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PEROXIDASE IN SUSPENSION CULTURES OF HAPLOPAPPUS GRACILIS

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SUMMARY

Peroxidase activity and isoenzyme composition of cells and culture medium were analysed in a cell suspension culture of *Haplopappus gracilis*. The results revealed marked changes in the activity and isoenzyme patterns of peroxidase during the culture cycle. During the exponential growth phase the peroxidase activity of the cells increased and a considerable amount of peroxidase was released into the culture medium. Several peroxidase isoenzymes in the medium increased in activity continuously throughout the whole exponential growth phase while others decreased during the second half of this phase. The decrease of the latter was caused by diffusion of the isoenzymes back into the cell walls as well as by inhibitors. During the stationary phase of the cell cycle only small changes occurred in the peroxidase activity and isoenzyme patterns.

1. INTRODUCTION

Studies on peroxidase in cell suspension cultures carried out to elucidate the role of this enzyme in growth and differentiation (see GASPAR et al. 1982) have been reported by several authors (e.g. KOSSATZ & VAN HUYSTEE 1976, ARNISON & BOLL 1976a, MÄDER et al. 1981, BREDEMEIJER et al. 1985). There are various ways to study the possible roles of peroxidase by using cell cultures. One approach is the induction of changes in the properties of the cell cultures such as growth pattern, cell form, aggregation and differentiation, by temperature treatments or by changing the growth hormone balance, and to establish whether these changes are correlated with alterations in peroxidase activity and isoenzyme pattern (DE JONG et al. 1968, ARNISON & BOLL 1976a, KING 1976, MASUDA et al. 1983). Another approach, i.e. the use of variant or mutant cell lines may also be a valuable method to study the role of peroxidase (CHIBBAR et al. 1984).

Several variant cell lines (Zn resistant and fermentation variants) have been isolated from cell suspension cultures of *Haplopappus gracilis* at our laboratory, to be used as markers (GILISSEN et al. 1985). We intended to use this material to investigate the role of peroxidase in growth and differentiation. The present work is a part of this investigation, and reports on the peroxidase activity and isoenzyme composition of cells and culture medium during the growth cycle of the wild type (Wt) cell line of *Haplopappus gracilis*.

2. MATERIALS AND METHODS

2.1 Cell cultures

Wild type (Wt) cell suspensions of *Haplopappus gracilis* (Nutt) Gray were maintained by subculturing weekly as described by GILISSEN et al. (1985). Samples for analyses were taken immediately after subculture and at various days after culture. Cell suspensions from two or three flasks per time were pooled and then cells were collected by suction on a Buchner funnel. The fresh weight of the cells was determined and one gram of sample was used for extraction as described previously (BREDEMEIJER et al. 1985). The culture filtrates were only centrifuged (18,000 g for 30 min) and used without further treatments for peroxidase assay and electrophoresis.

2.2 Peroxidase assay and electrophoresis

Peroxidase activity was measured by following the increase in absorbance at 436 nm at 25 °C which is due to oxidation of guaiacol in the presence of hydrogen peroxide and enzyme as described earlier (BREDEMEIJER et al. 1985) with one modification, i.e. 0.1 ml of H_2O_2 solution (0.8 ml perhydrol from Merck in 100 ml distilled water).

Peroxidase isoenzymes were separated by starch gel electrophoresis and detected by staining with benzidine and hydrogen peroxide (BREDEMEIJER et al. 1985).

3. RESULTS

3.1. Peroxidase activity during the growth cycle

The results on relative changes in fresh weight, total peroxidase activity of the cells and peroxidase activity released into the culture medium during the growth cycle are shown in *fig. 1*. During the exponential growth phase, the total peroxidase activity of the cells increased more rapidly than the fresh weight of the cells. Consequently, the peroxidase activity on a fresh weight basis increased gradually during the growth and reached a maximum at the beginning of the stationary phase. During this phase, the peroxidase activity of the cells changed only little.

The peroxidase activity in the culture medium increased only during the first half of the exponential growth phase. Thereafter, it decreased slightly. The peroxidase activity in the medium, at day 7, amounted to 54 per cent of the total activity in the culture.

