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A Quantitative *in vitro* Study of Non-specific Esterase in Carrot Taproot Explants Using a Simultaneous Azocoupling Method

LUDMILA BEDRNÍKOVÁ and K. BENEŠ

Institute of Experimental Botany, Czechoslovak Academy of Science, Praha*

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Abstract. A simultaneous azocoupling reaction used for *in situ* localization of non-specific esterase may be applied even for quantitative *in vitro* measurements. The developed standard procedure was verified with regard to substrate concentration, inhibitory effect of diazonium salt, incubation time, stability of resulting dye, relation between the amount of dye and the amount of material. On HW medium, non-specific esterase activity increases considerably both in phloem and xylem explants from *Daucus carota* L. taproot. The increase of enzyme is higher and appears earlier than the increase of fresh weight and dry matter, especially in the explants of phloem.

This paper is an extension of those by FOSKET 1964, FOSKET and ROBERTS 1965, BENEŠ *et al.* 1967 on callus initiation in carrot taproot explants and has 2 aims: 1. to prove whether it is possible to use for quantitative *in vitro* studies a simultaneous azocoupling reaction, used frequently for *in situ* localization, 2. if proved, to use that technique for measurements of non-specific esterase activity in carrot taproot explants during callus initiation.

Material and Methods

Experimental material was tissue discs and explants of carrot taproots (*Daucus carota* L. cv. Nantéská polodlouhá)**. To prove the method employed, tissue discs or homogenates of them taken under non-sterile conditions in the same way as when starting the culture of explants were used. The explantation for tissue cultures was performed in the following way. The middle third was taken from a selected washed root and transferred into a sterile box. All further handling was done aseptically. The root part was sterilized for 20 min by undiluted Famosept (Spofa)***, rinsed several times in H_2O and divided again into 3 parts about 1 cm thick (apical, middle and basal part). From these parts, cylinders were cut by a cork borer, at least 2 from xylem and 2 from phloem (both are secondary tissues). Using a device made

* *Address:* Ke dvoru 15, Praha 6 - Vokovice, Czechoslovakia.

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*** 2‰ hydrargyrum phenyl boricum in water

of razor blades and plastic plates the cylinders were cut into discs about 1 mm thick. 6 discs from each cylinder were placed into 1 Petri dish, the apical end on the surface of the medium. The same semi-solid medium was used as previously (HW and NA, BENEŠ *et al.* 1967) but with natural coconut milk, sterilized 2 × 30 min. at 100 °C in a vapor sterilizer. The whole medium was again sterilized in the same way. The cultures were kept in the dark at 26 °C. Fresh weight, dry matter and non-specific esterase activity were measured every second day, starting with the 1st day of culture up to the 17th, taking the day of setting cultures as 0. Three different roots were used in each experimental series. For measurements performed every second day, the explants of one part 1 cm thick of a given root were taken, *i.e.* 4 Petri dishes: 1. explants from phloem on HW medium (HWP), 2. xylem on HW (HWX), 3. explants from phloem on NA medium (NAP), 4. xylem on NA (NAX). Having 6 explants in each Petri dish, 3 were weighed (fresh weight) and then allowed to dry at 80° C up to constant weight (dry matter). The other 3 explants were used for measuring enzyme activity as given later. Since the differences are smaller between different 1 cm thick parts of the given root than between different roots, one part 1 cm thick of root No. 1 was used the 1st day, root No. 2 the 3rd day, root No. 3 the 5th day, root No. 1 the 7th day, root No. 2 the 9th day etc.

