

## Growth and peroxidase production in cultures of horseradish (*Armoracia rusticana*)

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### Introduction

Peroxidase, an enzyme of commercial importance to the diagnostic industry, is currently extracted and purified from the roots of field-grown horseradish plants (*Armoracia rusticana*) by conventional methods. It is, however, known that plant peroxidases occur in undifferentiated cells maintained in culture at a specific and total activity which may surpass that of the root (Shinshi & Noguchi, 1976). In this report we have examined cell cultures of the horseradish plant as a source of the enzyme.

### Materials and methods

'Hairy root' cultures were initiated and maintained on Murashige and Skoog medium (Flow Labs, Irvine, Scotland, U.K.) with 3% (w/v) sucrose (RM). Callus and cell suspension cultures were initiated and maintained on this medium containing 5 mg of 2,4-dichlorophenoxy acetic acid and 0.1 mg of kinetin/l (5D medium). 'Hairy root' cultures were initiated and maintained according to Hamill *et al.* (1986) using the *Agrobacterium rhizogenes* strain LBA9402. This carries the plasmid pRi1855 plus a disarmed binary vector pBIN19 containing the neomycin phosphotransferase gene.

To analyse peroxidase, the cells and culture filtrate were first separated by vacuum filtration. Cells (0.5 g fresh weight) were rapidly ground for 60 s in a mortar with 10 ml of 150 mM-phosphate-buffered saline (pH 6.2). The extract was then made up to 50 ml and centrifuged at 1000 g for 10 min to remove cell debris. The supernatant was decanted and assayed. Peroxidase was assayed by a modification of the method of Kay *et al.* (1967) using 0.125 mM-dimethoxybenzidine and 0.8 mM-hydrogen peroxide by monitoring the change in absorbance at 410 nm upon addition of 10  $\mu$ l of enzyme solution.

For histochemical staining, the cells were fixed for 5 min in 50% (v/v) methanol then stained with 4 vol. of diaminobenzidine (1 mg/ml) plus hydrogen peroxide (4 ml/l). Staining was absolutely dependent on the presence of both peroxide and diaminobenzidine, was apparent almost immediately and intensified over time.

Carbohydrate was determined by the phenol/sulphuric acid method of Clarke & Shannon (1976). Phosphate was determined by the phosphomolybdate method of Fiske & SubbaRow (1925). Ammonium was determined by Nessler's reagent as described by Vogel (1968). Nitrate was assayed by nitration of brucine according to Nicholas & Nason (1963).

The volumetric oxygen mass transfer coefficient ( $k_1a$ ) was determined by the method of Kato *et al.* (1975) with the modification that oxygen depletion was by purging with nitrogen. The relationship between  $k_1a$  and culture volume in shake flasks was established with 5D medium at a constant shaker speed (110 rev./min) over the range 10–200 cm<sup>3</sup> in 250 ml Erlenmeyer flasks:

$$\log k_1a = 1.8 - 0.00613 \text{ culture volume (ml)} \quad (r = 0.988)$$

This was used to vary  $k_1a$ . Growth was monitored daily by settled cell volume according to Gilissen *et al.* (1983), and at the end of the experiment, by fresh weight.

