Dynamics of indole-3-acetic acid oxidase activity in suspension culture of sunflower crown-gall

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Abstract

IAA oxidase activity was determined in several growth phases of a suspension culture of sunflower crown-gall. During the short phase of intensive growth (zero passage — PO) a negative correlation was noted between enzymatic activity and the rate of growth. IAA oxidase activity increased to a certain level is not a factor limiting cell division. For protraction of the phase of intensive growth (first passage — P1), however, a decrease in the activity of this enzyme seems indispensable. IAA oxidase activity in the tested culture is under the control of inhibitors present in the cells and medium. High enzyme inhibition was observed in PO cells during the phase of intensive growth and in P1 at the beginning and in the middle part of this phase. These results suggest that the auxin level determined in earlier studies in sunflower crown-gall culture is controlled by the IAA oxidase set. During the long phase of intensive growth (P1) this control is of negative feedback type.

Key words: crown-gall, sunflower, suspension culture, IAA-oxidase

INTRODUCTION

The excessive auxin content in crown-gall tumours and the ability of sterile tumour tissues to synthesise auxin in an *in vitro* system has been proved (Sequeira 1973, Davies et al. 1975). It was believed at first that the high auxin level in tumours is caused by the low IAA oxidase activity in their cells (Lipetz 1959). When it appeared, however, that tumour tissues release into the medium large quantities of enzymes decomposing IAA (Lipetz and Galston 1959, Witham

Abbreviations: IAA — indole-3-acetic acid; IAA-oxidase — indole-3-acetic acid oxidase; PVP — insoluble polyvinylpyrrolidone, Polyclar AT; PO — passage O; P1 — first passage.

and Gentile 1961), a high biosynthetic activity of the tumours was assumed as the cause of this high level. As regards genetic tumours, it is also considered that a derepression of genes of both IAA oxidase synthesis and of IAA-synthesising enzyme occurs (Kovács and Maliga 1973).

Stonier (1969) advanced the concept of "auxin protectors". These compounds produced by normal meristematic as well as tumour tissues, acting as antioxidants protect auxin. A relation was found between the development of crown-gall and protector activity (Stonier 1969). A positive correlation between the IAA level and protector activity in sunflower crown-gall cells in suspension culture was observed by Atsumi and Hayashi (1978). Syono (1979) noted simultaneously that tobacco callus strains with a tendency to anergisation are characterised by a lower activity of enzymes destroying IAA and a higher content of inhibitors of these enzymes.

These data are evidence of the existence of a complex system of auxin level regulation in tissues characterised by autonomous growth. This system comprises, on the one hand, IAA-degrading enzymes and factors influencing their activity, and on the other, auxin protectors. The mechanism of functioning of this system still remains obscure.

Earlier investigations of sunflower crown-gall culture (Chirek 1983) suggest that the growth activity of this culture depends on the auxin level (especially IAA) in the cells, and also on the specific equilibrium between auxin activity in the internal and the external cell medium. In order to gain a better knowledge of the factors regulating these relations the present paper presents the dynamics of IAA oxidase activity in the cells and medium of the same culture.

MATERIAL AND METHODS

Sunflower (*Helianthus annuus* L. cv. Borowski prażkowy) crown-gall suspension cultures were initiated from tissues maintained in agar culture since 1978. Murashige and Skoog (1962) liquid medium with an addition of thiamin in the amount of $1 \text{ mg} \cdot \text{dm}^{-3}$ was used. The method of culture and growth estimation have been described earlier (Chirek 1983). The determinations were performed in the zero passage PO — intermediate between tissue culture and cell culture and in the first passage P1 — representing cell culture proper.

IAA OXIDASE EXTRACTION

A weighed 2-g sample of cell mass filtered off from the medium was ground cold in a mortar with 10 cm^3 of 0.2 M Na-phosphate buffer, pH 6.1 (Hagen and Minch 1966). The homogenate was centrifuged at

4°C for 15 min at 15 000×g. The supernatant (S) was used immediately for IAA oxidase activity determination. Determinations were also performed in tenfold diluted supernatant (S-10) and in an extract prepared with the use of PVP (5 g of hydrated PVP per 1 g of cell mass). PVP hydration was done according to the procedure described by Knypl and Chylińska (1974).

IAA OXIDASE ACTIVITY DETERMINATION

The reaction mixture was prepared according to Galston and Dalberg (1954) in flasks wrapped in aluminium foil: 2.5 cm³ of Na-phosphate buffer (0.2 M, pH 6.1); 1 cm³ of 2,4-dichlorophenol (10⁻³ M); 1 cm³ of MnCl₂ (10⁻³ M); 1 cm³ of IAA (2×10⁻³ M); 4.5 cm³ of distilled water. After adjusting the temperature of the mixture to 30°C, 1 cm³ of enzymatic extract was added (final mixture volume 10 cm³, pH 6.1).