If not otherwise stated, throughout the whole work the following standard assay was applied, which is a result of a series of preliminary experiments not reported here. For every series of experiments sufficient quantities of the following solutions were prepared and stored: phosphate buffer pH 6·4 (10 g $NaH_2PO_4 . H_2O$ and 10 g $Na_2HPO_4 . 12 H_2O$ in H_2O to 1000 ml final volume; further only "buffer"), 1% α-naphthyl acetate in dimethylformamide, chloroform-ethanol 2 : 1 v/v. 0,5% Diazoechgranat GBC (Rohner) in buffer was freshly prepared before each use. One explant was homogenized in a glass homogenizer containing 5·5 ml of buffer. The homogenate was poured into a test-tube and the homogenizer washed with 10 ml of buffer, which was added to the homogenate. Then, 4 ml of diazonium salt solution were pipetted into the diluted homogenate. The incubation started with the addition of 0·5 ml of substrate solution followed by vigorous shaking of the entire mixture. The shaking was repeated several times during the incubation period, which lasted 20 min. at 22°C. To finish the incubation, the content of the test-tube was poured in a cylindrical separatory funnel containing 40 ml of chloroform-ethanol. The funnel was shaken thoroughly. When the funnel had been shaken several times, the watery phase became completely colourless. The organic phase was allowed to flow into a test-tube, left to clear and after about 2 hours, the dye content was measured using the Lange Modell IV colorimeter with the VG9 glass filter (green). The medium without substrate and the complete medium without homogenate containing corresponding amounts of dimethylformamide and buffer instead, were used as controls to exclude unspecific reactions due to phenolic substances and free napthol. The mean values of "without material" controls were subtracted from the mean of the readings taken.

For the following experiments proving the standard assay either discs of fresh tissue or homogenates of them were used; one ml of homogenate contained material of 1 tissue disc. Using the homogenate, the differences

between individual discs were excluded. One ml of this homogenate was added to 14·5 ml of buffer instead of homogenizing single discs separately.

The dependence of the amount of dye formed on substrate concentration was assayed as follows: into single test-tubes 0·5 ml was pipetted of 0·125%, 0·250%, 0·500%, 1·000%, 2·000% substrate solutions. Incubation was for 60 min.

The inhibitory effect of the diazonium salt used was tested comparing simultaneous azocoupling and postcoupling. In the latter case, diazonium salt solution was added 3 min. before the end of incubation, which lasted 40 min. altogether.

To follow the time course of the enzyme reaction, the incubation of single test-tubes was stopped after 5, 10, 20, 30, 40, 60 and 80 min. incubation.

Verifying the stability of the resulting dye, the absorbancy was measured repeatedly 1, 2, 3, 4 and 24 hours after the end of incubation. The coloured organic phase was kept free in the laboratory in a well closed test-tube.

The experiment on the relation between the a mount of dye formed and the amount of homogenate was of crucial importance. Keeping the final volume (20 ml) constant, 0·5; 1·0; 2·0; 3·0; 4·0 ml of homogenate was pipetted into the incubation medium. Incubation time 15 min.

Results

The preliminary experiments performed to develop the standard assay will not be reported here. Concerning those established to prove it, the results of one of several runs will be given of the following topics: the dependence of the amount of dye formed on substrate concentration, the inhibitory effect of diazonium salt, the time course of enzyme reaction, the stability of the resulting dye, the relation between the amount of dye formed and the amount of homogenate.

Fig. 1 proves that the amount of dye formed depends on substrate concentration. In the standard assay, that amount of substrate is used which results in the highest absorbancy. The addition of 2% substrate solution to the medium caused high turbidity.

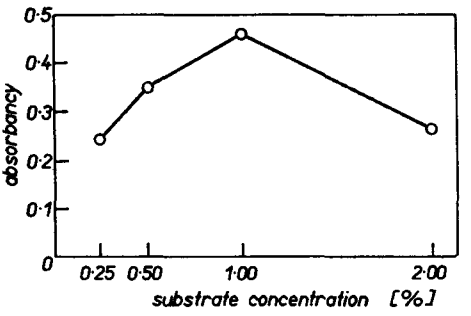

Fig. 1. The dependence of the amount of dye formed (absorbancy) on the concentration of substrate solution (%).