### Results and discussion

**Enzyme kinetics.** The enzyme displayed a  $K_m$  for peroxide of 1.25 mM and for dimethoxybenzidine of 0.25 mM.

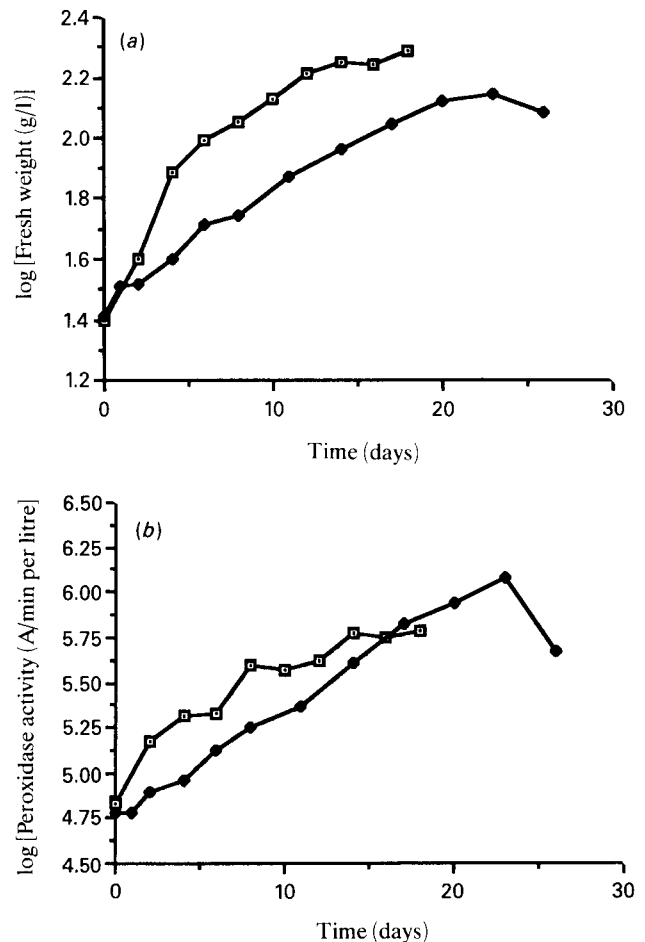


Fig. 1. Fresh weight (a) and peroxidase activity (b) through the growth cycle of 'hairy root' ( $\square$ ) and cell suspension ( $\bullet$ ) cultures

There were two peaks of enzyme activity at pH 4.8 and 5.4. The enzyme therefore can be easily measured and behaves like the enzyme extracted from roots (Kay *et al.*, 1967). It is therefore realistic to examine its production in culture. Histochemical staining of cells from a suspension culture shows that peroxidase is located predominantly in the cytoplasm of the cells and is concentrated in only certain cells within the cell population. This suggests that the peroxidase activity of the cell population may be increased either by selection for high yielding cells or by manipulation of the cells so that all cells produce enzyme.

**Growth and peroxidase activity.** Analysis of the growth (fresh weight) and peroxidase production of 'hairy root' cultures and cell suspension cultures are shown in Figs. 1(a) and 1(b). Cell suspension cultures grow more slowly than 'hairy roots', however, they are inherently more productive than 'hairy root' cultures in terms of peroxidase activity per gram fresh weight. For both the culture systems peroxidase activity parallels growth throughout the growth cycle. Given that peroxidase production is proportional to biomass, it is important to establish what constraints limit the rate and extent of biomass accumulation. Given sufficient mixing and aeration, biomass accumulation must be limited by the exhaustion of the supply of critical nutrients from the culture medium.

Measurements of the uptake of carbohydrate, phosphate, nitrate and ammonium in cell suspension cultures reveals that of these nutrients, only phosphate is depleted before the end of the growth cycle and may limit growth.

The factors limiting growth rate in plant cell cultures are not well understood; however, it has been shown that aeration of the cultures plays a part (Kato *et al.*, 1975). The results of a systematic study into the effect of  $k_a$  on growth of cell suspensions show that growth in the exponential phase of the cultures is proportional to  $k_a$  between 2.5 and 20, after which there is no significant increase. The fresh weight of the cultures in stationary phase is also related to  $k_a$  between 5 and 20.

*The future potential for production of peroxidase in culture.* The maximum biomass accumulation achieved so far is 324 g/litre fresh weight and was achieved by nutrient replenishment. If we combine this with the maximum specific growth rate achieved so far,  $0.45 \text{ day}^{-1}$ , we have a maximum biomass accumulation of around 150 g/day per litre. The protein content of the cells is currently 1% of fresh weight, giving 1.5 g of protein/day per litre. Peroxidase is 3–15% of protein,

so a maximum rate of production of 45–225 mg of peroxidase/day per litre is possible. The commercial viability of peroxidase production from cell cultures will depend upon whether or not these targets can be attained in large-scale fermentations.

