presented in his lecture a modification of Meudt and Gaines (1967) method which can also be used for sections. The products of the enzymatic oxidation of IAA are detected by an Ehrlich-like reagent. So far, however, Sagi has not published further results about his procedure. Trying to test the suitability of this method we based our experiments on the data presented in his lecture verified and supplemented by the author (Sagi, personal communication).

Seeds of *Lupinus albus* L. cv. Pflugs ultra were soaked in running tap water overnight and then allowed to germinate on perforated plastic plates covered with cotton—wool tissue. Plates were placed in closed glass jars containing water at the bottom and kept for two days in darkness at 25 °C. The jars were than transferred under continual light (fluorescent tubes Tesla, 5000 lx). Hypocotyls of 4 to 8 days old seedlings were excised and their upper part

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(3 to 5 mm under cotyledon insertion) was used to obtain 50 to 100 μm thick sections. Sections were prepared from non-fixed material using semiconductor freezing microtome Cryotom Tesla with additional cooling of the knife with solid CO_2. The material was sectioned frozen in 0.06 M phosphate buffer pH 5.6. The sections were transferred into the same buffer and used without any delay.

As mentioned, the incubation medium and detection reagent prepared according to Sagi were used. The incubation medium contained 50 μM of $MnCl_2$, 50 μM of *p*-coumaric acid and 2 mM of IAA in 1000 ml of 0.06 M phosphate buffer pH 5.6. IAA was dissolved in a minimum amount of ethanol, concentration of which in the medium did not exceed 3% v/v. The medium was always freshly prepared. $MnCl_2$ and *p*-coumaric acid solutions may be kept several days in a refrigerator. Fresh 0.5% dimethylaminocinnamaldehyde in 2 N HCl served as detection reagent. Free floating sections were incubated in the medium for 2 h on light. Then they were twice washed in 0.06 M phosphate buffer pH 5.6 and finally transferred into the detection reagent kept in a refrigerator in the dark. The positive reaction is expressed by a wine-red colour which starts to appear within a few minutes. Its density gradually increases for some time. The native preparations were made using a drop of the detection reagent.

The epidermis was heavily stained while the colour in the hypodermal layers of the cortex was much weaker. Distinct staining was also observed in the vascular bundles of the central cylinder especially in xylem. Analogical results were obtained in preliminary experiments with the hypocotyl and root tips of broad bean, *Vicia faba* L. cv. Chlumecký, and pea, *Pisum sativum* L. cv. Raman. The positive reaction in this material was also obtained in epidermis, hypodermal layers and vascular tissues of the central cylinder.

Further experiments were focused on (1) preservation of the colour product and obtaining of permanent slides, (2) elimination of false positive reaction by controls, (3) application of the incubation medium containing H_2O_2 and (4) examination of the use of fixed material.

The final colouring sooner or later disappears after dehydration in ethanol and mounting in Euparal (Pinnow, West Berlin). Similar results were obtained when dehydrated sections were mounted via xylen into damara or Canada balsam. Mounting of sections directly from the detection reagent into water media (Aquamount, Michrome, London; Hydramount, Gurr-Searle, High Wycombe, Bucks., and Synthetic gum, Koh-i-Noor, Městec Králové) also did not prevent the disappearance of the final colour. It also gradually disappears in 2 N HCl, H_2O and 0.06 M phosphate buffer pH 5.6. The best preservation of colouring was observed when sections were left in the detection reagent and kept in a refrigerator in the dark.

The validity of reaction was tested by heating of sections in water of 80 to 85 °C for 10 min, by treatment with KCN (10^{-3} M in the incubation medium) and by incubation of sections in medium lacking the substrate. All these tests gave negative results. The reaction was much weaker when sections were incubated in IAA containing medium but lacking either both or even only one activating component.

Addition of H_2O_2 (0.2 ml or 1.0 ml of 3% H_2O_2 per 10 ml of medium) resulted in a slight moderation of the reaction on sites which are heavily stained when H_2O_2 is absent.

Positive results were also obtained when frozen sections taken from fixed material were used (fixation in neutralized 10% formol for 6 h at room temperature, followed by washing material, sectioning and section preservation in 5% ethanol, see BENEŠ 1973).

The present results show that the IAA oxidase system can be detected by the described procedure. It is, however, necessary to note that reliability of results is conditioned by simultaneous application of controls, especially of the substrate lacking medium. The material used in this communication can be recommended as standard. The localization is possible to evaluate only on a cellular level. When the subcellular level is considered the diffusion of reaction products has to be taken into account. The diffusion cannot be completely eliminated also on the cellular level as suspected with the weak staining of the hypodermal layers. Another problem is, especially in the case of non-fixed material, the different solubility of individual fractions of IAA oxidase (SAGI 1972b). The failure to obtain permanent slides is a serious handicap to the procedure.

As found by PENNY *et al.* (1972), the growth promoting effect of externally applied auxin on lupine hypocotyl segments is limited by reaction of few cell layers on the hypocotyl surface. It is interesting that, using the present method, high IAA oxidase activity was found just in this critical region.

Oxidases and peroxidases are commonly detected by means of synthetic chromogenic substrates. Also in these cases it is possible to consider the activity of the IAA oxidase system. It should be stressed that the method of MEUDT and GAINES (1967) as modified and recommended by SAGI (1972a) and tested in the present experiments, involves oxidation of the natural substrate and detection of natural products of its oxidation. The use of this approach in histochemistry of this group of oxidoreductases is very rare. The present procedure is interesting from this point of view, too.

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