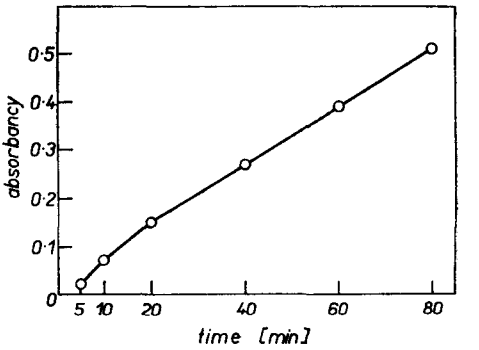

Fig. 2. Time course of enzyme reaction. The dependence of the dye formed (absorbancy) on the incubation time (min.).

The presence of diazonium salt caused an inhibition of enzyme activity. Using simultaneous azocoupling absorbancy 0·228 resulted in one experiment, and 0·266 in the other, while the corresponding values were 0·556 and 0·450 when adding the diazonium salt at the end of incubation.

Fig. 2 demonstrates the time course of enzyme reaction. The 20 min. incubation time is long enough to compensate for some inaccuracy at the

TABLE 1

THE STABILITY OF THE DYE FORMED. The values of absorbancy of solutions No. 1, 2, 3, 4, 5 are not seriously changed up to 24 hours after the end of incubation. The measurements of the controls "without material" taken simultaneously are also included.

No.	hour				
	1	2	3	4	24
1	0·32	0·33	0·33	0·32	0·30
2	0·23	0·23	0·23	0·23	0·22
3	0·26	0·26	0·27	0·27	0·26
4	0·27	0·28	0·28	0·27	0·26
5	0·26	0·27	0·26	0·26	0·24
Control	0·14	0·14	0·15	0·14	0·13

beginning of incubation, while the linearity still holds when increasing incubation time four times.

The results of an experiment on the stability of the dye formed are given in Table 1. No serious differences were revealed when the same solution was measured up to 24 hours after the end of incubation. The uniformity of individual tissue discs follows from the same Table.

Fig. 3 shows the results of an experiment on the relation between the amount of dye formed and the amount of material. It is apparently linear within the given limits.

It is necessary to say that controls of both types were highly uniform throught the whole work, giving an absorbancy of about 0·04 in the case of "without substrate" controls and about 0·13 in the case of controls "without material".

The increase of fresh weight, dry matter and non-specific esterase activity of phloem and xylem explants, cultured on HW and NA medium is shown in Figs. 4a, b, c. Neither phloem nor xylem explants form callus on NA

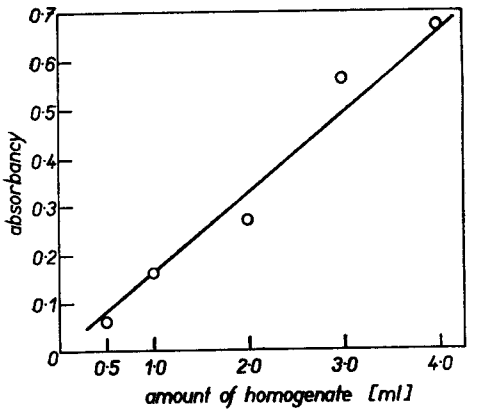

Fig. 3. The relation between the amount of dye formed (absorbancy) and the amount of homogenate(ml.).

medium, whereas on HW medium, callus is formed in both cases. Thus, both phloem and xylem respond in the same way to the culture medium, but phloem reacts somewhat earlier. In general, the increase of enzyme follows the increase of fresh and dry weight (see the differences between individual roots in the case of HWX, Fig. 4a, b, c). But the increase of enzyme activity precedes that of fresh and dry weight and is more vigorous: enzyme activity increases several times during 9 days of culturing of HWP whereas dry matter increases about twice and fresh weight still less. This may be seen in Fig. 5, which gives mean values of 3 independent series of experiments. The apparent decrease even of dry matter to the end of the period studied (see Fig. 4b) seems to be