3.2. Peroxidase isoenzyme patterns during the growth cycle

The 3 groups of peroxidase isoenzymes, i.e. A, B, and C, of the cells changed only quantitatively during the culture cycle (*fig. 2*). The most striking changes

took place immediately after inoculation into fresh medium and during the exponential growth of the cells. The isoenzymes of group B decreased strongly after subculture, while the others showed only small changes. During the exponential growth phase the peroxidase isoenzymes of group B increased again in activity and reached a maximum during the stationary phase. The peroxidase isoenzymes of group A increased in activity during the first two days after subculture and subsequently decreased again to the level observed at day 0. During the stationary phase, the cellular peroxidase isoenzymes showed no further changes for the period investigated (from 0 – 8 days).

The peroxidase isoenzyme patterns of the culture medium are shown in *fig. 3*. Immediately after subculture (t = 0) the activity of several peroxidase isoenzymes has decreased strongly as expected following dilution by 6 times with fresh medium. However, the isoenzymes A2 and B2 did not show a distinct decline in activity. With the onset of growth, the activity of all isoenzymes in the medium increased steadily during the exponential growth phase. The increase of group C continued until the cells reached the stationary phase. The isoenzymes of group A and B, on the contrary, began to decrease in activity during the second half of the exponential growth phase and the isoenzymes B3 and B5 even completely disappeared from the medium.

3.3. Fate of the peroxidase isoenzymes in the medium

In order to elucidate the process that caused the disappearance of the peroxidase isoenzymes B3 and B5 from the culture medium, various possibilities, such as degradation, inactivation, or absorbtion by cells were investigated.

Incubation of a cell-free culture filtrate from day 3 cultured for 4 days at 28 °C caused only an increase in activity of the peroxidase isoenzymes of group

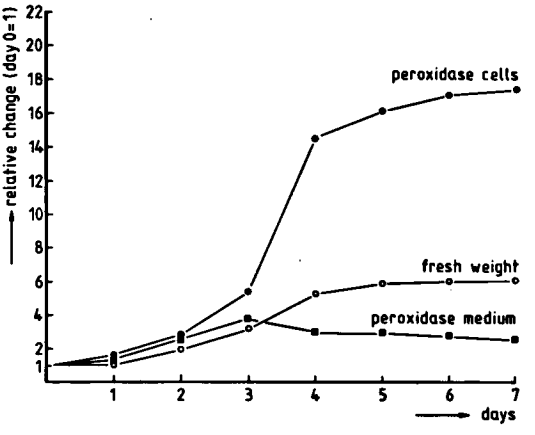

Fig. 1. Changes, relative to day 0, in fresh weight and peroxidase activity of the cells and the culture medium during the growth cycle of *Haplopappus gracilis* suspension culture. Absolute values for day 0: fresh weight, 1.27 g; cellular peroxidase, 78 enzyme units; medium peroxidase, 631 enzyme units.

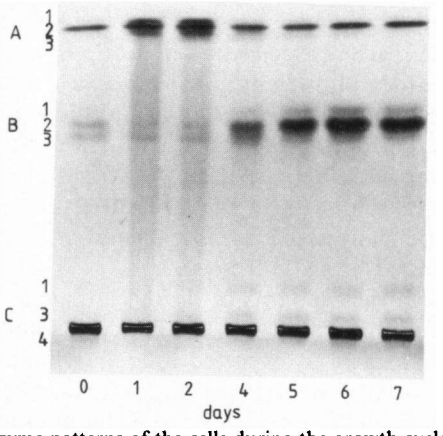

Fig. 2. Peroxidase isoenzyme patterns of the cells during the growth cycle of *Haplopappus gracilis* suspension culture. Day 7 represents the time when subculture was carried out.

A and some smearing of peroxidase activity (*fig. 4*). The activities of the other isoenzymes remained more or less the same, indicating that they were very stable. However, in the presence of cells the isoenzymes B3 and B5 disappeared (*fig. 3*: day 7). Consequently, a factor connected with the cells should be involved in the decrease of these isoenzymes in the medium.

To investigate the possibility of inactivition by a substance released from the cells after day 3, a culture filtrate from day 3 culture which still contained the isoenzymes B3 and B5 was mixed with a culture filtrate from day 5 or day 7 culture which did not exhibit activity of these isoenzymes, and subsequently incubated during 24 hrs at 28 °C. The results indicated that the isoenzymes were not inactivated (data not shown).