IAA oxidase activity was also determined in the culture medium (M) with the use of 1 cm³ of the culture filtrate as source of enzyme or 1 cm³ of buffer-diluted filtrate. When undiluted medium was used, the buffer amount in the reaction mixture was increased by 1 cm³ by decreasing correspondingly the amount of water. Incubation was run on a water bath at 30°C with continuous shaking. At 0 time and at 15-min intervals 1-cm³ samples of the mixture were collected, 2 cm³ of Salkowski reagent were added and after 30 min A₅₂₆ was read on a spectrophotometre Specol.

PROTEIN CONTENT DETERMINATION IN ENZYMATIC EXTRACT AND MEDIUM

To 3 cm³ of enzymatic extract 3 cm³ of 10 per cent trichloroacetic acid (TCA) was added for protein precipitation. The samples were centrifuged at 10 000 × g for 15 min, the sediment was successively washed with 70 and 96 per cent ethanol and dissolved in 5 cm³ of 1 N NaOH at 100°C for 10 min. After cooling and suitable dilution, protein was determined by the method of Lowry et al. (1951) with the use of bovine albumin as standard. Protein in the medium was determined analogously by adding 2.5 g TCA to 50 cm³ of medium (final TCA concentration 5%) for protein precipitation. The samples were centrifuged at 2400 × g for 15 min. The further procedure was similar as for the enzymatic extract.

All determinations were performed in three successive cultures of each passage. The results for the particular dates represent means from three or two determinations.

RESULTS

The growth of suspension cultures of sunflower crown-gall tumours in PO and P1 is shown in the diagram (Fig. 1). P1 is characterised by a longer phase of intensive growth as compared with phase PO, there-

fore, the cell numbers are higher (Fig. 1) and so are the fresh weight values (Figs. 2 and 3). In the 4th week of culture in PO the rate of growth is distinctly slowed down and passes to a stationary phase.

Fig. 1. Growth of sunflower crown-gall suspension cultures (passages: O — PO and 1st — P1)

IAA OXIDASE ACTIVITY

The enzymatic activity of the crude cell extract and the culture medium was tested during 60-90 min of incubation with IAA. Sometimes there occurred a lag phase in enzyme activity lasting about 15, less frequently 30 min. Extract dilution enhanced enzyme activity. The main activity of the enzyme was observed in the 30 min period (after the end of the lag phase), and the value of the IAA decomposed in this time, converted to 1 g of fresh cell mass or 1 mg of protein was adopted for comparison of the enzymatic activity of cells in various stages of culture growth. As regards the medium, enzymatic activity is given per 1 cm^3 of the medium and converted to 1 g of cells (assuming as basis the amount of the medium falling to 1 g of cells) and 10 μg of protein contained in the medium. Protein content in the cell extract and the medium is shown in Table 1.

Zero passage (PO). In cell extracts of 7-day culture (S) IAA oxidase activity was not found. It appeared in the phase of intensive growth and

Table 1

Protein content in cell enzyme extracts and in medium in the course of the growth cycle of sunflower crown-gall suspension culture

PO	Days of culture	7	14	21	28	35
	cell extract, mg · 100 mg ⁻¹ fr. wt	0.23	0.62	0.30	0.28	0.16
	medium, mg · 100 cm ⁻³	0.27	0.40	0.33	0.46	1.25
P1	days of culture	9	13	20	27	34
	cell extract, mg · 100 mg ⁻¹ fr. wt	0.20	0.18	0.19	0.19	0.10
	medium mg · 100 cm ⁻³	0.41	1.09	0.65	0.72	1.82

greatly increased in the slowing down of growth (progressive deceleration) and stationary phases (Fig. 2). Application of PVP which removes from plant extracts most phenol compounds, mainly o-diphenols — inhibitors of IAA oxidase (Knypl and Chylińska 1974), allowed to reveal IAA oxidase activity in the early period of growth (7th day) and its about tenfold increase (14th and 21st day, Fig. 2) when it was low. The efficacy of PVP in extracts from cells representing the phase of slowed down growth and the stationary phase decreased, and even some losses in enzyme activity were observed. This may have been due to the decrease in the number of enzyme inhibitors in the cell and protein loss in the enzymatic extract caused by PVP (Knypl and Chylińska 1974). Tenfold dilution of the enzymatic extract enhanced more than did PVP enzymatic activity of cells in the phase of intensive growth. The effect of dilution on cells in the stationary phase was weaker.

Culture medium (M) showed a low enzymatic activity in the phase of intensive growth and an increase in the stationary phase (Fig. 2). This activity decreased only towards protein in the stationary phase, this resulting from the increased protein level in the medium in this period (Table 1). Fivefold dilution of the medium enhanced enzymatic activity, more in the intensive growth than in the stationary phase.