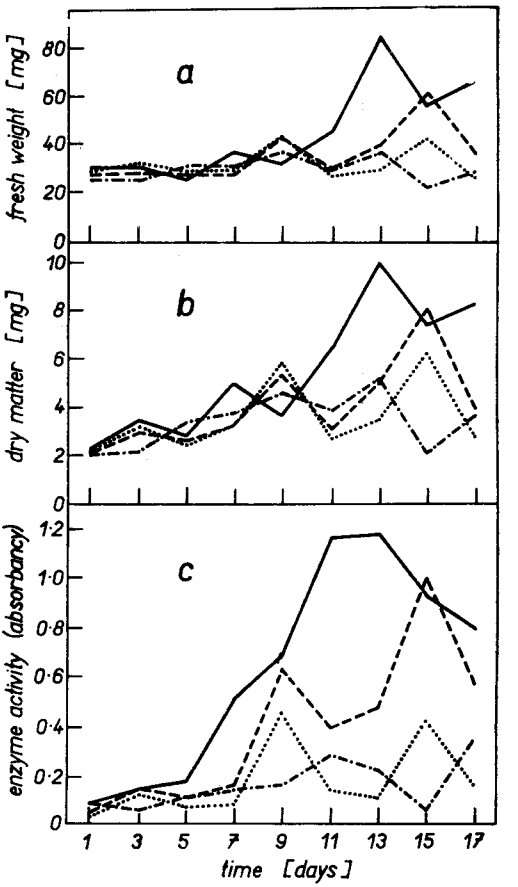

Fig. 4. The increase of fresh weight, dry matter and non-specific esterase activity of explants during culturing (days): a — fresh weight (mg), b — dry matter (mg), c — enzyme activity (absorbancy). —.—. — NAP, ... NAX, ——— HWP, — — — HWX. Results of one series of experiments.

due to variations of callus development. According to our preliminary results, the soluble proteins content follows the dry matter increase quite closely.

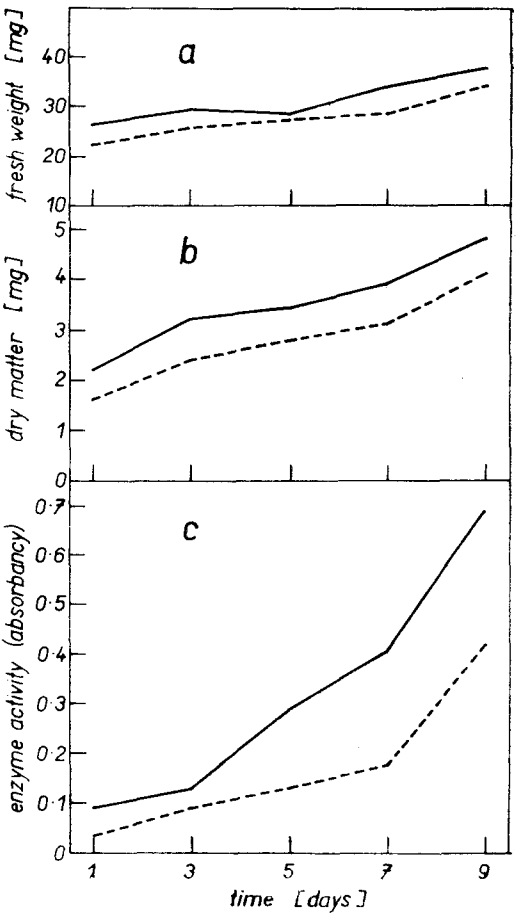

Fig. 5. The increase of fresh weight, dry matter and non-specific esterase activity at the beginning of cultivation (days): a — fresh weight (mg), b — dry matter (mg), c — enzyme activity (absorbancy). ——— HWP, — — — HWX. Means of 3 series of experiments.