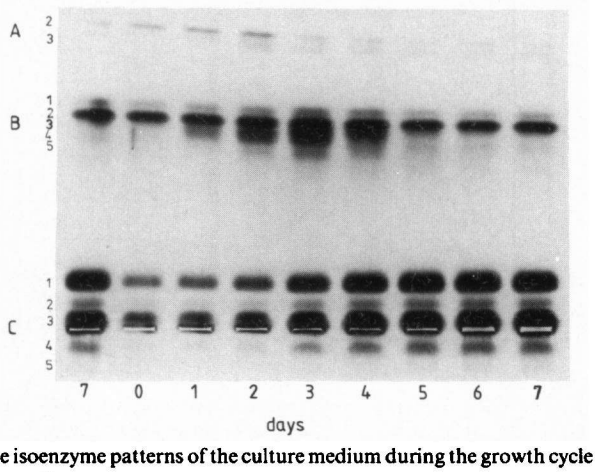

Fig. 3. Peroxidase isoenzyme patterns of the culture medium during the growth cycle of *Haplopappus gracilis* suspension culture.

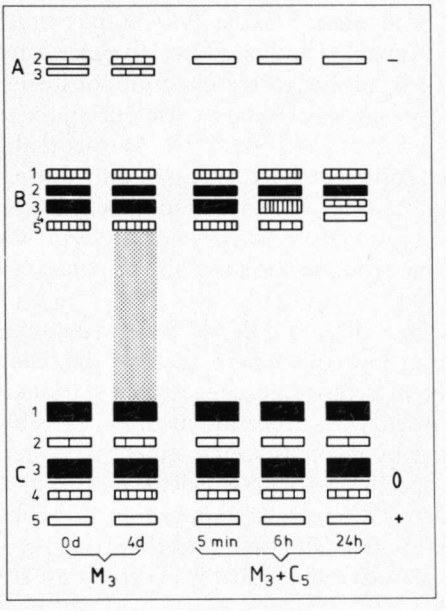

Fig. 4. Diagrammatic representation of peroxidase isoenzyme patterns of culture filtrate from day 3 culture (M3 : Od) of *Haplopappus gracilis* and the same culture filtrate after incubation for 4 days at 28 °C (M3 : 4d), or after incubation with cells of day 5 culture during 24 hrs (M3 + C5).

Finally, the possibility of removal of the isoenzymes from the medium by the cells was investigated. It appeared that incubation of cells (day 5), which did not release isoenzymes B3 and B5 anymore, in a culture filtrate (day 3) containing these isoenzymes caused a decrease of the B group in the medium, while the isoenzymes of group C remained unchanged (*fig. 4*). When cells of day 7 culture were used this decrease of B group isoenzymes was less pronounced (data not shown).

4. DISCUSSION

The activity and isoenzyme pattern of peroxidase in the cell suspension culture of *Haplopappus gracilis* changed during the culture cycle. In contrast with several other species, such as peanut (KOSSATZ & VAN HUYSTEE 1976), *Nicotiana* (MÄDER et al. 1981) and potato (BREDEMEIJER et al. 1985) the increase in total peroxidase activity of the cells was not similar to the increase of the fresh weight. The cellular peroxidase activity on a fresh weight basis increased during the exponential growth phase. In addition, the activity increased when expressed per milligram of extracted protein (data not shown) similar as has been observed for *Phaseolus vulgaris* during cell expansion (ARNISON & BOLL 1976b). The results support the view that peroxidase may be involved in cell wall expansion and development (ARNISON & BOLL 1976b, GASPAR et al. 1982).

The development of the peroxidase activity in the culture medium in *Haplopappus* was different from that in other species. In general, total peroxidase activity in the medium of cell cultures increases continuously during the exponential growth phase and for some species a decrease during the stationary phase has been reported (KOSSATZ & VAN HUYSTEE 1976, MÄDER et al. 1981). In *Haplopappus*, however, the peroxidase activity increased only during the first half of the growth phase. The subsequent decrease in total peroxidase activity was due to the decrease in activity of certain isoenzymes. It seems unlikely that this was caused by degradation processes because all peroxidase isoenzymes appeared to be very stable.

According to MÄDER et al. (1981) the decrease in medium peroxidase activity may be caused by absorbtion of the peroxidase by the cells or by diffusing back into the cell wall. The present results suggest that stationary phase cells indeed remove peroxidase isoenzymes from the medium, possibly by binding to the cell walls. Absorbtion by the cells seems unlikely, because cells growing in a medium containing specific peroxidase isoenzymes from another cell line did not absorb these isoenzymes (results unpubl.). In this context, it is interesting to mention the observation of NIEDERMEYER (1975) that peroxidase did not penetrate into cytoplasm of yeast cells incubated in peroxidase solution, but did penetrate the cell wall.