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## Development of an enzyme-linked immunosorbent assay for caffeine

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Caffeine is one of the most widely consumed pharmacologically active compounds. It occurs naturally and is readily extracted from coffee beans, tea leaves, kola nuts and cocoa beans. Caffeine is metabolized initially by demethylation in the body, the major metabolite produced is paraxanthine (1,7-dimethylxanthine). The pharmacological actions of caffeine include stimulation of the nervous system, respiration, cardiac muscle and skeletal muscle. Caffeine also acts on the renal tubules to produce a diuresis, relaxes smooth muscle and causes coronary dilatation. The main effect of caffeine on the central nervous system is to produce a rapid and clearer flow of thought and to allay drowsiness and fatigue. High doses of caffeine cause headache, tremors, nervousness and irritability. The measurement of caffeine ingested and present in the blood is therefore important. A number of methods have been developed to measure caffeine including gas-liquid chromatography, h.p.l.c. and radioimmunoassay. Many of these methods have drawbacks such as use of expensive equipment, time taken to perform and the use of radioactive tracers. We have developed an enzyme-linked immunosorbent assay for caffeine which is sensitive, quick to perform, and utilizes an enzyme-labelled compound which remains stable for a long period and is easy to produce.

The assay is a competitive assay which measures caffeine in plasma. The antiserum used in the assay was raised in a Suffolk sheep. The animal was immunized with a caffeine ester conjugated to ovalbumin by a modification of the mixed anhydride method (Erlanger *et al.*, 1957). The caffeine ester was prepared according to the method of Cook *et al.* (1976). The assay was carried out using a Probus quatro sample processor. A microtitre plate was first coated with ion-exchange

Abbreviation used: PBSGT, phosphate-buffered saline containing Tween 20 and gelatin (see text for composition).

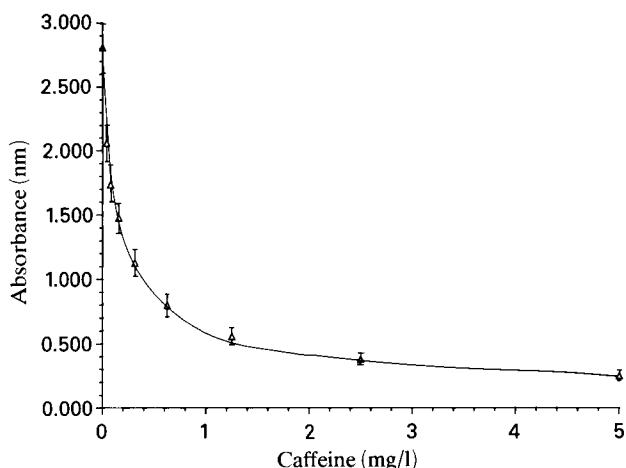


Fig. 1. Caffeine standard curve

Results are means  $\pm$  S.D.

purified caffeine antibodies (batch no. HP/S/3405-1; Guildhay Antiserum Ltd.) diluted with 0.1 M-bicarbonate/carbonate buffer (pH 9.6) to a concentration of 5  $\mu\text{g/ml}$ . The diluted antiserum was added to the inner 60 wells of the microtitre plate (200  $\mu\text{l/well}$ ) and incubated in a moist chamber at 37°C for 1 h. The plate was then washed in 0.15 M-phosphate-buffered saline (pH 7.4) containing 0.05% (v/v) Tween 20 and 0.1% (w/v) gelatin (PBSGT). All further dilutions and washing were carried out with this buffer unless otherwise stated. The samples/standards (5  $\mu\text{l/well}$ ) were then added to the appropriate wells. The samples were diluted 1:10 in PBSGT before addition to the microtitre plate. The standards were diluted in the range 0–10 mg/l (nine standards) in diluted caffeine-free plasma. The caffeine-free plasma was diluted 1:10 with assay buffer. After the addition of samples/standards, 95  $\mu\text{l}$  of PBSGT plus diluted peroxidase-labelled caffeine (100  $\mu\text{l/well}$ ) was added to the wells. Caffeine enzyme label was prepared using the caffeine ester coupled to peroxidase using the mixed an-