These results indicate that: (1) there exists an inverse relation between enzyme activity and culture growth rate in PO; (2) IAA oxidase inhibitors are present in the cells and medium. The amount or activity of these compounds is high in the intensive growth phase, decreasing in the stationary phase.

First passage (P1). Cells from P1 show a low IAA oxidase activity on the 9th day of culture, this indicating a decrease of its content as compared with that in the outset material (4-week PO culture). During the long phase of intensive growth characteristic for P1 (Figs. 1 and 3) IAA

Fig. 2. Changes in IAA-oxidase activity in sunflower crown-gall suspension culture in the course of growth — PO. S — enzyme extract; S-10 — enzyme extract 10 times diluted; S+ PVP — extract prepared with the use of PVP; M — medium; M-5 — medium 5 times diluted. * Calculated from medium quantity corresponding to 1 g of cells

Fig. 3. Changes in IAA-oxidase activity in sunflower crown-gall suspension culture in the course of growth — P1. Notations as in Fig. 2

oxidase activity increases about tenfold in the first period (9-20th day), then it is considerably depressed and increases once more in the end period of this phase when the rate of culture growth slows down. In the initial period of growth the greater part of the enzyme is released by the cells into the medium (Fig. 3). Tenfold dilution of the cell extract enhanced most enzyme activity (about 30 times) in the periods when

it was lowest (9th and 27th day of culture), less intensively (10 and 5 times) in the remaining periods. PVP applied in several samples proved but little efficient in enhancing enzymatic activity.

P1 culture medium exhibited a constant low enzymatic activity during the entire growth period, with a tendency to its increasing in the end phase of the culture (Fig. 3). Fivefold dilution of the medium increased this activity (5-2 times), indicating seemingly participation of the inhibitors in its regulation.

The different activity of undiluted and diluted enzymatic extracts (especially the cell ones) at several stages of growth may indicate the presence and significant role of inhibitors in regulation of the activity of the enzyme in the suspension culture P1 cycle.

Low enzyme activity in the cells seems to condition the start of the phase of intensive growth. An increase of this activity to a certain value is not a factor limiting cell division, however, the subsequent depression of IAA oxidase activity seems indispensable for prolongation of the phase of intensive growth in P1 as compared with that in PO (Figs. 2 and 3).

DISCUSSION

In earlier investigations of the suspension culture of sunflower crown-gall (Chirek 1983) a high auxin (including IAA) level was noted at the beginning of the intensive growth phase in PO, which progressively fell in the course of further culture. Comparison of these results with the IAA oxidase activity evaluated in the present study indicates a negative correlation between the activity of this enzyme and auxin level in the cells and culture growth rate. This suggests the control of the auxin level in PO cells by the IAA oxidase set.

In P1 of the culture characterised by a long intensive growth phase the total auxin level and IAA oxidase activity show a correlation based on negative feedback: the relatively low auxin level at the middle of the intensive growth phase (3rd week) is associated with a high IAA oxidase activity, and an increase in the auxin level in the 4th week coincides with a considerable depression of the activity of this enzyme. The successive increase of IAA oxidase activity occurs after stabilisation of a higher auxin level in the cells (5th week). This mechanism of auxin level regulation seems to condition the maintenance of intensive growth for a longer period, whereas in PO the IAA oxidase activity increase produced an irreversible fall of the auxin level in the cells, and, consequently, inhibition of their division.

In investigations on the culture of crown-gall cells from the sunflower cv. Giant Russian, Atsumi and Hayashi (1978) found a low IAA level in the phase of intensive growth of the culture and its accu-

mulation in the transition phase. IAA accumulation caused a depression of the auxin protectors level and an increase of IAA-decomposing enzymes activity. According to the authors, this is evidence of the existence in crown-gall cells of a negative feedback mechanism in auxin control.

The results of the present investigations indicate that a similar mechanism is in operation already during the phase of intensive growth of the culture (P1) and with a higher participation of IAA oxidase. In the period of adaptation of the culture (PO) this regulation occurs only in one direction. It seems also that IAA oxidase activity is controlled by inhibitors present in the cells and medium. A high enzyme inhibition was noted in PO cells in the phase of intensive growth and in P1 at the beginning and in the middle of this phase.

It was attempted to explain the process of anergisation of some tissues and cells in *in vitro* culture by changes in auxin metabolism regulation. The results obtained, did not, however, reveal a simple relation between the autonomy of growth and the endogenous auxin level, activity of enzymes degrading the latter and inhibitors of the enzymes. In normal and anergised cells with a similar capability of IAA synthesis a greater or smaller activity of IAA-degrading enzymes were detected (Mailard et al. 1976, Kevers et al. 1981, respectively). Autonomous growth was favoured, on the other hand, by a higher IAA oxidase inhibitor level (Mailard et al. 1976, Syono 1979).