A striking phenomenon observed in the *Haplopappus* cell culture was the maintenance of the activity of certain peroxidase isoenzymes in the medium in spite of subculture. One would expect a strong decrease in activity of all isoenzymes due to dilution with fresh medium, as observed in several other species (ARNISON & BOLL 1976b, BREDEMEIJER et al. 1985). Possibly, the decrease in activity caused by dilution was for certain peroxidase isoenzymes compensated by a rapid release from the cells, for example by a higher level of calcium in fresh medium (KEVERS et al. 1982). The decrease in activity of the cellular fraction of isoenzyme B2 caused by subculture supports this assumption. In the case of isoenzyme A2, the maintenance of activity may be due to dilution of an inhibitor because dialysis of medium caused an increase in activity.

In conclusion, it is clear that the activity and isoenzyme composition of peroxidase in the medium during the culture cycle not only depends on the rate of synthesis of peroxidase in the cells (CHIBBAR et al. 1984) and its secretion during cell expansion, but possibly also on changes in binding capacity of the cell walls and influence of medium constituents.

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REFERENCES

ARNISON, P. G. & W. G. BOLL (1976a): The effect of 2,4-D and kinetin on the morphology, growth,

and cytochemistry of peroxidase of cotyledon cell suspension cultures of bush bean (Phaseolus vulgaris cv. Contender). *Can. J. Bot.* **54**: 1847–1856.

— & — (1976b): The effect of 2,4-D and kinetin on peroxidase activity and isoenzyme pattern in cotyledon cell suspension cultures of bush bean (Phaseolus vulgaris cv. Contender). *Can. J. Bot.* **54**: 1857–1867.

BREDEMEIJER, G. M. M., H. C. J. BURG, K. SREE RAMULU & P. DIJKHUIS (1985): Release of peroxidase by cultured potato cells. *Acta Bot. Neerl.* **34**: 325–335.

CHIBBAR, R. N., R. CELLA, D. ALBANI & R. B. VAN HUYSTEE (1984): The growth of and peroxidase synthesis by two carrot cell lines. *J. Exp. Bot.* **35**: 1846–1852.

GASPAR, TH., C. PENEL, T. THORPE & H. GREPPIN (1982): *Peroxidases 1970–1980.* Université de Genève – Centre de Botanique, Genève.

GILISSEN, L. J. W., A. T. BARNEIX, M. VAN STAVEREN & H. BRETELER (1985): Variant cell lines of Haplopappus gracilis with disturbed activities of nitrate reductase and nitrite reductase. *Plant Physiol.* **78**: 658–660.

JONG, D. W. DE, A. C. OLSON, K. M. HAWKER & E. F. JANSEN (1968): Effect of cultivation temperature on peroxidase isoenzymes of plant cells grown in suspension. *Plant Physiol.* **43**: 841–844.

KEVERS, C., L. STICHER, C. PENEL, H. GREPPIN & TH. GASPAR (1982): Calcium controlled peroxidase secretion by sugarbeet cell suspensions in relation to habituation. *Plant Growth Regulation* **1**: 61–66.

KING, P. J. (1976): Studies on the growth in culture of plant cells. XX. Utilization of 2,4-dichlorophenoxyacetic acid by steady-state cell cultures of Acer pseudoplatanus L. *J. Exp. Bot.* **27**: 1053–1072.

KOSSATZ, V. C. & R. B. VAN HUYSTEE (1976): The specific activities of peroxidase and aminolevulinic acid dehydratase during the growth cycle of peanut suspension culture. *Can. J. Bot.* **54**: 2089–2094.

MÄDER, M., T. FÜSSL & E. WALKER (1981): On the synthesis of protoheme and peroxidase in suspension cultures of Nicotiana tabacum. *Z. Pflanzenphysiol.* **104**: 207–216.

MASUDA, H., H. FUKUDA & A. KOMAMINE (1983): Changes in peroxidase isoenzyme patterns during tracheary element differentiation in a culture of single cells isolated from the mesophyll of Zinnia elegans. *Z. Pflanzenphysiol.* **112**: 417–426.

NIEDERMEYER, W. (1975): Effect of cytochrome c and peroxidase on the plasmalemma of the yeast cell (Candida utilis). *Cytobiol.* **10**: 485–490.