Discussion

The development of callus tissue on carrot taproot explants has been described by several authors (see GAUTHERET 1959). Concerning our experiments, no hair-like formations (Wundhaare) appeared, visible to the naked eye (*cf.* BARTOŠ 1954, PETRŮ 1954). On HW medium, the formation and increase of callus was continuous without any signs of necrosis, whereas no callus was formed up to 45 days of culturing on NA medium.

It seems that there is a direct relationship between the development of the histochemical azocoupling methods for *in situ* localization of hydrolytic enzymes and the biochemical methods for measuring hydrolase activity with phenol esters as substrates. Together with many azocoupling methods for *in situ* studies, methods of the same type for *in vitro* measurements were elaborated, making possible a parallel assay on a slide and in the test-tube, sometimes using even the same incubation medium. While this possibility was fully used by some authors (NACHLAS and SELIGMAN 1949a, b, SELIGMAN and NACHLAS 1950, GOMORI 1953, RAVIN and SELIGMAN 1953, KRAMER *et al.* 1963, 1964), other authors have argued against the use of

azocoupling for quantitative enzyme assay (LAGUNOFF and BENDITT 1961). The fact that in vitro azocoupling methods are quoted not only in handbooks of test-tube histochemistry (GLICK 1961/1963) but even in manuals of biochemistry and enzymology respectively (COLOWICK and KAPLAN 1955, BERGMEYER 1965) demonstrates that the arguments against the use of azocoupling methods for quantitative test-tube assays have not been accepted. The possibility of parallel slide and test-tube assay seems to be an important advantage of azocoupling methods. The methods of the Gomori-Takamatsu type lack of this possibility and a colorimeter test-tube assay of indigogenic type has been developed relatively recently (TSOU and SU 1965). Indoxyl esters were used for *in vitro* studies in Holt's laboratory but the measurements were manometric (see BENEŠ 1971, in preparation). From the point of view of enzyme assay only, the non-coupling methods with naphthyl esters as substrates are more rational and simple (FRIEDMAN *et al.* 1966, LAGUNOFF and BENDITT 1961).

It is the aim of the present paper to prove whether a method used for non-specific esterase localization could be used even for test-tube measurements, not to develop a quantitative assay as such. Thus, it is in connection with some of the mentioned papers from Seligman's laboratory and should be taken as an extension of our previous work, based on the results of FRIEDE and KNOLLER 1965, performed in Roberts' laboratory (BENEŠ *et al.* 1967).

The term non-specific esterase is used with some doubt in this paper. Taken broadly, the term esterase designates the whole subclass of hydrolytic enzymes. In the narrow sense, it comprises enzymes hydrolyzing esters of organic acids (fatty acids, carbonic acids — hence carboesterase, carboxylesterase). If not otherwise stated, the term esterase is used here in the latter sense. Within esterases (as defined in this way) different groups can be distinguished. Since different criteria are used for this purpose, the classification and terminology is rather obscure. Concerning substrates, non-specific and more or less specific esterases were distinguished. In this sense the designation non-specific esterase is apparently used. The azoesterases or azolesterases were distinguished from others; they attack N-containing substrates more or less specifically. Lipases were defined as esterases preferring long chain substrates, being more active on substrates in suspension than in solution. The question was also raised, whether some esterases do not act differently on glycerides and on "simple" esters. In contrast to the term aliesterase the term arylesterase was used for enzymes preferring esters of phenol, naphthol *etc.* PEARSE (1960) used the name aromesterase. Recently, the use of activators and inhibitors is the most important tool for esterase classification. An attempt to bring together results and views of different approaches to esterase classification was performed by PEARSE (1960). There are several more recent attempts of this sort in histochemical literature, taking into account different molecular forms of enzymes (ERÄNKO *et al.* 1962, 1964, MIETKIEWSKI and MALENDOWICZ 1967, VOGEL 1967, YOSHIMURA *et al.* 1969). Otherwise, see SUMNER and SOMMERS 1953, HOFFMANN-OSTENHOF 1954, DIXON and WEBB 1958, BOYER, LARDY, MYRBÄCK 1959/1963, FLORKIN and STOTZ 1965, BARMAN 1969, AUGUSTINSSON 1964, HOFSTEE