In the light of these data elucidation of the problem of auxin autonomy requires further investigations on the dynamics of enzyme activity, the isoenzyme spectrum and their inhibitors. It also seems necessary to take into account the role of cellular secretion in the regulation of the intracellular pool of both auxins and enzymes and their inhibitors.

REFERENCES

- Atsumi S., Hayashi T., 1978. The relationship between auxin concentration, auxin protection and auxin destruction in crown-gall cells cultured *in vitro*. Plant Cell Physiol. 19: 1391-1397.
- Chirek Z., 1983. Changes in auxin activity in sunflower crown-gall suspension culture in the course of growth. Acta Soc. Bot. Pol. 52: 285-294.
- Davies F. S., Shiao-Lim Chen Man, Noodén L. D., 1975. Auxin synthesis in crown-gall tumor tissue: a comparison of three putative precursors. Physiol. Plant. 33: 39-41.
- Galston A. W., Dalberg L., 1954. The adaptive formation and physiological significance of indoleacetic acid oxidase. Amer. J. Bot. 41: 373-380.
- Hagen G. L., Minch J., 1966. An improved technique for the extraction of auxin destroying enzymes in plant tissues. Plant Physiol. 41 (suppl.): xli.
- Knypl J. S., Chylińska K. M., 1974. Removal of IAA-oxidase inhibitors from carrot root extracts by grinding with insoluble polyvinylpyrrolidone. Biochem. Physiol. Pflanzen 166: 333-343.

- Kevers C., Coumans M., De Greef W., Hofinger M., Gaspar Th., 1981. Habituation in sugarbeet callus: auxin content, auxin protectors, peroxidase pattern and inhibitors. *Physiol. Plant.* 51: 281-286.
- Kovács E. I., Maliga P., 1973. Indoleacetic acid oxidase regulation in genetically tumorous and normal tobacco plants and their tissue cultures. *Acta Bot. Acad. Sci. Hung.* 18: 315-322.
- Lipetz J., 1959. A possible role of indoleacetic acid oxidase in crown-gall tumour induction. *Nature* 184: 1076-1077.
- Lipetz J., Galston A. W., 1959. Indoleacetic acid oxidase and peroxidase activities in normal nad crown-gall tissue cultures of *Parthenocissus tricuspidata*. *Amer. J. Bot.* 46: 193-196.
- Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Mailard F., Pilet P. E., Zryd J. P., 1976. Auxin dependence and auxin oxidase of cultured sycamore cells. *Experientia* 32: 841-842.
- Murashige T., Skoog F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- Sequeira L., 1973. Hormone metabolism in diseased plants. *Ann. Rev. Plant Physiol.* 24: 353-380.
- Stonier T., 1969. Studies on auxin protectors: VII. Association of auxin protectors with crown-gall development in sunflower stems. *Plant Physiol.* 44: 1169-1174.
- Syono K., 1979. Correlation between induction of auxin-nonrequiring tobacco calluses and increase in inhibitor(s) of IAA-destruction activity. *Plant Cell Physiol.* 20: 29-42.
- Witham F. H., Gentile A. C., 1961. Some characteristics and inhibitors of indoleacetic acid oxidase from tissue cultures of crown-gall. *J. Exp. Bot.* 12: 188-198.

*Dynamika aktywności oksydazy kwasu indolo-3-octowego
w kulturze zawieszinowej crown-gall słonecznika*

Streszczenie

Określono aktywność oksydazy IAA w kilku fazach wzrostu kultury zawieszinowej crown-gall słonecznika. Podczas krótkiej fazy intensywnego wzrostu (pasaż zerowy — PO) stwierdzono ujemną korelację pomiędzy aktywnością enzymatyczną a tempem wzrostu. Wzmoczenie aktywności oksydazy IAA do pewnego poziomu nie jest czynnikiem ograniczającym podział komórek; jednak dla przedłużenia fazy intensywnego wzrostu (pasaż pierwszy — P1) wydaje się niezbędny spadek aktywności tego enzymu. Aktywność oksydazy IAA w badanej kulturze znajduje się pod kontrolą inhibitorów występujących w komórkach i podłożu. Wysoką inhibicję enzymu stwierdzono w komórkach PO podczas fazy intensywnego wzrostu oraz w P1 — na początku i w środkowym okresie tej fazy. Uzyskane wyniki sugerują, że oznaczony w poprzednich badaniach poziom auksyn w kulturze crown-gall słonecznika jest kontrolowany przez zespół oksydazy IAA. Podczas długiej fazy intensywnego wzrostu (P1) jest to kontrola typu ujemnego sprzężenia zwrotnego.