1964. In recent papers dealing with non-specific esterase in plant material from different viewpoints but systematically, the problem of classification and terminology was not raised (see *e.g.* SCHWARTZ *et al.* 1964, SCHWARTZ 1965, DESBOROUGH and PELOQUIN 1967). It was regarded necessary to mention this problem here. It is generally known that the substrate used in the present work may be hydrolysed by peptidases. On the other hand, it is worth mentioning that Fremy discovered pectase (*i.e.* pectin esterase) just in carrot (KARTÉSZ, see SUMNER and SOMMERS 1953). Many plant esterases which may hydrolyse α-naphthyl acetate are known. It seems less probable that the activity revealed is caused by some specific esterases or by lipases. The presence of an enzyme similar to acetylesterase from citrus fruits or to esterase from wheat germ seems to be more probable.

Concerning the results of experiments on verification of the standard assay, the optimal — under the given conditions — substrate concentration is used.

The inhibitory effect of diazonium salt does not seem to be an acute hindrance in the use of the standard assay. But it must be taken into account when assuming the increase of enzyme activity as new enzyme synthesis (see later): if the newly formed enzyme is of different molecular form, it may be differently sensitive towards the diazonium salt.

The incubation time was made long enough in respect to incubation time inaccuracies, when processing more samples in the same run. No shortage of substrate and diazonium salt was revealed when prolonging incubation time up to four times the standard.

The stability of the dye was verified. Other characteristics of the dye are known (FRIEDE and KNOLLER 1965). The clearing of the slight turbidity of final dye solution may cause difficulties in some cases.

The results of the experiments on the relation between the amount of dye formed and the amount of material proved the possibility of using the standard assay for non-specific esterase activity measurements.

In a series of papers, enzyme activity was measured in maturing and differentiating tissue, mostly of root tips. In the present work, non-specific esterase activity was followed in material comprising a considerable part of meristemizating and dedifferentiating cells. While many papers deal with the comparison of original and callus tissue, few only are devoted to the study of callus formation. It is of interest that concerning callus initiation, no considerable differences were seen between phloem and xylem in the present study, which corresponds to Gautheret's findings (see BEERMAN *et al.* 1966). It seems that the increase of non-specific esterase activity is quite similar to that of acid phosphatase, reported previously (BENEŠ *et al.* 1967). Since fresh weight and dry matter were followed together with non-specific esterase, it is possible to conclude that in spite of the same general tendency, the increase is not proportional: the enzyme increases earlier and more. Enzyme synthesis seems to be involved but no proof is given here. An increase in hydrolytic enzyme activity (regardless of synthesis or activation) does not seem to be a general phenomenon of the first days of culturing explants (BESEMER 1968).

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LUDMILA BEDRNÍKOVÁ, K. BENEŠ, Ústav experimentální botaniky ČSAV, Praha: **Kvantitativní *in vitro* stanovení nespecifické esterasy v explantátech z kořene mrkve simultánní azokopulační metodou.** — Biol. Plant. **13** : 224—233, 1971.

Bylo zjištěno, že simultánní azokopulační reakce běžně používané k stanovení nespecifické esterasy *in situ* lze použít i ke kvantitativnímu stanovení *in vitro*. Byla ověřena optimální koncentrace substrátu, inhibiční účinek diazoniové soli, vhodná délka inkubace, stálost vzniklého azobarviva a posléze vztah mezi množstvím barviva a množstvím enzym obsahujícího materiálu. Bylo zjištěno, že během 17 dní kultivace na HW mediu aktivita sledovaného enzymu silně vzrůstá u explantátů ze sekundárního floemu i xylemu. Tento přírůstek aktivity enzymu začíná dříve než přírůstek čerstvé váhy a sušiny a je zprvu značně vyšší.